Engineered cell surfaces: fertile ground for molecular landscaping

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The cell surface contains a wealth of information that determines how cells interact with their environment. Methods for directing the cell surface expression of novel protein-based and oligosaccharide-based epitopes are stimulating new directions in biotechnology and biomedical research.

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Introduction

The boundary between biology and chemistry has eroded in recent years. The integration of these disciplines has brought an increasingly molecular perspective to bear on biological systems. Once thought of as intractable, biomolecules are now manipulated as compounds with defined structures and chemical reactivities. The logical extension of this view is to approach cells as complex machines whose architecture and metabolic pathways can be understood and manipulated at the molecular level. This vision has led to the development of the rapidly expanding field of cellular engineering which, even at this early stage, has already produced innovations such as cell-based biocatalysts [1–3] and biosensors [4,5].

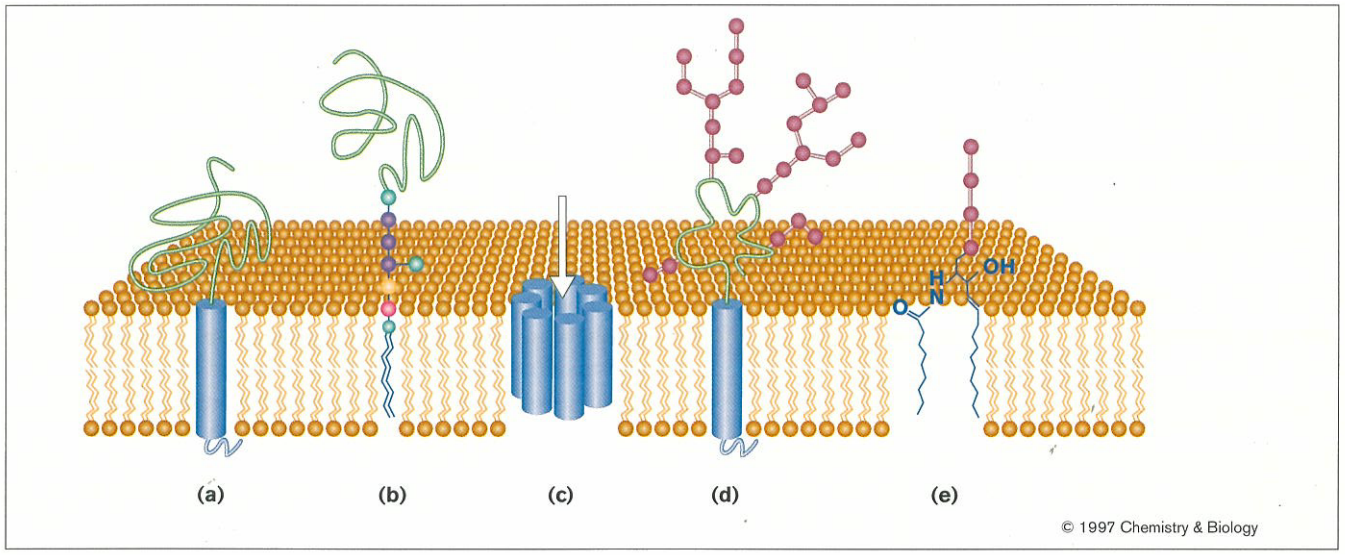
The processes governed by cell-surface molecules are fundamental to many biological phenomena and have attracted attention to the cell surface as an appealing target for engineering. Methods for controlling the display of membrane-associated epitopes offer a multitude of opportunities for studying cellular adhesion, signal transduction and cell–cell recognition events. In addition, cell-surface molecules are uniquely accessible to the outside world and have been recognized as convenient hosts for epitope libraries [6]. Here we summarize current approaches to cell-surface engineering and discuss present challenges and future directions. Due to the limited space available and the rapid expansion of this area of research, we are unable to cite all of the pertinent literature. We have chosen a few representative examples, and in some cases review articles, to highlight the salient topics in cell-surface engineering.

