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Research review paper

Cell detachment: Post-isolation challenges

Qin Zheng [a], Samir M. Iqbal [b,c,d,e,f,*], Yuan Wan [a,g,**]

[a] Department of Oncology, The Second Affiliated Hospital of Southeast University, Nanjing, Jiangsu 210003, PR China
[b] Nano-Bio Lab, University of Texas at Arlington, Arlington, TX 76019, USA
[c] Department of Electrical Engineering, University of Texas at Arlington, Arlington, TX 76011, USA
[d] Nanotechnology Research Center, Shimadzu Institute for Research Technologies, University of Texas at Arlington, Arlington, TX 76019, USA
[e] Department of Bioengineering, University of Texas at Arlington, Arlington, TX 76010, USA
[f] Joint Graduate Studies Committee of Bioengineering Program, University of Texas at Arlington and University of Texas Southwestern Medical Center at Dallas, University of Texas at Arlington, Arlington, TX 76019, USA
[g] Ian Wark Research Institute, University of South Australia, Mawson Lakes, Adelaide, SA 5095, Australia

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ABSTRACT

Rare cells already have become established indicators for disease diagnosis, to help track prognosis, and in developing personalized therapy. Numerous techniques have been developed to effectively and specifically detect and sort rare cells and cell isolation techniques have gained much attention among researchers in the last few decades. Recent developments in nanotechnologies and microfluidics have been used with great promise towards these goals. The research emphasis has also shifted from simple detection with microfluidic devices to comprehensive isolation, collection and subsequent analysis with integrated and automated systems. The first challenge in post-isolation analysis is cell detachment from substrates, while keeping cells viable and unperturbed. In this review, various methods used for cell detachments are discussed. For effective cell sorting, the detachment is identified as critical criteria for selecting substrates and methods.

Contents

* Correspondence to: S.M. Iqbal, 500 S. Cooper St #217, Box 19072, University of Texas at Arlington, Arlington, TX 76019, USA. Tel.: +1 817 272 0228; fax: +1 817 272 7458.
** Correspondence to: Y. Wan, Ian Wark Research Institute, University of South Australia, Mawson Lakes, Adelaide, SA 5095, Australia. Tel.: +61 8 830 23415; fax: +61 8 830 23683.
E-mail addresses: smiqbal@uta.edu (S.M. Iqbal), yuan.wan@unisa.edu.au (Y. Wan).

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1. Introduction

Cells with less than 1000 count in a milliliter of sample are defined as rare cells (Dharmasiri et al., 2010). These include circulating tumor cells (CTCs), circulating endothelial cells, hematopoietic stem cells, HIV infected CD4 + T cells and many others. Rare cells have garnered significant interest from the scientific and clinical communities as these may provide invaluable information for disease diagnostics, prognostics and therapeutics (Baum et al., 1992; De Coppi et al., 2007; Hansford et al., 2007; Iverson et al., 1981; Nagrath et al., 2007; Peterlin and Trono, 2003; Simpson, 1993; Singh et al., 2003; Toma et al., 2001; N. Uchida et al., 2000). To efficiently isolate rare cells in a viable state, a number of approaches have been reported, including devices that rely on mechanical forces, dielectrophoresis, optical interactions, magnetic sorting, flow cytometry, and microfluidic channels. Previous reviews have discussed many of these rare cell isolation approaches (Dharmasiri et al., 2010; Yu et al., 2011). Recently, nanostructured substrates have been promisingly employed in rare cell isolation; these not only provide better isolation sensitivity and specificity but also can keep captured rare cells viable on the surface, while requiring very little effort in sample preparation (Chen et al., 2011; Kim et al., 2010, 2012; Lee et al., 2012; Park et al., 2012; Sekine et al., 2011; Wan et al., 2012b; Wang et al., 2009, 2011; N. Zhang et al., 2012). Few devices have been tested with real patients' body fluids for rare cell isolation, and encouraging results have been observed with ligand functionalized nanostructured substrates (Wang et al., 2011; N. Zhang et al., 2012).

There is great opportunity in not just rare cell enumeration and isolation but also to be able to do further analysis like whole cell culture and molecular profiling. These may guide personalized therapy and track cell genotype during treatment. For this, the captured rare cells should remain viable and detached for subsequent analysis (den Toonder, 2011). For example, CTCs hold the key in understanding the biology of metastasis and heterogeneity among tumor cells (Yu et al., 2011). If perturbations occur during the isolation process, stem cells may not remain suitable for cell-based therapy anymore (Chen et al., 2010). Similarly, viable HIV infected CD4 + cells would better reveal the dynamics of T cell population if these stay in the native state (Hazenberg et al., 2000). Currently, most analysis on captured cells is done by directly lysing cells on the device surface for downstream enzymatic reactions to measure DNA, mRNA, miRNA, ncRNA, and proteins. With high definition cell assays, such as CellSearch™ system, cell counting and downstream characterization requires fixing of cells that renders subsequent whole cell culture and analysis impossible (Marrinucci et al., 2012). However, the value of captured rare cells is much more than that; for example, FACS analysis can simultaneously analyse approximately 18 different proteins, which can provide better and more comprehensive understanding of protein expression at the single cell level (Bar-Even et al., 2006; Newman et al., 2006; Sachs et al., 2005). Thus, detachment and collection of captured rare cells from substrates without perturbing the microenvironment is a pressing issue.

In order to detach cells from substrates, two major adhesive forces need to be overcome. These are (i) interactions between the receptors and capture ligand, and/or (ii) focal adhesion. Cells themselves can detach from surfaces during their migration by complex signal regulation and participation of multiple proteins, however cells cannot be harvested by this active detachment. On the contrary, by means of external forces, cells can be effectively lifted off in a passive manner. Theoretical models predict a logarithmic dependence of mechanical detachment on adhesive forces (Bell, 1978; Dembo et al., 1988), and data have been obtained to experimentally test these models (Kuo and Lauffenburger, 1993; Wang and Lin, 2007). It is now of utmost importance to find reasonable ways to overcome the adhesive forces while keeping cells viable and unperturbed.

Traditionally, enzymatic treatment for cell detachment is most common. This process digests extracellular matrix (ECM) proteins and cells are released. This treatment is invasive as other cell surface proteins (ion channels, receptors, cell-to-cell junction proteins, etc.) are also digested (Keizer et al., 1988). As an alternative, a temperature induced cell detachment method, based on the fact that ECM generally adheres to a hydrophobic surface rather than highly hydrophilic surface, was also developed (McAuslan and Johnson, 1987). Although the need for less invasive cell harvesting methods is not discounted by the community, only a few works like electricity-induced, pH change-induced, and light-induced methods have approached this issue (Hong et al., 2013). Only a few almost non-invasive cell detachment methods such as those using aptamers have been shown for cell detachment. It is thus necessary to summarize these available cell detachment technologies and have a comparison among them. This review introduces cell detachment strategies and their respective mechanisms, limitations, practical applications, and future directions. We define a few criteria for selecting ideal substrates for rare cell capture and detachment.

2. Cell detachment strategies

The cell detachment strategies span a wide range, including cell biology, molecular biology, biomaterial science, tissue engineering, and electrical engineering. Techniques developed from different backgrounds emphasize different purposes and practical applications. For example, in tissue engineering complete cell sheet lift-off is preferred; contrarily, laser mediated technique can precisely detach a targeted single cell while aptamer functionalized substrates show promise in the capability of surface regeneration. Therefore, a simple comparison of cell detachment efficiency and viability of lifted-off cells among these techniques may downplay the real foci of the respective works. We introduce various cell detachment approaches and discuss their respective advantages and limitations. This can help define various benchmarks for selecting or developing suitable materials and/or methods for reaching ideal cell detachment in future works. Cell detachment conditions, releasing times, detachment rates and cell viability rates for typical approaches are summarized in Table 1.

