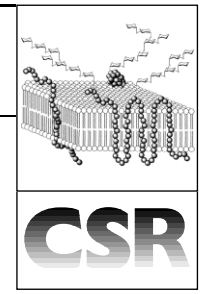
Chemical modification of mammalian cell surfaces



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The mammalian cell surface is a highly heterogeneous chemical environment with proteins, carbohydrates, lipids and composite molecules controlling vital cell functions. Chemical modification of this environment is a challenge due to the complexity of the surface chemistry and the fragility of the cell. Here, we review recent attempts to perform targeted, non-genetically controlled, changes to cell surface chemistry. Potential applications of cell surface engineering are presented.

1 Introduction

The cell membrane of mammalian cells is a complex composite of lipids, proteins and carbohydrates. These components work together to generate the sophisticated functions of the membrane, such as selective uptake of molecules into the cell, specific communication between the cell membrane and the extracellular matrix and direct contact with neighbouring cells.

The well-known lipid bilayer structure forms the barrier between the cell cytoplasm and its environment. The protein and carbohydrate components introduce selective interaction sites. Fig. 1 provides an overview of the different components of the cell membrane.

The cell membrane is a dynamic structure that turns-over its chemical constituents and changes its overall composition over time in response to its environment. For example, during development of a tissue the individual cell components influence tissue morphogenesis *via* changes in carbohydrate and protein handles on the outer surface of their membranes. This is demonstrated by the Notch receptor, which is involved in determining cell type in development. The addition of fucose residues to key extracellular residues of Notch by a fucosyltransferase called Fringe results in altered binding patterns for Notch receptor ligands and, hence, can alter cell morphology.[1] Detailed reviews of the structure and function of the mammalian cell membrane are available.[2]

The heterogeneity of the chemical structure of cell membranes makes them a challenging environment within which to introduce non-native chemical species. The challenge is further complicated by the need to ensure that chemical modification does not induce undesirable changes in the pattern of cell behaviour. However, despite the difficulties, the area of cell surface engineering has seen considerable progress in recent years. This review will highlight the broad range of approaches

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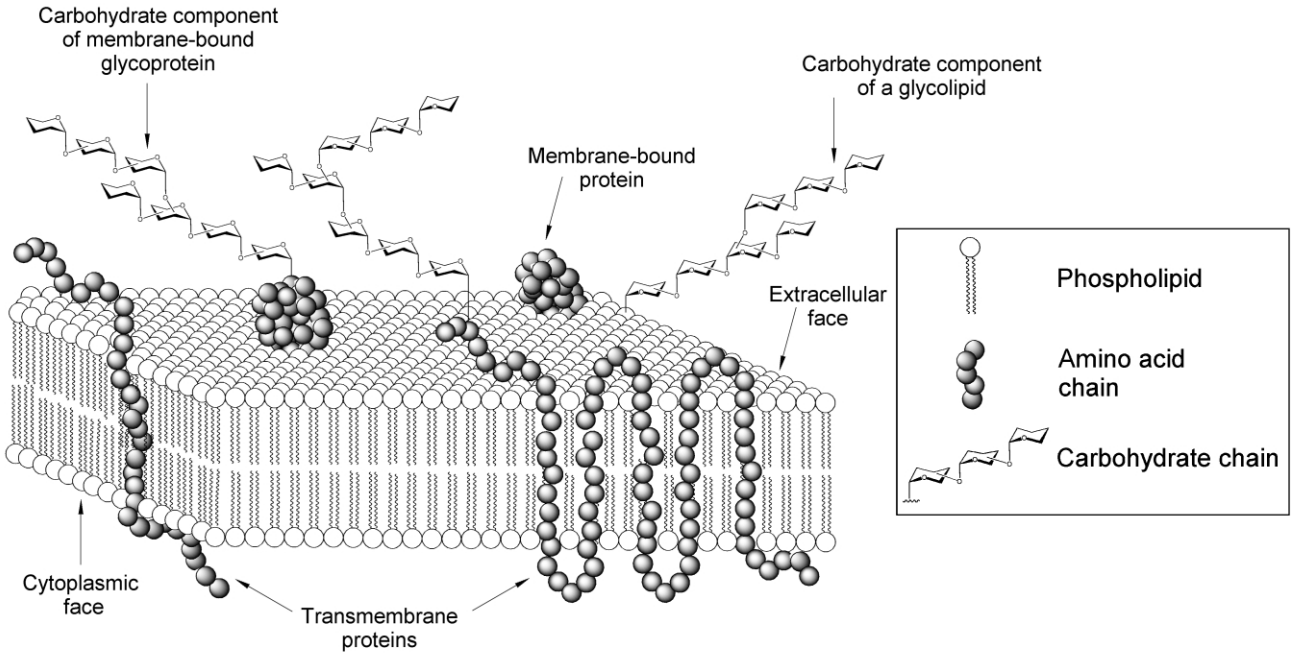


Fig. 1 A schematic representation of the eukaryotic cell membrane illustrating the associated lipid, glycolipid, protein and glycoprotein structures.

to alter cell surface chemistry using non-genetic methods. The review begins by considering applications of surface engineered cells.

2 Applications of surface engineered mammalian cells

The fundamental importance of cell surface interactions to cell and tissue function both *in vivo* and *in vitro* means that chemical approaches to control these interactions have a wide range of applications. The principal application of many of the techniques reviewed below is pure academic interest in the effect of cell surface modification on cell function. For example, a key technique for altering the expression of cell surface sugars is metabolic engineering.[3,4] This technique, reviewed in Section 3.4, enables non-natural carbohydrate residues, usually modified sialic acids, to be expressed in cell surface glycoforms by simply feeding the cells with a synthetic, non-natural precursor. This is a valuable tool to investigate the tolerance of biosynthetic enzymes to modifications in their substrates and the effects of slight structural modifications to cell surface oligosaccharide on events such as virus binding and cell adhesion. However, metabolic engineering and the other techniques of cell surface chemical engineering reviewed here also have potential pharmaceutical and biomedical implications, which are now being investigated. These can be broadly divided into two main areas:

2.1 Drug delivery

An increasing number of modern drugs and drug delivery systems consist of large molecules such as peptides and DNA. Delivery to a particular organ or cell type and subsequent uptake by the target can be severely hampered by the lack of specific receptors or transport mechanisms for the drug. However, by chemically engineering cell surfaces, it is possible to enhance their interactions with drugs and drug delivery systems. For example, Lee *et al.* have decorated cell surfaces with a synthetic adenovirus receptor using a metabolic engineering approach.[5] This artificial receptor facilitates the entrance of adenovirus into cells that are normally resistant to infection by this virus, potentially enabling gene therapy *via* such a strategy. Martin and Peterson have recently used the concept of surface engineering to insert synthetic receptors into cell surfaces for the uptake of exogenous proteins, controlling the selective permeability of the cell membrane to large drug molecules.[6] In addition, Liu *et al.* have described a method of tagging tumour cells by metabolic incorporation of a non-natural sugar into the polysialic acid molecules on the cell surface. By adjusting the dose and timing of administration of the non-natural sugar they were able to control the targeting of an antibody, specific for the altered polysialic acid, and hence selectively killed tumour cells.[7]

2.2 Tissue engineering and cell-based therapies

The aim of tissue engineering is to promote the regeneration of tissues from populations of cells. As cell-to-cell and cell-to-matrix interactions are of huge importance in the development or repair of a tissue, there is considerable potential for the use of cell surface engineering as a tool in this field.