The mechanisms used to landscape cell surfaces can be broadly divided into three main categories: first, recruitment of the cell's genetic machinery for epitope expression; second, external delivery of novel epitopes to cell surfaces; and third, metabolic incorporation of epitopes into cell-surface molecules. The most prevalent cell-surface biopolymers are proteins and oligosaccharides, the latter typically being presented in the context of membrane-associated glycoproteins or glycolipids. As a result, these molecules have been the targets of most of the cell-surface engineering efforts to date (Figure 1).

Genetic approaches to protein epitope expression

Transfection of cells with genes encoding novel protein epitopes is a straightforward strategy for engineering cell surfaces. The challenge in this area is to identify a mechanism for directing the protein of interest to the outer surface of the membrane (or cell wall in the case of yeast, Gram-positive bacteria and plant cells). In eukaryotic cells,

Figure 1



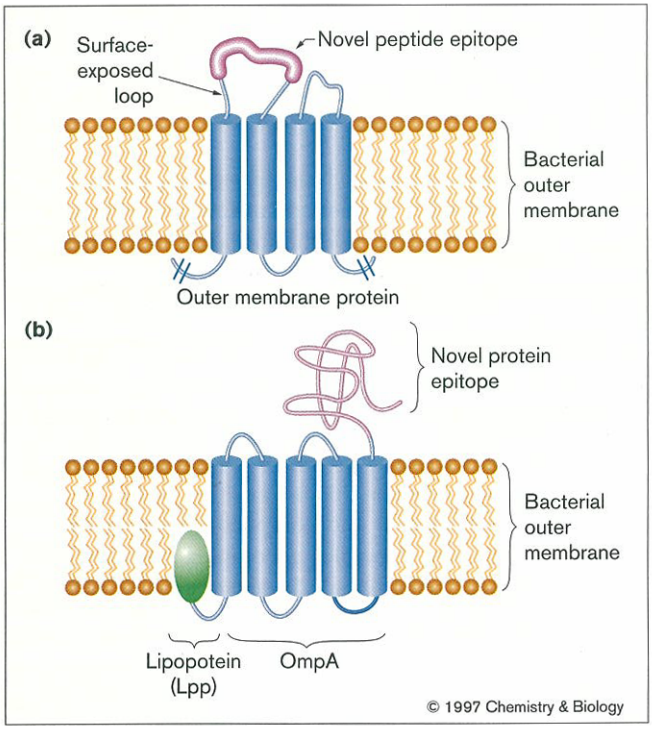
The landscape of the cell surface and opportunities for molecular engineering. **(a)** Membrane-associated protein. Novel protein or peptide epitopes (shown in green) can be expressed on cell surfaces as fusion proteins with a membrane-spanning domain (or cell-wall-anchored domain in the case of yeast, plant cells and Gram-positive bacteria) from another protein (shown in blue). **(b)** Glycosyl-phosphatidylinositol (GPI)-anchored protein. Proteins can be directed to the cell surface in eukaryotic cells as fusions with a consensus sequence for the attachment of a GPI anchor (shown in blue). GPI-anchored proteins can also be coated onto cell surfaces exogenously by passive adsorption from the extracellular milieu. **(c)** Pore-forming protein. Proteins from the extracellular environment can insert into the plasma membrane to form organized functional assemblies, such as gated pores that regulate membrane permeability. **(d)** Membrane-associated glycoprotein. New oligosaccharide epitopes (shown in pink) can be engineered by the expression of glycosyl-transferases, by exogenous enzymatic remodeling, or by the metabolic incorporation of unnatural monosaccharides. **(e)** Glycolipid. Oligo-saccharide epitopes (shown in pink) on glycolipids can be altered similarly to glycoproteins. New oligosaccharide epitopes can also be delivered to cell surfaces by passive adsorption of hydrophobic glycosides from the extracellular milieu.

this transformation can be accomplished by appending the gene encoding the protein epitope of interest to that encoding an appropriate leader sequence and membrane-spanning domain, often borrowed from another cell-surface-associated molecule. For example, Rieder *et al.* [7] used this technique to express a fusion protein composed of a virus-binding antibody and the membrane-associated molecule ICAM-1 on the surface of eukaryotic cells. This epitope acted as a novel cell-surface receptor, conferring upon its host cell the ability to be selectively infected by foot-and-mouth disease virus.