2.1. Active cell detachment

In cell biology, adhesion of cells to ECM is a key step in the regulation of cell morphology, migration, proliferation and differentiation (Ridley et al., 2003). Integrin heterodimers on cell membrane act as the predominant receptors to recognize ECM ligands, to form focal adhesions, and to mediate cell adhesion to ECM (Mitra et al., 2005). On the contrary, during migration, numerous proteins such as microtubules, Kinesin-1, phosphatases, focal adhesion kinase, Calpain-2, ZF21, etc. are involved in the disassembly of focal adhesion complexes. Therefore, cells can be released from ECM by reducing regional adhesive forces (Nagano et al., 2012). However, in rare cell capture and release, it might affect the functionalities of the cells while detaching the captured cells from substrate by regulation of biomolecules in cytoplasm, since the attachment mechanisms and regulation pathways are not fully known. Hence, passive detachment of cells could be more efficient and provide more manipulative power.

2.2. Flowing fluid mediated detachment

The shear stress generated by a controlled fluid, a measure of tangential force per unit area, is widely applied to detach the attached cells. The cells detach from the microfluidic device surfaces when the hydrodynamic force can overcome the cell adhesive force (Fig. 1). The effects of flow rate, flow acceleration, and flow type in cell detachment have been studied before (Abu-Reesh and Kargi, 1989; Cheung et al., 2009; Lu et al., 2004). Technically, applying flowing fluid to attached cells is not difficult; a well-sealed microfluidic flow-through device and a flow-rate controllable syringe pump can satisfy the basic configurations of cell detachment. However, the major issue is on choosing the suitable shear stress to attached cells. Various factors, such as antibody

Table 1
Comparison of the cell isolation and detachment approaches.

Property	Material	Cell types	Treatment conditions	Capture efficiency	Releasing time	Detachment rate	Cell viability
Shear stress	Flowing fluid	Various	Laminar flow with high flow rate; turbulent flow with high flow rate; flow acceleration	N/A	Few seconds	>50%	Poor
Temperature	PNIPAAm–PEG (Ernst et al., 2007)	Fibroblasts	Decreased temperature from 37 to 25 °C; at a flow rate of 560 μm s^{-1}	N/A	30 min	80%	N/A
	PNIPAAm–PDMS Ma et al. (2010a)	COS cells; mesenchymal stem cells	Decreased temperature from 37 to 20 °C	N/A	10–20 min	N/A	89% and 97%, respectively
	PNIPAAm–Neutravidin-antibody (Gurkan et al., 2011)	CD4+	Decreased temperature from 37 to <32 °C facilitated by ~10 μL fluid	>90% from buffer	<10 min	59%	94%
	PNIPPAm–BSA–biotin-antibody (Liu et al., 2013)	MCF7	Decreased temperature from 37 to 20 °C	>95% from buffer	20 min	99%	>95%
	PNIPAAm–polystyrene (Tang et al., 2012)	MFC and BAEC	Decreased temperature from 37 to 20 °C, at a shear stress of 9.4 dyn cm^{-2}	N/A	~1 h	~100%	N/A
pH	Chitosan (Y.-H. Chen et al., 2012)	HeLa	Increased pH from 6.99–7.2 to 7.65	N/A	~1 h	>90%	>95%
	PAH/PSS (Guillaume-Gentil et al., 2011)	Mesenchymal stem cells	Decreased pH from 7.4 to 4.0	N/A	2–3 min	N/A	>95%
EDTA	Alginate hydrogel-RGDS (Plouffe et al., 2009)	Fibroblasts	Rinsed with 50 mM EDTA solution	~8% from buffer	15 min	97%	~80%
	Alginate-PEG-antibody (Hatch et al., 2011)	Endothelial progenitor cells	Rinsed 50 mM solution of EDTA at 10 μL min^{-1}	~30% from buffer	10 min	N/A	~90%
Ions	HA/PLL (Zahn et al., 2012)	C_2C_{12} myoblast cell sheets	5 mM of ferrocyanide	N/A	<5 min	~100%	>90%
Light	Poly(NSP-*co*-MMA) (Higuchi et al., 2004)	Mesenchymal stem cells	UV 365 nm, 950 μW/cm^2	N/A	4 min	>90%	>98%
	Polyketal-co-polyacetal (Pasparakis et al., 2011)	3 T3	He–Xe lamp, 450 W, 50 mJ cm^{-2} at 248 nm	N/A	<5 min	Single cell release	>80%
	Photosensitive *o*-nitrobenzyl (Shin et al., 2011)	Molt-3 T-lymphocytes	UV 365 nm, 1.2 W/cm^2	N/A	0.5 s	>90%	>95%
	Gold nanoparticles (Kolesnikova et al., 2012)	Fibroblasts	2 W CW diode laser, 532 nm, 500–1000 mW	N/A	2–5 min	Poor	Poor
	TiO_2 nanoparticles (Hong et al., 2013)	MC3T3-E1	365 nm, 1.4 mW/cm^2, total energy: 3360 mJ/cm^2	N/A	20 min	>90%	>97%
	Carbon nanotubes (Sada et al., 2011	HeLa	Near infrared laser, Nd: YVO_4, 1064 nm, 20 Hz, 4–6 mW	N/A	4 ns	Single cell release	Limited
Electrodes	Conductive metal substrate (Inaba et al., 2009; Wildt et al., 2010; Zhu et al., 2008)	Various	Voltage scan from −0.5 to −1.3 V	N/A	1–10 min	~100%	~100%
Enzyme	Trypsin	Various	0.05–0.25% trypsin	N/A	up to 1 h	~100%	85–98%
	Exonuclease I/aptamer (Chen et al., 2011)	T lymphocytes	Incubated at 37 °C	N/A	50 min	97%	90%
	Exonuclease I/aptamer (Zhao et al., 2012)	CCRF-CEM	Incubated at 37 °C	12% from whole blood	10 min	68%	66%
	Endonuclease/aptamer (Li et al., 2013)	CCRF-CEM	Incubated at 37 °C	N/A	30 min	~99%	~98%
Aptamer	RELease/aptamer (Wan et al., 2012a)	hGBM cells	Incubated at 37 °C	44% from buffer	30 min	92%	N/A
	RELease/aptamer (Z. Zhang et al., 2012)	CCRF-CEM	Incubated at 37 °C	~100% from buffer	10 min	95%	99%
	Aptamer (Zhu et al., 2012)	CCRF-CEM	Increased temperature from 37 to 48 °C; rinsing at 5 μL min^{-1}	~100% from buffer	2 min	80%	~95%

type, receptor density on cell membranes, substrate geometry, etc. determine the cell adhesive force; therefore, it is impractical to precisely calculate the shear stress which would be required to detach the attached cells. Most of time, a rough estimate of force is calculated from theoretical models which can detach 50% of cells from the surfaces. It is followed by fine adjustment in real detachment work. Hence, this detachment strategy has low efficiency and many limitations (Kwon et al., 2007).

