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Review Article

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Plenty more room on the glass bottom: Surface functionalization and nanobiotechnology for cell isolation

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ABSTRACT

Surface functionalization is a widely adopted technique for surface modification which allows researchers to customize surfaces to integrate with their research. Surface functionalization has been used recently to adapt surfaces to integrate with biological materials specifically to isolate cells or mimic biological tissues through cell patterning. Cell isolation and cell patterning both can be integrated with extant techniques or surfaces to customize the research to whatever needs to be tested. Substrates such as metals, biologically mimicking surfaces, environmental responsive surfaces, and even three-dimensional surfaces such as hydrogels have all been adapted to allow for functionalization for both patterning and isolation. In this review we have described both the advantages and disadvantages of these techniques and the related chemistries to better understand these tools and how best to apply them in the hope that we can further expand upon the research in the field.

1 Introduction

As the famous physicist Richard Feynman once immortalized in his lectures [1], "*There's plenty more room at the bottom*". This optimistic vision calls for technologies to study systems at the micro- and nanoscale. An important extension of this would be to study biological systems at the micro- and nanoscales. Indeed, several of the basic biological units—cells, organelles, and proteins—are found at this scale; so, studying their dynamics requires tools that can probe these spaces.

An emerging approach for studying biological systems "at the bottom" is surface functionalization, which can introduce moieties onto a material to promote cell adhesion [2, 3]. Adhering cells to surfaces enriches these cells for further biological analysis and can broadly be described as targeting either their physical characteristics or plasma membrane components. The former is well described by Song et al.

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[4]; wherein physical characteristics, such as size, charge, and density, are used to separate cells. Since cells of different lineages may share similar physical characteristics, this approach may not provide the specificity needed for common cell-lineage-based biological characterizations [5–8]. Conversely, cell enrichment, based on components found on the plasma membrane that define cell lineage, (e.g., cluster of differentiation/CD markers) [9, 10] and state [11] are highly utilized [9, 10, 12–21]. This review will focus on affinity-based approaches for surface functionalization.

Affinity-based approaches aim to pull down targeted cells. However, it is not enough to simply have cells adhere to a capture surface. Eliminating non-specific or non-targeted cell binding is paramount to maintaining the fidelity of downstream analyses. Non-specific cell attachment leads to inflated estimates of cell capture [22]. Incorrect cell capture estimates then affect downstream analyses by inflating or deflating measurements of biomarkers [23]. To overcome non-specific binding, researchers have investigated several techniques, such as bovine serum albumin (BSA) blotting [20, 24–26], increasing specificity using avidin and biotin protein family interactions [18, 24, 26–36], engineering highly specific aptamers [37–49], and applying poly(ethylene glycol) (PEG) brushes to repel non-specific binding [29, 49, 50]. Mitigating non-specific binding enables high purity, which further enables the isolation of rare cell-like circulating cancer cells [22]. Many of the affinity-based enrichment techniques we describe in this review utilize these approaches for eliminating non-specific binding.

Affinity-based cell enrichment techniques can be further divided into two primary techniques: cell patterning and cell isolation. Cell patterning pulls cells onto the surface [51] for further analysis of protein [23, 52–60], gene [61], or cellular responses to stimuli [62, 63], such as drugs [64–67] (Fig. 1). In contrast, cell isolation combines the cell pull-down portion of cell patterning with release of the captured cells, enabling off-surface experimentation [30, 31, 34, 40, 68, 69]. Both approaches can also be used for negative enrichment, wherein the cells captured on the surface assist in depleting a population from the larger sample. Thus, affinity-based surface functionalization enables a wide-range of cell investigations [70–74].

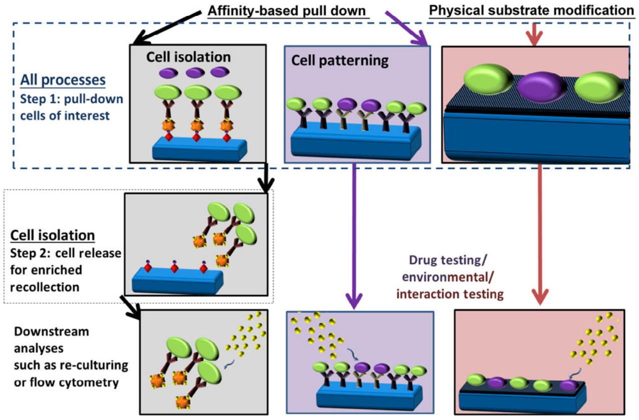

Figure 1 Schematic comparison of surface functionalization techniques. Affinity-based pull-down techniques can be subcategorized into cell isolation and patterning techniques, which differ in their cell release steps for isolation. Physical substrate modification does not inherently use affinity-based pull-down and is merely a physical modification of the surface which changes its physical properties, such as roughness or stiffness.

As researchers seek to pattern or isolate cells, several disciplines can be employed and various materials can be used that can result in several new approaches. Indeed, an advantage of surface functionalization is its disciplinary convergence, wherein techniques from chemistry and materials science [75, 76], biology [77–79], electrical engineering [80, 81], and even physics [82, 83] can be applied to modify a surface. As a result, practically any material can be functionalized, from metals [84–86] to polymers [14, 76, 87, 88] and even biological materials [18]. Using these convergent techniques and materials, several cell patterning and isolation approaches have been developed for studying biological systems, including: surface plasmon resonance (SPR) [64, 89], which quantifies the kinetics of molecular interactions; photolithography [79], which enables the construction of layered integrated systems at the micro- and nanoscales; and "smart" materials [90], like thermally responsive polymers used for appending chemical conformational changes to surfaces and substrates that originally are static and unresponsive. We have merely begun to scratch the surface of what can be accomplished via surface functionalization, as there are innumerable applications [17, 28, 30–32, 64, 79, 88–111].

In this review, we will present three primary approaches for surface functionalization: (1) inorganic self-assembled monolayers consisting of thiols and silanes, (2) organic extracellular matrices (ECM) mimicking surface functionalization, and (3) unusual substrates and adsorbed protein surfaces. In the context of each affinity approach, we discuss how they are applied to cell patterning and cell isolation and present the advantages and disadvantages of each. We then present the opportunities that exist to adapt the functionalization to one's own needs.

2 Techniques for surface functionalization

2.1 Surface functionalization via inorganic self-assembled monolayer chemistry

A widely adopted surface functionalization methodology is the self-assembled monolayer (SAM) [64, 82, 89, 91, 94, 112–117]. As the name implies, SAMs independently self-orient into monolayers on surfaces [118] and substrates. As both surfaces and substrates become functionalized in these processes, in this manuscript, we will use these terms interchangeably. These inorganic SAMs are applied based on the type of surface that is used, and can be tailored in composition and length to correspond with the functionality desired [112]. Common SAMs include: (1) thiols and (2) silanes.