One major area of interest for cell surface engineering is blocking the cell-to-cell recognition that triggers immune rejection of a foreign cell or tissue. For instance, the transplant of pancreatic islets, the insulin-producing units of the pancreas, from a donor to a patient is a potential cure for insulin-dependent diabetes. The major problem with this therapy is the immune rejection of the islets by the host. However, in an effort to combat this, Panza *et al.* have encapsulated pancreatic islets in poly(ethylene glycol) (PEG), which has a protein and cell repellent action, thus blocking the binding of immune cells to the foreign tissue.[8]

Another area of great interest in tissue engineering is the repair of nerve damage, particularly to the spinal cord, with recent results suggesting that cell surface engineering can be used to assist such nerve regeneration. By metabolically engineering the surface of neurons with a modified sialic acid, Büttner *et al.* have demonstrated that the length of neurites can be more than doubled compared to control cells.[9]

Our own group has also reported the use of cell surface engineering to induce rapid cell aggregation. The structure and function of a tissue or organ is significantly influenced by intimate cell–cell and cell–matrix interactions. By generating

three-dimensional clusters of cells, we promote these interactions and also decrease the mobility of individual cells, which is beneficial for preventing migration from the site of injection in cell-based therapeutics. This aggregation technique is based on cell crosslinking using avidin-biotin bridges built on specifically oxidized cell surface sites.[10]

The remainder of this review will focus on the methods used to engineer the molecular landscape of the cell surface and highlight areas of significant potential for biomedical applications.

3 Chemical strategies to engineer cell surfaces

3.1 Insertion of molecules into cell membranes

The lipophilic nature of the mammalian cell membrane has been exploited by a number of groups in order to display bioactive molecules, both naturally occurring and synthetic, on the cell surface. To achieve this insertion, a fatty tether is attached to the biomolecule of interest and, when applied to the cell, the fatty moiety incorporates into the membrane, leaving the biomolecule exposed on the cell surface. This has been achieved using two main classes of compound, namely GPI-anchored proteins and cholesterol-tethered compounds.

3.1.1 GPI-anchored proteins. Rather than spanning the lipid bilayer, a number of naturally occurring cell surface proteins are covalently linked to lipids during post-translational modification and are thus anchored directly to the membrane. Amongst these are the glycosylphosphatidylinositol-(GPI) anchored proteins, which include some cell surface receptors, enzymes and antigens.[11] As depicted in Fig. 2, this structure has a conserved core throughout nature, consisting of ethanolamine phosphate, a trimannoside unit, glucosamine and the inositol phospholipid anchor. Various substituents on the carbohydrate residues also exist and contribute to the function of the anchor.

When GPI-anchored proteins are removed from a cell membrane and subsequently applied to other cells, they efficiently insert into the host membrane conferring their full biological functionality.[12] This has been demonstrated with a number of GPI-linked proteins, with the major work to date concerned with the treatment of paroxysmal nocturnal haemoglobinuria (PNH). This disease, caused by a deficiency in a class of GPI-anchored protein, affects red blood cells and results in haemolysis, thrombosis and bone marrow failure. It has been demonstrated that GPI-anchored peptides that suppress complement-mediated lysis, such as decay accelerating factor (DAF) and CD59, can be effectively incorporated into erythrocytes from PNH patients, protecting them from lysis *in vitro*.[13] Another potential therapeutic application for GPI-anchored proteins is tumour immunotherapy. Many tumours evade the immune response by lacking one of the two signalling factors necessary to stimulate T-cells. It has recently been demonstrated that these missing co-stimulatory factors can be transferred to the surface of tumour cells using the GPI anchor. When EG7 tumour cells were treated with GPI-linked B7-1 (GPI-B7-1), a co-stimulatory adhesion molecule, the cells stably incorporated the peptide on their surfaces.[14] To investigate the potential for immunotherapy using GPI-B7-1, mice were immunized with tumour cell membranes in the presence or absence of interleukin-12 (IL-12), a cytokine involved in T-cell and natural killer cell activation. The animals were then challenged with intact EG7 tumour cells and the incidence of tumours measured. Mice that had been immunized with GPI-B7-1-treated membranes showed no incidence of tumours, whereas, of those that were injected with untreated EG7 membranes, nearly 75% developed cancer. In the absence of GPI-B7-1, IL-12 exhibited no anti-tumour effect (Fig. 3).

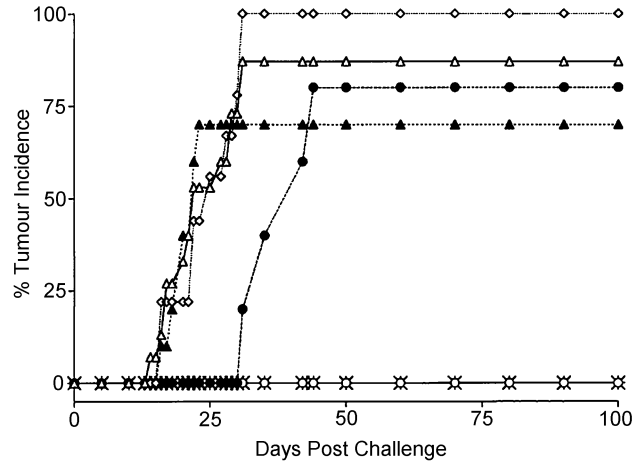


Fig. 3 Demonstration of the potential for the use of GPI-anchored proteins in tumour immunotherapy. Mice were immunized with buffer (△), IL-12 (◇), EG7 membranes (▲), EG7 membranes + IL-12 (●), EG7 membranes treated with GPI-linked B7-1 (○) or EG7 membranes treated with GPI-linked B7-1 + IL-12 (*). Following immunization, the animals were challenged with EG7 tumour cells. Reprinted with permission from R. S. McHugh *et al*. ref. 14. © 1999 the American Association for Cancer Research.

These examples demonstrate that protein transfer using GPI anchors has great therapeutic potential for disorders and diseases where cell surface molecules are aberrant. However, work in this area has almost exclusively involved the use of purified GPI-linked proteins from natural sources. If synthetic GPI anchors were readily available, it would be possible to link these *via* their terminal amine functionality to peptides or other bioactive molecules using standard coupling chemistry. To date, a small number of GPI anchors found in both prokaryotes and eukaryotes have been synthesized. However, until recently, there was no variable strategy for the synthesis of different GPI anchors and the attachment of a peptide had been largely overlooked. Using the retrosynthesis depicted in Scheme 1, Pekari and Schmidt successfully synthesized and fully characterized the GPI anchors for rat brain Thy-1 and scrapie protein in their water-soluble and lipidated forms.[15] More recently, Xue *et al.* have demonstrated the first total synthesis of a GPI-anchored peptide, the sperm CD52 GPI anchor coupled to a dipeptide CD52 fragment.[16] The efficiency of these strategies and the use of versatile building blocks should enable other GPI anchors to be synthesized and potentially used for the transfer of therapeutic biomolecules to cell surfaces.

3.1.2 Cholesterol-tethered molecules. A method of cell surface engineering analogous to the use of GPI anchors is the transfer of cholesterol-tethered molecules into the lipid bilayer. As with GPI anchoring, the insertion of cholesterol into the cell membrane has the potential to be used for any biomolecule of