The shear stress may also affect cell viability and function during the detachment process. Normally, cells attached on the surface require an extremely high detachment force. The spreading and flatness of cells may also further increase the threshold (W. Chen et al., 2012; Kwon et al., 2007; Wan et al., 2011), while certain cells can inherently resist high shear stress. More importantly, vulnerable cells such as tumor cells might be damaged by extremely high shear stress. When shear stress is applied on cells, the observed cell deformation is dramatic (Fig. 1(A)) (Cheung et al., 2009; Wankhede et al., 2006), and the deformation can cause a rise in cell membrane tension and surface area. When the shear stress reaches a critical point for a given cell, the cell will be damaged (Born et al., 1992). Moreover, turbulent shear may cause a higher degree of damage than laminar shear for the same shear level and exposure time (Abu-Reesh and Kargi, 1989). Furthermore, shear stress also can significantly disturb the cell microenvironment (Fig. 1(B)). For example, shear stress can induce cell differentiation (Wang et al., 2005), enhance cell proliferation, adhesion and migration (Albuquerque et al., 2000; Chowdhury et al., 2010). Thus, utilizing shear stress to detach cells may neither keep cells viable nor maintain cells under the native status. To sum up, low detachment efficiency and potential damage to cell viability and functions are major disadvantages of using shear stress mediated cell detachment. The

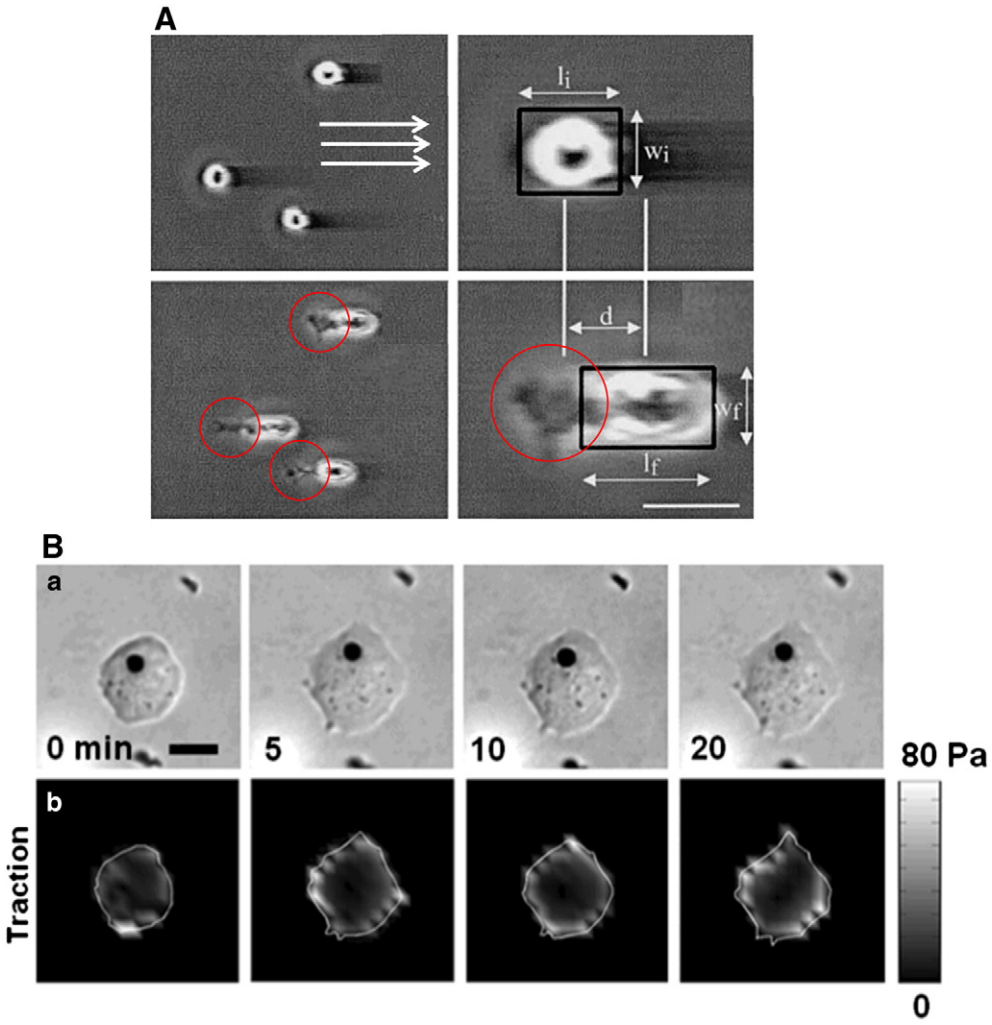

Fig. 1. (A): Cell deformation before detachment from substrate under applied shear stress (Goldstein and DiMilla, 2002). The hydrodynamic shear was generated by the flow to the right (white unidirectional arrows). Red circles show suspected cell fragments (dark area) after cell detached from original attached place. The displacement and spreading were found to be mutually exclusive. (B): The temporally captured micrographs show the effects of the stress. (a) Spreading of mouse embryonic stem cells from left image to the right. (b) Corresponding increase in the cell tractions at the cell periphery within a few minutes of the application of the stress (Chowdhury et al., 2010).

major advantages are simplicity and inexpensive nature of pressurized flow. If the target cells are sturdy enough and shear stress has an insignificant effect on their functions, utilizing shear stress to detach cells could be the best option.

2.3. Stimuli-responsive polymers mediated detachment

Stimuli-responsive polymers respond dramatically to external stimuli such as changes in temperature, pH, ionic strength, solvent composition, light, mechanical force, electric and magnetic fields, the presence of chelating species, and others. These polymers can respond to external stimuli by changing their microstructures from collapsed to expanded as well as by reversibly returning to their initial state (Alarcon et al., 2005). Hydrogels, for example, can entrap large quantities of water or biological fluids. These mimic the natural living environment, which results in excellent biocompatibility and mechanical properties. Controlling the surface energy of stimuli-responsive polymers can modulate properties such as hydrability, solvability, biomolecular adsorption, and cell adhesion. These materials are playing an increasing part in a diverse range of applications, such as drug delivery, diagnostics, tissue engineering and smart optical systems, as well as biosensors, microelectromechanical systems, coating and textiles (Stuart et al., 2010). Here, we present few stimuli-responsive polymers that have been used in cell detachment applications. Light-responsive polymers will be discussed in part of light induced detachment.

2.3.1. Thermo-responsive polymers

Cell sheet lift-off technique emerged first in tissue engineering. Cells are cultured on a thermo-responsive surface which enables reversible cell adhesion to and detachment from the surface by controllable hydrophobicity of the surface (Shimizu et al., 2009). Poly(N-isopropyacrylamide) (PNIPAAm) is a widely used thermo-responsive polymer in tissue engineering applications. PNIPAAm undergoes a hydration switch at its lower critical solution temperature (LCST) of 32 °C. The PNIPAAm molecules are hydrophilic below the LCST but become hydrophobic above the LCST due to dehydration of the molecules. This change is reversible with temperature (Fig. 2). Cells generally prefer hydrophobic surfaces compared to hydrophilic ones for attachment. Thus, cells can adhere to PNIPAAm at 37 °C to form confluent cell sheets that can be lifted off from PNIPAAm surface by decreasing the temperature to 20 °C (Elloumi-Hannachi et al., 2009). Grafting density of PNIPAAm is the primary factor in controlling cell adhesion and detachment. For example, cell adhesion and detachment are only observed on PNIPAAm when grafted density is around 1.4 μg cm^{-2} (thickness 15–20 nm) (Akiyama et al., 2004). Cell adhesion can be inhibited when the grafting density of the PNIPAAM layer is larger than 2.9 μg cm^{-2}

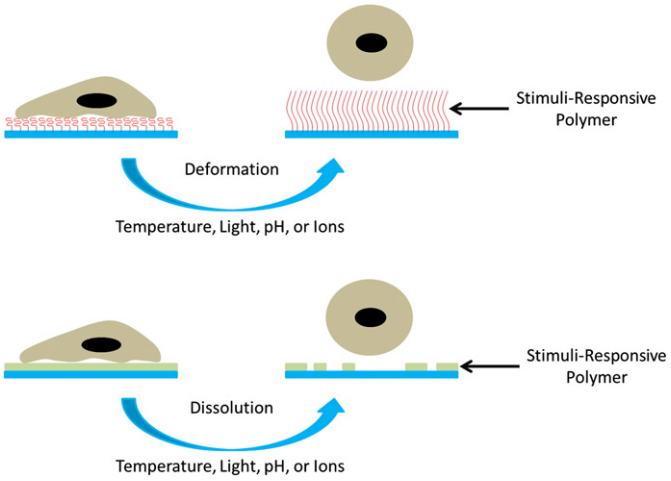

Fig. 2. Cell detached from stimuli-responsive polymer surface. The changes in temperature, light, pH or ionic concentration can either cause polymers to switch from hydrophobic to hydrophilic or dissolve polymers.