Silane SAMs, which consist of silicon and oxygen groups, are used on silicon-based surfaces such as glass or silicon wafers. These silanes are appended with unique functional groups, depending on the experimental design [113] (Fig. 2). Because glass and silicon surfaces are relatively cheap and inert, silicon-based devices are gaining popularity, paralleling the greater adoption of glass-based microfluidic devices in literature [119].

Thiol SAMs contain sulfur and hydrogen groups (SH). Thiol SAMs are often functionalized on metal surfaces, like copper [120], palladium [82, 83, 121], platinum [83, 86], mercury [83, 118], gold [115, 117, 122–125], and silver [83, 85, 120, 126]. Similar to silanes, thiols can have functional groups to tune the intended surface chemistry [83]. Owing to the large number of potential surfaces, thiol chemistry is one of the most commonly adapted SAM [82, 112, 115, 117, 126, 127].

Gold and silver can also be functionalized with SAMs made of selenides or selenoates, which both consist of selenide groups [112]. Thus, substrates such as gold and silver can be functionalized with different SAMs depending on the necessary experimental design to enable more complex functionalization schema. These widely used SAM functionalization methodologies mentioned herein are by no means exhaustive and are meant to provide the basis for the chemistries expanded on within this paper.

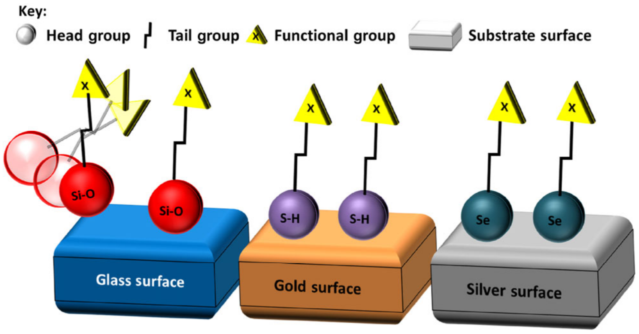

Figure 2 Schematic of self-assembling monolayers on different substrates. These chemicals self-orient themselves such that the head group binds to the substrate without the need for complex experimental design. Thus, these materials expose the functional groups on the tails for further chemical modification and reaction. There are other examples of substrate and self-assembled monolayer pairs, but silane, thiol, and selenide chemistry are among the most widely used in the literature.

2.1.1 *Thiol chemistry applications and design*

Thiol SAM-functionalized metal substrates are very well characterized in the field since metals have been used for a variety of applications, including affinity-based pull-down. A Web of Science literature comparison shows gold was approximately three times more commonly listed as a key term than glass in publications regarding cell release, cell patterning, and even functionalization (Fig. 3). Each blue node is proportionally sized based on the amount of publications within each category, while each green satellite orb indicates the number of publications that include each type of substrate or functionalization to show their relative contributions within the category. Each of the orbs has been organized into three columns that are ordered by total number of publications within the cell isolation, cell patterning, and generic functionalization groups as literature spaces for comparison. This format shows the spaces in which these key terms are used and the applications of thiol,

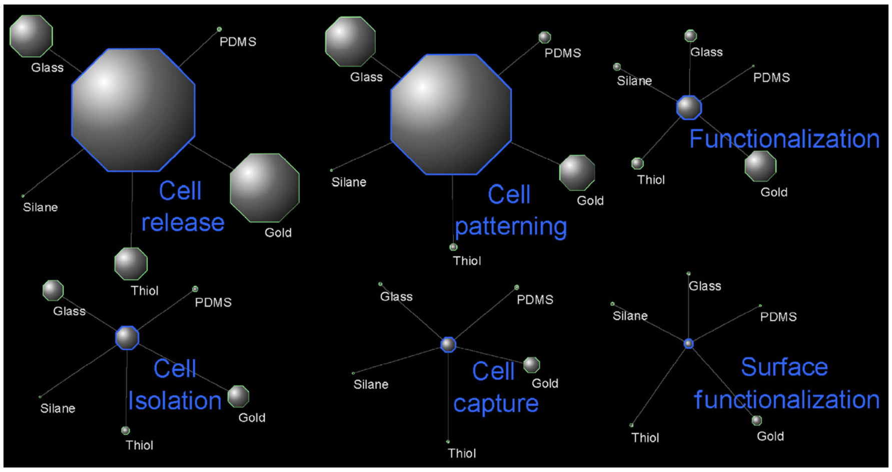

Figure 3 Schematic representation of the literature contribution of affinity-based surface functionalization. Each blue node has been proportionately sized and organized from left to right based on its footprint within literature. The columns represent cell isolation, cell patterning, and surface functionalization, respectively, showing the prevalence of different types of surface functionalization within each literature field, as represented by the green orbs. The green orbs represent the proportionate amount of papers within each larger blue node category. Within the cell isolation and surface functionalization categories, gold is the most prevalent substrate; however, in cell patterning, this trend shifts, such that glass is the most prevalent key term.

silane, and substrates for each subfield of literature. Therefore, for any discussion on SAMs, it is important to begin with the most prominently used material—gold—to understand how they can be integrated with the desired experimental design. As we discuss affinity-based pull-down, we will also cover both cell patterning and cell isolation techniques using thiol chemistries by first discussing how thiols allow for both tunable cell patterning and integration with extant quantitative technologies, followed by cell isolation and recollection.

2.1.1.1 *Thiol SAMs enable tunable cell patterning*

Self-assembled thiols allow for tunable cell patterning on gold and other metallic-based substrates. Thiol-based methods have been used in microfluidic systems to pattern cells [114, 128–130]. Li et al. has used thiols to direct and aid in the deposition of ECM proteins by using the gold surface as the cathode. They used $HS(CH_2)_{11}(OCH_2CH_2)_6OH$, more commonly known as E_6, to activate the surface for deposition with more typical cell patterning materials, such as ECM and fibronectin proteins. After functionalizing the gold surface with the E_6 thiol, the ECM and fibronectin solutions could be used as the electrolytic solution for electrochemistry. Through the introduction of an electric potential, the E_6 thiol was desorbed and replaced with the ECM proteins selectively—fully activating those regions for cell adhesion. As gold is extremely conductive of electrical charges, they were able to easily and specifically activate only the environments for cell adhesion. Conversely, thiol chemistry can also be used to pattern regions to prevent cell binding [64, 79]. Jiang et al. micro-printed two different types of thiols: 1) $HS(CH_2)_{11}(OCH_2OCH_2)_3OH$ ($C_{11}EG_3$) and 2) $HS(CH_2)_{17}CH_3$ (C_{18}), which differed in their cell adherence properties. The $C_{11}EG_3$ thiols resisted both protein deposition and cell attachment. In contrast, the C_{18} thiol served both for protein absorption and directed cell attachment. Through selectively patterning of regions with the different thiols, the cells could be confined into directed patterns. Using a small cathodic voltage pulse of –1.2 V for 30 s, they were then able to release the EG_3 thiols from the surface of the gold, enabling migration of the cells into the defunctionalized region. Jiang et al was then able to observe differential migration capabilities across cell types through the release of these thiols. Thus, thiol chemistry can be used to tune the activation or deactivation of surfaces for cell patterning.