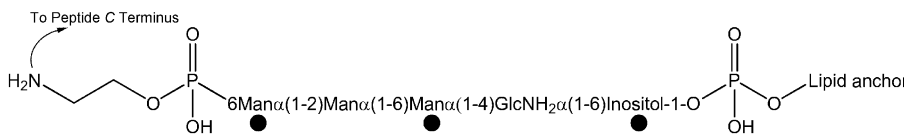
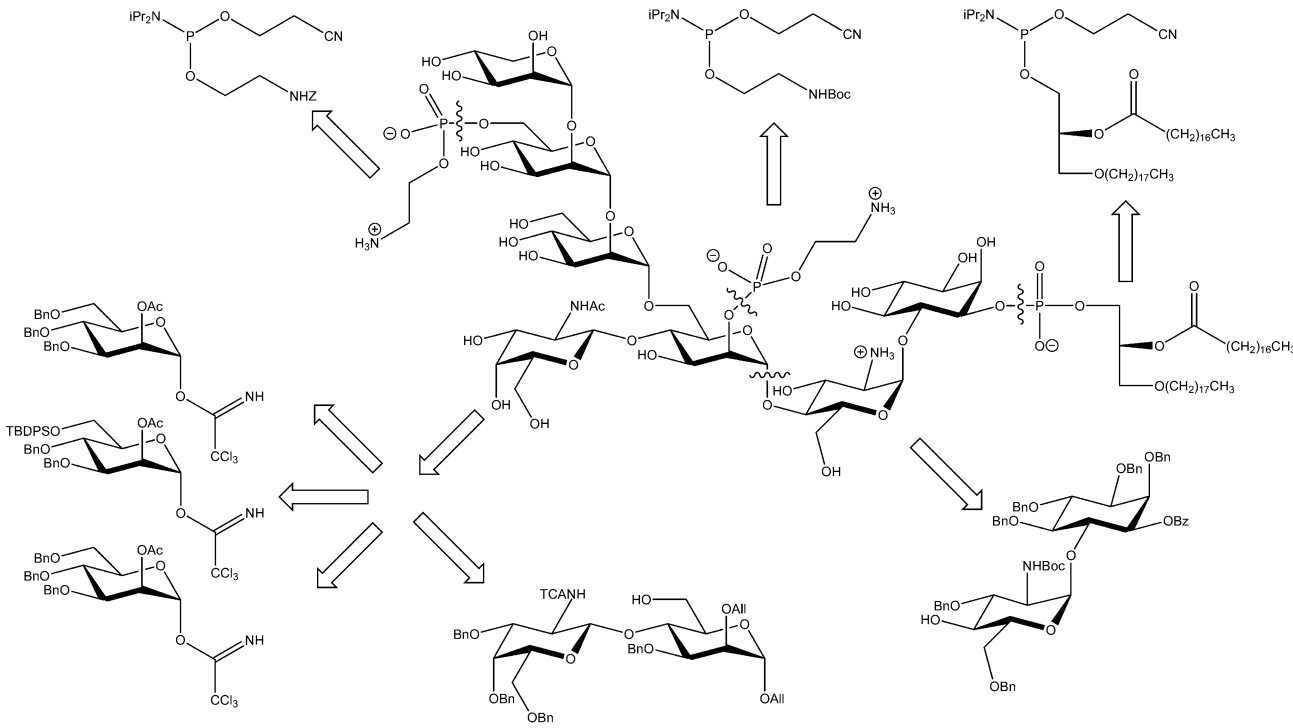


Fig. 2 The conserved core structure of the GPI anchor with the locations of common substitutions (●).



Scheme 1 Retrosynthesis of the rat brain Thy-1 GPI anchor.

interest. However, recent work by Hussey *et al.* has utilized the mechanism by which some viruses and bacterial toxins gain entry to eukaryotic cells to develop a novel peptide delivery system. Normally, these viruses and toxins bind to low molecular weight receptors composed of an oligosaccharide headgroup linked to ceramide. These receptors are located in sphingolipid-rich sub-domains of the plasma membrane, which also contain high levels of cholesterol, and binding of the infectious agent stimulates clathrin-mediated endocytosis *via* a poorly understood mechanism, transferring the complex into the cell. Peterson has demonstrated that this mechanism can be utilized to provide artificial receptors on cell surfaces, potentially assisting the uptake of therapeutic agents. Initially, *N*-cholesterylglycine-fluorescein, a fluorescent "memtigen" (membrane-anchored antigen), derived from 5-aminofluorescein and 3β-cholesterylamine was synthesized (Fig. 4). When

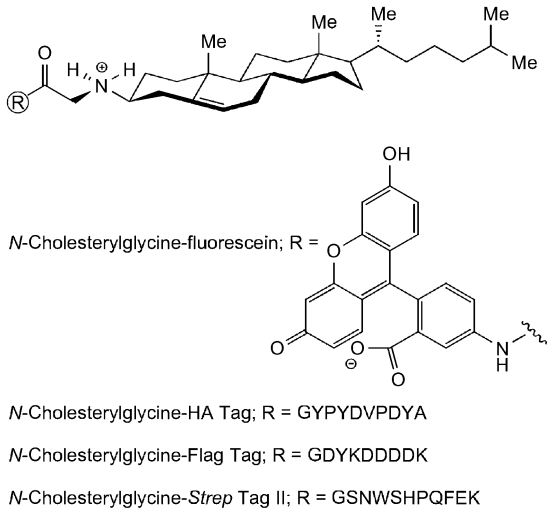
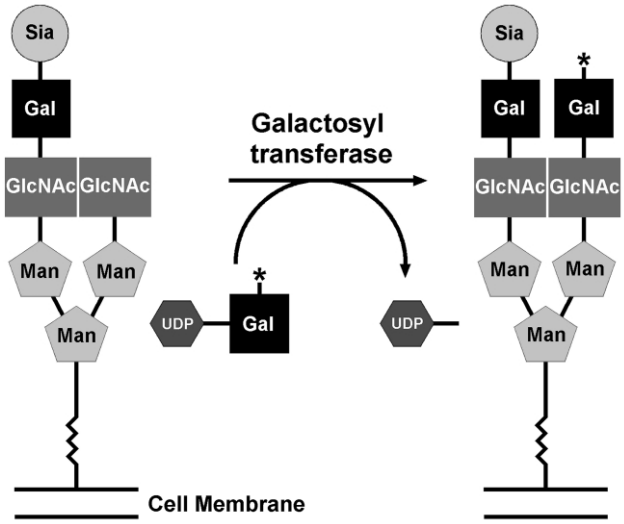


Fig. 4 Structures of *N*-cholesterylglycine-fluorescein and protein-binding non-natural receptors used in "synthetic receptor targeting" studies.

this molecule was incubated with Jurkat lymphocytes at 10 μM, all of the cells exhibited an intense green fluorescence on their membranes after one hour.[17] To examine the ability of this molecule to act as an artificial receptor, cells were then incubated with an antibody against fluorescein that possessed a red fluorescent marker. Within four hours, > 99 % of cells exhibited intracellular red and green fluorescence, which was shown to be a result of endocytosis of the fluorescein–antibody complex. To extend this technique of "synthetic receptor targeting", more complex non-natural receptors consisting of protein-binding peptides tethered to 3β-cholesterylamine were synthesized (Fig. 4). Insertion of these molecules into the lipid bilayer and subsequent treatment with antibodies against the peptide sequences resulted in their clathrin-mediated endocytosis.[6] Using this method, a functional enzyme, β-galactosidase, was delivered to and subsequently exhibited activity in Jurkat cells, suggesting that this technique may be of use as a drug or gene delivery system.

