Minireview

Strategies for cell membrane functionalization

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Abstract

The ability to rationally manipulate and augment the cytoplasmic membrane can be used to overcome many of the challenges faced by conventional cellular therapies and provide innovative opportunities when combined with new biotechnologies. The focus of this review is on emerging strategies used in cell functionalization, highlighting both pioneering approaches and recent developments. These will be discussed within the context of future directions in this rapidly evolving field.

Keywords: Functionalizing, cells, membrane, biomaterials

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Introduction

The demand for cell functionalization

New biotechnologies, such as organ-on-a-chip[1] and 3D bioprinting,[2] are providing researchers with increasingly innovative approaches to studying disease, engineering tissue and promoting in vivo regeneration. These biotechnologies often demand unnatural functions from cells, for instance, in cell therapy, we would ideally like to target cells to a particular area of the body, often to promote an unnatural response within a hostile environment, while being able to visualize the entire process in vivo.[3,4] Cells were never intended for use in allogeneic therapy,[5] nor were tissues meant to be engineered in an incubator,[6] or embryos in a test tube.[7] Such applications are greatly removed from how a cell is programmed to function within an organism, which limits cells to pre-defined functions (e.g. cell-specific signaling pathways[8] and surface markers[9]) and imposes tight constraints based on physiological conditions (e.g. temperature[10] and oxygen tension[11]). Indeed, we are now at a stage where the *cell itself* could be considered the major restrictive factor, thus, effective methods to re-engineer cells are required to keep up with the rapid pace of biotechnological development.

An emerging strategy to overcome these limitations is cytoplasmic membrane modification, which can be used to either supplement the existing capabilities of a cell, or provide entirely new, non-native functionality. This *cell functionalization* approach has allowed us, for instance, to provide cells with additional binding sites[12] and nutrients,[13] protection in harsh environments,[14] increased adhesion to scaffolds[15] and magnetic contrast.[16] Compared to genetic modification, these strategies are simpler, faster and can be used to deliver a greater variety of materials to a wider range of cells. The scope of this review will cover both the active and passive delivery of soft biomaterials (e.g. proteins, biopolymers and carbohydrates) with a specific focus on non-specific membrane binding and instances where cells have been augmented with added functionality. We will present a selection of both pioneering and recent approaches to cell functionalization, discuss their relative merits, and conclude by considering the challenges and future directions of this exciting new field.

The cell membrane as an addressable canvas

The cytoplasmic membrane was first identified by Wilhelm Pfeffer in 1877, who proposed a *membrane theory* that suggested cells were filled with an aqueous solution contained by a physical, semi-permeable barrier.[17] In 1925, Gorter and Grendel famously used a Langmuir trough to demonstrate that the cytoplasmic membrane of erythrocytes was only two molecules thick.[18] This led to the well-recognized *phospholipid bilayer model*, in which the cytoplasmic membrane was considered a lamellar bilayer stabilized by hydrophobic interactions between the fatty acid tails of the constituent phospholipids. It is now known that up to 1000 different lipids contribute to the bilayer structure, which has a thickness of approximately 75 Å.[19] This lipid sandwich supports a wide array of proteins, which can be embedded within the bilayer (integral), loosely bound to the surface (peripheral) or attached *via* a lipid anchor (lipid-bound). This was elegantly portrayed in Singer and Nicolson's *fluid mosaic model* in 1972, which depicted proteins laterally