2.1.1.2 *Thiol SAMs enable integration of cell patterning with extant quantitative technologies*

Researchers such as Jiang et al. and Dillmore et al.

were able to integrate thiol surface functionalization with extant quantitative technologies, including SPR spectroscopy or photolithography, to facilitate further adaptability to their cell patterning modalities. SPR was used in the capture chip to quantify deposition of protein as the cells migrated into the unthiolated gold regions [64, 89]. The ability to quantify protein deposition enables cell migration patterns to be determined in response to multiple chemical stimuli. Indeed, Jiang et al. were able to show an abolishment of migration after treatment of the cell surface with different drugs that inhibit migration [64]. Taking this thiol-based cell patterning a step further, Dillmore et al. patterned photocleavable thiols with a reactive conjugate [114]. This conjugate then attaches to ligands or cellular binding motifs required for cellular attachment, such as the tripeptide binding domain RGD. As RGD consists of arginine, glycine, apartate peptides it gets its name from the abbreviations of those peptides. As it is a frequent target of several integrins, the RGD domain is a common cell binding motif. Using a light source, or even a photolithographic mask, the surface could be adapted to allow for attachment of cells to specific regions. Another combinational methodology uses both the conductive properties of gold and adaptability of thiol chemistry. Gold surfaces have been patterned with hydroquinone that can be oxidized with an electrical current through the substrate. Once oxidized, the hydroquinone reacts with a conjugate that can then be attached to a ligand of choice [129]. This can be integrated with a functionalization scheme that has hydroquinone on multiple sections of the gold surface, wherein only the regions that are electrically active will be oxidized and promote cell attachment. This allows for selective activation of regions depending on the experimental design, further increasing the ability to customize the surface via functionalization. Thus, surface functionalization enables integration of the gold substrate system with a variety of existing technologies and experimental techniques.

2.1.1.3 *Cell isolation enabled through electrical conductivity of thiol patterned substrates*

While the above chemical functionalization has been used for cell patterning, these modalities can be integrated with supplementary chemistry to allow for recollection of the isolated cells. Their recollection would allow them to be analyzed downstream or subsequently passaged. As such, thiol-based cell isolation typically harnesses the electrical conductivity of the gold or other metals used as substrates to release the bound thiols from the surface. By introducing –1.2 V to the gold surface, the thiol-modified molecules can be desorbed from the surface [64, 80, 129, 130]. Since the cells were attached directly to the thiol, desorption results in the release of the cells from the surface, which can be re-cultured as large sheets, individualized cells, or even analyzed separately using other downstream techniques [19, 55, 56]. Cell isolation is therefore enabled through integration of the thiol SAM-patterned substrate with electrically stimulated release of cells from the functionalized, gold surface.

2.1.2 *Silane chemistry applications and design*

Another commonly used type of SAM is silanes, which self-assemble spontaneously on substrates that contain silicon. As both glass (silicon dioxide) and poly(dimethyl siloxane) (PDMS) are very versatile and well-established as modalities for microfluidics, they are very prominently used in the literature, as evidenced by the Web of Science literature search footprint consisting of thousands of papers (Fig. 3). Glass has been widely adopted for functionalization due to some advantages, such as its optical clarity and low cost (Fig. 3) [119]. In microfluidic devices, glass is typically bonded to PDMS (an elastomeric substrate that contains additional silicon groups) [16, 37, 59, 94, 105, 119, 127, 131–133], and can be further functionalized with silane SAMs. We show that silane functionalization can be used to 1) pattern cells, 2) integrate with extant technologies, and 3) isolate cells through reactive additives and environmental changes on silicon-containing substrates and microfluidic devices.

2.1.2.1 *Silane SAMs enable patterning of lowly abundant, circulating cells*

Amino silanes such as (3-aminopropyl) triethoxy silane (APTES) have been integrated with affinity-based capture modalities for cell patterning of circulating cells that are low in abundance. Both cell isolation and cell patterning focus on rare cell types of interest to allow for the most application of their devices. This is based on the idea that if the devices can resolve cell pull-down of lowly abundant cells in solution, then their devices would easily resolve them when they are expressed in high abundance. One example is circulating tumor cells (CTCs) owing to the vital information they can provide about tumoral activity [4, 12, 37, 134–141]. Functionalization schemes have targeted CTCs in a variety of modalities. CTC separation modalities have been developed on glass and PDMS that have been functionalized with self-assembled chlorinated silanes and multiple epoxy layers so they can be conjugated with anti-EpCAM antibodies to isolate the CTCs [105]. Similarly, several iterations of the NanoVelcro system have been developed, which use silane chemistry to functionalize silicon nanowire substrates with streptavidin and biotinylated anti-EpCAM for pull-down of CTCs [61]. Both techniques use antibodies as a targeting technique with the surface functionalized silanes acting as the tether to hold the antibody/cell complexes on the surface.

2.1.2.2 *Silane SAMs enable integration of cell patterning with other extant technologies*

In addition, silane-functionalized surfaces have been integrated with alternative architectures and modalities to widen the ability of the technology to pattern cells. Saneinejad et al. implemented glass-based silane functionalization with sputtered gold to create a hybridized system for patterning cells. As previously described, the gold could be functionalized with thiols to pattern PEG-repulsive or peptide-adhesive regions for cell growth based on the configuration of the chemistry, while the silane can allow for the opposite set of proteins to be used for patterning [142]. This would allow for tuning of the cell-binding sites and allow for separate regions if multiple cell-binding locations are needed. Saneinejad et al. also implemented a different capturing technology using silane chemistry that differed from the more typical antibody targeting method. Saneinejad et al. used folded sequences of DNA, called aptamers, to target cells of interest. These aptamers allow for directed affinity pull-down of cells without using specific antibodies because the sequences could be perfectly tailored to fit the receptor in question [37, 38, 41, 43, 46, 48, 49, 95, 143–145]. Mahmood et al. even experimented with directly conjugating aptamers specific to the epidermal growth factor (EGF) receptor to the silanes. This method pulled down glioblastoma cells to investigate differences in behavior between the cancer cells and the healthy ones [68]. These alternate architectures and modalities conferred the ability to pattern or test cells through integration with the silane SAMs and expanded the applications for both technologies and for cell patterning.