Proteins can also be directed to the plasma membrane by the addition of a glycosylphosphatidylinositol (GPI) anchor attachment consensus sequence at the carboxyl terminus (such proteins can also be released from the plasma membrane by co-expression with a GPI-specific phospholipase) [8–11]. Compared to fusion with a transmembrane domain from a different protein, the addition of a GPI anchor sequence is a minor modification and is less likely to cause gross structural alterations. This approach has been successful in the identification of Rubella virus glycoproteins responsible for cell-surface binding, for example [12].

The practice of cell-surface protein engineering has recently been expanded beyond basic research applications and into the biotechnology arena. With the advent of high-throughput screening methods using fluorescence activated cell sorters (FACS), the display of epitope libraries on the surface of microbes and yeast has become an attractive strategy for identifying high-affinity ligands, complementing similar strategies based on phage display [13]. Several protein fusion-based strategies for cell-surface display have been pursued. In Gram-negative bacteria such as *Escherichia coli*, peptides of less than 60 amino acids in length have been incorporated into the cell-surface-exposed loops of a variety of outer membrane proteins (Omps) (Figure 2a) [14–16]. Peptide epitopes have also been localized to the cell surface by their incorporation into the structural proteins of pili and flagella [17–19]. Unfortunately, the introduction of larger polypeptides and proteins disrupts the structure and localization of these delivery vehicles, restricting the utility of this approach. The development of a chimeric vector composed of the outer membrane localization signal from the major lipoprotein of *E. coli* (Lpp) fused to a segment of the outer membrane protein OmpA (Lpp–OmpA) has led to the successful display of proteins of up to 54 kD in size (Figure 2b) [20,21]. Enzymes such as β-lactamase and libraries of antibody fragments have been presented on bacterial outer membranes using this technology.

Figure 2



Expression of novel peptide and protein epitopes on outer membrane proteins (Omps) in Gram-negative bacteria. **(a)** The expression of short peptides (<60 amino acids) can be accomplished by insertion into surface-exposed loops. **(b)** Larger proteins can be presented in the context of a fusion protein with an Lpp–OmpA hybrid (adapted from [6]).

In Gram-positive bacteria, proteins can be targeted to the surface of the organism as fusions with cell-wall-associated molecules [22,23]. The cell-wall-associated domain of protein A from *Staphylococcus aureus* has been used as a vehicle for the presentation of an 80-amino-acid malaria-derived peptide antigen on the surface of *Staphylococcus carnosus* [22]. Mice immunized with a *Staphylococcus xylosus* variant expressing human respiratory syncytial virus antigens on cell-wall-associated molecules produced a specific antibody, suggesting that it may be possible to use these bacteria in live vaccines [23].

Cell-surface engineering in yeast has been accomplished using fusion vectors with cell-wall-associated proteins such as the mating adhesion protein α-agglutinin or hsp150 delta-carrier protein, the latter also leading to secretion [3,13,24–26]. Schreuder *et al.* [24] were the first to demonstrate this approach by targeting the enzyme β-galactosidase to yeast cell walls. The enzyme had increased proteolytic stability compared to the free enzyme, an advantage for cell-based biocatalysts. Recently, Tanaka and coworkers [26] have taken this technology one step further by engineering yeast cell surfaces to add a catalytic activity that confers a selective advantage. They delivered the enzyme glucoamylase from *Rhizopus oryzae* onto the cell surface in the context of an α-agglutinin fusion protein, enabling the yeast to use starch as a carbon source and to grow on previously unusable media.

