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Immobilisation on Polystyrene of Diazirine Derivatives of Mono- and Disaccharides: Biological Activities of Modified Surfaces

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Abstract—The potential of surface glycoengineering for biomaterials and biosensors originates from the importance of carbohydrate–protein interactions in biological systems. The strategy employed here utilises carbene generated by illumination of diazirine to achieve covalent bonding of carbohydrates. Here, we describe the synthesis of an aryl diazirine containing a disaccharide (lactose). Surface analysis techniques [X-ray photoelectron spectroscopy (XPS) and time of flight secondary ion mass spectroscopy (ToF-SIMS)] demonstrate its successful surface immobilisation on polystyrene (PS). Results are compared to those previously obtained with an aryl diazirine containing a monosaccharide (galactose). The biological activity of galactose- or lactose-modified PS samples is studied using rat hepatocytes, Allo A lectin and solid-phase semi-synthesis with α-2,6-sialyltransferase. Allo A shows some binding to galactose-modified PS but none to lactose-modified surfaces. Similar results are obtained with rat hepatocytes. In contrast, sialylation of lactose-modified PS is achieved but not with galactose-modified surfaces. The different responses indicate that the biological activity depends not only on the carbohydrate per se but also on the structure and length of the spacer.

Introduction

Recently developed biomaterials aim to mimic biological surfaces to obtain defined and controlled interactions with biomolecules and living cells.[1,2] Plasma membranes display arrays of receptors that interact with specific ligands. Among these interactions, carbohydrate–protein interactions play a key role in a number of regulatory processes in cells.[3–6] Thus, oriented surface immobilisation of carbohydrates ('surface glyco-engineering') allows one to mimic the specific interactions that occur naturally at cell surfaces and can create a matrix on which specific cellular functions of cultured cells might be investigated.

Carbohydrate immobilisation has been achieved by various techniques[7,8] (copolymerisation, coupling with divinyl sulphone, coupling to CNBr-activated substrates, reductive amination, bisorane method, etc.). Photo-immobilisation provides a versatile tool with respect to the substrate (organic and inorganic) and allows one to easily create microdomains of biorecognition with addressable printing, using mask-assisted lithography techniques. The photoreagents most often used for photo-immobilisation of biomolecules are

Abbreviations: MAD, 4-(2,5-dioxo-2,5-dihydropyrrol-1-yl)-*N*-[3-(3-trifluoro-diazirin-3-yl)phenyl]butyramide; MAD-Gal, 4-(3-D-galactopyranosylsulfanyl-2,5-dioxopyrrolidiny-1-yl)-*N*-(3-(3-trifluoromethyl-3*H*-diazirin-3-yl)phenyl]butyramide; ToF-SIMS, time-of-flight secondary ion mass spectrometry; XPS, X-ray photoelectron spectroscopy; mamu, milli atomic mass unit, MS; UHV, ultra high vaccum; PS, polystyrene; Allo A, allomyrina dichotoma; RCA, ricinus communis agglutinin; PBS, phosphate buffer saline; BSA, bovine serum albumin; CMP-Neu 5 Ac, cytidine 5′-monophospho-*N*-acetylneuramic acid; NR, neutral red; MTT, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CMF, crude membrane fraction; EROD, ethoxyresorufin de-ethylase; ASGP-R, asialoglycoprotein receptor; PVLA, poly (vinbenzyl-β-D-lactonamide); DCC, *N,N′*-dicyclohexylcarbodiimide

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arylazides, trifluoromethyl-aryl diazirines and benzophenones.[9] These reagents generate very reactive intermediates upon light activation. Their interaction with the support material leads to the formation of covalent bonds.[9] Thus, the synthesis of molecules containing carbohydrate and photoactivatable domains makes it possible to achieve covalent binding of biologically active carbohydrates.

The synthesis and characterisation of an aryl diazirine containing a galactose photoreagent [4-(3-D-galactopyranosylsulfanyl-2,5-dioxopyrrolidin-1-yl)-*N*-(3-(3-trifluoromethyl-3*H*-diazirin-3-yl)phenyl]butyramide (MAD-Gal)] **1** was previously reported (Scheme 1).[10] Using a masking technique, a specific pattern of immobilised carbohydrate was laid down on diamond and confirmed with surface analysis techniques [X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ions mass spectrometry, (ToF-SIMS)]. However, the biological activity of the modified surface remained to be probed. Accordingly, the objective of the present study is to investigate the biological activity of the photo-immobilised galactose and to compare it with the properties of surface-photo-immobilised lactose prepared in an analogous manner. These engineered surfaces are expected to be of interest for the design of biosensor devices. The biological functionality of the surfaces is tested with a defined substance (Allo A lectin) and with intact cells (rat hepatocytes), both of which were expected to bind to galactose residues. Allo A lectin was reported to have a specific affinity for galactose residues especially as part of lactose (3.1 mM inhibits haemagglutinin activity) and *O*-nitrophenyl β-D-galactopyranoside (inhibitory at 12.5 mM).[11] Hepatocytes express on their surface the asialoglycoprotein receptor which is responsible for the clearance of abnormal galactose-terminated serum glycoproteins.[12,13] Most serum glycoproteins carry terminal sialic acid residues and a penultimate galactose residue. When desialylated, the exposed galactose residues of the glycoprotein can interact with the asialoglycoprotein receptor, initiating removal of the glycoprotein from the circulation by endocytosis. Subsequently, the incorporated protein is hydrolysed in lysosomes.[14–17] Moreover, it was considered of interest to develop a photoactivatable precursor for the semi-synthesis of more complex carbohydrates. The transfer of sialic acid by α-2,6-sialyltransferase[18] to galactose residues to a solid support was studied to develop our approach to solid phase synthesis.

The present paper describes the synthesis of a 6-(β-D-galactopyranosyl-(1→4)-β-D-glucopyranosylsulfanyl)hexanoic acid [3-(3-trifluoromethyl-3*H*-diazirin-3-yl)phenyl]-amide **8** (Scheme 2). Its immobilisation on polystyrene (the material most often used in biological assays) is demonstrated with surface analysis spectroscopic methods XPS and ToF-SIMS. The biological activity of the polystyrene-modified surfaces is probed with the lectin Allo A, primary rat hepatocytes, and α-2,6-sialyltransferase and compared with MAD-Gal modified polystyrene.[10]

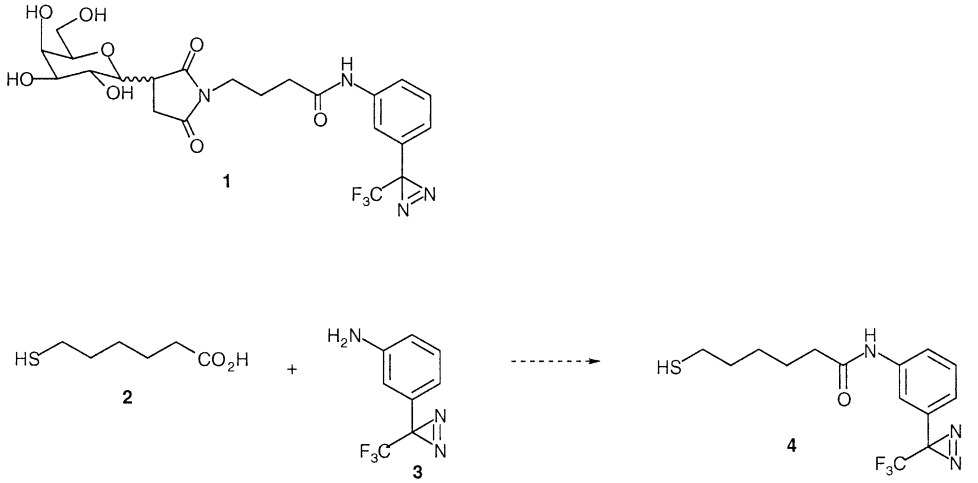
Results

Synthesis of 6-(β-D-galactosyl-(1→4)-β-D-glucopyrano sylsulfanyl)hexanoic acid [3-(3-trifluoromethyl-3*H*-diazirin-3yl)-phenyl]amide 8