2.1.2.3 *Silane SAMs enable cell isolation through reactive additives*

Complex systems have been constructed to facilitate cell release through additives, such as biotin, exonucleases, and even antisense RELease molecules, to recollect cells captured by the silane-based surfaces. One such system is our lab's Secondary Anchor Targeted Cell Release system (SATCR) [30, 31]. This system uses a SAM of an aminosilane, which attaches to the glass and PDMS surface. We then bind a desthiobiotin protein to the amine group on the APTES layer. Desthiobiotin is a functional analog of biotin. Biotin family members bind exclusively to avidin family members, and are known to be one of the most selective and powerful natural binding partners [26, 27, 146–152]. The capture surface uses streptavidin to pull down biotinylated antibodies on the functionalized surface. Release is initiated by outcompeting binding of the desthiobiotin-conjugated protein to streptavidin with a more effective binding partner, such as biotin. Through the introduction of 4 mM biotin, the cells are released from the surface [30]. This system allows for customizable capture and release using different biotinylated antibodies that target specific cells of interest, but is still limited by the binding strength of the antibody. To address this, integrated aptamers have been introduced to improve binding affinity between the cellular targets and their silicon functionalized capture surface [37, 38, 40, 41, 44, 49]. Using mercapto silane, the aptamer is conjugated to silicon nanowires to promote cell capture. The cells are released through the introduction of an exonuclease, which degrades the aptamers and releases the cells from the wires for recollection [40]. Furthermore, aptamers allow for reversible binding and release, as interactions with a complementary sequence of DNA will bind to the aptamer and release bound cells non-destructively through conformational changes within the aptamer structure. The aptamers can be used to functionalize surfaces beyond plain glass. This was evidenced by Zhang et al., who used silane chemistry to add a hydrogel onto a glass surface and then functionalized it with aptamers targeting CCRF-CEM cells, a human lymphoblastic leukemia cell line. These aptamers were then exposed to complementary strands of DNA, which allowed for non-destructive release of the cells from the hydrogel surface. In addition, the hydrogels could be regenerated since binding of the complimentary strands was reversible [38]. It is also possible to use specific endonucleases to cleave or destabilize aptamers to release cells from the capture surface [49]. Li et al. functionalized a glass surface with silanes before bonding it with a hydrogel that was also functionalized with aptamers [49]. These aptamers were also specific to the CCRF-CEM cells, but contained specific BamHI and KpnI endonuclease recognition sequences that cleaved the aptamers to release the cells from the hydrogel surface. BamHI is able to release 99% of captured cells in this way, while a trypsin-based control was only able to release 80%; however, trypsin is a non-specific enzyme that will cleave cell surface proteins and destroy the cells themselves if left unchecked. The authors note that these cleavage regions and endonucleases can be customized [49], which can help avoid cellular damage or cleavage of cell surface receptors. Similarly, aptamer-conjugated silanes were implemented into a microfluidic device, which used an assay with pits to facilitate the pull-down of cells via aptamers attached to glass beads [37]. These cells were subsequently released using an antisense RELease molecule that would unravel the aptamer structure. These techniques use aptamers to supplant the use of antibodies in the capture modality and avoid antibody affinity issues that could limit cell capture. Thus, through the addition of reactive additives, cell isolation systems can reliably release the cells of interest for enriched recollection.

2.1.2.4 Silane SAMs enable cell isolation through environmentally-induced release

Environmental changes such as temperature, pH, light, charge, and magnetism have been used to promote cell release rather than adding a release-inducing molecule. This affords the advantage of affecting the total capture modality without worrying about the concentrations of releasing agents in the system. Furthermore, these systems have been designed to limit forces upon the cells and maintain substrate biocompatibility to avoid changing cellular expression systems. Moreover, while all these described techniques are integrated with a silane-based system, the chemistries can be adapted to other substrates for customized experimental designs.

Temperature: Thermal energy has been used to release cells from functionalized surfaces. NanoVelcro cellular release system of Lin et al. does not use protein binding competition to release cells, but instead uses environmental changes to release cells from the aptamers attached to biotin streptavidin complexes [61]. Like the SATCR system, the NanoVelcro system uses the interaction between streptavidin and biotinylated aptamers to release the cells of interest for recollection. Through the conjugation of a thermally reactive polymer, poly(N-isopropyl acrylamide) (pNIPAM), to the capturing modality, the NanoVelcro CTC system was able to release cells. When the temperature was lowered to 4 °C, the pNIPAM would unravel and release the cell receptors from the biotinylated anti-EpCAM antibodies. Thus, the conformational changes that released the cells from the surface were triggered by the thermal changes, which opens the door to designing future adaptive functionalization schema.

pH: Systems even exist that respond to both environmental pH and glucose to release cells of interest from mercapto silane-functionalized, silicon nanowires [153]. Silicon nanowires are functionalized by silane chemistry, which adds polymer brushes to the surface. These polymer brushes pull down human luminal breast cancer MCF7 cells, as these cells overexpress sialic acid within their plasma membranes. At a physiological pH of 6.8, the brushes can capture and isolate MCF7 cells onto the silicon nanowires; increasing pH to 7.8 causes binding competition between the cells and glucose in the system, as the polymer brushes are attached to both. The conformational changes within the polymer brush structure are induced because of the change in pH, and these changes allow the substrate to respond to the stimulus and ultimately release the cells. Thus, this functionalization could be applied to other glass and silicon-based surfaces to include pH-induced conformational changes for various cell types by simply substituting the polymer brush used to target the cells.

Light: Photo-responsive linkers have been implemented to release cells with the addition of specific frequencies of light [58]. A photo-cleavable linker responsive to 330 nm photo irradiation was implemented with an anti-HEL-IgG antibody to release cells of interest. Once light was introduced into the system, the linker was cleaved, and the cells were released from the surface. This system could feasibly be multiplexed with many photo-cleavable linkers to parallelize the capture of cells. Thus, the introduction of multiple targeting modalities or antibodies with spectrally different photo-linkers could allow for several disparate cell types of interest to be simultaneously isolated from a sample. Furthermore, if cleavage of the linkers occurred with different wavelengths of light, the cells could be imaged together and released simultaneously or sequentially. This would enable for very selective downstream analysis and separation. Thus, light-responsive photo-linker release provides further flexibility for designing functionalization-based approaches.