Genetic approaches to oligosaccharide epitope expression

Oligosaccharides are not under direct genetic control, but are the products of the expression of several genes in concert. The biosynthesis of oligosaccharides often follows a particular order of assembly which is dictated by the specificities of glycosyltransferases and their compartmentalization along the secretory pathway. Consequently, engineering the expression of novel epitopes through genetic manipulations is not a straightforward affair. Nonetheless, several groups have gained insight into the biological roles of specific carbohydrate structures by the engineered expression or deletion of glycosyltransferases within the cell, leading to the presence or absence of the carbohydrate structure on the cell surface [27,28]. The power of this approach has been dramatically demonstrated by Lowe *et al.* [29], who discovered that transfection of fucosyltransferase-deficient COS-1 cells with the cDNA for an α1→3 fucosyltransferase conferred E-selectin binding activity. This observation suggested early on that fucose would be an important component of the selectin ligands, which has since been shown to be the case. Glycosyltransferase expression has also been used to modulate the metastatic activity of tumor cells. The enzyme N-acetylglucosaminyltransferase V (GnT-V) is responsible for extending a mannose residue on N-linked oligosaccharides with a β1→6GlcNAc branch, a cell-surface modification that correlates with high metastatic potential. N-Acetylglucosaminyltransferase III (GnT-III) attaches a bisecting β1→4 GlcNAc residue to the same mannose residue, thereby precluding GnT-V activity. Taniguchi *et al.* [30] overexpressed GnT-III in a metastatic cell line, and found that this suppressed metastasis, presumably by preventing the expression of the β1→6GlcNAc branch.

External delivery of protein epitopes to cell surfaces

Although genetic approaches have obvious advantages, many cells, such as primary cultures or those grown *in vivo*, cannot be readily transfected. Moreover, the range of epitopes that can be expressed genetically is limited to a large extent by the natural biopolymer subunits and the specificities of the biosynthetic enzymes. These restrictions have prompted the development of exogenous methods for cell-surface engineering.

One of the mildest methods for introducing proteins onto cell surfaces is the passive adsorption of GPI-anchored proteins from the extracellular milieu [8]. Using this technique, the amount of protein displayed on the surface can be readily controlled, and multiple GPI-anchored proteins

can be presented concurrently or in series on the same cell surface. Although cells engineered in this manner cannot replicate their remodeled state, their service in the functional analysis of cell-surface proteins is well documented. One of the first proteins studied by this method was decay accelerating factor (DAF), a component of normal human erythrocyte membranes that is deficient in patients suffering from paroxysmal nocturnal hemoglobinuria (PNH) [31]. Its function as a suppressor of complement-mediated lysis was elucidated when it was discovered that incorporation of DAF into erythrocytes from PNH patients protected them from destruction. More recently, 'artificial' antigen-presenting cells have been generated by delivering GPI-anchored class I MHC-antigenic peptide complexes to cell surfaces [32,33].

Proteins that form suitable hydrophobic assemblies can insert directly into the plasma membrane without the aid of a GPI anchor. For example, Russo *et al.* [34] engineered a metal-binding variant of *Staphylococcus aureus* α-toxin which inserted into membranes and formed Zn^{2+}-gateable 20 Å pores in living cells. The transport of small molecules across the plasma membrane of these engineered cells was controlled by the reversible addition of a metal chelator, offering an approach to the selective permeabilization of cell membranes.

External delivery of oligosaccharide epitopes to cell surfaces

As discussed above, the control of oligosaccharide expression by genetic methods suffers from a number of limitations. Several genes must be simultaneously expressed to obtain a complex oligosaccharide epitope, which may then be only a minor component of a heterogeneous mixture of glycoforms present on the cell surface. In contrast, the delivery of exogenous oligosaccharides to cell surfaces would allow control over the expression level and uniformity of the target epitope. A classical method for the mild attachment of carbohydrate moieties to cell surfaces is the passive adsorption of hydrophobic glycosides from the extracellular milieu, analogous to GPI-anchored protein adsorption [35–37]. This method has been used to investigate the binding of bacterial lipopolysaccharide (LPS) to macrophage LPS-binding proteins [38,39] and to elucidate the role of the ganglioside G_{M1} as a ligand for a bacterial toxin [40].