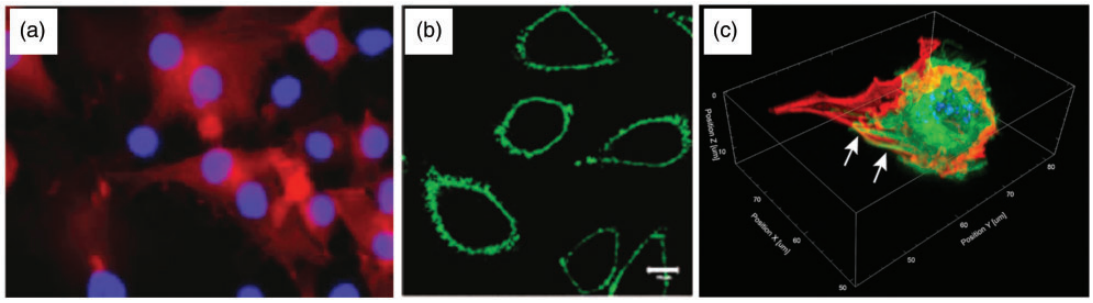

Figure 1 Fluorescence microscopy images of functionalized cells. (a) An example of cell surface chemistry, with human foetal osteoblasts (nuclei-labeled blue with DAPI) metabolically labeled with L-azidohomoalanine were conjugated to a biotinylated alkyne that was subsequently visualized using fluorescent streptavidin (labeled red).[28] Reprinted (adapted) with permission from Borcard et al. Bioconjugate Chemistry 22, 1422-32 Copyright 2011 American Chemical Society. (b) An example of non-covalent membrane labeling, in which a polyethylene glycol/oleyl chain was used to anchor proteins such as GFP (labeled green) into NIH3T3 cells.[29] Reproduced with kind permission from John Wiley and Sons: Kato et al.[29] (c) An example of an extended cellular coating, whereby matrix proteins including fibronectin (labeled red) were used to ''shrink wrap'' C2C12 cells (nuclei-labeled blue with DAPI, actin fibres labeled in green and indicated with arrows).[30] Reproduced with kind permission from Springer Science + Business Media: Palchesko et al.,[30] Figure 4(e). (A color version of this figure is available in the online journal.)

diffusing throughout a dynamic phospholipid bilayer, considered to be an isotropic fluid.[20] An important advance on this model was the incorporation of lipid microdomains, which were proposed as non-equilibrium, two-dimensional aggregates of phospholipids and proteins, essential in membrane trafficking and turnover.[21] Finally, an often overlooked component of the cytoplasmic membrane is the glycocalyx, a layer of glycans present on the outer membrane leaflet that is used by cells to interact with the extracellular environment.[22] These components form the basis of the current understanding of the cytoplasmic membrane structure.

A cell biologist will (correctly) consider the phospholipid bilayer a protective structural barrier, with the proteins and carbohydrates as functional components regulating mass transport,[23] adhesion[24] and signaling.[25] An alternative view of the cytoplasmic membrane is that of a canvas of addressable molecules and moieties, which can be exploited as targets for cell functionalization. Carolyn Bertozzi memorably described the cell membrane as a ''sea of functionality,''[26] and when viewed from this perspective, the phospholipid bilayer becomes a dynamic hydrophobic continuum into which lipid anchors may be inserted, while proteins, glycans and phospholipid head groups are simply a collection of chemically addressable functional groups. Having said this, a multitude of factors must be considered when designing a cell surface modification strategy. First and foremost is the maintenance of cell viability, which necessitates functionalization methodologies that employ aqueous conditions, physiological pH and ionic strength, as well as ambient temperature and pressure. Even under these cell-amenable conditions, the introduction of membrane-active chemical species can still lead to cytotoxicity through processes such membrane thinning or hole formation,[27] while blocking or modifying specific glycans can also be detrimental to cell function. Second, the temporal persistence of the exogenous material must be considered. Unlike genetic modification, which can be used for long-term transgene expression, cell functionalization strategies are intrinsically transient due to membrane turnover and mitosis that continuously dilute membrane-bound species. Finally, the spatial location and orientation of the membrane guest molecule can be important for certain applications, for instance, a targeting antibody may require a linker to project it away from the membrane surface into extracellular space, while a receptor protein or glycan will need to transduce signals across the membrane bilayer. Fortunately, there exists an array of well-developed cell functionalization strategies that cater to different, individual requirements. The remainder of this review will discuss the relative merits and notable successes of three broad approaches; cell surface chemistry, non-covalent membrane labeling and extended cellular coating (Figure 1).