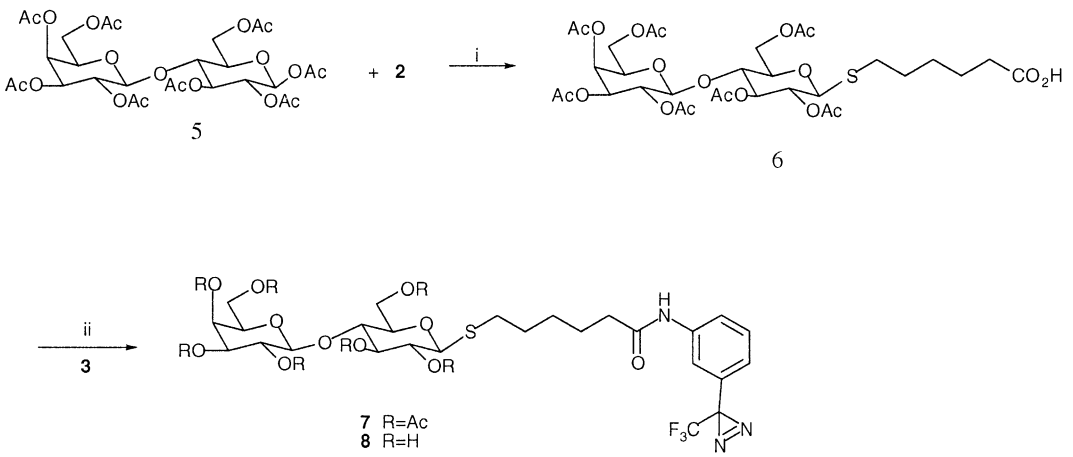
Lactose octa-acetate **5** was first coupled to 6-mercaptohexanoic acid **2** to give 6-(2,3,4,6-tetra-*O*-acetyl)-(1→4)-β-D-2,3,6-triacetyl-glucopyranosylsulfanyl)hexanoic acid **6** using BF_3–Et_2O. It was then linked to 3-(3-trifluoromethyl-3*H*-diazirin-3-yl)-phenylamine **3** via amide formation to give the protected lactose derivative **7**.

Deprotection of derivative **7** gave compound **8** with 30% yield.

The overall yield in the synthesis was 4.3%. In Figure 1 is shown the UV absorption spectrum of the product exhibiting characteristic diazirine adsorption band at 357 nm.[19] Figure 1 further illustrates conversion of the diazirine into carbene, as the absorbance decreased with increasing light exposure at 357 nm, demonstrating exposure time-dependent photoactivation.



Scheme 1. Structure of MAD-Gal and synthesis scheme of the crosslinker **4** by the condensation of 6-mercaptohexanoic and with 3-(3-trifluoromethyl-3H-diazirin-3-yl)phenylamine **3**.



Scheme 2. Reagents: (i) $BF_3{\cdot}Et_2O$; (ii) $ClCO_2Et/Et_3N$; (iii) NaOMe/MeOH. Lactose aryl diazirine synthesis is illustrated. Lactose octaacetate **5** was first coupled to 6-mercaptohexanoic acid **2** to give 6-(2,3,4,6-tetra-*O*-acetyl)-(1-4)-D-2,3,6-triacetyl-glucopyranosylsulfanyl)hexanoic acid **6** using BF_3–Et_2O. It was then linked to 3-(3-trifluoromethyl-3*H*-diazirin-3-yl)-phenylamine **3** via amide formation to give the protected lactose derivative **7**.

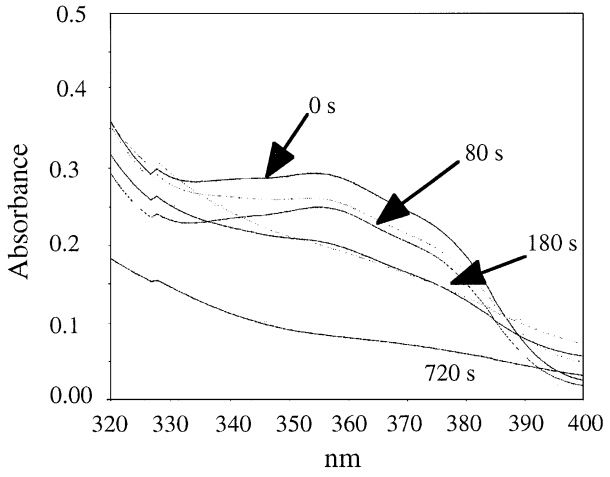


Figure 1. The absorbance of the diazirine functions at 357 nm is displayed as a function of irradation time. It illustrates conversion of the diazirines into carbenes as the absorbance diminished with increasing light exposure at 357 nm, demonstrating exposure time-dependent photoactivation.

Surface analysis

Light activation at 357 nm of the diazirine leads to a reactive carbene and a covalent bond is generated with the surface provided that close contact between the surface and the carbene is obtained. Reaction of aryl diazirine containing molecules with various substrates has previously been described.[19–23] In the case of a diamond substrate, fluorine indicates the presence of MAD-Gal.[23] It is thus expected that immobilisation of MAD-Gal or lactose aryl diazirine on polystyrene should result in an increase of the surface fluorine content.

In Table 1, XPS atomic percentages of samples at different steps of MAD-Gal surface modification on polystyrene are displayed. The significant detection of oxygen on sample A can be explained by the γ-ray sterilisation of the sample.[24] Fluorine was only detected on sample C with XPS indicating that molecules are at the surface after photoactivation and the final washing. The result is confirmed with ToF-SIMS where F^- normalised intensity (Table 1) was eight times higher on sample **C** than on sample **B**. The residual level of molecules observed on sample B by ToF-SIMS is below the XPS instrument detection limit. Other characteristic ions such as CF_3^- confirm the immobilisation of MAD-Gal similarly to what has already been observed in previous studies on diamond.[22,23] For lactose aryl diazirine, fluorine atomic percentage and F^- normalised intensity (Table 2) illustrated in the same way the successful immobilisation of the molecule at the surface of polystyrene.

Figure 2 shows that ToF-SIMS F^- normalised intensity and XPS fluorine atomic percentage increased as a function of the concentration of MAD-Gal solution used for surface immobilisation on polystyrene. The intensity of F^- and F atomic% are related to the surface density of photobonded molecules, and thus MAD-Gal density increased with the concentration of applied MAD-Gal solution. This observation confirms previous results obtained with photobonded 4-(2,5-dioxo-2,5-dihydropyrrol-1-yl)-*N*-[3-(3-trifluoromethyl-3*H*-diazirin-3-yl)phenyl]butyramide (MAD) on silicon nitride.[21]

Binding of Allo A lectin

Galactose residue availability at the surface of polystyrene was probed with the β-galactose specific lectin Allo A. Biotinylated lectin was used. The amount of surface lectin was measured with ^{35}S streptavidin. Retained radio-activity relates to the amount of radioactivity in the radiolabelled streptavidin deposited. This represents an arbitrary standard but is used to indicate the relative levels of radioactivity remaining after the various treatments of the modified surface. The results are illustrated in Figures 3 and 4 for MAD-Gal and lactose modified polystyrene surfaces, respectively. For the two sets of data, the percentages of radioactivity for samples **A** are similar (1.2 and 2.5%). For samples **B**, the signal remains very low, even though it is slightly higher in the case of MAD-Gal.