The context in which carbohydrate epitopes are expressed (e.g., extended from the plasma membrane on glycoproteins, or presented in clusters) may be an important factor in determining recognition on the cell surface. Thus, it would be best to introduce novel oligosaccharide epitopes in a manner that mimics their native environment. Ideally, these epitopes would be appended to existing glycoconjugates. Remodeling of cell-surface glycoconjugates has been achieved with the use of glycosyltransferases and their sugar nucleotide substrates. Some of the most exciting work in this area has been motivated by the observation that certain glycosyltransferases are permissive for unnatural substrates, enabling the enzymatic attachment of unnatural sugars onto cell surfaces and the expansion of the cell-surface carbohydrate repertoire.

Sialyltransferases and fucosyltransferases appear to be particularly relaxed with respect to substrate specificity, and both have been exploited for the introduction of unnatural carbohydrate epitopes onto cells. Brossmer and coworkers [41] decorated cells with 9-azido and 9-amino sialic acid analogs by first removing natural sialic acids enzymatically, and then reinstalling the unnatural analogs with a sialyltransferase and the corresponding synthetic CMP-sialic acid derivatives. In a landmark study, Hindsgaul, Palcic and coworkers [42] demonstrated that a fucosyltransferase from human milk will transfer fucose analogs with C6 substituents of enormous size and complexity onto cell-surface glycoconjugates. In principle, a complex epitope of any type could be delivered to cells in one enzymatic step. As a demonstration, these researchers synthesized a derivative of GDP-fucose carrying a blood group B trisaccharide and enzymatically transferred the complex structure onto cells [42,43] (Figure 3). Erythrocytes of blood type O were decorated with the blood group B trisaccharide in this way and displayed the B phenotype in a standard blood-typing assay.

The power of this strategy is further highlighted in a recent study of the relative E-selectin and L-selectin binding affinities of synthetic oligosaccharides [44]. The oligosaccharides were displayed on chinese hamster ovary (CHO) cells using the fucosyl transfer approach, and were found to have different relative binding activities from similar oligosaccharides in solution. This observation suggests that the context in which ligands are presented can affect the outcome of receptor-binding studies, and underscores the importance of cell-surface engineering technology for studying cell-surface receptor interactions.

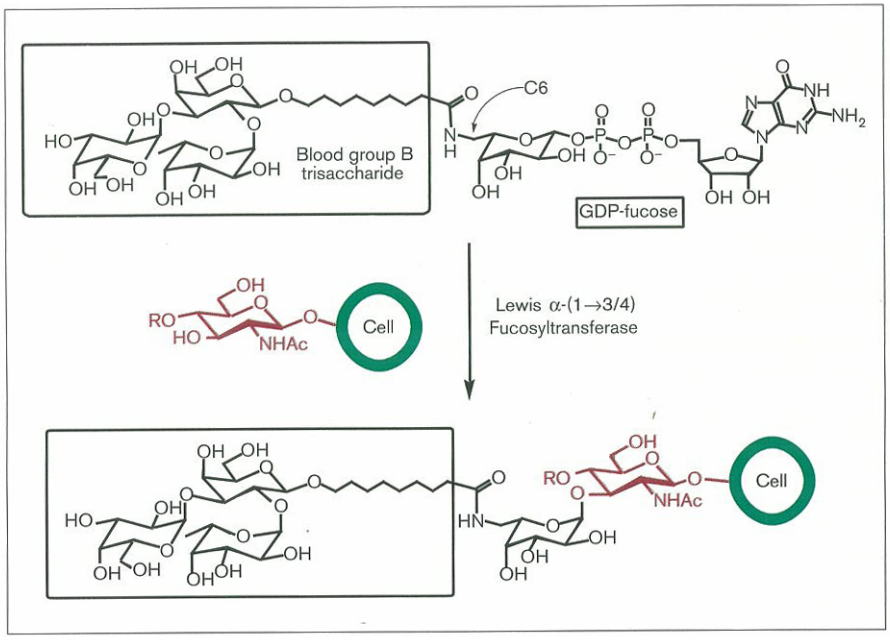
Metabolic delivery of novel epitopes to the cell surface

It is now possible to create new cell-surface epitopes via metabolic incorporation of unnatural precursors into plasma membrane components. Oligosaccharides are particularly amenable to this type of metabolic engineering since the enzymes and transport proteins in certain biosynthetic pathways are tolerant of unnatural substrates [45]. Cells will take up monosaccharide analogs from the extracellular environment and process them into cell-surface glycoconjugates. In contrast, the pathways for protein biosynthesis are more restrictive and only the most conservatively modified amino acids are accepted [46–50].