Covalent cell surface chemistry and bio-orthogonal labeling

Designing chemical syntheses under cytocompatible conditions is challenging, as cell viability has to be prioritized over more common objectives, such as reaction yield and rate. With this in mind, an attractive strategy is to use bio-inspired enzymatic reactions that have evolved to work under physiological conditions. For example, the McEver and Wohlgemuth Groups used α-1,3-fucosyltransferase to enzymatically modify selectin ligands with guanosine diphosphate fucose, in order to present the glycoprotein sialyl Lewis X (sLeX) on cord blood cells[31] and human mesenchymal stem cells (hMSCs).[32] Here, promoting the surface expression of sLeX was shown to mediate a non-native cell rolling response to endothelial selectins. Cell rolling was also targeted by the Karp Group in 2008,[101] who used biotinylation of cell-surface amines to streptavidin-link biotinylated sLeX (Figure 2(a)). This report was preceded by a very similar ''ProtEx'' technology, developed by the Shirwan Group in 2005,[33] which showed that streptavidin fusion proteins could persistently label cells in vitro and in vivo. This approach has been used to enhance graft survival with CD95L,[34,35] inhibit cancer cell growth with CD80[36,37] and produce whole-cell vaccines bearing GM-CSF/TNFα co-stimulators.[38] Despite being less abundant than amines, thiol groups present on cysteine-bearing proteins are an attractive target for cell surface chemistry, and their capacity for click chemistry reactivity has been used,

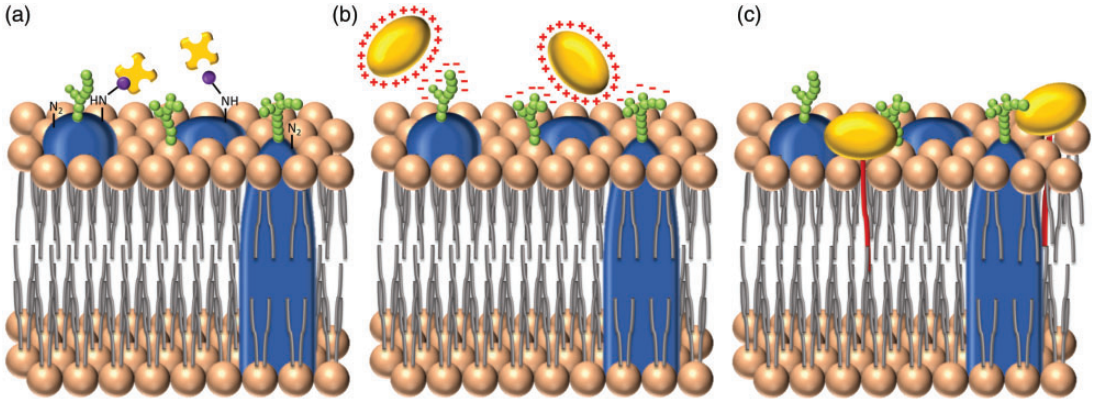

Figure 2 Three broad approaches to cell membrane functionalization. (a) The first method is direct surface chemistry, performed on functional groups present on the cell membrane. Here, for instance, amine groups present on membrane proteins have been biotinylated (purple) to allow the addition of streptavidin (yellow). This approach is commonly used to deliver species labeled with streptavidin or biotin.[87] (b) The second method is to increase the cationic surface charge of the exogenous species to facilitate attractive electrostatic interactions with negatively charged moieties present predominantly within the glycocalyx. (c) The third strategy uses hydrophobic interactions between a conjugated lipid tail and the phospholipid bilayer, to anchor the exogenous species to the cell membrane. (A color version of this figure is available in the online journal.)