(thickness >30 nm) due to incomplete dehydration of PNIPAAm chains. On the other hand, when the density of PNIPAAm layer is too small (thickness <15 nm), attached cells fail to detach from the surface (K. Uchida et al., 2000). Recently, PNIPAAm have been copolymerized with several other polymers (Cao et al., 2005; Eeckman et al., 2004; Kong et al., 2009; Maeda et al., 2006; Nitschke et al., 2007; Reed et al., 2010; Schmaljohann, 2005; Schmaljohann et al., 2003; Tsuda et al., 2005; Yang et al., 2010), resulting in a wide range of LCSTs and different mechanical properties for easy-handling of cell experiments. The technical detail of copolymerization can be found elsewhere (Roy and Gupta, 2003). Here, we only introduce application of PNIPAAm for cell detachment in microfluidic devices. In 2007, Oliver and coworkers embodied a PNIPAAm-poly(ethylene glycol)-thiol copolymer into microfluidic devices on gold substrates to control mouse fibroblast detachment (Ernst et al., 2007). When temperature decreased from 37 °C to 25 °C in a microfluidic device, approximately 97% of cells on the surface changed to a spherical morphology and about 80% of these cells were lifted off in a flow. Ma et al. coated PNIPAAm on different thicknesses of polydimethylsiloxane (PDMS) substrates, and observed that 89.1% of cultured COS7 cells and 97.2% human mesenchymal stem cells were harvested. In comparison, 80.6% of COS7 cells and 75.7% stem cells were harvested by trypsin digestion (Ma et al., 2010b). Umut et al. immobilized Neutravidin followed by biotinylated antibody on PNIPAAm coated microfluidic channels; after target cells were captured in blood samples, Neutravidin–antibody–cell complexes were flushed out of the surface by quickly cooling down the whole device below 32 °C (Gurkan et al., 2011). The releasing efficiency of captured CD4+ was approximately 59% with 91% specificity and 94% viability under an optimized flow rate; similar results were observed in trials of CD34+ cell capture and release. They further prepared a thermoresponsive chip by using a thermoelectric module to control its local temperature at 37 °C in a selected zone within a microfluidic channel, while keeping temperature in the rest zones at 31 °C (Gurkan et al., 2012). Consequently, target cells could be effectively captured and released in pre-defined areas by manipulation of local temperature in a microfluidic system. Recently, Liu et al. coated PNIPAAm on silicon nanopillars and further introduced antibody onto PNIPAAm by the anchor biotin-BSA for MCF7 cell isolation (Liu et al., 2012). It was reported that 98.8% of captured MCF7 cells were released from thermoresponsive nanostructured surfaces with minimal decrease in cell viability (<1%). In another report, larger shear forces (9.4 dyn cm^{-2}) could effectively lift off MFC or BAEC cells (almost 100%) from a PNIPAAm grafted tissue culture polystyrene surface within 1 h in microchannels at 20 °C (Tang et al., 2012). Other polymers such as pluronic, xyloglucan, hydroxybutyl chitosan, methylcellulose, poloxamers and elastin-like polypeptides (Brun-Graeppi et al., 2010; Higuchi et al., 2006) have been demonstrated to be promising thermoresponsive polymers; however, none of these have been studied or applied for cell attachment/detachment.

2.3.2. pH-responsive polymers

The key element of pH-sensitive polymers is the presence of ionizable weak acidic or basic moieties attached to a hydrophobic backbone (Fig. 2). Upon ionization, the coiled chains extend dramatically, responding to the electrostatic repulsions of the generated charges (anions or cations) (Bajpai et al., 2008). It was reported that chitosan surface becomes negatively charged when medium pH is higher than 7.4. Since ECM proteins have a net negative charge at physiological pH, cells therefore were repelled by charge exclusion (Yeh and Lin, 2008). Chen et al. showed that over 90% of cells rapidly detached from chitosan surface within 1 h when pH increased to 7.65 with >95% viability (Y.-H. Chen et al., 2012). Polyelectrolyte multilayers (PEM), composed of weakly ionic forces between poly(allylamine hydrochloride) and poly(acrylic acid) (PAH/PAA), swell substantially in physiological conditions to present richly hydrated surfaces. These could be used for cell detachment as well (Mendelsohn et al., 2002). The other kinds of pH-responsive polymers are acid-degradable ones. PEM composed of poly(allylamine hydrochloride) and poly(4-styrene sulfonate) (PAH/PSS) can be hydrolysed when pH decreases from 7.4 to 4.0, and therefore cells can be released with >95% cell viability (Guillaume-Gentil et al., 2011).

2.3.3. Alginate hydrogel

Aqueous alginate can easily form hydrogel at room temperature by adding a certain concentration of calcium salt (Fig. 2). It can be then dissolved with the same ease of appearance of chelator molecules such as ethylene diamine tetraacetic acid (EDTA). It is widely used in cell encapsulation, cell transplantation, and tissue engineering applications (Augst et al., 2006). In 2005, Braschler et al. first presented a method for in situ alginate hydrogel formation by adjusting concentrations of calcium and EDTA, and achieved cell entrapment and release in microfluidic device (Braschler et al., 2005). For efficient capture of primary rat cardiac fibroblasts, a peptide was conjugated to sodium alginate (Plouffe et al., 2009). In a microfluidic device peptide functionalized alginate showed a two-fold increase of cell adhesion compared to unmodified alginate, and 97% of captured cells were released from the former group compared to 69% from the latter; furthermore, there was no significant difference in cell viability (around 80%) after release versus pre-injection. In their latest work, premixed poly(ethylene glycol) (PEG) and antibodies were grafted to alginate hydrogel which enhanced endothelial progenitor cell (EPC) capture efficiency (Hatch et al., 2011). Around 10×10^4 EPC (isolation efficiency was close to 30%) were recovered by chemically chelating PEG incorporated alginate hydrogel with 74% purity; for comparison, approximately 4×10^4 EPC were recovered from non-PEG incorporated alginate hydrogel with only 25% purity. Nevertheless, EDTA as a chelating agent binds to calcium and prevents joining of cadherins between cells and formation of cell clumps. It also detaches adherent cells and inactivates a range of metallopeptidases. Cell viability and function can be , however, affected by the chemical reagents (Suárez-González et al., 2010).

2.3.4. Other stimuli-responsive polymers

Ion-induced cell detachment has been achieved from PEM consisting of hyaluronic acid and poly-L-lysine (HA/PLL) which was hydrolysed by increasing ferrocyanide concentration (Fig. 2) (Zahn et al., 2012).