Promiscuity in the sialic acid biosynthetic pathway has been studied in some detail, leading to new insights into

Figure 3

Enzymatic transfer of a complex trisaccharide epitope onto cell-surface glycoconjugates [42].



the biological functions of cell-surface glycoconjugates. Sialic acid residues, which are biosynthesized from *N*-acetylmannosamine (ManNAc), terminate a large fraction of mammalian glycoconjugates and serve as ligands for a number of viral and bacterial receptors. Several *in vitro* studies have hinted at the permissive nature of the enzymes in this pathway, especially with regard to the *N*-acyl substituent on *N*-acetylmannosamine. Reutter and coworkers [51,52] used this system to introduce unnatural *N*-propanoyl, *N*-butanoyl and *N*-pentanoyl sialic acids onto cells by feeding them the corresponding unnatural mannosamine precursors (Figure 4a). The engineered cells showed altered ability to be infected with polyoma virus, which uses sialic acid as a ligand for binding prior to infection [53]. These researchers also found that the presence of unnatural cell-surface sialic acids represses contact inhibition in cultured fibroblasts, suggesting a role for sialic acid in this critical event [54].

In our own research group, rather than focusing on unnatural sugars as altered carbohydrate epitopes for the study of biological function, we have used oligosaccharide pathways as vehicles to introduce novel chemical reactivity onto cell surfaces. For example, cells treated with *N*-levulinoylmannosamine (ManLev), an unnatural precursor to sialic acid that bears a ketone group, express the corresponding keto-sialic acid on cell-surface glycoconjugates (Figure 4b) [55]. The ketones can be selectively ligated with hydrazide-, hydroxylamine- or thiosemicarbazide-conjugated molecules, allowing the cell surface to be chemically remodeled with a variety of epitopes. This approach combines aspects of both metabolic engineering and external chemical delivery and can, in principle, be extended to a number of different biosynthetic pathways and functional groups.