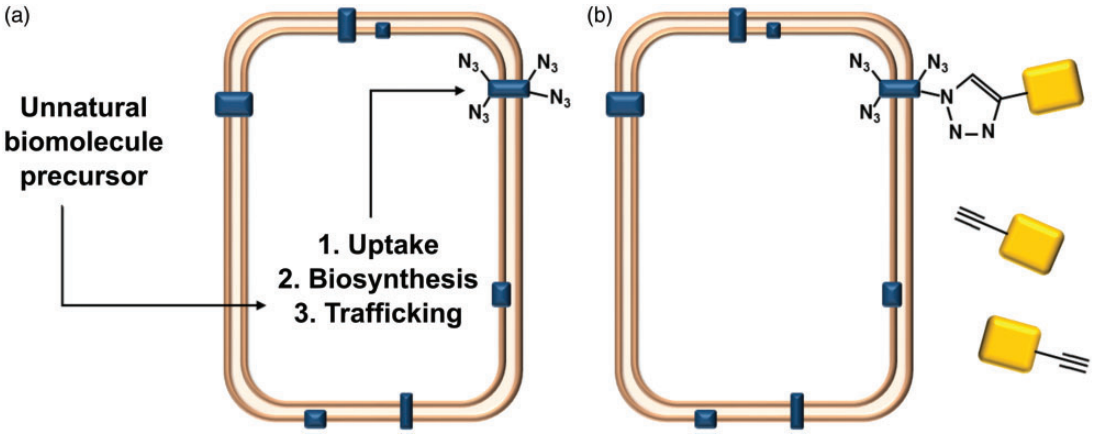

Figure 3 Metabolic labeling and biorthogonal chemistry. (a) Unnatural biomolecular precursors, included as cell media additives, can be taken up by cells and become incorporated into lipids, carbohydrates or proteins (blue), including those at the cell membrane. (b) Metabolic labeling can be used to present reactive groups that can bind a secondary species (yellow). This is usually mediated by orthogonal click chemistry, in this example, an alkynated secondary species is bound to a cell metabolically labeled with azide groups. (A color version of this figure is available in the online journal.)

for instance, in the binding of maleimide-functionalized, drug-loaded liposomes.[39] Overall, these direct cell surface modifications represent an excellent approach, albeit one that is restricted by constrained reaction conditions and a limited number and range of addressable groups.

A major breakthrough in this field was the development of ''bio-orthogonal'' chemistry, which was pioneered by the Bertozzi Group in 1997 (Figure 3).[40] This built upon an established technique known as ''metabolic labeling,'' whereby culture medium supplemented with certain non-canonical amino acids or monosaccharides allowed the incorporation of new functional groups into the proteome or glycome.[41,42] While metabolic labeling has been used in its own right as a functionalization tool to modulate virus–cell interactions,[43] the Bertozzi Group extended the scope of this technology by introducing ketones as a reactive base for click chemistry reactions.[40] This two-step, bio-orthogonal approach provided rapid kinetics with high specificity (i.e. no side reactivity) under physiological conditions, as well as great versatility. Indeed, azides, alkynes, thiols and methacryloyls have been successfully incorporated into cell surface glycans,[44–47] amino acids[15,28,48,49] and lipids,[50] with applications that include the selective killing of cells,[51] drug conjugation,[46] cell-surface click gelation[52] and artificial adhesion to 2D or 3D substrates.[15,45] Metabolic labeling and bio-orthogonal strategies still suffer from limitations associated with tightly regulated biosynthetic pathways (more of an issue for amino acids than glycans), interference from specific metabolic pathways (a particular issue with ketone labeling), cytotoxicity arising from certain mediators (such as copper ions in certain azide–alkyne reactions) and the necessity to include a compatible, ''clickable'' functional group on the secondary reactive species.[53] Most of these issues can be overcome with careful experimental design, and metabolic labeling coupled with bio-orthogonal bio-conjugate chemistry remains an elegant approach to cell

functionalization. Furthermore, metabolic labeling is the only approach discussed in this review, other than ProtEx, that has been effectively performed in vivo.[54]

Non-covalent interactions with the cytoplasmic membrane

An extremely facile approach to cell functionalization is to generate a membrane-active biomaterial in isolation, rather than trying to perform in situ chemical reactions at the cell surface. Perhaps, the simplest approach is to generate a cationic molecule that will interact with anionic proteoglycans present within the cell glycocalyx (Figure 2(b)). One of the first examples of this approach was reported in 1972, when Danon et al.[55] showed that chemically cationized ferritin could effectively contrast label cell membranes for electron microscopy. This approach used a relatively simple chemical reaction, whereby acidic amino acids on the protein shell were converted into non-native cationic residues *via* carbodiimide-mediated nucleophilic addition of reactive diamines. This approach was very recently applied to the superparamagnetic protein magnetoferritin, where hMSCs were contrast labeled for magnetic resonance imaging, using incubation periods as short as 1 min.[16] The magnetization of hMSCs was reduced when the biosynthesis of sulfated proteoglycans was significantly inhibited, which was evidence that these anionic glycocalyx species play a major role in mediating the electrostatic binding of chemically cationized magnetoferritin.