The stimuli-responsive polymers mentioned above have been synthesized, studied and applied in tissue engineering; however, most of these have not yet been integrated into microfluidic systems. Summarizing the stimuli-responsive polymers mentioned above, cells have been detached from polymer substrates by changing hydrability (e.g. PNIPAAm), degradability (e.g. PAH/PSS) or solvability (e.g. Alginate

hydrogel). Although the efficiency of cell detachment from polymer surfaces can reach 80–90% in 30–70 min, there are several issues that need to be considered. First, many of these polymers were designed for complete cell sheet lift-off rather than single cell detachment; these lifted cells off from the polymer surface by utilizing "mechanical forces" generated from stimuli-induced changes, but not by destroying cell–cell connections and/or receptor–ligand interactions. In previous studies, cell clusters rather than individual cells could be easily detached from surface, probably due to the internal tension in the cell cluster (Ernst et al., 2007). In the case of single cells, when the mechanical energy is not sufficient to induce cell detachment, external force is generally required. It is possible to detach single cells from photodegradable polymer surfaces in laser-mediated model with a mask, but the specificity of rare cell isolation and detachment remains a challenge. This approach also faces other problems that will be discussed later. Second, the potential cytotoxicity cannot be neglected as well. In some situations, some polymers are biocompatible; however the degraded and dissolved fragments may cause cytotoxicity. For example, when the temperature is decreased, the PNIPAAm layer eventually dissolves which might introduce a cytotoxicity risk (Vihola et al., 2005). On the other hand, after polymer degradation or dissolution, fragments that were connected previously to the immobilized ligands might be uptaken via receptor mediated endocytosis. In pH-responsive polymer applications, regulation of pH value is achieved by diffusion of protons or hydroxyl groups that may result in local pH values dramatically changing and eventually becoming harmful to cells. High concentration of ions might also influence normal proliferation and cellular functions in ion-responsive polymer cases. Third, polymers which are good for cell sheet detachment in tissue engineering might not be suitable for cell capture and detachment in microfluidic devices. Some polymers such as alginate hydrogels have inherent disadvantages such as high level of non-specific binding and limited amount of sites for conjugating antibodies. These factors significantly reduce the cell capture specificity and efficiency. It is well established that the number of immobilized ligands can affect the cell isolation efficiency (Phillips et al., 2008). The higher the number of ligands, many more cells can be captured. The receptor and ligands have one-to-one instantaneous binding mechanism which means that many more immobilized ligands undoubtedly can increase the odds of binding. Thus, more ligands are helpful in rare cell capture, and this may not be achievable on some polymers. Fourth, grafting a uniform and thin polymer layer on substrates requires special equipment and expertise. Finally, the real-life application of stimuli-responsive polymer in rare cell capture and detachment relies on the development pace of polymer science.

2.4. Light induced detachment

The light induced cell detachment generally includes light-responsive polymers, light-sensitive molecules, and light-irradiation nanoparticle mediated cell lift-off. These approaches can provide simple and effective ways for cell detachment. In one example, 375 nm ultraviolet (U)V was used to irradiate the photosensitive surface of nitrobenzospiropyran and methyl methacrylate copolymers on poly (NSP-*co*-MMA)-coated glass substrate. The copolymer switched from hydrophobic to hydrophilic due to the change between two isomeric states of spiropyran (Fig. 2). The work showed that platelets and mesenchymal stem cells (KUSA-A1) were thus easily detached from these surfaces (Higuchi et al., 2004). The 90% of attached KUSA-A1 cells were released with >98% viability.

Photodegradable polymer is another kind of light-responsive polymer, and the photolysis mechanism involves the formation of zwitterion intermediates and their subsequent transformation into carbonyl and hydroxyl products (Wang et al., 2007). A copolymer of polyketal and polyacetal has been used as photodegradable substrate for laser mediated cell detachment (Pasparakis et al., 2011). The cells could be completely removed as a result of polymer ablation, and about 80% of detached cells were viable. By using appropriate masking techniques, this approach can precisely handle selective cell detachment.

Cells have also been detached by UV induced cleavage of antibody anchors containing photosensitive *o*-nitrobenzyl groups that were photocleavable. Attached cells were exposed to 365 nm UV and 90% of cells were detached after 1 min exposure followed by gentle rinsing, without compromising cell viability (Shin et al., 2011).

Recently, Kolesnikova et al. have reported fibroblast cell detachment from 15 nm thick gold nanoparticle coated surfaces by laser irradiation. They concluded that the reactive oxygen species which could damage cell membrane generating from illumination of gold nanoparticles were the reason of cell detachment. The cell density, cell age, laser power, and nanoparticle patterning had effects on cell detachment, making the whole lift-off work complicated (Kolesnikova et al., 2012). Instead of gold nanoparticles, TiO_2 nanodots under 365 nm UV irradiation have also been used for cell detachment (Hong et al., 2013). Approximately 90% of MC3T3-E1 cells on a nanodot coated surface were detached after 20 min of UV irradiation. For comparison, trypsin released 85% of attached cells. UV mediated cell detachment kept more than 97% of cells viable with 0.6% necrosis and 2% apoptosis. Near infrared laser mediated irradiation is the induction of an effective photoacoustic effect. The shockwave generated from the irradiated single-walled carbon nanotube can result in cell detachment (Sada et al., 2011). However, cell membrane can be destroyed by the shockwave, and therefore cell viability is limited in this approach.

Light can induce 90% of adherent cell detachment, and it is possible to detach specific cells from substrates. However, their detachment mechanisms may reveal few issues that may limit application. Photosensitive molecules or nanoparticles and external light sources are not common; in other words, expensive equipment, safety storage of photosensitive materials, and experienced experimental skills are required. It may be impractical to integrate all the components into a small automated analysis device for point of care. Reactive oxygen species emanated from laser illuminated gold nanoparticles can also directly damage cell membrane. Photoacoustic effect generated under UV irradiation can expand pores on cell membranes that may cause potential invasion of virus and/or bacteria (Lee et al., 1999). Therefore the essential trade-off to high cell detachment efficiency is loss of viability of detached cells.

2.5. Electrochemical detachment

The electrical manipulation of cell detachment has also emerged recently; the release efficiency is close to 100%. This kind of approach contains two essential parts: self-assembled monolayers (SAM) and a conductive metal substrate. Ligands for cell capture and attachment are first coated on the conductive metal substrate, and then attached cells may be detached by a number of mechanisms (Fig. 3). In the case of Fig. 3(A), antibodies or peptides were immobilized on SAM of alkanethiol coated gold substrate or optically transparent indium tin oxide substrates to capture target cells, and then captured cells were released by electrochemical desorption of proteins from electrode surfaces (Guillaume-Gentil et al., 2008; Inaba et al., 2009; Kim et al., 2009; Wildt et al., 2010; Zhu et al., 2008).

Compared to stimuli-responsive polymers, electrochemical methods can achieve cell detachment in just a few minutes. However, it requires elaborate fabrication of sensor features and specialized coating. Moreover, there is no precise data available to reveal cell viability after detachment; alkanethiol molecules may remain bound to the cell membrane proteins of the detached cells, and these may adversely influence and affect the detached cells. Although detached cells can proliferate vigorously in culture media, the current and voltage applied in cell detachment might affect cells to a certain extent. It was noticed in a study that cells could not be quickly lifted-off when high voltage was applied for a short time. The SAM desorption was inefficient in this case to get the desired efficiency of detachment. The cell detachment