In the case of MAD-Gal (Fig. 3), radioactivity measurements of sample **C** demonstrated that Allo A lectin

had a higher binding for the surface grafted with galactose residues than polystyrene (**A**) and that this affinity is a function of MAD-Gal surface density. Furthermore, inhibition with asialofetuin of lectin binding on immobilised galactose was observed (Fig. 3I). It confirms that this interaction was specific and not owing to a variation in physisorption (related to a change in surface energy).

Table 1. XPS atomic percentages and ToF-SIMS relative intensities for the different steps of MAD-Gal immobilisation on PS

At percentages (%)	Sample **A**	Sample **B**	Sample **C**
N	bdl	bdl	0.28±0.26
F	bdl	bdl	0.45±0.16
S	bdl	bdl	bdl
C	93.7±0.6	94.1±1.0	95.5±1.2
O	6.1±0.6	5.9±1.0	3.8±0.8
ToF-SIMS			
Corrected total intensity (counts)×10^4	110.4±1.8	106.4±12.9	132.2±15.7
F^- normalised intensity ‰	0.9±0.13	26±4	205±53
CF_3^- normalised intensity ‰	0.008±0.003	0.2±0.08	1.2±0.5

XPS and ToF-SIMS analysis of MAD-Gal grafted PS. Atomic percentages of the elements were observed with XPS. bdl means below detection limit. Three areas were measured per sample. Sample **A** corresponds to the plain polystyrene, sample **B** corresponds to the surface on which MAD-Gal was deposited and washed without light activation and sample **C** is the surface on which MAD-Gal was deposited, light activated and washed. MAD-Gal is expected to be observed only on sample C. Fluorine and nitrogen are signatures of the molecule and are only observed on sample **C**. ToF-SIMS normalised intensity of selected ions characteristic of MAD-Gal molecule (F^- and CF_3^-) are also displayed. Normalised intensity is the ratio of the absolute intensity of the selected ion over the total corrected intensity which is the total intensity minus H^- intensity. Four areas were measured per sample. MAD-Gal was expected to be observed only on sample **C**. Characteristic ions were observed on sample **C**, not observed on sample **A** while sample B exhibited slightly higher intensities than sample **A.**

Table 2. XPS atomic percentages and ToF-SIMS relative intensities for the different steps of lactose aryl diazirine immobilisation on PS

At percentages (%)	Sample **A**	Sample **B**	Sample **C**
N	bdl	0.34±0.20	0.51±0.49
F	bdl	bdl	1.18±0.4
S	bdl	bdl	0.18±0.16
C	97.79±0.36	94.53±1.27	88.77±0.54
O	2.27±0.47	5.13±1.08	9.35±0.49
ToF-SIMS			
Corrected total intensity (counts)×10^4	16.3±4.5	49.6±5.4	43.7±10.7
F^- normalised intensity ‰	1.10	2.72±0.42	63.17±21.78
CF_3^- normalised intensity ‰	0.13	0.20±0.08	0.65±0.07

XPS and ToF-SIMS analysis of lactose aryl diazirine grafted PS. XPS atomic percentages are displayed for samples **A**, **B** and **C**. Sample **A** corresponds to as received polystyrene. Sample **B** corresponds to polystyrene on which a methanolic solution of lactose aryl diazirine was deposited, and no light activation was performed before the final washing. This surface should be similar to sample **A**. Sample **C** corresponds to polystyrene on which a methanolic solution of lactose aryl diazirine was deposited and light activation was performed before the final washing. The molecule should be present at the surface of the material. Three areas were analysed per sample. ToF-SIMS normalised intensities of sample **A**, **B** and **C** are also displayed. Normalised values were calculated by dividing the absolute intensity of secondary ions by the corrected total intensity (=total intensity from which intensity of H^- was subtracted). Three areas were analysed per sample. Lactose aryl diazirine observed only on sample **C**.

In the case of lactose modified polystyrene surfaces (Fig. 4), the radioactivity only reaches the value of approximately 3%, which is comparable to sample **B** in the case of MAD-Gal experiments but almost 7 times lower than for the corresponding sample **C**. Surface analysis demonstrated that the surface density of the two molecules were almost similar. Thus, no specific affinity was demonstrated for lactose modified polystyrene surfaces.

Binding of primary cultured rat hepatocytes

On the cellular level, accessibility of the galactose and lactose residues was tested with primary cultured rat hepatocytes. Binding to surface galactose residues did

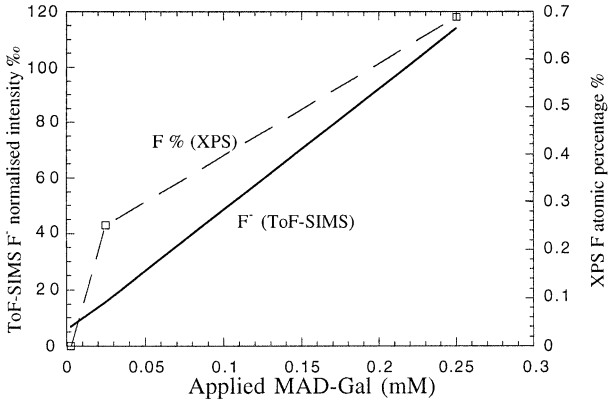


Figure 2. F^- normalised intensity values [absolute intensity of F^-/(total intensity−H^- intensity)] and fluorine atomic percentages are displayed as a function of the MAD-Gal concentration used for immobilisation. The intensity of surface characteristics signatures increases with increasing concentration of MAD-Gal.

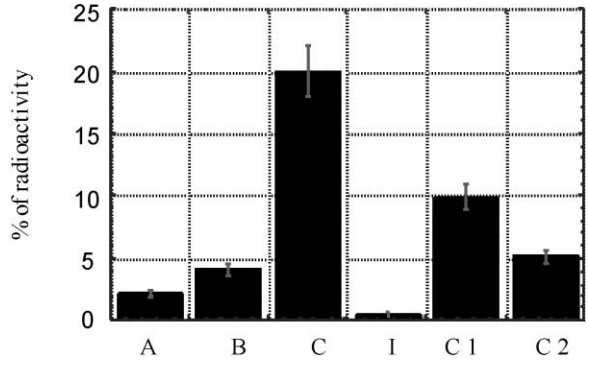


Figure 3. Binding of biotinylated Allo A lectin to MAD-Gal derivatised PS surface. The derivatised PS surfaces were incubated with Allo A lectin and then extensively washed to remove physisorbed lectins. [^{35}S] streptavidin was added and the surfaces were again washed to remove excess streptavidin. Finally, radioactivity was measured by scintillation counting. The radioactivity is higher on sample **C** and increases with the concentration of the MAD-Gal solution (C, C1 and C2 corresponding to 0.25, 0.025 and 0.0025 mM, respectively) used for immobilisation. This illustrates the higher binding of streptavidin to the surface **C**. **I** corresponds to incubation of MAD-Gal grafted PS with asialofetuin and Allo A lectin. 100% corresponds to the total added radioactive streptavidin.

not affect xenobiotic metabolism or viability as indicated by high EROD activities and protein content (as an indicator of number of attached cells). Both are comparable to those obtained with the reference substrate CMF (data not shown). Figure 5 shows that NR uptake of hepatocytes cultured on MAD-Gal coated polystyrene surfaces increased in proportion with the density of galactose molecules on the surface. MTT formation (not shown) and NR uptake increased up to a concentration of 2.5 mM galactose and showed no saturation effect (Fig. 5). NR uptake was reduced, when lectins (RCA) were added most probably because RCA competes with asialoglycoprotein receptors for terminal galactose residues of MAD-Gal (Fig. 5).