Electrical current: In addition to light energy, electrical energy has been used to release captured cells. MCF7 cells were isolated using a biotinylated antibody conjugated to functionalized nanofiber mats on glass [81]. These cells were then stimulated by 20 cycles of electrical current from –0.8 − +0.5 V. The electrical current caused the capture surface to be repulsed by the charged nanofibers and resulted in cell release from the nanofibers. The change in voltage was also noted to not sharply disrupt cell function or viability [81, 154–157]; thus, this stimulation should not cause cell death. As this technique uses the electrical conductivity of the silicon nanowires as the basis for releasing cells, it can be appended to other conductive metal substrates using chemistries that are not silicon-based. In those cases, alternative chemical schema could be designed for specific substrates. In this manner, electrical current can be applied to substrates beyond silicon to reliably release cells.

Magnetism: Magnetism can also be used to isolate and release cells in a microfluidic device [158]. Magnetic nanoparticles were first functionalized with silanes and then with an anti-EpCAM antibody to pull down the cells of interest to the surface of the microfluidic device. The magnetic field held the labeled cells while the other cells flowed through. When the magnet was turned off, the cells were released and could be collected. This technique is similar to typical magnetic bead isolation techniques used to isolate cells, such as the Dynabead® system [159–161]. However, unlike magnetic bead isolation, this technique uses a microfluidic device to bind the cells statically, rather than with a column as is more typical for magnetic bead isolation. Moreover, as this technique uses environmental changes to facilitate release rather than directly targeting the capturing modality, there is no need to worry about depletion of small molecules, competitive binding of releasing agents, or rebinding of the released cells. Therefore, these magnetic-isolated cells can be released reliably without relying on concentration gradient changes of small molecules that diffuse within the solution or device.

2.2 Organic ECMs that mimic functionalized surfaces and hydrogels

In contrast to the previously described inorganic surface functionalization methods, organic functionalization schemas use surface-independent chemistries and often seek to recapitulate the *in vivo* environment. Organic-based functionalization schemas may also avoid potentially toxic or non-physiological effects from non-inert, inorganic functionalizations. Indeed, there is a pressing need for biocompatibility in *in vitro* environments [30, 31, 162, 163].

As ECM chemistry does not utilize SAMs, the functionalization schema tend to be more complex than the monolayer chemistries mentioned above. These substrate-independent functionalization schema can be applied to both solid substrates [108] as well as hydrogels [164–166] to mimic three-dimensional (3D) cellular environments more physiologically. Since these surfaces more closely resemble the cell's native environment, they are optimized for biocompatibility and can recapitulate the cell's physiological expression patterns. This has been shown in two dimensions with glass functionalized with chitosan molecules [108], which are subsequently conjugated with antibodies onto the chitosan. The adipose stem cells that were tested would not have natively attached to the chitosan without recognizing the antibody, which allowed for selective cell patterning. We discuss two-dimensional (2D) smart surfaces, 2D hydrogels, and 3D hydrogel environments, as they recapitulate physiology for the cells patterned or released from the surface. As such, these techniques show the adaptability of surface functionalization, as it enables several disparate, and potentially multidimensional substrates, to reach similar experimental capabilities.

2D smart surfaces: The integration of 2D "smart surfaces" with ECM-mimicking surfaces enables thermally-stimulated release of cells. "Smart surfaces" are substrates that can respond to stimuli dynamically [36, 90, 111, 167, 168]. They have been integrated with a variety of substrates, including plant based polysaccharides such as xyloglucan [168]. Xyloglucan surfaces expressing RGD domains have been used to isolate human malignant skin melanoma A375 cells through the creation of stable films on glass 24-well plates [168]. As the A375 cells overexpress the integrin for binding to the RGD domains, the cells were able to be cultured readily on the xyloglucan-functionalized surfaces. Once captured, cells were released through heating the well plate to room temperature, showing the ability of these ECM-mimicking "smart" surfaces to respond to stimuli.

2D hydrogels: 2D hydrogels have been used to improve cellular physiology and binding and reduce inflammation. Bridging the gap between the 2D glass-based system and the 3D hydrogel environment is hybrids, such as 2D hydrogel systems that are surface functionalized with heparin to promote cell adherence and proliferation [3]. These systems improve the system's biocompatibility through the addition of external molecules. For example, heparin has previously been integrated with materials to make implantation of hydrogels, such as poly(methyl methacrylate), more biologically inert and to reduce inflammation [169]. Moreover, as glycosaminoglycans such as hyaluronic acid are expressed natively within the body [170], they are popular bases for hydrogel creation. Endothelial progenitor cells have been pulled down upon these hyaluronic acid hydrogels through functionalization with CD34 antibodies via EDC (carbodiimide) conjugation. These carbodiimides act as catalysts in order to conjugate amines and carboxylic acids together. Hydrogels consisting of heparin methacrylate have been tested against hyaluronic acid methacrylate hydrogels to compare the benefits of heparin's heightened affinity on cell adhesion to the top of the hydrogel with the heparin-modified hydrogels; it was observed that the former promoted significantly higher cell growth both with and without the antibody functionalization. Improving cell adhesion, while reducing inflammation, is crucial for representing the physiology of systems, as inflammation greatly alters expression levels of cellular receptors and impacts the proliferation and migration of certain cell types. As such, the utility that surface functionalization affords in reducing inflammation and improving cell adhesion shows the adaptability that surface modification can confer.

3D hydrogels: 3D hydrogel systems recapitulate physiology and can be integrated with micro-printing modalities to polymerize cells encapsulated within the hydrogel. They have been created to represent cellular environments more faithfully. This has been done with *in vitro* work using hydrogels, such as Matrigel [107, 171–173], to recapitulate the 3D physiology. Since Matrigel composition varies inconsistently by lot number, it is not an ideal system for functionalization. Developing consistent systems that would serve as a substrate for surface functionalization would allow for recapitulation and selective patterning of cells within a 3D cellular environment, enabling investigation of co-culturing interactions between different cell types. We examine below 3D aptamer-functionalized hydrogels and 3D Sterolithographic Apparatus (SLA)-printed hydrogels as viable methodologies and substrates for functionalization.