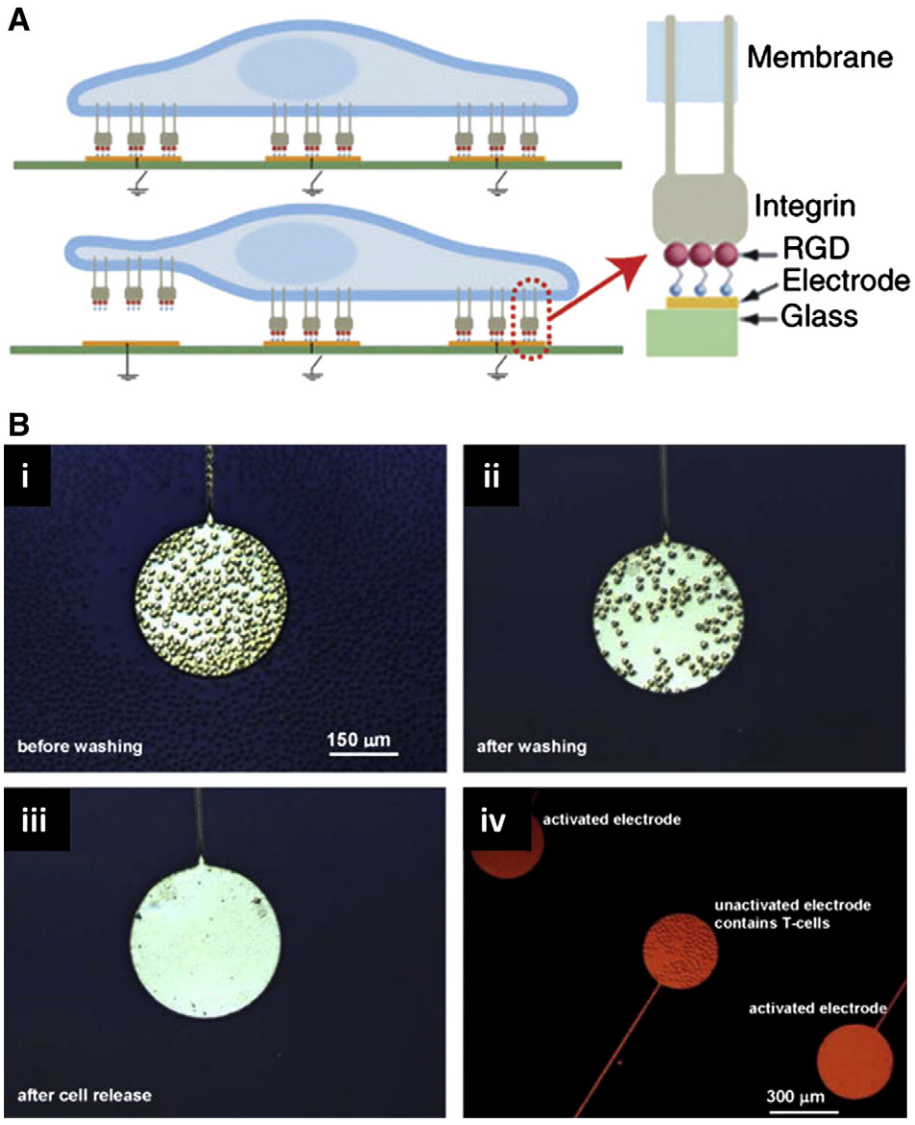

Fig. 3. Release of cells from antibody modified microelectrodes. (A) Mechanism of cell detachment from electrodes via application of a voltage pulse. The voltage results in electrochemical desorption of the arginine–glycine–aspartic acid (RGD)-terminated thiols, and results into the detachment of the cells (Wildt et al., 2010); (B): The optical micrographs from (i) to (iii) show cells captured on microelectrodes and released by application of negative potential followed by gentle agitation of the surface. (iv) shows an array of individually addressable microelectrodes and the selective release of cells by activation of specific microelectrodes (Zhu et al., 2008).

in electrochemical methods may partly rely on cell membrane contraction during the detachment (Inaba et al., 2009). The relationship between voltage and the degree of cell membrane contraction has not been established so far. Anyway, light and electrochemical induced cell detachment may have strong influence on cell viability and function thus these methods are not suitable for applications like stem cell sorting and recollection.

2.6. Enzyme digestion mediated detachment

Trypsinization is still the most commonly used method for cell detachment from surfaces. It can resuspend cells by cleaving proteins binding the cells to the surface. Normally, cells are resuspended by exposure to 0.25% trypsin in 0.02% EDTA at 37 °C for a few minutes followed by trypsin deactivation. In microfluidic devices, 0.05% to 0.25% trypsin has been successfully used for detaching captured cells with ~100% efficiency (Adams et al., 2008; Dharmasiri et al., 2009, 2011; Takayama et al., 1999). However, long trypsinization may damage the cell membrane (Tsai and Wang, 2005). Although many cells tolerate trypsin digestion for a short time, it still can adversely affect cytoskeleton proteins such as integrins that are involved in regulating cell adhesion, stability and elasticity (Canavan et al., 2005). Further, some epitopes of the cell membrane are particularly sensitive to trypsin and have been known to degrade after trypsinization (Keizer et al., 1988). Percentages of viable cells were seen to significantly decrease from 98% to 85% when trypsinization time went from 5 to 60 min, and the cytotoxic effects appeared after 20 min of trypsinization (Brown et al., 2007; Foglieni et al., 2001).

Recently, nuclease(s), another kind of enzyme, has been used to cleave nucleotides by breaking phosphodiester bonds and thus detaching cells from aptamer functionalized surfaces. Aptamers are oligonucleotides that fold into unique 3D conformations akin to proteins, allowing these to specifically bind to a specific target molecule, and these have been shown to have affinities and specificities that are comparable with those of antibodies. In addition, these are more stable than antibodies. Therefore, aptamers have been used in cell labeling studies, in activating cell signaling pathways, and in cell isolation and detection (Wan et al., 2010). These can be easily synthesized, reversibly denatured, and decomposed by nuclease (Herr et al., 2006). Chen et al. immobilized DNA aptamers on silicon nanowire arrays to efficiently and specifically capture T lymphocytes, and employed exonuclease I

to degrade single-stranded DNA aptamers. It was reported that about 97% of captured cells were released from aptamer grafted nanowires after 50 min incubation at 37 °C. On the contrary, only 74% of cells were released from aptamer grafted plain silicon surface. The significant differences might have stemmed from the steric effects which could have impeded the free diffusion of exonucleases to the binding sites between cells and aptamers. It would have been more difficult for exonucleases to diffuse below cells that were intimately attached to plain silicon surfaces. This lift-off method could keep, on average, 90% of cells viable (Chen et al., 2011).

Single-stranded DNA molecules containing repetitive aptamer domains that could specifically recognize protein tyrosine kinase were prepared by long rolling circle amplification and immobilized on microfluidic device surfaces. With exonuclease I treatment at 37 °C for 10 min, an average 68% of the captured cells were released from the surface with an average viability of 66%. For comparison, 87% of cells incubated with exonuclease in solution stayed viable (Zhao et al., 2012). Li et al. prepared aptamer functionalized polyacrylamide hydrogel for cell isolation, and used endonuclease to release captured cells. Their aptamer was designed to carry an exogenous endonuclease-recognizing cleavage site comprised of nucleotides, and thus the release was sequence-specific. The results showed that approximately 99% of captured cell were released at 37 °C for 30 min with endonuclease treatment. Further, compared to trypsin, endonuclease released 15% more captured cells within 10 min. The differences putatively came from reduced steric hindrance by aptamers on hydrogel surfaces that made the cleaving of cell receptor on cell membrane much easier (Li et al., 2013). The low cell viability indicated that nucleases could have damaged the cell recognition surfaces.

Adherent cells can be detached in a few minutes with enzymes that can directly and effectively cleave receptors or antibodies. However, both trypsin and exonuclease or endonuclease can damage cell membrane. Moreover, the detachment process may also rely on experimental experience. The concentration of enzymes, digestion time, and enzyme deactivation all need to be optimized; and these parameters need to be adjusted among individual cells due to their heterogeneity. Even if some enzymes do not do serious damages to the cells, it is still critical for some very sensitive cells (such as hematopoietic stem cells) to maintain high levels of viability and their cell phenotypic characteristics when exposed to enzymes.

2.7. Aptamers mediated detachment

Aptamers have some distinct characteristics. For example, these can hybridize with the complementary sequences, and can be made to change conformations under certain conditions of temperature, pH or salt concentration. As mentioned above, aptamers show high affinity to target molecule by adopting specific conformation; therefore, aptamers might lose affinity and specificity by simply alternating their conformation. An anti-sense oligonucleotide (RELease) was used to hybridize with RNA aptamer, thus completely opening its hairpin structure, and finally reducing its affinity to target molecule (Fig. 4) (Wan et al., 2012a). In the device, glass beads were functionalized with anti-epidermal growth factor receptor (anti-EGFR) aptamers and these were arranged in ordered array of pits in PDMS channel. The tumor cells were captured from the flow. After cell capture, glass beads were first released from the pits by flipping over the microfluidic device. The captured cells were then detached from glass beads with the RELease sequence. Flow cytometry revealed that 76% of aptamers could be released by competitive hybridization. In cell release from glass beads, results showed that on average 92% of captured cells could be released from glass bead surface after RELease hybridization. On the other hand, only 69% of captured cell could be released by soft resuspending. Zhang et al. adapted the same strategy by using a complementary oligonucleotide to change aptamer conformation, and thus causing the release of cell from aptamer functionalized surfaces (Z. Zhang et al., 2012). In this work around 95% of cells were released within 10 min, and 99% of the released cells were viable. These two independent studies have demonstrated that using complementary oligonucleotides to change aptamer conformation and releasing cells from surfaces is a non-destructive and effective way.