A complementary approach, developed by the Liu Group at Harvard University, used aggressive site-directed mutagenesis to produce ''supercharged proteins'' possessing an unnaturally large number of charged residues.[56] Here, a thermodynamically stable variant of the green fluorescent protein (GFP) bearing a theoretical net charge of +36 was shown to efficiently interact with membrane proteoglycans and was used to deliver proteins and DNA to a range of different cells.[57–59] Both chemical cationization and supercharging, however, involve making widespread modifications to the surface of a protein, which can lead to conformational changes in secondary and tertiary structure and subsequent loss of biological activity. In general, however, cationization represents a sound approach for delivering robust proteins (e.g. ferritin, GFP) to the cell membrane, however, this approach is likely to be challenging for more structurally sensitive proteins, while certain cationic species have also been shown to induce cytotoxicity via membrane thinning and hole formation.[27]

An alternative strategy to induce artificial membrane binding is to use a hydrophobic moiety to anchor a species to the phospholipid bilayer (Figure 2(c)). A pioneering example was introduced by Kim and Peacock,[60] who decorated hybridoma cells with anti-mouse antibodies using palmitate protein A. This advance was achieved by exploiting the ability of the palmitate lipid to intercalate with the cytoplasmic membrane, and the affinity of protein A for the Fc region of antibodies. Using palmitated proteins as a scaffold ensures that any bound antibodies are oriented away from the cell surface, which effectively presents the binding paratope. A decade later, this technology was adopted by the Dennis Group, who used palmitate protein G and tissue-specific antibodies to target chondrogenic progenitor cells to cartilage,[61] as well as hMSCs to endothelial cells,[12] the colon[62] and infarcted heart tissue.[63] In 2000, the Tykocinski Group broadened this approach beyond antibodies by expressing fusion proteins containing the immunoglobulin Fc region, which could specifically bind membrane-anchored palmitate protein A.[64] Variations on this ''protein transfer'' technique have been used to induce cell-rolling by functionalizing MSCs with CD162,[65] as well as eliciting anti-tumor responses using an array of co-stimulators and cytokines, including CD80,[66,67] CD254,[68,69] CCL21,[68,69] CD95L[68] and CD137L.[68,69]

In 1995, the Selvaraj and Tykocinski Groups introduced a biomimetic method known as ''cell surface painting'' using proteins recombinantly-tagged with glycosylphosphatidylinositol (GPI).[70,71] The GPI tail, present in many native membrane-bound proteins, inserts into lipid raft domains in the cytoplasmic membrane and anchors the fusion protein to the cell, without the need for any intermediary species. Importantly, the original function of the anchored protein is retained, which allowed cells to be painted with a wide range of proteins, including CD80,[70–75] CD86,[71,72] CD1,[76] IL-12,[77] TIMP-1,[78] TCR,[79] CCL5[80] and the I-domain of CD11a.[81] A major drawback to both protein transfer and cell surface painting is the reliance upon fusion proteins, which limits versatility, and can be time-consuming to prepare at sufficient quantities. With this in mind, a number of groups have used liposome-based delivery vectors to present antigens[82] and synthetic membrane receptor mimics,[83] increase the cellular association of GPI-anchored proteins[84] and provide binding sites for secondary species, such as biotinylated SLeX.[85] Liposome-based approaches, however, are often limited by poor encapsulation efficiency, particularly with large biomolecular species.[86]

The limitations surrounding fusion proteins and liposomes can be circumvented in several ways, for instance, by using synthetic glycoprotein analogues[87] or metal-chelating lipids bearing nickel nitriloacetic acid (NTA) bound to polyhistidine-tagged proteins.[88] Another, bioconjugation approach involves the direct covalent coupling of lipids to proteins (lipidation) to display hydrophobic tails such as myristyl,[89,90] palmityl,[90,91] stearyl[90,92] or oleyl.[93–95] Here, the membrane affinity of lipidated proteins can be tuned to some extent by increasing the molecular weight of the lipid,[96] while membrane persistence can be increased by using branched lipid tails.[29] A limitation of protein lipidation, however, is the requirement of organic co-solvents or detergents to prevent protein aggregation. A new technology that circumvents these issues is ''cell priming,'' which uses chemically cationized proteins electrostatically conjugated to a responsive poly(ethylene glycol) (PEG)-based surfactant corona. Conformational rearrangement of the amphiphilic polymer surfactant promotes protein stability and aqueous solubility (due to the hydrophilic PEG segment),[97,98] and mediates membrane tethering for around one week in culture (via the hydrophobic tail).[13] Myoglobin conjugates retained their oxygen-binding capacity[99] and were delivered to hMSCs to provide an in situ oxygen reservoir to enhance the production of matrix fibres at the