Aptamer functionalized 3D hydrogels: Another advantage of using 3D hydrogels is the ability to functionalize them to improvement upon their native characteristics, such as release time and distribution of ligands or cell attachment within the hydrogel matrix. Hydrogels functionalized with aptamers allow for cell capture or even sustained response and storage of ligands through reversible binding to the aptamers [38, 47]. As such, these techniques can also be used for both cell patterning and cell isolation based "catch and release" of cells within the hydrogel, similar to the 2D methodologies mentioned above [38, 49]. Release can be implemented through the introduction of complementary DNA sequences to the aptamer, releasing cells from the hydrogel without damaging either the cells or hydrogel systems [38]. In contrast, ligands can be targeted by the aptamers instead of the cells. This allows for the storage of ligands to potentially modulate release dynamics. This has been studied for both platelet derived growth factor BB (PDGF-BB) [174] and vascular endothelial growth factor (VEGF) [47] by measuring release percentages and cell viability within the hydrogels. Both of these ligands are crucial for the creation and maintenance of blood vessel in the process of angiogenesis. ECM-mimicking scaffolding, such as peptides [42] or gelatin [47], can then be used to pattern cells within the hydrogel, as previous researchers have shown that pure, unfunctionalized PEG hydrogels lack several physiological characteristics such as biomolecular cues, scaffolding, or even signaling molecules [42]. Thus, 3D hydrogels have been created using gelatin- and aptamer-functionalized PEG hydrogels [47], creating a scaffolding for cells adherence. While pure PEG hydrogels were unable to promote cell attachment, the gelatin- and aptamer-functionalized PEG hydrogels adhered to cells, which was further improved when the ratio of gelatin to PEG was increased. In addition, this hydrogel was able to create a macroporous and permeable hydrogel system using carbon dioxide bubbles to create pores within the matrix. This resulted in a system that could transfer nutrients, which was verified by measuring sequestration of VEGF [47]. When the hydrogel was not functionalized with the aptamer, the uptake of VEGF was minimal (~ 5% absorbed), showing the permeable nature of the gel matrix [47]. When the gel was functionalized with the aptamer, the uptake was ~ 92%. Thus, when the aptamer-loaded hydrogel and the non-aptamer-functionalized gels were exposed to both cells and VEGF, the fluorescence intensity associated with cell viability was 8 times smaller in the non-aptamer functionalized system; this implied that the sequestered VEGF allowed for its sustained delivery to the cells [47].

SLA printed 3D hydrogels: While the aptamer-functionalized systems showed the importance of substrate modification to modify cell growth, developing architectures that could promote multiple co-cultured cells types requires spatial differentiation that mimics the physiological striations within a biological system. Thus, substrate modification serves as the basis for implementing full 3D hydrogel system of Raman et al., which more fully mimics physiological environments for cellular systems [166]. This system is notable as it encapsulates and polymerizes the hydrogel with cells already laden in the system rather than seeding the cells after polymerization. Indeed, this system eliminates the problem of cells settling to the bottom of the gel through a clever inversion of typical fabrication setups. The hydrogel was polymerized via UV light in a high-resolution, custom-built, projection stereolithographic apparatus and that could polymerize different layers into a multi-material structure using alternating resin types. This design is similar to other previously mentioned functionalization schema in which additional layers of chemicals were used to add properties to a material. Furthermore, the different encapsulated cell types could allow for co-culture to further recapitulate the cell microenvironment. These systems all allow for the creation of more complex physiological extracellular environments regardless of substrate functionalization.

2.3 Alternative surface functionalization: Unusual substrates and adsorbed surfaces

Alternative methodologies for surface functionalization exist, which use 1) unusual substrates, such as native tissue to pattern or isolate cells, or 2) molecular adsorption, such as thermal polymer surface coatings. Functionalization of native tissue is a stark departure from typical surface functionalization using silicon or metallic substrates. As the native tissue has been de-cellularized and is a scaffold for amine-terminating proteins, they can easily be targeted by surface functionalization chemistries. Similarly, adsorption or coating of proteins or molecules upon a surface differs from surface conjugations—which we have described above—as it does not involve interactions stronger than simple covalent attachment. As these are covalently attached, the interaction between the adsorbed protein and molecules are not as strong as the aforementioned chemistries. Despite the weaker adhesion, adsorption of proteins has been used extensively with different microfluidic devices for applications, such as antibody adsorption on PDMS surfaces [16, 133]. These adsorbed antibodies pull down cells of interest to the surface for quantification, allowing for patterning or organization of the cells of interest. We describe the functionalization of native proteins, the weaknesses of adsorbing proteins, as well as the process of applying thermal polymer coatings to surfaces.

Native protein functionalization: As a potential evolution of protein absorption as it relates to surface functionalization, native proteins from organs have been used as structural targets for functionalization [18]. Aortic valve tissue has been de-cellularized to become the substrate for surface functionalization. The aortic valve was functionalized with biotin to pull down avidin-conjugated fluorophores and antibodies using the free amine groups on these proteins. Using native tissue for functionalization allows the existing physiological architecture to be adapted, improved, or customized. Functionalizing native tissue affords the flexibility of using natively adsorbed proteins as the targets, while still using more complex chemistry to orient the capturing modality. The functionalization of native tissue enables patterning of cells onto these previously de-cellularized tissues and can be used to potentially restore function to the tissue using cells or tissue from specific patients for transplantation. Therefore, native protein functionalization represents a further evolution of surface functionalization, which begins to interface with patient-specific disease treatments.

Weaknesses of adsorption: One distinct weakness of protein or molecule adsorption is that they are not specifically tethered, and thus they lack an established orientation [175]. Orientation is crucial in molecular and protein-binding scenarios, as most proteins have specific binding regions in which they interact with other proteins. When proteins are conjugated randomly, some proteins will have their binding regions hidden or occluded. This would result in the inability of another protein or molecule to bind to it. Studies even show that oriented antibody immobilization with long spacers increased the signal intensity by as much as 10-times [176, 177]. This distinct weakness is why SAM chemistry is preformed despite it being more complex. Despite this, SAM guarantees orientation of the molecules or proteins, assuring that all proteins retain function when bound correctly. To circumvent the orientation issue, protocols may use an excess of protein during immobilization, such that, even if some percentage is non-functional, there is still enough functional material. Thus, adsorption of molecules and proteins allows for more facile functionalization of cells of interest, though it cannot control the spatial orientations of the immobilized targets.

Thermal polymer coating: Coating thermally-responsive polymers onto substrates allows the facile appending of "smart" function to a variety of substrates for cell isolation. As the polymer is covalently attached to a substrate, the substrate itself does not matter. The thermally responsive pNIPAM polymer coated on polystyrene dishes via laser irradiation creates a responsive system that can alternatively culture and release cells depending the investigator's needs [178, 179]. As the cells do not require the pNIPAM to be in a specific orientation to attach, it is a non-issue whether they are oriented correctly or not. However, as the temperature is lowered to 4 °C, the surface will release the cells for recollection. Integration of this type of polymer coating with other substrates enables greater adoption of "smart materials". Therefore, the pNIIPAM surface enables both cell isolation as well as greater customization of substrate-independent surface functionalization using molecular adsorption.