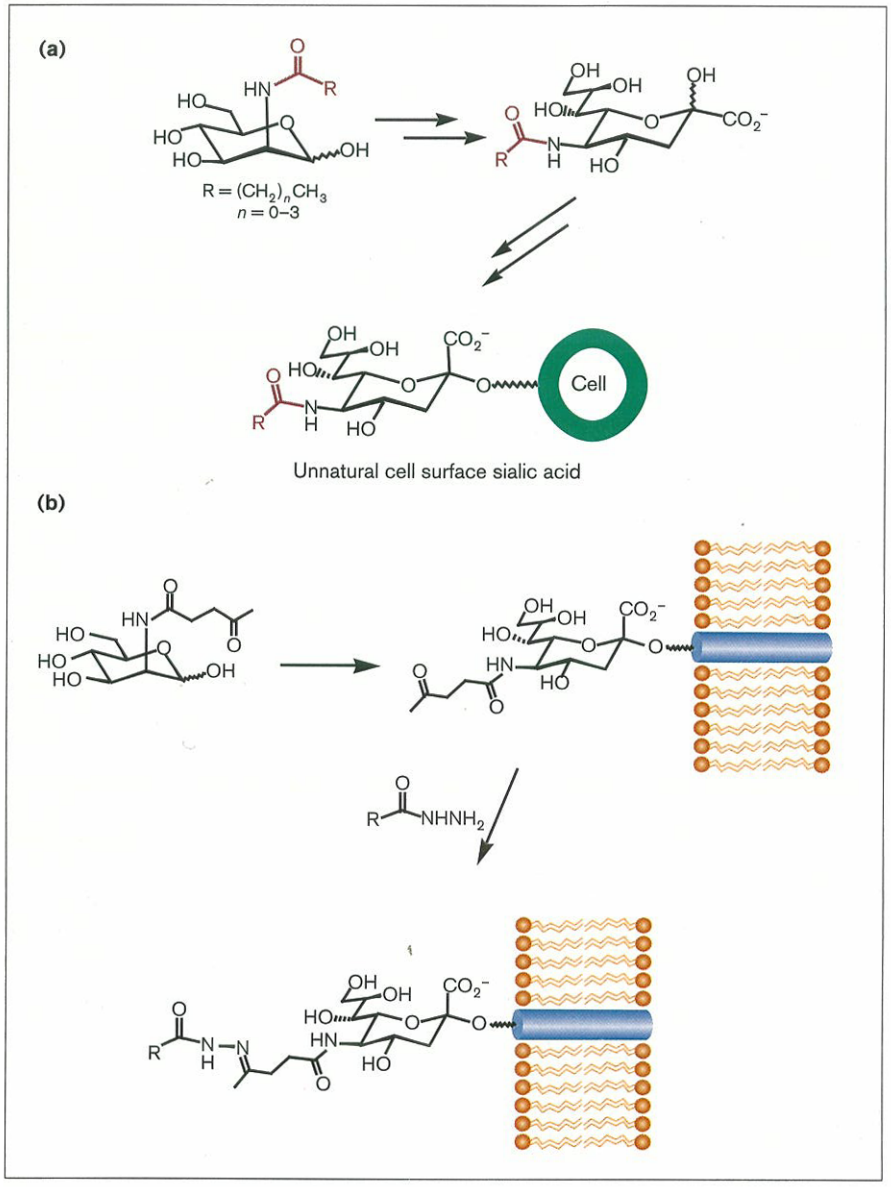
Future directions

The diverse array of strategies for cell-surface engineering represents the accumulation of increasingly powerful tools for manipulating cells at the genetic and biochemical level. Advances in these technologies will also produce new, multifaceted tactics for landscaping cell surfaces. The potential of these techniques is tremendous. Working together, chemists and biologists will be able to develop methods that combine genetic and metabolic engineering to introduce a range of unnatural epitopes onto cells without the restrictions imposed by nature's biosynthetic machinery and metabolic building blocks.

The boundaries of genetically controlled epitope expression are constantly being expanded by revolutionary technologies such as *in vivo* unnatural amino acid mutagenesis [56]. The ability to evolve enzymes with new specificities also has far-reaching implications for metabolic engineering [57]. For example, unnatural metabolic precursors and complementary biosynthetic enzymes could be combined to produce cells with rationally designed surfaces. These cells would produce progeny capable of creating similar unnatural surfaces.

The opportunities afforded by cell-surface engineering are also rapidly expanding. The engineered adhesion of cells to synthetic matrices is of significant interest in biosensor and bioreactor design, and in the field of tissue engineering. At

Figure 4



Metabolic incorporation of unnatural monosaccharides into cell-surface glycoconjugates. **(a)** Mannosamine derivatives with unnatural acyl groups (R) are taken up by cells, converted to the corresponding sialic acids, and presented on the cell surface in the context of glycoconjugates [53]. **(b)** Metabolic incorporation of a chemically reactive functional group into cell-surface sialic acids for chemical remodeling of cell surfaces. The ketone group, derived from *N*-levulinoylmannosamine (the sialic acid precursor), can be covalently ligated with hydrazide-functionalized epitopes [55].

present, research in these areas has focused on tailoring material surfaces to the adhesion preferences of the cell [58,59]. With improved cell-surface engineering technology, we may soon be able to tailor cell surfaces for preferred material substrates. Cells endowed with novel catalytic or receptor-binding activity may find use in tissue targeting and remodeling. And as the entire genome sequences of increasingly complex organisms continue to be defined, the idea of designing living cells with new properties comes into the realm of possibility.

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