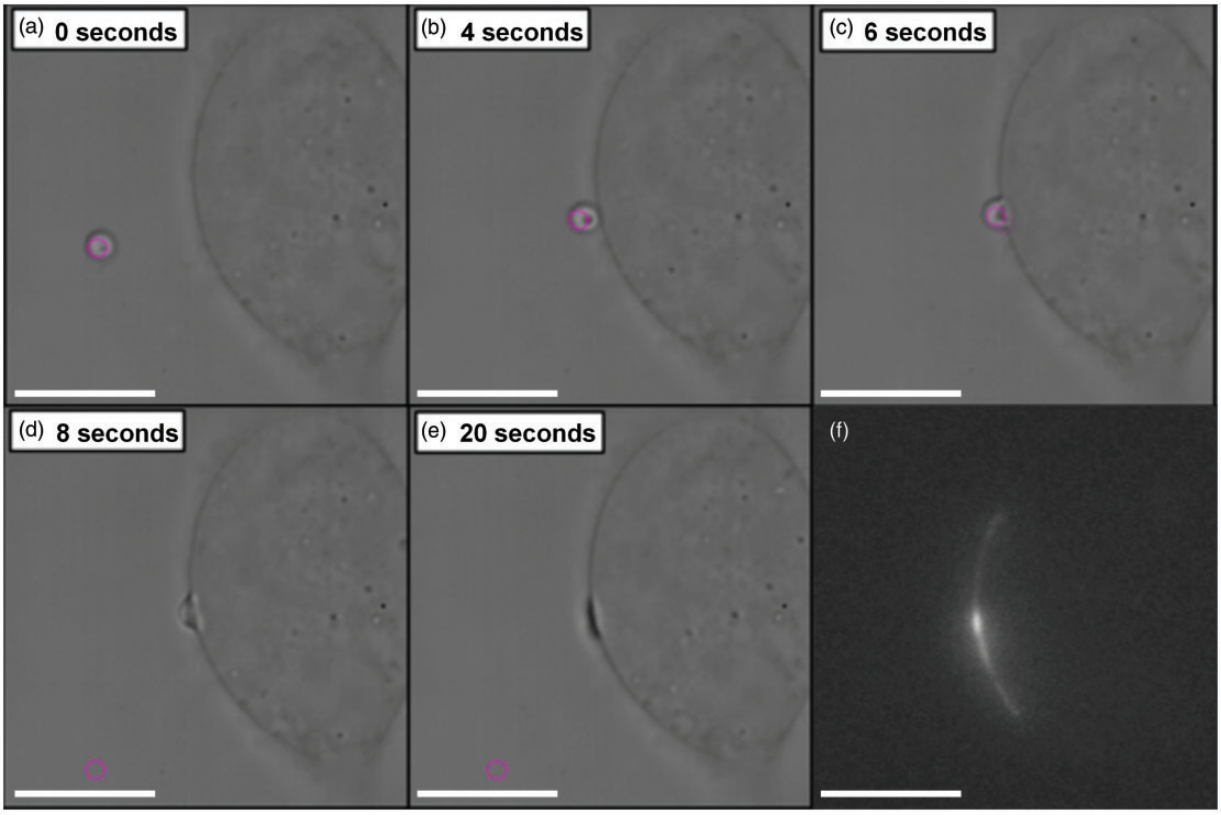

Figure 4 Cell paintballing using coacervate microdroplets. Armstrong et al. recently demonstrated that membrane-free coacervate microdroplets can be actively loaded with biomaterial payloads of protein or nucleotides, and then delivered to the cell membrane using optical tweezers.[113] (a)–(e) Time-lapse bright field microscope images showing an optical trap (pink circle) maneuvering a GFP-loaded coacervate microdroplet toward a human mesenchymal stem cell to initiate a targeted fusion event. (f) Fluorescence microscopy revealed fluorescence emission from the GFP payload present at the site of delivery

centre of engineered cartilage constructs.[13] Importantly, both cell priming and protein lipidation necessitate careful modification of the protein surface, as aggressive bioconjugation strategies can lead to denaturation and subsequent loss of biological function.[100] With this in mind, orthogonal or site-specific modifications are an attractive option, however, these approaches are not feasible for all proteins.