3 Advantages/disadvantages of surface functionalization

3.1 Advantages of surface functionalization

Surface functionalization has many advantages for substrate modification, as it can customize and integrate different materials. Surface functionalization can also impart utility to otherwise non-functionalized platforms. While there are many researchers that do adsorb proteins to glass without functionalization [16, 24], surface functionalization preserves the functional orientation of the protein, which allows for more efficient usage of the adhered protein. Furthermore, surface functionalization can allow for customizable passivation or inactivation of surfaces, which simple protein adsorption cannot do [64, 116]. Surface functionalization can also enable layered architectures of proteins for more specific applications, such as cell recollection [30, 31, 58]. Through the use of specific tail groups and established conjugation chemistries, such as EDC/NHS coupling (which uses another activating agent NHS with the EDC catalyst to improve yield), proteins and chemical moieties can be conjugated to each other to increase functionality [3, 30, 31, 104, 180]. Even more still, functionalization can be used to change the surface properties of a material by altering its reactivity. The addition of oxide or amine groups to a surface changes its hydrophobicity, which is very useful when patterning cells or binding proteins. All these customizations can allow for a more interactive and responsive system. These changes have been appended onto surfaces to produce smart materials [166, 181] that are able to respond to environmental changes [111, 167]. Thus, surface functionalization enables the addition of both reactivity and responsiveness to surfaces and materials through the chemistries that it utilizes. These advantages and disadvantages are summarized in Table 1.

3.2 Disadvantages of surface functionalization

Despite all the applications and advantages surface

Table 1 Advantages and disadvantages of surface functionalization applications

Application	Advantages	Disadvantages
Inorganic self-assembled monolayers	• Easy to functionalize self-assembled monolayers	• Substrate choice limits self-assembled monolayer choice
	• Preserves orientation	• Self-assembled monolayer choice limits chemical methodologies
	• Customizable through end group chemistries	• Harder to make more biocompatible without more complex reaction schema appended to monolayer functionalization
	• Established chemistries	• Risk of cross-reactivity with complicated reaction schemas
	• Enables both cell isolation and cell patterning	
	• Utilizes with well characterized materials such as glass or pure metals	
	• Integratable with extant techniques such as microfluidics or SPR	
Organic ECM mimicking systems	• Mimics physiological system and may be potentially more biocompatible	• May still use harsh chemicals to functionalize and risk cross-reactivity downstream
	• Avoids potential toxicity from inorganic non-inert functionalization	• More complex chemistry than self-assembling monolayer chemistry to create extracellular scaffolding for cells
	• Limited by fewer substrate functionalization restrictions	• Requires specific chemistries to bind extracellular proteins to substrate
	• Integratable with extant technology like 3D hydrogel systems	
Native protein functionalization	• Functionalizes natural tissue and architecture	• May not be able to specifically determine location within tissue for patterning
	• Biocompatible and mimics physiology	• May be difficult to measure or quantify within functionalized tissue natively
	• Can re-culture or co-culture cells upon extant ECM scaffolding to restore or append functionality to existing tissue	
Protein adsorption	• Functionalizes with more facile chemistries	• Proteins will not have controlled orientation, so all they may not be fully functional
Thermal polymer coating	• Does not require orientation	• Must be fully crosslinked, or will result in cell toxicity
	• Enables "smart" responsive systems	• Requires laser irradiation or other complex covalent bonding techniques
	• Substrate independent	

functionalization affords, there are three main weaknesses: 1) substrate limitation, 2) methodology limitations, and 3) cross-reactivity. These three disadvantages all constrain the chemistries available for surface functionalization; however, there are ways to mitigate or circumvent some of these disadvantages through chemical and reaction design. Moreover, one of surface functionalization's greatest strengths is customizability—so while it is true that certain design constraints exist, and it is important to work within them, the amount of possible customization within the space is large enough to have some applications in practically every experimental setup. It is important to understand the limits of every tool to fully understand how to utilize them most effectively. With proper experimental design and substrate selection, surface functionalization can be used to further expand the applications and functionality of the experimental setup.

Substrate limitations: One disadvantage of surface functionalization is that certain chemistries are limited by the chosen substrates. When using glass surfaces, SAM modification is restricted to silanes, as they self-assemble most effectively on that substrate. Similarly, as mentioned above, gold tends to be restricted to primarily thiol and selenoate SAMs. This limits the chemistries, as it requires one specific type of head group to react with it. Of course, this does not constrain the choice of tail groups that are possible, as anything from amines [31] to disulfides [115] can be appended onto thiol head groups. This is more of an issue if the thiols that are used require a higher level of chemical safety, such as a chemical hood or fume hood. Similarly, certain silanes can be dangerous as well, and being restricted to their use can be limiting. Another important thing to note is that there are numerous functionalization techniques, such as the organic ECM mimicking chemistries, that involve more complex chemistry and are not limited to specific substrates. As such, they completely ignore this disadvantage, and sacrifice ease of functionalization for substrate specificity. Therefore, while it is true that substrate selection limits the number of possible chemicals that can react with the surface, this does not mean that there are no options to choose from, as there are numerous customizations possible for the tail group SAMs despite the head group options being chemically constrained.

Methodology limitations: Another weakness of surface functionalization is the limitation on the number of possible methodologies for a single substrate. As the functionalizing chemicals that can react with a substrate are limited, so too are the reaction schemas limited by the functionalized material. Thus, there may be a limited number of methodologies that can be used to apply a certain type of functional group to a specific substrate. Some substrates, such as metals, are extremely well established in literature and are less limited in this respect; however, the fact that most metals can only be used in schemas that involve thiol chemistry does put constraints on what can be applied to it. Even organic ECM-mimicking chemistries still require detailed functionalization methodologies that are much more rigid and constrained due to the reactants and protocols involved. Despite these limitations, surface functionalization allows for vast customization within those design constraints. For instance, in the thiol chemistry example, while the metal substrates are limited to thiol SAMs if using self-assembled chemistries, the tail groups can be changed to match whichever chemical functional group is necessary. This can be used to add amine groups for reactions that involve further conjugation with carboxylic acids [31] or vice versa, as carboxylic acids could be attached to the thiol and react with amine groups for other subsequent functionalizations. The number of combinations for possible functional groups can be much further complexed by the number of reacting agents that can interact with them. Thus, while it is true that the possible chemical reactions are limited for each substrate and functionalizing pair, there is still a great deal of depth to which chemistries can be chosen within that space.