3.2 Reactions using exogenous enzymes

The use of enzyme-catalyzed chemical transformations in organic synthesis is now widely recognized as a viable alternative to traditional methods, particularly in carbohydrate chemistry, where enzymes are widely employed in the formation of glycosidic bonds. Such reactions can be performed on the cell surface, utilizing the existing surface glycoforms as acceptors for reactions with an exogenously applied glycosyltransferase and appropriate activated sugar donor. For example, cells or isolated membranes can be readily radiolabelled by incubation with bovine galactosyltransferase and UDP[^{14}C/^{3}H]galactose as depicted in Scheme 2. The activated, labelled sugar donor is transferred to the acceptor structure, in this case *N*-acetylglucosamine residues, resulting in radioactively labelled glycoconjugates.[18] However, for altering the chemical landscape of cells in order to influence cellular function, the transfer of natural monosaccharide residues to surface glycoconjugates is not sufficient. Work involving enzyme-catalyzed glycosidic bond formations has demonstrated that certain enzymes, especially some sialyl- and fucosyltransferases can tolerate analogues of their natural substrates that possess various substituent groups. Hence, application of these tolerant enzymes and unnatural sugar donors to cell surfaces results in the transfer of the modified carbohydrate to existing glyco-



Scheme 2 Simple cell surface engineering by application of exogenous galactosyltransferase and its radiolabelled substrate. Labelled, activated galactose (UDP-Gal) is transferred to terminal *N*-acetylglucosamine (GlcNAc) residues in existing cell surface glycoforms. The other carbohydrate residues depicted are mannose (Man), and sialic acid (Sia).

forms. A striking example of this was demonstrated by Srivastava *et al.* using a fucosyltransferase, Le-FucT, isolated from human milk. In nature, this enzyme uses GDP-fucose as an activated donor to transfer a single fucose residue to particular carbohydrate acceptor sequences. It was shown, however, that Le-FucT can tolerate very large substituents at the C-6 position on the fucose ring, demonstrated by the successful transfer of a synthetic GDP-fucose analogue that contained the blood group B antigen (Fig. 5). Incubation of this fucose derivative and Le-

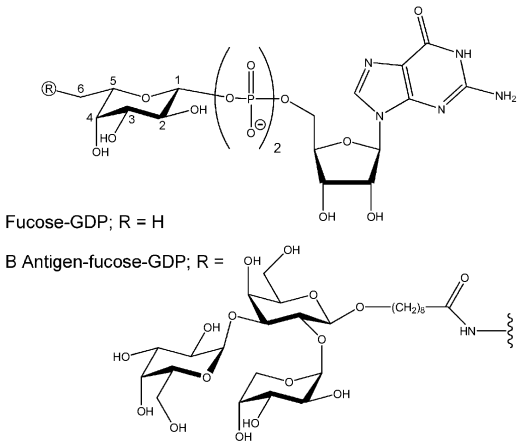


Fig. 5 The structures of fucose-GDP, the natural substrate of the Le-FucT fucosyltransferase, and an example of the huge substitutions tolerated by the enzyme at the C-6 position.

FucT with type O erythrocytes resulted in its transfer to suitable donors within cell surface glycoproteins, conferring the B phenotype to the cells as demonstrated by agglutination with an anti-B blood group antibody.[19]

Sialyltransferases have also been utilized for the chemical engineering of cell surfaces. However, unlike the use of Le-FucT, existing sialic acids must be enzymatically cleaved from cell surface glycoconjugates before transfer of the artificial sugars. Using such a procedure, Herrler *et al.* investigated the transfer of various sialic acids with substituents at the C-9 position to erythrocytes (Fig. 6). All analogues tested were incorporated into cell surface glycoconjugates using rat liver Galβ1,4GlcNAc α2,6-sialyltransferase, but various functional alterations were observed when the cells were then incubated with the influenza C virus, which usually binds to cells *via* sialic acids with an *O*-acetyl group attached to the C-9 position.[20]

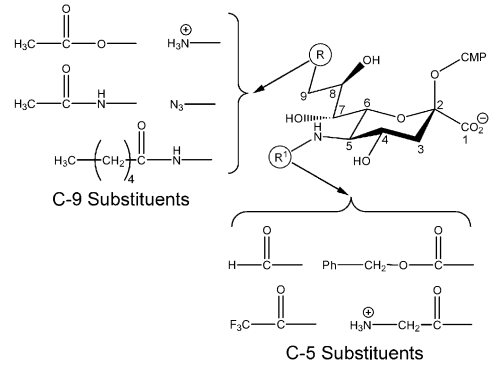


Fig. 6 Structures of sialic acids with C-5 and C-9 substituents tolerated by various sialyltransferases.

Transfer of 9-azido-, 9-amino- and 9-hexanoylamido-substituted sialic acids to erythrocyte surfaces abolished recognition by the virus. The only cells which displayed virus binding were those displaying an *O*- or *N*-acetyl group at C-9, suggesting that this moiety is a vital for the recognition process. More recently, Gross and Brossmer have demonstrated that four different sialyltransferases are capable of utilizing synthetic sialic acids with various C-5 substituents[21] (Fig. 6). Although this demonstration was not performed using cells, it increases the range of structural groups tolerated by sialyltransferases for transfer to cellular sialoglycoconjugates. However, the exogenous application of tolerant glycosyltransferases and sugar donor analogues has recently progressed into and, to a certain extent, been superseded by the utilization of endogenous metabolic machinery to engineer cell surfaces.

3.3 Inhibition of biosynthetic pathways

Since the molecular complexity and functionality of the cell surface is greatly influenced by the glycosylation of proteins and lipids, inhibition of carbohydrate metabolism presents an alternative strategy for manipulating the surface chemistry of cells. The numerous steps required to convert a monosaccharide to an active sugar donor and subsequently transfer it to a growing oligosaccharide chain necessitate a diverse complement of enzymes. This, in contrast to peptide biosynthesis for example, enables the inhibition of specific enzymes, giving the potential to subtly manipulate surface glycosylation. The development of potent and selective glycosylation inhibitors is currently of great interest for a number of therapeutic applications, with oligosaccharyl transferases, glycosyltransferases and glycosidases the target enzymes.[22–24] Inhibitory molecules range from natural products, such as carbohydrate mimetic alkaloids from plants and microorganisms,[25] to specifically designed synthetic drugs. Most inhibitors exert their effects by competing with the natural enzyme substrates (sugar donor or acceptor species), acting as transition state analogues of the enzyme–substrate complex or behaving as decoys for glycoside biosynthesis.[26]

Since the structures of cell surface glycoforms are important in recognition and signalling common therapeutic targets of glycosylation inhibitors include cancer and autoimmune diseases. For example, castanospermine (Fig. 7), a naturally

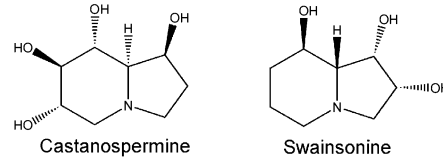


Fig. 7 Structures of the alkaloid glycosidase inhibitors castanospermine and swainsonine.