In the case of lactose modified polystyrene, no increase in the NR uptake was observed (Fig. 6).

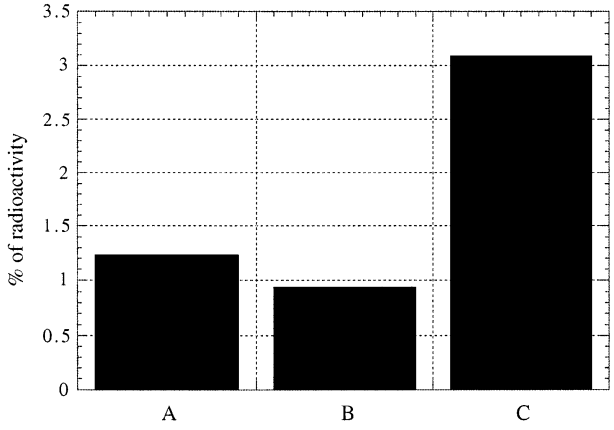


Figure 4. Binding of biotinylated Allo A lectin to aryl lactose derivatised PS surfaces. Derivatised surfaces were incubated with Allo A lectin and then extensively washed to remove physisorbed lectins. [^{35}S] streptavidin was incubatedand the surfaces were again rinsed to remove excess streptavidin. Finally, radioactivity was measured by scintillation counting. The radioactivity is higher on sample C but in comparison with Figure 3, it can be concluded that no specific affinity was observed in this case.

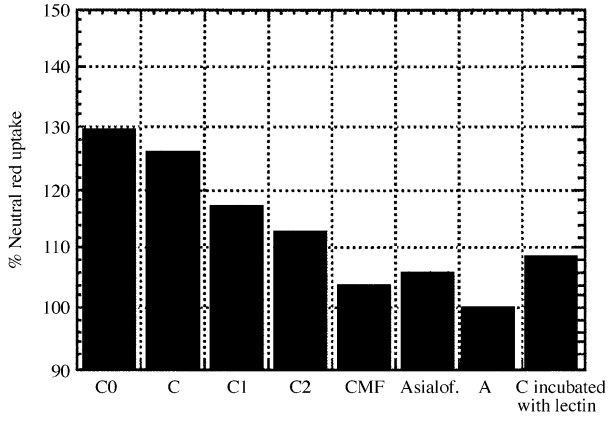


Figure 5. NR uptake is related to the lysosomal activity. The NR uptake decreased with the decreasing of surface galactose residues (C0, C, C1 and C2 corresponding to 2.5, 0.25, 0.025 and 0.0025 mM, respectively). The NR uptake was similar to that of surface **A** (plain polystyrene) when the surface were incubated with a lectin (RCA) that protects the galactose residues. Hepatocytes possess higher NR uptake on asialofetuin coated surfaces than on surface A, but lower than on MAD-Gal grafted PS. 100% corresponds to surface A.

Enzym-catalysed sialylation of immobilised galactose

Experiments in solution (data not shown) demonstrated that transferase activity was 30 times slower with MAD-Gal than with the natural substrate while it was only 2 times slower with *N*-acetyllactosamine pNp than with lactose aryl diazirine. It was assumed that this difference was due to the succinimidyl group of MAD-Gal. Accordingly solid phase semisynthesis experiments were carried out on lactose modified polystyrene only, as illustrated in Figure 7. As shown in Figure 7, transfer of radioactive NeuAc to immobilised lactose proceeded linearly up to 4 h under the experimental conditions. Control including zero time incubation and a no enzyme (uncontrolled attachment due to CMP-Neu5Ac decomposition) control afforded background incorporation level only (approx. 20 cpm).

Discussion

Initially, it was planned to synthesise the crosslinker **4** by condensation of 6-mercaptohexanoic acid with 3-(3-trifluoromethyl-3*H*-diazirin-3-yl)phenylamine **3** (Scheme 1). This crosslinker **4** would have a thiol function for

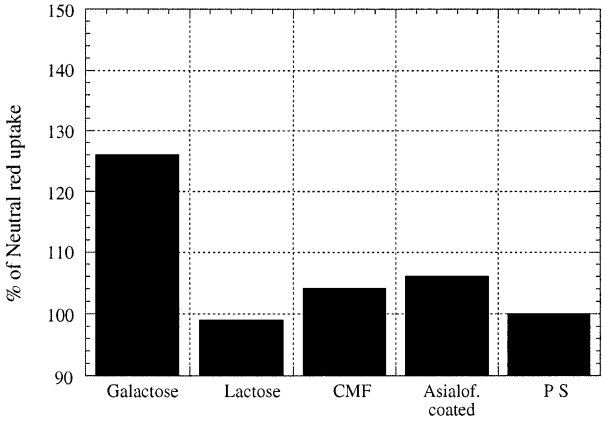


Figure 6. Primary rat hepatocytes were incubated on PS modified with aryl lactose diazirine. No effect of the surface was observed.

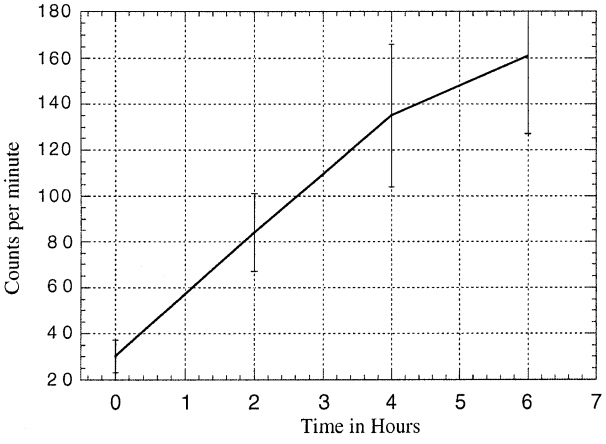


Figure 7. PS modified with aryl lactose diazirine was incubated with a-2,6-sialyltransferase. Incorporation of radioactive NeuAc was linear for 4 h.

coupling with oligosaccharides and a diazirine function for carbene-mediated surface photo-immobilisation.

However, model reactions with aniline demonstrated that coupling of lactose octa-acetate with 6-mercapto-hexanoic acid phenylamide was not successful with BF_3-Et_2O (60% recovery of the starting thiol) using conditions that have been successful in other cases.[25,26]

The successful procedure investigated is illustrated in Scheme 2. Condensation of a carboxylic acid with the amine requires the activation of the carboxylic group.[27] Activation of thioglycoside **6** with *N*,*N*′-dicyclohexyl-carbodiimide (DCC)/hydroxy benzotriazole was not successful. The lack of reaction may have been owing to steric hindrance by the 3-(trifluoromethyl)-diazirinyl group or to the inductive effect of the 3-trifluoromethyl-3*H*-diazirin-3-yl group since DCC/hydroxy benzotriazole activation gave an 80% yield of the condensation product with aniline (data not shown).