The affinity binding between aptamers and target molecules can be strongly temperature dependent. Target cells can be captured with aptamer functionalized surface followed by a moderate temperature change of the surface, produced on-chip, to reversibly disrupt the cell–aptamer interaction, allowing release and elution of viable target cells for downstream analysis (Zhu et al., 2012). In such a device, aptamer grafted PDMS layer was heated up to 48 °C, and approximately 80% of captured cells were released after rinsing with PBS at 5 μL min^{-1} flow rate for 2 min. At a higher flow rate of 5 μL min^{-1} and at different temperatures (from 30 to 48 °C), it was reported that a higher number of cells were detached at the elevated temperature. The greater loss of binding between the aptamers and the cells was probably due to temperature-dependent changes in the conformational structure of aptamers. The device was also regenerative; decreasing the temperature from 60 °C to room temperature helped aptamers gradually form original conformation. The device was thus available for cell capture again.

Recently, aptamers in cell capture and detachment have shown some outstanding properties. As oligonucleotides, these recognize and bind to protein via specific 3D conformation; therefore altering this conformation can release aptamers from cell membrane protein, and further release cells from the substrate. The change can be achieved by hybridizing aptamer oligonucleotide with its complementary chain, or changing environmental temperature, pH, or ionic concentration. By way of competitive hybridization, 92–95% of adherent cells can be detached and almost 100% cells can stay viable due to the rapid degradation of releasing oligonucleotides within the endosomal and lysosomal compartments after entering into cell plasma via energy-dependent endocytosis pathways or by direct translocation (Ciftci and Levy, 2001). The other advantage of this detachment is regeneration. Fresh aptamers can be introduced to substrate surface via nucleic acid hybridization. Using very high temperature to change 3D conformation of aptamer for cell detachment is in dispute. The high temperature has been shown to significantly change viability of CCRF-CEM cells. In vitro studies have demonstrated that human cell lines and tumor cells can be significantly damaged when environment temperatures get around 45–53 °C (Jolesz and Talos, 2005; Walsh et al., 2007).

2.8. Other cell detachment approaches

Lectin can specifically and reversibly bind to mono- and oligosaccharide carbohydrate structures that are present on the surfaces of mammalian cells. It has been used for capturing T and B lymphocytic leukemia. Once cells were captured, these could be released with the use of their equivalent inhibiting sugars (Vickers et al., 2011; Zheng et al., 2007). However, the capture efficiency and specificity were too poor to be applied in practical isolation setups. Cells attached on peptide conjugated magnetite cationic liposomes can be easily lifted off by removing magnet (Ito et al., 2005). The potential adverse effect might be from cytotoxicity of residual magnetic nanoparticles. Removable micropellets also can be used for efficient detachment of cells from microfluidic device surfaces. Mechanical forces have been shown to collect cells attached to micropellets (Quinto-Su et al., 2008; Wang et al., 2008).

3. Discussion and future outlook

Negative enrichment where undesired cells are captured, while leaving the target cells in the flow stream can be the best way to avoid cell detachment altogether (Green and Murthy, 2009). If there is no singular biomarker (for example not all CTCs express the epithelial cell adhesion molecule antigen because of heterogeneity) that distinguishes

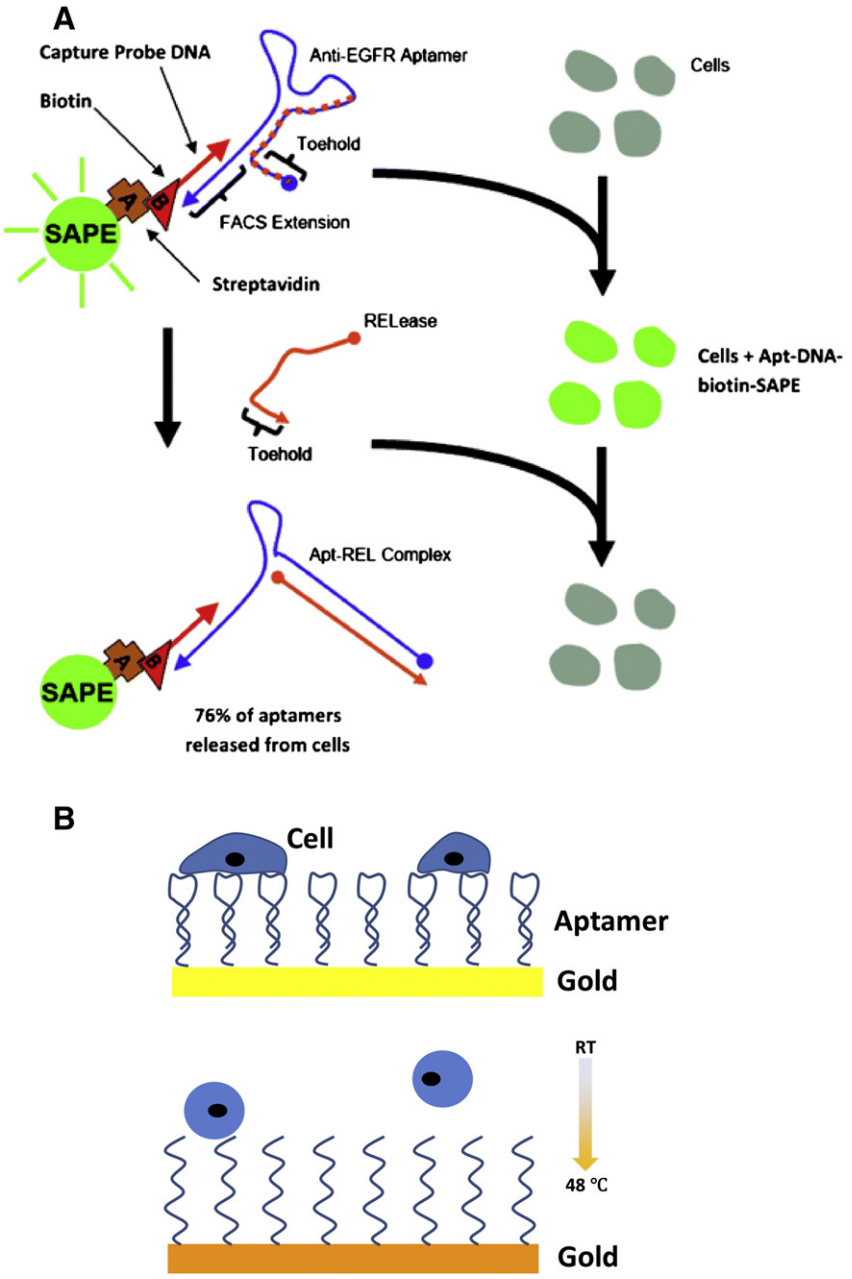

Fig. 4. (A): Cell detachment via competitive hybridization of RNA aptamer with RELease oligonucleotide (Wan et al., 2012a). The capture probe DNA is used to anchor the whole complex to the surface of a chip. Here it shows a streptavidin–biotin complex that is carrying fluorescently labeled SAPE. When the RELease molecule is provided, it can get into the secondary structure of the aptamer starting from the Toehold. Once the aptamer secondary structure is disrupted it can no longer stay bound to the cancer cells and thus SAPE light is washed away from the cells. In case a surface binding amine is use in place of the streptavidin–biotin–SAPE complex, the aptamer stays bound to the surface while the cancer cells are released and separated after washing non-adherent normal cells; (B): Schematic depicting specific capture of targets cells by the surface immobilized aptamers and ultimate release by increasing temperature of the chip from room temperature to 48 °C. The increase in temperature results in the conformation of the aptamer (hairpin loop in this example) ultimately resulting in the loss of the binding efficiency of the aptamer and thus release of the cells.