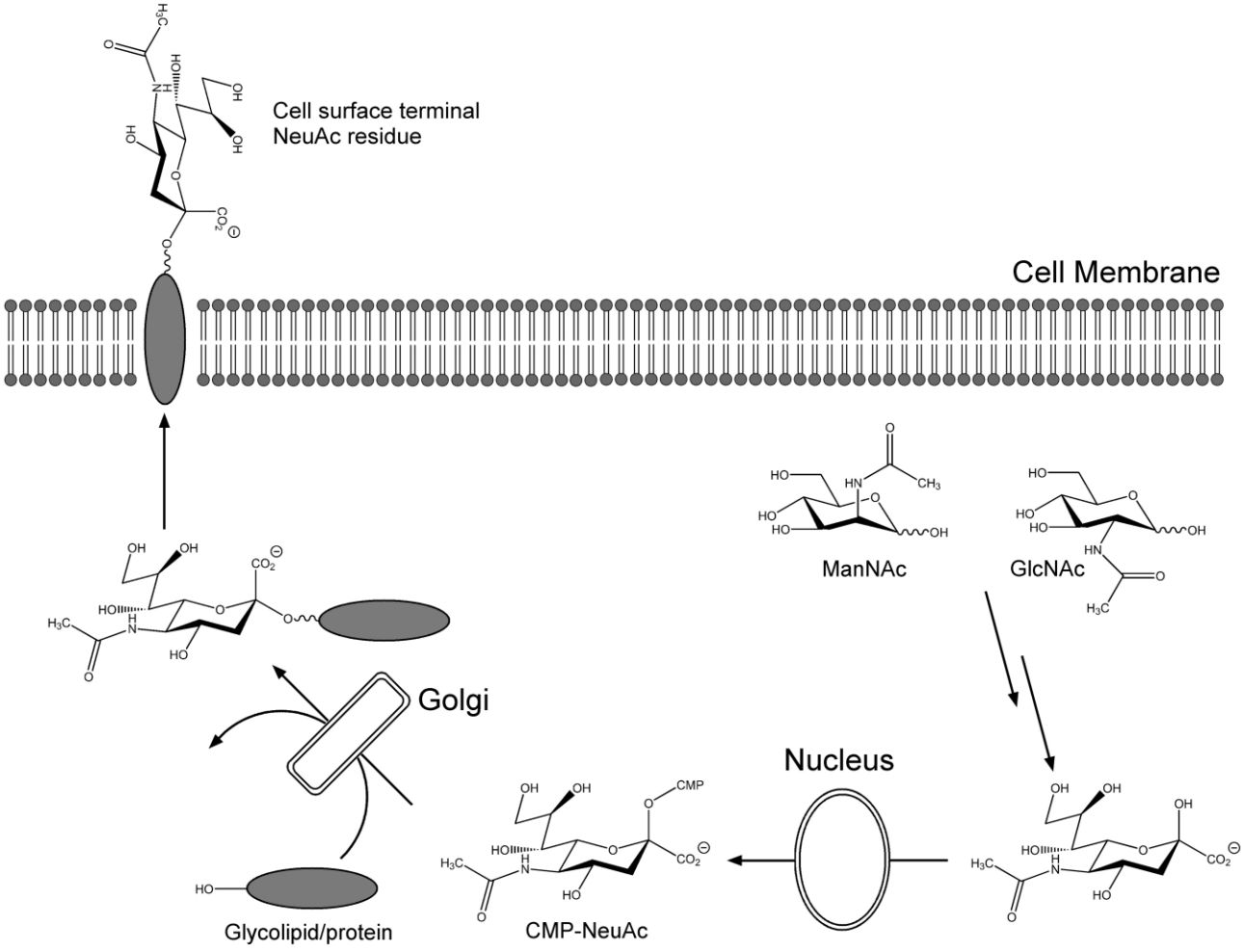
occurring indolizidine alkaloid, has recently been shown to prevent experimental autoimmune encephalomyelitis (EAE), a

murine model of multiple sclerosis, by altering cell surface glycosylation.[27] This disease is caused when T-cells infiltrate the central nervous system and are presented with myelin antigens, causing them to release interleukin-2 (IL-2). IL-2 in turn, binds to the IL-2 receptor on T-cells and causes an autocrine clonal expansion and autoimmune attack. The subsequent demyelination of nerves results in disrupted nerve transmission manifested as symptoms such as numbness, tremors, weakness, pain, seizures and paralysis. Castanospermine inhibits glucosidase enzymes, which are important in the processing of oligosaccharides in the early stages of the *N*-linked glycosylation pathway. Inhibition of glucosidases, rather than preventing *N*-glycosylation, results in "immature" cell surface *N*-linked glycoforms instead of more complex structures. This quantitative reduction in cell surface glycosylation, when applied to the EAE model, reduced signal transduction, *via* the IL-2 receptor in a concentration-dependent manner, preventing clonal expansion of T-cells and autoimmune attack. A similar alkaloid, swainsonine, has undergone clinical trials as an anti-tumour compound, exhibiting a similar mechanism of action.[28] Increased *N*-glycosylation has been shown to contribute to cancer metastasis and swainsonine (Fig. 7), by inhibiting Golgi α-mannosidase II, reduces complex oligosaccharide structures thereby slowing the development of tumours.

3.4 Metabolic engineering

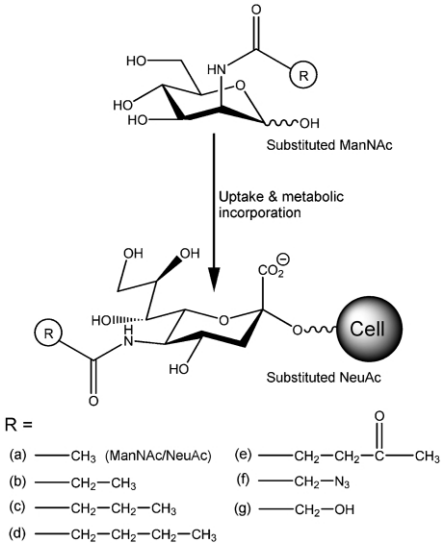
An alternative strategy available for altering the chemical functionality of cell surfaces is metabolic engineering. Certain enzymes involved in the biosynthesis of cell surface molecules are tolerant to a degree of structural variability in their substrates. This means that cells can be incubated with unnatural synthetic precursors of cell surface moieties, which are taken up and metabolized, resulting in the incorporation of the unnatural structures on the exterior of the cell. The best-known example of this is the incorporation of unnatural sialic acid precursors into cell surface glycoforms. Sialic acids are the most common terminal sugar residue on the cell surfaces and have been demonstrated to be important in such functions as cell adhesion and recognition. On mammalian cells, the most common sialic acids are *N*-glycolyneuraminic acid (NeuGc) and *N*-acetylneuraminic acid (NeuAc), although NeuGc is absent from human cells. The biosynthesis of these sugars is a multistep pathway, which begins with *N*-acetylglucosamine (GlcNAc) or *N*-acetylmannosamine (ManNAc) as precursors. These sugars are converted to CMP-NeuAc, the activated sugar donor, which is transferred to glycolipids or glycoproteins by a membrane-bound sialyltransferase in the Golgi before the glycoconjugate is exported to the cell surface (Scheme 3). NeuGc is synthesized by the conversion of CMP-NeuAc to CMP-NeuGc by CMP-NeuAc hydroxylase, if present, and is subsequently transferred to cell surface glycoforms in the same manner as NeuAc.

It was demonstrated by Kayser *et al.* that the enzymes in this pathway can tolerate *N*-acyl substituents of sialic acid precursors, resulting in the expression of modified sialic acids with C-5 substituents on the cell surface.[3] For example, when applied to rats *in vivo*, the *N*-propanoyl analogues of GlcNAc and ManNAc were taken up by cells and metabolically incorporated into cell surface glycoconjugates as *N*-propanoylneuraminic acid (NeuPr). This sialic acid, which does not occur naturally, was incorporated in the membrane glycoproteins of all organs tested, with *N*-propanoylmannosamine (ManNPr) a more



Scheme 3 The biosynthesis of NeuAc from ManNAc or GlcNAc and its subsequent transfer to cell surface glycolipids or glycoproteins.

effective substrate than the equivalent glucosamine derivative. In addition to ManNPr, a number of different *N*-acylmannosamines have now been synthesized and metabolically incorporated into the cell surface glycoforms of a variety of cell types.[4] These include *N*-butanoyl-, *N*-pentanoyl-, *N*-glycolyl-, *N*-levulinoyl- and *N*-azidoacetylmannosamine (Scheme 4). The



Scheme 4 Metabolic engineering of cell surface glycoforms using synthetic ManNAc derivatives. These compounds are taken up by cells and metabolized to the corresponding non-natural sialic acids.

latter two ManNAc derivatives contain chemically reactive functionalities and will be covered in more depth later in this review.