According to Collioud et al.,[19] 4-maleimidobutyric acid can be successfully coupled to 3-(3-trifluoromethyl-3*H*-diazirin-3-yl)phenylamine using mixed anhydride activation of the carboxylic acid with ethyl chloroformate. However, the coupling reaction of the thioglycoside **6** with 3-(3-trifluoromethyl-3*H*-diazirin-3-yl)phenylamine under the same conditions failed. Nevertheless, at a lower temperature (−65 °C) and with three equivalents of triethylamine, the reaction proceeded successfully to give the amide **7**. Deprotection of **7** gave **8** with a lower yield than expected. This could suggest that either some material is lost by adsorption on Celite or/and Amberlyst or that **7** may contain impurities.

In conclusion, 6-(β-D-galactosyl-(1→4)-β-D-glucopyranosylsulfanyl)hexanoic acid [3-(3-trifluoromethyl-3*H*-diazirin-3yl)-phenyl]amide **8** was successfully synthesised, albeit in low yield. Better yield may be achieved using other synthetic routes (by activation of the glycosyl donor with trichloroacetimidate[28,29] or by silylation followed by coupling using ZnI_2[30]).

Upon light activation of the diazirine, a reactive carbene is generated and may lead to covalent bonding to the substrate if close contact is allowed. Fluorine can be related to the presence of the molecule. XPS atomic percentages and ToF-SIMS relative intensities (Tables 1 and 2) demonstrate the successful grafting of the two molecules to PS surface.

The extent of grafting of the two molecules is of the same order of magnitude and is estimated between 10^{12} and 10^{13} molecules cm^{-2} (corresponding to the detection limit of XPS). According to ToF-SIMS F^- normalised intensity, MAD-Gal grafting is higher than lactose aryl diazirine while, according to XPS, it is the opposite. However, owing to the complexity of secondary ion creation (possible difference in matrix effects), one should rather consider XPS data to compare grafting extents, which are then only slightly higher for lactose aryl diazirine.

The biological availability of the terminal galactose residues was tested with Allo A lectin and primary rat hepatocytes. Allo A showed affinity for MAD-Gal modified PS. Although, Yamashita et al.[11] and Umetsu et al.[31] demonstrated that hemagglutination was not inhibited by simple carbohydrates such as D-glucose, D-galactose, Adachi et al.[32] reported a similar test with galactose coated polystyrene surfaces [thin film coating with a lactonamide containing polymer (PVLA)]. In a competitive inhibition study with asialofetuin ($1\ mg\ ml^{-1}$), they obtained 70% inhibition. With MAD-Gal coated polystyrene, 100% inhibition was obtained using the same concentration of asialofetuin. This suggests that in this case, either the galactose surface density or the binding of Allo A for surface grafted galactose was lower.

In the case of lactose modified PS, Allo A did not exhibit affinity for the terminal galactose. Therefore, the difference in lectin binding could be attributed to differences in affinity of the lectin for the different terminal galactose residues at the surface.

On the cellular level, an effect was also observed only in the case of MAD-Gal modified surfaces. MTT formation and NR uptake were increased. NR uptake showed to be dependent upon galactose surface density. This effect on NR uptake needs further explanation which may relate to an unknown intracellular effect of immobilized asialoglycoprotein receptors on lysosomal activity as well as MTT formation. ASGP-R (asialoglycoprotein receptor) found as surface receptors of hepatocytes is expressed on their sinusoidal membranes[33] when the cells are kept at high cell density[34] similar to the ones chosen in the present investigation. It might well be, that binding of ASGP-R to galactose induces endocytosis and degradation processes (lysosomal activity) comparable to the effects obtained by asialoglycoproteins in an intact organ. This would explain why galactose coating increases NR uptake without altering the viability of hepatocytes.

No effect was observed for lactose modified surfaces. The literature is well documented on ligands that have been used for the study of the asialoglycoprotein receptor.[35–41] The structure of the lactose aryl diazirine should not impair this interaction according to the literature. In consequence, it remains to be clarified why hepatocytes interacted with MAD-Gal modified polystyrene, while they did not with lactose modified polystyrene.

In the case of enzym-catalysed sialylation, incorporation of NeuAc was observed for only lactose modified surfaces and proceeded linearly up to 4 h. Based on the estimation of 10^{12}–10^{13} molecules per cm^2, the estimated incorporation yield for 6 h incubation is in the range of 4–40%. According to Yamada et al.,[42] the extent of galactosylation is dependent upon the primer arm in polymer supported semisynthesis with bovine galactosyl transferase. These authors reported a yield of 30% in the case of short-arm primer and a nearly quantitative yield with longer-armed primer for 24 h incubation

times.[42] In our case, the incorporation (on a linear approximation) was lower than the one observed by Yamada et al. The difference might be related to the spacer structure. Accordingly, further investigation should focus on the spacer design to increase the transferase efficiency. The efficiency of the transfer reaction may also be hampered by increasing concentration of CMP which inhibits sialyltransferase by competitive inhibition.[43]

Conclusions

Diazirine-containing photoreagents containing mono- and disaccharides were successfully synthesised and immobilised on polystyrene. Biological activity of the modified polystyrene was demonstrated with Allo A lectin, primary rat hepatocytes and α-2,6-sialyltransferase. Different responses of lectin Allo A and hepatocytes to the galactose and lactose modified surfaces indicate that the biological activity depends not only on the carbohydrate but also on the mode of biomolecule presentation (structure of the spacer). Improvement of the biological interactions will require more complex structures (cluster, spacer design, oligosaccharides).

Experimental

Materials

Biotinylated Allo A lectins (Allo A-I MW 65000, Allo A-II MW 66500 g/mol) were purchased from Oxford Glycosystems (Oxford, UK). Radiolabelled [^{35}S] streptavidin from Amersham, UK, (M_r 60,000 g mol^{-1}, specific radioactivity 1.35 TBq mmol^{-1}, radioactive concentration 3.70 MBq m^{l-1}) was used to detect lectin binding. Radiolabelled (^{14}C) CMP-Neu5Ac (Amersham, UK) was used to probe the enzymatic reaction. The sialic acid was labelled at positions 4, 5, 6, 7 and 9. (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Fluka (Switzerland). CYP 1A1 was purchased from Amersham Life Science (UK). Liquid scintillation measurements were performed with ultima gold scintillation liquid (Packard) on a Tri-Carb 2300tr scintillation beta counter from Packard (Switzerland). Dry toluene was purchased from Fluka (Switzerland).

Falcon multiwell (non tissue culture treated, Heidelberg, Germany) or ELISA plates (Nunc, Switzerland) were used for XPS analysis, biomolecule and cell assays. These PS dishes are γ-ray sterilised by the manufacturer. The plates were used as received. For ToF-SIMS experiments, polystyrene (M_r 30000 g/mol, monodispersed, kindly provided by the polymer laboratory (LP-DMX) of EPFL) was spin cast on a silicon wafer (5×5 mm) from a 30 mg mL^{-1} solution (in dry toluene) in order to limit charging of the sample during analysis. Spin casting conditions were 65 s at 5000 rpm (acceleration 1000 r min^{-1} s^{-1}).