Cross-reactivity: Lastly, one concern of using complex chemistry to functionalize substrates is the risk of cross-reactivity (chemical reactions of the materials with other typically innocuous chemicals downstream). Most SAMs are inert and non-reactive; however, with surface functionalization schema that may involve more complex chemistries such as substitution reactions, it is possible that either the functionalizing chemical itself can react with another downstream chemical or a reaction byproduct can re-react with it. For example, during the functionalization of a non-standard material, such as polyvinyl chloride [88], chlorine groups released from silanization can result in their reaction with other downstream reactants. This reaction could create byproducts that could damage the cellular or functionalized system or whatever the system being investigated. If the cross reactivity results in toxic byproducts, it could damage the isolated cells [182, 183] and alter the expected results, which would invalidate the study entirely. It is also possible that these reaction byproducts are more dangerous than the expected reaction products because it would be fruitless to isolate cells with surface functionalization techniques only to have the functionalization materials alter or damage the cells. Many sequenced functionalization protocols involve long incubation steps with excess reactants to try to fully react with each subsequent layer. In this way, any reaction byproducts can be fully reacted and removed from the system before interaction with cells. Many multi-step functionalization protocols [31] also involve long and frequent wash steps to be increasingly sure that no unreacted materials remain after each functionalization step. Coupled with the extra reactant washes, this may also help remove any of the toxic reactants or products from the system before exposure to the cells. However, even this cannot completely remove the risk of cross-reactivity, and thus strict chemical design is paramount to fully optimize the surface functionalization protocol. Even organic ECM-mimicking functionalization protocols use techniques such as irradiation [63, 184] or acids (such as HCl) to functionalize surfaces [77]. Increasingly, researchers have characterized surface reactions using chemical techniques, such as X-ray photoelectron spectroscopy (XPS) [88] (which uses X-ray irradiation to measure the number of electrons and their kinetic energy to measure the composition and empirical formulas of the materials tested) and Fourier transform infrared spectroscopy (FTIR) [88] (which uses multiple frequencies of light and Fourier transforms for data processing to inform about the absorbance at each frequency) to verify reaction byproducts and ensure that there are no unaccounted byproducts. Therefore, cross-reactivity is a distinct limitation in more complex surface functionalization schemas; however, it can be mitigated through careful chemical and reaction design, multiple wash steps, and characterization.

3.3 Substrate chemistry selection

When deciding which substrate chemistry is ideal for a project, the most important things to consider are the parameters that are worth studying in the system that is being designed. Based on the experimental design, certain parameters will be tested or investigated. Deciding which parameters to study, optimizes the system for measurement of those parameters. For instance, if the system design requires optically clear material for binding quantification, then a substrate that is optically clear will be required for surface functionalization. Similarly, if multiple systems can resolve those parameters, then the parameterization decisions can be left to personal preference. For example, if a researcher values material cost as a parameter, a cheaper system like silicon or glass as the substrate may be sufficient for organic SAM functionalization; thus, gold or other pure metal-based functionalization is not necessary if the system does not need to be integrated with another technique, such as a cleavable system. Silicon substrates such as glass are easily obtained, cheap, and disposable. Moreover, silane chemistry has been very well established, and there is a wealth of literature on it. Silicon-based nanowires have even been adapted for higher conductance and more metal-like properties for silicon-based systems. However, gold is easier to implement parameters such as electrical conductance. If the testing modality requires an electrical conduction or integration with a measurement modality such as SPR, using thiol chemistries will allow for the addition of alternate functionality to the metal surface for customization. These systems could conversely use organic ECM-mimicking functionalization techniques for biological applications as well if the experiments require it. Furthermore, since both the tail group and the functional group on both thiol and silane chemicals can be customized completely, any sort of chemical functionality could be appended—from amine groups for conjugation via NHS and EDC or even specialized protein tags such as streptavidin or biotin. This affords complete customizability based on which parameters are necessary for each system. Ultimately, each material has associated specialties and weaknesses, so choosing a substrate to optimize the experimental design should be the first step in deciding which surface chemistry SAM to utilize.

3.4 Further applications for surface functionalization techniques

Surface functionalization can be applied to practically any substrate to improve selectivity, stability, or compatibility of different surfaces. By tuning the hydrophobicity of surfaces, cell capture or culturing can be performed more effectively and with more physiological environments [81, 114, 127, 142]. This research can be integrated with imaging modalities to functionalize fluorophores or even quantum dots with chemistries such that they bind to aptamers or antibodies [20] to enable multiplexing of signals [20, 27]. Surface functionalization can even be applied to improve biocompatibility of materials that are implanted into the body [77] to resist degradation, rejection, and reduce inflammation. As previously mentioned, the most important parameter for deciding whether to implement surface functionalization into a new research modality being considered is to determine which functions need to be imparted onto the substrate and for which purpose. From there, choosing the substrate and the subsequent chemistries will come much more easily, as the form will fit the function.

Surface functionalization can also be used to alter or improve substrate properties such as roughness, which increases the surface area to improve pull-down percentages. While this technique does alter the surface of the substrate using chemicals, it is completely independent of affinity-based pull-down (Fig. 2). As a result, these techniques can be used in conjunction with the above-described affinity-based pull-down chemistries to pattern or isolate cells. For example, Mahmood et al. used acids to micro-rough the surface of the glass prior to amino silane functionalization. The increase in surface area allowed for higher interaction between the functionalized capturing moiety and the cells of interest—CTCs—and thus allowed for higher isolation percentages than a flattened glass version would. The micro-roughed surface was functionalized with anti-EGFR antibodies to pull down CTCs [143] after roughing the surface with acids. In contrast, Diéguez et al. acid-roughened the PDMS surface of the capture device, rather than the glass, before conjugating with CD45 antibodies [185], which also showed improvements in cell pull-down. The antibodies were conjugated to both the glass and PDMS surfaces, so the increase in surface area was relegated exclusively to the PDMS surface. For both modalities, the surface roughness increased the surface area for greater cell capture, while the silane functionalization pulled the cells down to the surface and tethered them to the surface.

4 Conclusions

This review discusses the different methodologies for surface functionalization, including SAMs, ECM-mimicking substrates, and adsorbed protein functionalization as well as the applications of each of these techniques in a variety of science fields. Through the presentation of our laboratory's research and of the many luminaries in several disparate fields, we demonstrate that surface functionalization is a very powerful tool for appending utility to substrates that may not natively contain such capabilities. This functionality can be easily designed to interface with different substrates to improve biocompatibility for biological research or interface and integrate with measurement modalities for quantification of specific research parameters. Thus, surface functionalization can be implemented by researchers of practically any field based on their applications. Through the flexibility and customization that functionalization affords, hopefully, we can begin to chip away at the vast glass bottom left for us—harkening back to the spirit of what Dr. Feynman spoke of in his lectures urging us to embrace the unknown so long ago. It is only through this acknowledgment of the long distance that we have yet to go, and the things that we have yet to know, that we are able to move forward and truly revolutionize the field.

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