This approach has now been exploited to structurally alter the cell surface sialic acids of a number of cell types, with functional results that have potential therapeutic applications. For instance, Jennings and co-workers have demonstrated that tumour cells can be targeted for immunotherapy in this way. Certain important cancers express cell surface α2–8 polysialic acid. Incubating such tumour cells with ManNPr resulted in the *N*-acetyl groups of cell surface α2–8 polysialic acids being substituted with *N*-propanoyl groups. The resulting α2–8 *N*-propanoylated polysialic acid could then be targeted by a monoclonal antibody (mAb 13D9), which specifically recognizes polysialic acids that contain NeuPr. Treatment with this antibody induced cell lysis in a manner dependent on the concentration of ManNPr with which the cells were incubated (Fig. 8). When ManNPr and mAb 13D9 were applied to an *in vivo* solid tumour model, metastasis of the tumour cells was controlled, although the tumours were not destroyed.[7]

Examples also exist of the potential benefits of metabolically engineering sialic acids for the regeneration of damaged nervous tissue. Collins *et al.*, have described the engineering of cell surface sialic acids of a neuroblastoma–glioma hybrid cell line (NG108-15). Of interest was the effect of this engineering on the binding of myelin-associated glycoproteins (MAG) to the cells. This protein is a sialic acid binding lectin, involved in stabilizing the myelin sheath around nerve axons and its binding has been implicated in the failure of injured nerves to regenerate. It was shown that incubation of these cells with *N*-glycolylmannosamine pentaacetate resulted in the expression of NeuGc on the cell surfaces, with the five acetate groups removed by intracellular esterases. After 5 days incubation, this sialic acid, which is not normally expressed by this cell type, constituted 70–80% of the total cell surface sialic acid component. In addition, the binding of MAG was almost completely abolished in treated cells, while binding remained unchanged in cells incubated with ManNAc tetraacetate as a negative control.[29] Another example of nerve regeneration was

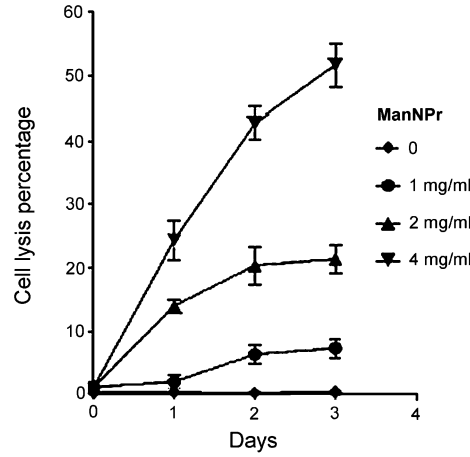
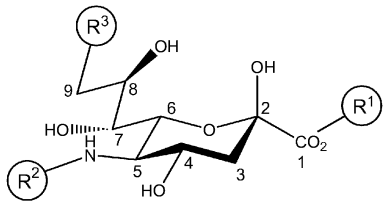


Fig. 8 Immunotargeting tumour cells by metabolic engineering of their cell surfaces. RBL-2H3 tumour cells were incubated with different concentrations of ManNPr for 3 days and subsequently incubated with a monoclonal antibody that recognizes propanoylated α2–8 polysialic acid. Reprinted with permission from T. M. Liu *et al.* ref. 7. © 2000 the American Society for Biochemistry and Molecular Biology.

reported by Büttner *et al.*. When PC12 cells, a rat neuronal cell line, and primary small cerebellar granule cells were treated with ManNPr, a significant increase in neurite outgrowth (as much as >120%) was observed compared to control cells.[9] This result was dependent on the growth substrate employed, with laminin- and collagen I-coated dishes more effective than poly-D-lysine, suggesting the involvement of integrin receptors, in which sialic acid acids are important mediators of binding. It was also shown for the first time that this metabolic engineering approach caused the expression of a number of cytosolic proteins to be altered, with most downregulated. The mechanism for this phenomenon has not yet been elucidated although, as sialic acid is the only monosaccharide known to be activated in the nucleus, the presence of the non-natural CMP-NeuPr may be affecting transcription.

The importance of sialic acids in cell adhesion molecules and cell surface receptors means that metabolically altering the structure of the surface sialic acid complement is also likely to affect cell recognition and adhesion events. This was effectively demonstrated by Keppler *et al.*, who incubated kidney epithelial cells and a B lymphoma cell line with *N*-propanoyl-, *N*-butanoyl- and *N*-pentanoylmannosamine and then examined the ability of two polyoma viruses to bind to the cells. All three precursors were metabolized into the corresponding unnatural cell surface sialic acids by both cell types and were shown to influence virus binding. Depending on the *N*-acyl substituent, virus infection was either inhibited by up to 95% or increased by up to 7 times that of control cells.[30] This was attributable to cell surface virus receptors expressing the elongated *N*-acylsialic acids, thus altering binding affinities of the viruses and this has since been demonstrated with several other viruses. Alterations in cell–cell interactions by metabolic delivery of *N*-acylsialic acids has also been observed in human fibroblasts, where the normal contact-dependent inhibition of cell growth was abolished by incubation with ManNPr.[31]

A very recent development in the metabolic engineering of cells is the use of unnatural sialic acid analogues. It was widely believed that an efficient sialic acid uptake mechanism was absent from eukaryotic cells. However, Oetke *et al.* have now established that exogenous NeuAc is readily taken up by both human cell lines and primary cells, then incorporated into glycoconjugates.[32] Subsequent work has demonstrated that a number of synthetic sialic acid analogues with distinct, varied substitutions at the C-1, C-5 or C-9 positions are readily taken up and expressed on mammalian cell surfaces, by conversion to the CMP-sialic acid and transfer to glycoconjugates by sialyltransferases (Fig. 9).[33] The use of such compounds has



Sialic Acid	R^1	R^2	R^3
NeuAc	H	CH_3CO	HO
9-Deoxy-NeuAc	H	CH_3CO	H
9-Iodo-NeuAc	H	CH_3CO	I
9-Thio-NeuAc	H	CH_3CO	HS
9-SCH_3-NeuAc	H	CH_3CO	CH_3S
9-SO_2CH_3-NeuAc	H	CH_3CO	CH_3SO_2
5-*N*-Fluoroac-NeuAc	H	FCH_2CO	HO
5-*N*-Trifluoroac-NeuAc	H	CF_3CO	HO
5-*N*-Thioac-NeuAc	H	CH_3CS	HO
NeuAc-Me-ester	CH_3	CH_3CO	HO
NeuAc-Et-ester	CH_3CH_2	CH_3CO	HO

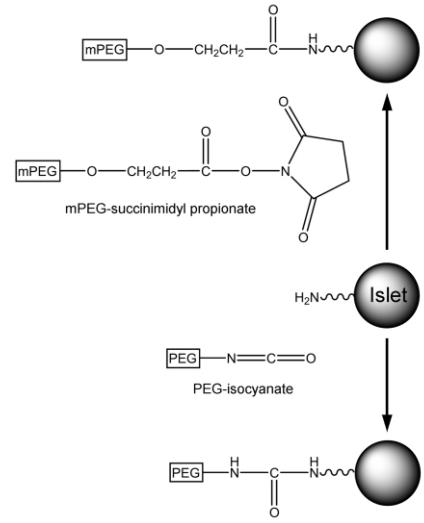
Fig. 9 Synthetic NeuAc derivatives that have been successfully used to metabolically engineer cell surfaces.

certain advantages over the sialic acid precursor analogues such as ManNPr. To date, these derivatives have only been able to deliver substituents at the C-5 position of sialic acid due to the enzymes early in the pathway being intolerant of C-9 substituents. In addition, the mannosamine derivatives only contain six of the carbons present in the resultant sialic acids (C-4 to C-9), which precludes the possibility of incorporating substituents at all possible points on the molecule. The initial biological results with these new sialic acid analogues reveal that, as with the *N*-acylmannosamine derivates, biological activity is dictated by the type of substitutions present. For example, the binding of a sialic acid-binding lectin, CD22, to a human B lymphoma cell line was enhanced following treatment with *N*-glycolylneuramininc acid, but abolished by treatment with 9-iodo-*N*-acetylneuraminic acid. This early data opens another avenue for the metabolic engineering of cell surfaces, with perhaps the incorporation of substituents on all carbons of sialic acid being possible. This combined with the techniques described below for the covalent ligation of biomolecules to cell surfaces has enormous potential for modulating cell behaviour.