Extended cellular coatings

An entirely different approach to cell functionalization is to wrap or patch cells with thin polymeric microsheets. For instance, agarose, carrageenan or low-methoxy pectin biopolymers have been used to generate a 50-μm thick gel veneer around newly fertilized toad eggs.[102,103] This process is an excellent example of a single-cell *coating* (rather than gel encapsulation) and was shown to be effective at preventing microbial infection and improving post-hatching survival rates. This study, however, was demonstrated using relatively large cells (diameter = 1–1.5 mm) and has not been applied to smaller cells (diameter <100 μm). Palchesko et al.[30] reported a more advanced, microscale technology that used extracellular matrix protein sheets to ''shrink wrap'' endothelial cells, myoblasts and cardiomyocytes. This global coating of functional biomolecules was shown to be effective at modulating the structure, adhesion and behavior of the coated cells and was used in the study of cell–matrix interactions.

In 2008, the Rubner Group reported that photolithography and layer-by-layer assembly could be used to generate cell-binding patches of polymer[104] or mucin/lectin.[105] These so-called ''cell backpacks'' were persistently attached to one side of T-cells and monocytes, respectively, and have been used to magnetize cells,[104] promote non-native cellular assembly,[106] provide resistance to phagocytosis[107] and deliver therapeutics by ''hitchhiking'' on the surface of monocytes.[108] The major limitation of this approach is the requirement for time-consuming, layer-by-layer deposition of polymeric material, however, this was recently addressed by the Guan Group, who used microcontact printing as a simpler, cheaper and higher throughput method for generating cellular backpacks.[109,110]

Discussion and new developments

The strategies discussed above could be broadly considered as passive labeling technologies, whereby cells exposed to bulk media, reagents or biomaterials are functionalized in an indiscriminate fashion. Recently, there has been a focus on more controlled approaches that allow targeted delivery of discrete biomaterial payloads to specific areas of individual cells. In 2011, for example, the Cojoc Group reported on a new approach whereby liposomes were maneuvered

toward the surface of individual neurons using optical tweezers.[111,112] The optically trapped liposomes were then ruptured, using an external pulse of ultraviolet radiation, which released proteins and chemical stimuli that directionally stimulated adjacent neurons. A similar approach was developed by the Perriman Group to optically deliver membrane-free coacervate microdroplets to the cytoplasmic membrane of MSCs (Figure 4). Here, the coacervate microdroplets were optimized to undergo spontaneous fusion with the cell membrane, without the need for external stimulation.[113] Moreover, the coacervate microdroplets could be pre-loaded with biomolecules (e.g. proteins, nucleic acids or small molecular dyes), allowing cells to be ''paintballed'' with discrete patches of functional payload. While these approaches are intrinsically low throughput, with respect to total cell number, they represent extremely powerful diagnostic and experimental tools for site-specific or single-cell functionalization.

In summary, it is clear that rational reconfiguration of the cytoplasmic membrane is a highly effective pathway to endow cells with new functionality to enhance cell-based biotechnologies. Indeed, the rapid pace of biotechnological advance makes this an opportune moment to add an extra dimension to the host of cell-based therapies at our fingertips, whether this is targeting cells to diseased tissues or tumors, engineering whole cell vaccines, interfacing cells with materials for bioelectronics and biosensing, regenerative medicine or disease modeling. What is surprising, is that cell functionalization remains an under-exploited tool, particularly when contrasted with the success of transfection ''toolkits'' that have made genetic manipulation a routine undertaking. This disparity may be rationalized, in part, by the inherently interdisciplinary nature of cell functionalization, which draws on expertize from disparate fields of synthetic chemistry, materials science, biochemistry and cell biology, and generally necessitates a more considered, bespoke approach. To this end, we hope that this review will serve as an aid to a greater understanding of the subtle differences between strategies, encourage the adoption of current techniques, and inspire the development of new cell functionalization methodologies.

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DECLARATION OF CONFLICTING INTERESTS

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