PBS (Phosphate buffered saline, 150 mM NaCl, 50 mM phosphate, pH 7.2–7.4) and cacodylate buffer (pH 6.8, 1 M) were prepared on site. Chemical ionisation was performed with ammonia as the ionising gas on a Nermag R-10-10 quadrupole spectrometer. Electrospray ionisation was also used. The analyses were carried out on a VG Platform (micromass) single quadrupole MS in the negative mode, courtesy of the University of Bern. The samples were dissolved in MeOH/H_2O (1/1, v/v) containing 1% of triethylamine. The major ions were all singly charged.

Accurate mass spectra of carbohydrate derivatives were obtained using a Fourier transform ion cyclotron mass spectrometer (Bioapex, Bruker Daltonics, USA) equipped with a 94 Tesla magnet (Magnex, Abingdon, UK). ^{1}H NMR and ^{13}C NMR spectra were obtained using either an AC-P-200 spectrometer (4.7 T) or an AC-P-250 (5.9 T) spectrometer equipped with an auto sampler. $CDCl_3$ and CD_3OD (Glaser, Switzerland) were used as solvents.

XPS analysis was carried out under UHV conditions (10^{-9} Torr) using a PHI 5500 system equipped with a hemispherical analyser and a non-monochromatised Mg K_α (14.0 kV, 350 W) radiation source. The analysed area was 0.12 mm^2, allowing the acquisition of three spectra on each sample. Spectra were taken at a 45° take-off angle. Pass energies used for survey scans and high resolution elemental scans were 93.5 and 23.5 eV, respectively. Spectra were referenced in the C1s spectrum to C–H/C–C at 285 eV (PS). The spectrometer was calibrated by using Cu $2p_{3/2}$, Ag $3d_{5/2}$ and Au $4f_{7/2}$ at 932, 368, 84 eV, respectively. Data reduction was performed by applying the PHI-Access Software 5.2.[44] Absolute areas were calculated using a parabolic integration routine. Background was calculated using a trapezoid area after subtracting a Shirley–Sherwood background. The corrected area was normalised to time and to the unit step to give the intensity in counts s^{-1} eV^{-1}. Instrumental sensitivity factors (PHI sensitivity factors) were 0.296, 0.711, 0.477, 1.000, 0.339 for carbon (C_{1s}), oxygen (O_{1s}), nitrogen (N_{1s}), fluorine (F_{1s}) and silicon (Si_{2p}), respectively.[44] Atomic percentages were calculated using the above sensitivity factors S_x and the relation: $X\% = (I_x/S_x)/(\Sigma\ I_i/S_i)$. Sample charge-up was observed for polystyrene substrate and was compensated by low energy electrons (maximum of 12 eV).

The ToF-SIMS system used in this study was a Trift 1 mass spectrometer from PHI-EVANS & Associates (described in detail elsewhere[45,46]) equipped with a pulsed FEI Ga^+ ion gun operated at 15 kV. Sample surfaces were biased ±3 kV with respect to the grounded extraction electrode for positive and negative mode SIMS, respectively. The 800–1700 pA DC ion beam current was pulsed at 5 kHz repetition rate (8 ns pulse width, measured with the unbunched beam). The analysed area was estimated to 84×84 μm^2. The analysis was performed under so-called 'static' conditions with an ion dose on the order of 10^{12} ions cm^{-2}. All spectra were collected using high mass resolution conditions (bunched beam). The mass resolution obtained for $Si^{+/-}$ on a Si wafer was $m/\Delta m > 3600$ in the positive mode and >2400 in the negative mode. Charge compensation was performed with a pulsed 20 eV electron gun.

ToF-SIMS spectra were recorded for each sample on three different sample spots and in each mode (positive and negative). Values displayed are mean values and standard deviations from negative mode data. ToF-SIMS data were analysed using Cadence software from Charles Evans & Associates. Mass calibration was performed using similar calibration peaks for a meaningful comparison. Accurate calibration was achieved using hydrocarbon peaks such as CH^-, C_2H^-, C_3H^-, and C_4H^- (in the negative mode) as well as low intensity characteristic PDMS (polydimethylsiloxane) peaks such as $C_5H_{15}O_4Si_3^-$. The standard deviation on all the detected peaks was less than 10 milli-atomic mass unit (mamu). It must be noted that the data treatment was based on intensity values measured in mass windows which were identical for all the samples. The residual intensity measured in some cases should be considered as noise but was not arbitrarily subtracted in the data presentation. ToF-SIMS peak values were normalised by dividing the absolute peak intensity of secondary ions by the corrected total intensity, that is the total intensity from which intensities of $H^{+/-}$ were subtracted due to limited reproducibility of their absolute values.

Methods

6-Mercaptohexanoic acid 2. A mixture of ε-caprolactone (13.6 g, 120 mmol), thiourea (8.4 g, 110 mmol) and hydrobromic acid 48% (9.8 g, 120 mmol) was heated (~120 °C) with stirring in an oil bath for 9 h, then cooled to room temperature. Sodium hydroxide solution (50% m/v) was added until a clear solution was obtained. This solution was further heated (~100 °C) for 3 h, cooled to room temperature and acidified with sulphuric acid (aqueous solution 50% v/v) until a clear solution was obtained (pH ~1). The clear solution was extracted with diethyl ether (3×l00 cm^3). The combined organic phase was washed with water (3×l00 cm^3), dried ($MgSO_4$) and the solvent was evaporated under reduced pressure. The remaining yellow oil was purified by distillation under reduced pressure (135–140 °C, ~1 mm Hg) to give the acid **2** as a colourless oil (3.76 g). δ_H (250 MHz; $CDCl_3$) 1.34 (1H, t, $J=7.9$ Hz, *SH*), 1.37–1.51 (2H, m, CH_2), 1.57–1.71 (4 H, m, 2×CH_2), 2.37 (2 H, t, $J=7.3$ Hz, CH_2COOH), 2.53 (2 H, dt, $J=7.9$ Hz, 7.0, $HSCH_2$), 10.4 (1 H, br, COOH); δ_C (62.90 MHz; $CDCl_3$) 23.92, 24.21, 27.56, 33.41 ($HSCH_2$), 33.75 (CH_2COOH), 179.92 (COOH); *m/z* (+CI) 166 ($[M+NH_4]+$, 1.7%), 148 ($[M]^+$, 0.8), 130 (2.8), 115 (2.8), 102 (3.5).