3.5 Covalent ligation to cell surface chemical groups

A final technique that enables the chemical modification of cell surfaces is the application of direct covalent reactions. There are two means of achieving this. Firstly, chemical functionalities on the cell surface such as amines or thiols can be used to ligate suitably reactive molecules. The second approach involves generating cell surface functional groups that are not normally present on cells and directing molecules with complementary reactivity towards them.

3.5.1 Reactions with native cell surface species. Although there are numerous commercially available reactive probes for labelling cells *via* their exposed functional groups, attempts to alter the behaviour of living cells or tissues using this technique have been extremely limited. However, two recent examples in the literature suggest that this may be a viable technique in the area of tissue engineering, where implants are particularly susceptible to recognition and destruction by host immune cells. This may be reduced or eliminated by encapsulation of the implanted cells in a biocompatible polymer, such as poly(ethylene glycol) (PEG). Rather than simply embedding cells in a polymer matrix, amine-reactive PEGs have been used to covalently attach the polymer to the extracellular proteins surrounding pancreatic islets as a possible mechanism to shield them from immune targeting when transplanted as treatment for insulin-dependent diabetes mellitus. PEG was attached to the surface of pancreatic islets by incubation for up to one hour with either PEG-isocyanate or monomethoxy-PEG-succinimidyl propionate (Scheme 5).[8,34] This resulted in the islets being

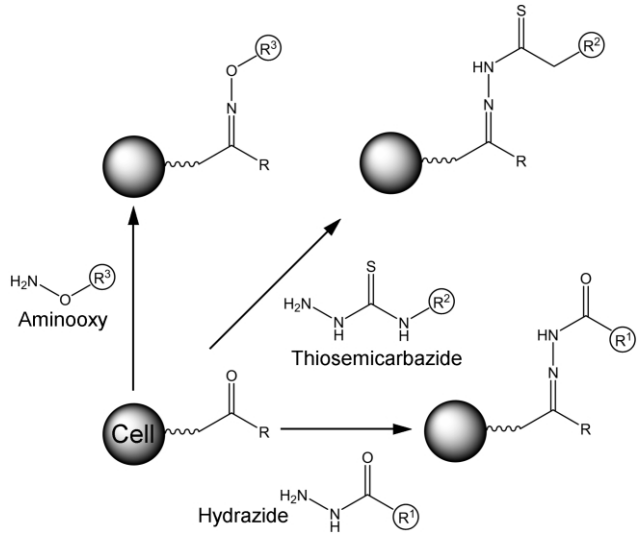


Scheme 5 The poly(ethylene glycol) encapsulation of pancreatic islets by reaction of free surface amines with amine-reactive PEG species.

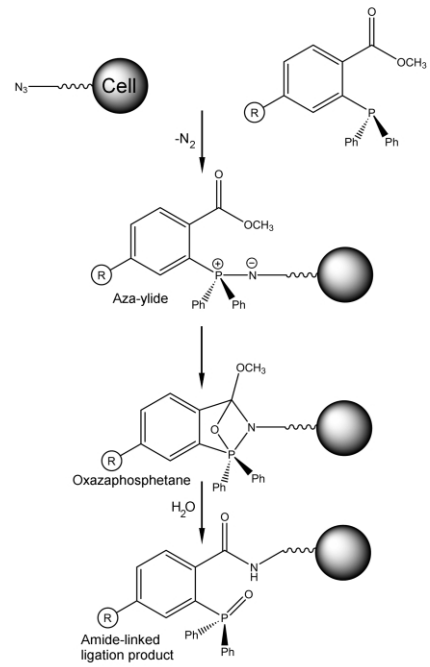
completely surrounded by PEG molecules and did not significantly affect cell viability, insulin secretion or response to glucose challenge in comparison to untreated cells. Such a technique offers real potential for applications such as this, where non-specifically altering cell surface architecture is acceptable. However, this lack of specificity when targeting natural cell surface functionalities with reactive molecules could be a major drawback for some applications. For example, covalently binding an amine-reactive probe to all free cell surface amines is likely to severely disrupt the function of the cell, especially if the binding sites of receptors and enzymes are blocked. However, other approaches exist to overcome this problem.

3.5.2 Reactions with non-native cell surface species. To enable a more selective approach, the generation of non-native reactive groups at specific sites on cell surface molecules is an alternative approach for engineering the molecular landscape of the cell. Current attention is focused on the generation of two distinct types of chemical groups, reactive carbonyls (in the form of aldehydes and ketones) or azides. These species are not normally found on the surface of cells and therefore can be used to chemoselectively ligate suitably functionalized molecules to them. The introduction of cell surface aldehyde and ketone groups enables biomolecules with hydrazide, aminooxy or thiosemicarbazide functionalities to be selectively ligated (Scheme 6).[35] The generation of azides on cell surfaces, however, necessitates an alternative strategy for the chemoselective ligation of molecules of interest. To achieve this, Bertozzi and co-workers employed a modification of the Staudinger reaction, which occurs between an azide and a phosphine, to ligate a triarylphosphine to non-native cell surface azides, generating a stable amide-linked adduct (Scheme 7).[36,37] The incorporation of these functional groups has been achieved using three alternative methods, namely application of exogenous enzymes, direct chemical reaction and metabolic engineering.

3.5.2.1 Generation of cell surface aldehydes. For many years, aldehyde groups have been routinely introduced at specific sites



Scheme 6 Chemoselective ligation reactions of cell surface aldehydes and ketones.

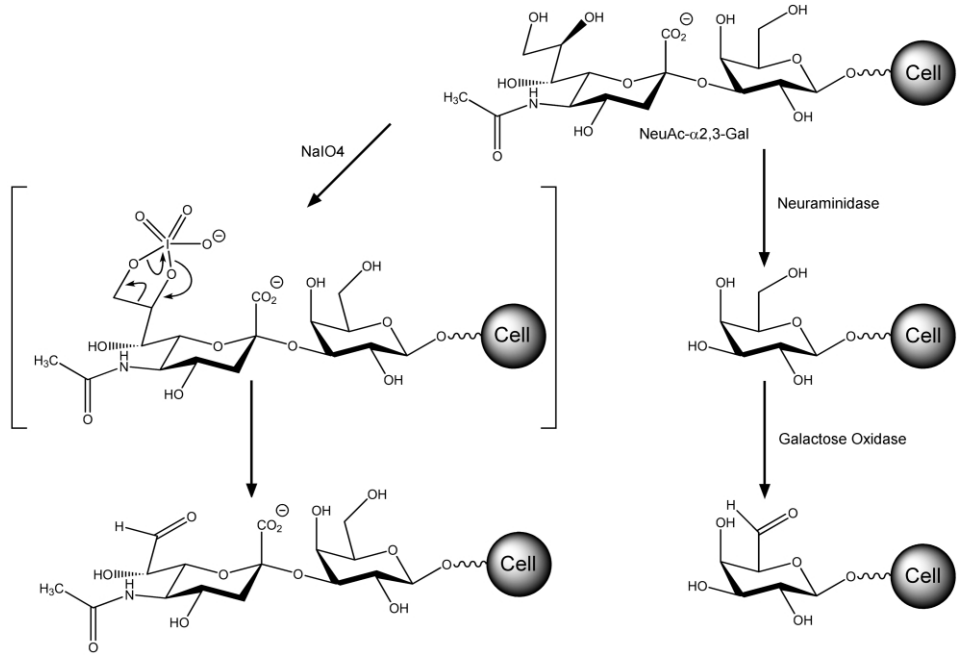


Scheme 7 Reaction of cell surface azides with triarylphosphine species *via* the modified Staudinger reaction.