the target cells, isolation by negative enrichment could be an alternative tool. On the other hand, it is the only way for identifying rare cells that have not yet been discovered; and these undiscovered cells may provide valuable information about surface antigen expression that can further guide the development of targeting aptamers or antibodies. However, it requires abundant antibodies against all unwanted cells and the target cell must be known in the starting cell sample, otherwise neither isolation specificity nor efficiency can meet isolation requirements. Therefore, positive enrichment as an intuitive way will still be the major tool for rare cell isolation. Taking advantage of negative and positive enrichments, as a trade-off, would help in achieving reliable rare cell isolation results in the future. By rough separation of target cells from non-target ones with negative enrichment first (as a "crude separation") can significantly increase isolation efficiency and specificity with a ligand functionalized substrate (as a "fine separation") in the following positive enrichment. High isolation efficiency and specificity can significantly increase the chance of obtaining pure target cells during cell detachment.

The future cell detachment works may pivot heavily around two directions: aptamers and nanostructured polymers. Aptamers have been shown to have affinities and specificities that are comparable to those of antibodies, with the added advantage of being highly stable (Wan

et al., 2010). Grafting aptamers on nanostructured substrates has shown ultrasensitivity and considerable specificity in rare cell isolation (Chen et al., 2011; Wan et al., 2012b; Z. Zhang et al., 2012). Therefore, specific biomarkers of rare cell membranes should be further explored, and corresponding aptamers targeting these biomarkers also should be selected. Aptamer hybridization-mediated cell detachment also has achieved high efficiency with high viability. Its key feature is the oligonucleotide hybridization; while the release oligonucleotide has minimal effect on cell viability. Thus, although aptamer discovery and development takes up considerable time compared to the development time for antibody or other protein production, it is still worthwhile to explore novel aptamers considering their outstanding advantages in cell capture and detachment. In aptamer, the "active motif" for target binding is reunited with unique properties of nucleic acid. Aptamers thus can be viewed as antibody–oligonucleotide chimeras. Antibody–oligonucleotide chimera mediated cell detachment may be better suited until more aptamers become available (Fig. 5). Antibody–oligonucleotide chimeras after surface immobilization can be used for cell isolation; (1) followed by cell detachment via nuclease treatment, or (2) via denaturing hybridized double strand structure, or (3) free energy based detachment by longer release oligonucleotide through competitive hybridization. Beyond these three proposed detachment approaches, numerous novel approaches are bound to emerge that will improve both detachment efficiency and cell viability.

More work is needed on nanostructured polymers as well. Nanostructured substrates have emerged as promising platforms such that these provide better isolation efficiency and specificity while keeping captured cells viable. So far, nanostructured surfaces have been prepared on substrates like silicon, glass, quartz, etc.; however, these materials have mechanical properties that are not conducive for cell attachment, and thus may affect cell viability to some extent. In future work, a combination of nanostructure with stimuli-responsive polymer, which biomimic human tissue, could be a potential research direction. Nanostructures can be directly formed on polymer surface (Sekine et al., 2011), or by coating polymer on nanostructured solid substrate (Liu et al., 2013). As mentioned before, in traditional 2D substrate single cell might not be lifted off from stimuli-responsive polymer surface due to insufficient "mechanical forces" generated from stimuli-induced changes. Stimuli-responsive polymers with nanostructure characteristics are expected to also facilitate cell detachment from surface via relatively larger cell-polymer contact area. Nevertheless, none of these nanostructured substrates have unequivocally shown clinical validity or utility as most of the methods are still in lab settings. Although a few devices have shown that rare cells can be directly captured from patients' body fluid, more testing should be done towards clinical applications. None of stimuli-responsive polymers with nanostructures have been studied either.

Finally, current detachment methods also can be combined to facilitate cell viability. For example, shear stress can facilitate captured cell detachment from stimuli-responsive polymer surfaces; potential from electrodes can easily destroy tertiary structure of aptamer and release cells; potential also can destroy double strand structure between capture probe DNA and aptamer (Fig. 5) and thus can be used to release cells from aptamers; temperature could be precisely and quickly regulated if grafting thermo-responsive polymer on electrodes in microdevices; thermo and pH dual responsive polymer, such as poly(amidoamine) dendronized poly (2-hydroxylethyl) methacrylate, which has been studied in controlled drug release (Chang et al., 2011) could be effective materials for cell detachment; and so on.

To sum up, detachment of captured cells for cell culture or subsequent analysis is the first and major challenge in post-isolation works. Before selecting suitable substrates for rare cell sorting and detachment, the following criteria could be considered. (i) The potential substrates must provide sufficient sites for ligand immobilization, which can ensure acceptable rare cell isolation specificity; (ii) tunable topography or ease in surface coating (nanostructured surfaces) for better isolation efficiency; (iii) the potential substrates should be biocompatible and have suitable mechanical properties that mimic in vivo environment; (iv) the substrate can be integrated into a point-of-care system; (iv) captured cells must be kept on surface with minimal influence on their viability and functions; (v) the cell detachment process should be handled easily with high detachment efficiency.

4. Summary

In this review, we have introduced the strategies involved in rare cell detachment from substrates for further whole cell culture and/or analysis. The respective advantages and disadvantages have been discussed and some suggestions, specially focused on the combined areas of molecules (e.g. aptamers) and materials (novel polymers), have been provided to better achieve dual purposes of rare cell manipulation: isolation and detachment. Compared to aptamer mediated cell isolation and detachment, other methods including flowing fluid, stimuli-responsive polymer, light, electrodes, and enzymes facilitate cell detachment only by physical or chemical ways. These methods cannot perform cell isolation functions unless surfaces carry ligands for cell isolation purpose. Thereby, ligands grafting on surfaces may pose other technical challenges, such as polymer surface coating characteristics (e.g. thickness of PNIPAAm), insufficient immobilization loci (e.g. alginate gel), and inconsistent sample to sample variation (e.g. due to surface charges), etc. In contrast, aptamers can carry out both cell isolation and cell detachment, providing relatively simpler operations without significant disturbance of cell microenvironment. This unique property enables the creation of novel and better cell detachment methods. The biocompatible polymers which can provide abundant ligand immobilization sites and offer better mechanical properties for cell attachment can further maintain cell viability to some extent. In conclusion, specific adhesive ligands, creative substrate designs, and smart biomaterials might dramatically change the trend of rare cell sorting and detachment in the future. A portable, easy to use and inexpensive system can significantly influence early stage diagnosis, therapy monitoring, and cell based therapy.

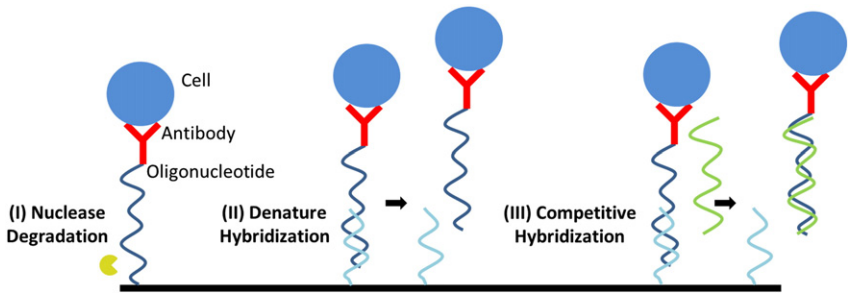

Fig. 5. Proposed methods for cell detachment with antibody–oligonucleotide chimeras.

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