6-(2,3,4,6-Tetra-*O*-acetyl)-β-D-galactopyranosyl-(1→4)-2,3,6-triacetyl-β-D-glucopyranosylsulfanyl)hexanoic acid 6. To a stirred mixture of lactose octaacetate 2.72 g (4 mmol), 6-mercaptohexanoic acid (0.48 g, 3.2 mmol) and dry CH_2Cl_2 (10 mL) under N_2, BF_3 diethyl etherate (0.85 g, 6 mmol) were added dropwise. The mixture was stirred for 2 h at room temperature under nitrogen, diluted with CH_2Cl_2 (200 mL) and washed with HCl (1 M, 3×100 mL) and water (1×100 mL). The organic phase was dried ($MgSO_4$), and the solvent was evaporated at reduced pressure to give a yellow oil. Purification required two separations by flash chromatography (CH_2Cl_2/MeOH 20:1 and 10:1) to give the derivative **6** (1.65 mg, 67%). δ_H (250 MHz; $CDCl_3$) 1.34–1.46 (2 H, m, CH_2), 1.54–1.67 (4 H, m, 2×CH_2), 1.96 (3H, s, $COCH_3$), 2.02 (3H, s, $COCH_3$), 2.03 (6H, s, $COCH_3$), 2.04 (3H, s, $COCH_3$), 2.09 (3H, s, $COCH_3$), 2.13 (3H, s, $COCH_3$), 2.32 (2H, t, $J=7.4$ Hz, CH_2), 2.54–2.68 (2H, m, CH_2), 3.58–3.61 (1H, m, 5′-H), 3.75 (1H, t, $J=9.4$, 4′-H), 3.85 (1 H, t, $J=6.6$ Hz, 5-H), 3.98–4.19 (m, 3H, C-6 and 6b′-H), 4.44 (1H, d, $J=9.2$ Hz, 1-H), 4.84–4.96 (2H, m), 5.07 (1H, dd, $J=6.2$ Hz), 5.18 (1H, t, $J=9.4$ Hz, 4′-H), 5.32 (1 H, d), δ_C (62.90 MHz; $CDCl_3$) 20.37, 20.50, 20.61, 20.71, 23.96, 27.89, 29.17, 33.54, 60.68, 62.13, 66.49, 68.95, 70.15, 70.53, 70.85, 73.63, 76.04, 83.35 (1-C), 100.91 (1′-C), 169.01 ($COCH_3$), 169.59 ($COCH_3$), 169.68 ($COCH_3$), 170.07 ($COCH_3$), 170.30 ($COCH_3$), 178.35 ($COCH_3$, or CH_2COOH). IR 1665 cm^{-1}. Found *m/z* 789.2246. $(C_{32}H_{46}O_{19}S+Na)^+$ requires *m/z* 789.2246.

6-(2,3,4,6-Tetra-*O*-acetyl)-β-D-galactopyranosyl-(1→4)-2,3,5-triacetyl-β-D-glucopyranosylsulfanyl)hexanoic acid [3-(3-trifluoromethyl-3*H*-diazirin-3-yl)phenyl]amide 7. *m*-[3-(Trifluoromethyl)diazirine-3-yl]aniline **3** was stored as *N*-[3-(3-Trifluoromethyl-3*H*-diazirin-3-yl)phenyl]formamide to avoid oxidation of the amine function. *N*-[3-(3-Trifluoromethyl - 3*H* - diazirin - 3 - yl)phenyl]formamide (120 mg) was dissolved in methanol (3 cm^3). Concentrated HCl (1 mL) was added. After 10 min, the mixture was cooled to 0 °C and neutralised with NaOH (6 M, 3 mL). The aqueous phase was extracted with ethyl ether (5×4 mL). The combined organic phases were washed with deionised water (5 mL) and dried $MgSO_4$). The solvent was evaporated under reduced pressure to give *m*-[3-(trifluoromethyl)diazirine-3-yl]aniline **3** as a yellow oil which was used without further purification. A solution of thioglycoside **6** (383 mg) in dry DMF (9 mL) was cooled to −20 °C in a salt-ice bath under nitrogen. Triethylamine (209 μL) was added dropwise. The mixture was further cooled at −60 °C in a dry ice/acetone bath. Ethyl chloroformate (80 μL in 1.1 mL of dry DMF) was added. The reaction was allowed to proceed for 30 min. A solution of the *m*-[3-(trifluoromethyl)diazirine-3-yl]aniline (above) in dimethylformamide (1.1 mL) was added dropwise. The mixture was allowed to stand for 5 h and 35 min at room temperature. The reaction mixture was poured on to a mixture of NaCl solution (1 M, 40 mL) and ice and was extracted with diethyl ether (4×15 mL). The combined organic phases were washed sequentially with HCl (1 M, 2×10 mL), saturated $NaHCO_3$ (2×10 mL) and deionised water (2×10 mL). The organic phase was then dried over $MgSO_4$. The solvent was evaporated under reduced pressure. The residual oil was purified by flash chromatography (1.5×16 cm) on silica gel 60 (0.015–0.04 mm, column dimensions 1.5×16 cm) with ethyl acetate-hexane (1:1, 200 mL) as eluent to give the amide **7** (71 mg), R_f 0.1 [ethyl acetate/hexane (1:1)]. δ_H (200 MHz; $CDCl_3$) 1.37–1.54 (2H, m, CH_2), 1.54–1.85 (4H, m, 2×CH_2), 1.96 (3H, s, $COCH_3$), 2.03 (3H, s, $COCH_3$), 2.04 (3H, s, $COCH_3$), 2.05 (3H, s, $COCH_3$), 2.06 (3H, s, $COCH_3$), 2.13 (3H, s, $COCH_3$), 2.15 (3H, s, $COCH_3$), 2.34 (2H, t, $J=6.9$ Hz, CH_2), 2.54–2.72 (2H,

m, CH_2), 3.58–3.68 (1H, m, C-5′), 3.81 (1 H, t, $J=9.4$ Hz, C-4′), 3.87 (1H, t, $J=6.6$ Hz, C-5), 4.00–4.22 (m, 3H, C-6 and C-6b′), 4.44 (1H, m, C-1), 4.88–5.02 (2H, m), 5.15 (1H, m), 5.21 (1H, m), 5.32 (1H, d), 7.08 (1H, m), 7.3 (1H, m), 7.5 (2H, m, aromatic). δ_C (50.3 MHz; $CDCl_3$) 20.51, 20.63, 20.79, 20.96, 24.78, 27.74, 29.21, 29.81, 37.28, 60.76, 61.96, 66.61, 69.17, 70.22, 70.75, 70.98, 73.77, 76.07, 76.39, 76.81, 77.03, 77.66, 83.66 (C-1), 101.07 (C-1′), 117.36, 120.75, 121.89, 129.63, 129.92, 138.90, 168.99 ($COCH_3$), 169.67 ($COCH_3$), 169.87 ($COCH_3$), 170.07 ($COCH_3$), 170.13 ($COCH_3$), 170.34 ($COCH_3$), 170.72 ($COCH_3$), 171.43 (O=C–N). MS ESI: calcd: 948.26 m/z, exp: 948.20 m/z, additional peaks at 822 (M−H-3 Ac), 780 (M−H-4 Ac), 738 m/z (M−H-5 Ac).

6-(β-D-Galactosyl-(1→4)-β-D-glucopyranosylsulfanyl)-hexanoic acid [3-(3-trifluoromethyl-3*H*-diazirin-3yl)-phenyl]amide 8. To a solution of the acetylated glycoside **7** (71 mg) in dry methanol (8 mL) was added dropwise, under nitrogen, a solution of sodium methoxide in methanol (0.12 mM, 0.6 mL, 0.6 equiv). After 9 h the mixture was neutralised with Amberlyt 15, and filtered over Celite. The organic solvent was evaporated under reduced pressure to give thioglycoside **8** (14.3 mg). δ_H (200 MHz; MeOH-d_4) 1.49–1.69 (2H, m, CH_2), 1.69–1.89 (4H, m, $2\times CH_2$), 2.46 (2H, t, $J=7.2$ Hz, CH_2), 2.73–2.95 (2H, m, CH_2), 3.44–4.09 (12H, m, carbohydrate, methanol), 4.39 (1H, C-1′), 6.94–7.16 (1H, m, aromatic), 7.39–7.55 (1H, m, aromatic), 7.64–7.76 (2H, m, aromatic). δ_C (50.3 MHz; MeOH-d_4) 26.25, 29.35, 30.70, 30.75, 37.80, 62.08, 62.5, 64.43, 70.30, 72.54, 74.10, 74.83, 77.09, 77.94, 80.4, 80.5, 87.08, 105.04, 118.63, 122.20, 122.65, 130.73. Quaternary carbons (CF_3, C=O, C aromatic–N, C aromatic–C) are not observed owing to the low signal/noise ratio. MS ESI (M−1): Found m/z 678.1894. $(C_{26}H_{36}F_3N_3O_{11}S+Na)^+$ requires m/z 678.1921. TLC in CH_2Cl_2/MeOH (2:1) of the deprotected compound **8** (Scheme 2) gave a single spot on TLC (R_f 0.37).