in cell surface glycoconjugates for their detection, characterization and purification. Little attention, however, has been given to the utilization of these to engineer living cells. The first method by which this may be achieved is the application of exogenous galactose oxidase, which oxidizes terminal galactosyl and *N*-acetylgalactosaminyl residues. These sugars are often the penultimate residues in surface glycoforms and are not easily recognized. In order to oxidize these, the terminal monosaccharide residue must be cleaved and, since this is invariably sialic acid, neuraminidase treatment precedes the application of galactose oxidase (Scheme 8).[18]

A simpler method for the introduction of aldehydes is the oxidation of sialic acid residues with sodium periodate.[38] Although a relatively crude technique, it is rapid, concentration-dependent and selective for the vicinal diol present in sialic acid when employed under mild conditions (Scheme 8). This method has been used to ligate mono- and oligosaccharide hydrazides to the surface of living erythrocytes *in vitro*, functionally altering O-type cells to react with either anti-A or anti-B blood group sera.[39] More recently, we have demonstrated that aldehyde groups can be effectively introduced into the cell surface glycoconjugates of adherent cell monolayers by mild periodate oxidation. This had no significant effect on cell viability, number or morphology. Using this procedure, we also attempted to induce cellular aggregation, a particularly important concept in tissue engineering where three-dimensional reconstruction of tissues is the ultimate goal.[10] By oxidizing cells in suspension and then ligating biotin hydrazide to the resulting aldehyde groups, we effectively biotinylated the entire cell surface. Utilizing the high affinity of avidin for biotin and its four binding sites, flooding the biotinylated cell suspension with avidin induced cells to aggregate to structures in the millimetre-sized range (Fig. 10).

3.5.2.2 Generation of cell surface ketones and azides. As discussed previously, the metabolic delivery of modified sialic acid precursors is a powerful technique for the engineering of cell surface sialic acids. Bertozzi and co-workers have recently



Scheme 8 The generation of cell surface aldehydes by mild periodate oxidation and the application of exogenous enzymes to existing cell surface glycoforms.

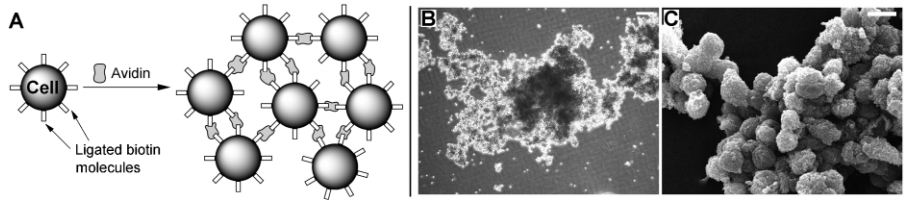


Fig. 10 The induction of multicellular aggregation by cell surface engineering. (A) Schematic representation of the aggregation concept, where biotinylated cells in suspension are cross-linked by addition of avidin. (B and C) Phase-contrast and scanning electron microscope images of L6 myoblasts aggregated using this technique. Bars = 200 μm and 10 μm, respectively. No aggregation was observed when avidin was added to non-biotinylated cells (not shown).

added a new dimension to this technique by the development of functionalized mannosamine derivatives, which enable ketone and azide groups to be metabolically delivered to cell surfaces.[37,40–42] These compounds, *N*-levulinoylmannosamine (ManLev) and *N*-azidoacetylmannosamine (ManNAz) (Scheme 4 (e) and (f)), have been shown to be effectively metabolized into their corresponding cell surface sialic acids in numerous cell types, with no adverse effects on cell viability. It has also been demonstrated that uptake of the per-acetylated derivatives of these compounds, Ac_4ManLev and Ac_4ManNAz, is greatly enhanced compared to the parent compound due to increased membrane permeability. Therefore, the acetylated sugars can achieve the same level of incorporation as the free sugar at a much lower concentration. Interestingly, studies have also shown that using ManLev, the level of cell surface ketone expression is dependent on species. Rodent cells have a much lower propensity to incorporate ManLev than primate cells, possibly due to variations in the permissiveness of the enzymes in the sialic acid pathway to structural modifications.[42]

A wealth of research is now emerging using ManLev and ManNAz as a means of ligating bioactive molecules to cell surfaces, with the major benefit of metabolically decorating cell surfaces in this manner being its biocompatibility for *in vivo* applications. For instance, as many cancers are highly sialylated, ManLev or ManNAz could be used to generate ketones or azides on tumour cells, allowing effective targeting by drugs, antigens or labels for diagnostic purposes. Indeed, an aminooxy derivative of a contrast agent widely used in magnetic resonance imaging has been synthesized and shown to distinguish between ManLev-treated cells with a high (tumour) or low (normal) sialic acid complement.[43] It has also been demonstrated that, like the novel cholesterol- and GPI-tethered receptors described above, ManLev can be used to engineer novel receptors for gene transfer applications. NIH-3T3 cells, a mouse fibroblast cell line, are normally resistant to adenovirus infection. However the metabolic incorporation of cell surface ketones by incubation with ManLev enabled the ligation of biotin hydrazide followed by a NeutrAvidin-conjugated anti-adenovirus antibody. When these cells were challenged with adenovirus, virus particles bound to the novel viral receptors and were internalized by the cells, resulting in a 50-fold enhancement of gene transfer using a recombinant adenovirus expressing β-galactosidase.[5] One further example of the utility of this method is the ability to raise antibodies against the modified sialic acids. *N*-Levulinoylneuraminic acid (SiaLev), the modified sialic acid generated by cells following ManLev treatment, was conjugated to a protein and injected into rabbits to illicit an immune response. The resultant antibodies were shown to bind to both immobilized SiaLev and to cells pre-incubated with ManLev. This binding was specific to SiaLev, whether present in *O*- or *N*-linked glycoproteins and only background binding was seen in cells pre-treated with *N*-pentanoylmannosamine rather than ManLev. Antibody binding was also shown to induce complement-mediated cell lysis in a cultured tumour cell line, suggesting that this may be a valuable method for tumour immunotherapy *in vivo*.[44]

It is apparent that there are numerous potential applications for the metabolic introduction of chemically reactive cell surface groups. One added benefit of this technology is that the generation of cell surface ketones by ManLev treatment and azides by ManNAz treatment can be performed concurrently and that the reactions using either hydrazides or the Staudinger ligation can be performed using complementary conditions. The ability to incorporate such orthogonal reactive groups expands the possibilities of this technique.

4 Conclusions and outlook

The outer membranes of most eukaryotic cells comprise a complex molecular environment, rich in reactive functional groups that are available for further modification. For example, simple covalent conjugation of reactive PEG species to any available cell surface amine (present as the ε-amino groups of lysine side-chains) of pancreatic islets cells may provide a robust technique to shield the normally immunogenic cell surface of transplant tissue whilst maintaining normal metabolic function.[8,34] However, within this review we have clearly served to illustrate how the chemical biologist has now successfully developed a repertoire of alternative techniques for the controlled and reproducible modification of this cell surface milieu and furthermore, we have exemplified the possible application of these procedures in the fields of biomedical and tissue engineering research.

Whether one follows the often synthetically complex generation of cholesterol or GPI based anchors for insertion of naturally occurring or synthetic biomolecules into the cell membrane,[6,12–16] or the use of metabolic engineering using modified monosaccharide precursors[3,37,40–42] to generate novel reactive functional groups, the arena of mammalian cell surface remodelling appears to offer a safe and effective means of manipulating membrane structures *in vivo*, *ex vivo* and *in vitro*. As our understanding of the importance and role of cell–cell and cell–matrix molecular interactions increases, our ability to mimic, enhance or attenuate these processes will ultimately rely more and more upon our ability to redecorate the mammalian cell surface in a controlled, reliable and reproducible manner. The techniques highlighted within this review provide a good indication that we have begun to realise this objective. However, there remains a clear requirement to dissect the metabolic processes of the eukaryotic cell further, in order to enhance our current repertoire. In so doing, this technology will ultimately provide a platform for truly exciting contributions to biology, biotechnology and medicine.

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