Immobilisation of MAD-Gal 1 and 6-(β-D-galactosyl-(1→4)-β-D-glucopyranosylsulfanyl)hexanoic acid [3-(3-trifluoromethyl-3*H*-diazirin-3yl)-phenyl]amide 8 on polystyrene. Immobilisation on PS was carried out as described elsewhere.[23] Briefly, four different polystyrene sample types were prepared for further experimental characterisation. Volume of the deposited solution was a function of sample area.

Droplets of a MAD-Gal or lactose aryl diazirine solution (in ethanol and in methanol, respectively) were deposited on the PS samples. Following the experiments, various PS types and various concentrations were used: 10 μL of a solution (0.25 mM) on spin-casted PS for ToF-SIMS; 25 μL of solution (0.25 mM) on PS cut out from falcon well for XPS; 45 μL of solution (0.25 mM) on individual Elisa wells for Allo A and solid-phase semi-synthesis; 80 μL of solution (0.25 mM) on Falcon non tissue culture treated plates for rat hepatocytes. Moreover, in the case of XPS and Allo A, the effect of the MAD-Gal solution concentration on grafting extent was studied with 0.25, 0.025 and 0.0025 mM solutions.

The solvent was evaporated for 2 h at 30–40 mbar before irradiation of the samples (350 nm, 0.95 mW cm^{-2}, 25 min). The samples were washed four times in water (HPLC grade). Samples are referred to as **A**, **B** and **C**. Sample **A** is the pristine polystyrene. Sample **B** is polystyrene with MAD-Gal deposited and washed. This sample treatment does not include light exposure. Physisorbed molecules should be removed by washing. Sample **C** is the polystyrene surface on which the molecules were deposited. The surface was then photoactivated and washed. Covalently attached molecules are expected to remain bound to the surface, by contrast with sample **B**.[23]

Lectin binding to lactose or galactose modified polystyrene

Lectin binding studies were performed on MAD-Gal (**1**) or lactose aryl diazirine (**8**) modified ELISA plates. Individual wells were coated with BSA (1 mg/mL, 100 μL) in PBS and 1 μL of a 500 μg ml^{-1} biotinylated Allo A lectin (500 μg mL^{-1}, 1 μL) in PBS was added. For inhibition tests, incubation was performed in BSA solution (1 mg mL^{-1} in PBS, 90 μL), followed by asialofetuin (10 mg mL^{-1}, 10 μL) and Allo A lectin (500 μg mL^{-1} 1 μL). The samples were incubated for 1 h at room temperature with agitation. The samples were washed subsequently three times with Tween (0.02% in PBS, 100 μL, 5 min, agitation), three times with PBS (100 μL, 5 min, agitation), and once with deionised water (100 μL, 5 min, agitation).

Commercial [^{35}S] radiolabelled streptavidin solution was used to quantify surface bound biotin–Allo A. The stock solution was diluted in water (1:100). Aliquots (50 μL) of the solution were added to each well. The plate was incubated for 1 h at room temperature. The samples were washed three times with Tween 0.02% in PBS (100 μL, 5 min, agitation), three times with PBS (100 μL, 5 min, agitation) and once with deionised water (100 μL, 5 min, agitation). For radioactivity measurements, individual wells were placed in scintillation vials, and dissolved in toluene (30–40 min). Scintillation fluid (5 mL) was added and radioactivity was measured by scintillation counting. One hundred percent of radioactivity corresponded to the radioactivity of 50 μL of the diluted streptavidin solution. Triplicates of samples **A**, **B** and **C** were measured.

Isolation and cultures of primary rat hepatocytes

The reagents used for hepatocyte isolation and cultures were identical to those described earlier.[47] The hepatocytes were isolated by two-step in situ liver perfusion[47] from male Sprague–Dawley rats [Zur: SD (Crl:CD®)], (Institute of Toxicology, Swiss Federal Institute of Technology, Zürich, Switzerland; age 54±2 days; 240–270 g body weight) kept under controlled environmental conditions (12 h light–dark cycle, diet No. 3430 KLIBA, Kaiseraugst, Switzerland, food and water ad libitum) as

reported.[47] Isolated hepatocyte showed a viability of >85% (assessed by Trypan Blue exclusion in samples without bovine serum albumin). The contamination with non parenchymal cells was less than 0.5%. The hepatocytes were seeded into 24-well non tissue culture plates at densities of 50×10^3 cells cm^{-2}, in William's medium E (Sigma) without phenol red, supplemented with dexamethasone (100 nM), insulin (10 nM), L-glutamine (2 mM), selenium (30 nM), aprotinin (1 μg mL^{-1}) and penicillin/streptomycin (100 IU mL^{-1}). The cells were cultured for 5 days at 37 °C at 5% CO_2 in air. The influence of the surface graft on cellular functions was assessed by comparing cellular functions in uncoated and coated wells on the same 24-well plates. Coating includes a well-established extracellular matrix as reference [Crude Liver Membrane Fraction (CMF) 30 μg and 0.3 μg collagen in 0.5 mL PBS overnight at 4 °C] as described elsewhere[48] and asialofetuin (1 mg in 0.5 mL PBS overnight at 4 °C) as a mimic of the galactose-coated surface. To some galactose modified wells, additionally 5 μg of a galactose binding lectin (from *Cytisus scoparius*, Sigma, St Louis, USA) was applied in 0.5 mL PBS for 1 h at room temperature.

Hepatocyte function

The binding of hepatocytes to the engineered polystyrene surfaces was investigated in four ways, each of which is related to specific changes in cell function as a result of the hepatocyte surface interactions: This includes lysosomal activity by neutral red (NR) uptake (neutral-red assay[49]), the total protein as an estimation of attached cells (cell density, BCA assay, Pierce, Rockford, IL, USA), the total cellular bioreductive capacity or viability by using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT-assay[50]] and an estimation of the activity of a representative cytochrome P450 enzyme (CYP 1A1) involved in the oxidative metabolism of xenobiotics using ethoxyresorufin de-ethylase activity (EROD-activity[50]).

After 24 h (attachment period) and after 5 days in culture (end of the experiment), the four parameters were determine simultaneously.

Protein content. After removal of the medium, the BCA-solution (Pierce, Rockford, IL, USA) was applied directly to each well for 20 min and the developing colour (depending on the amount of total protein) was measured at 550 nm (Hamilton HR 7000 microplate reader).

MTT assay. The tetrazolium salt (1.2 mM) was applied in culture medium to each well and incubated for 30 min (hepatocytes) at 37 °C. The medium was replaced with ethanol [90% (v/v)] in HEPES–buffer (50 mM, pH 8.0). The amount of soluble MTT-formazan formed was measured at 550 nm (Hamilton HR 7000 microplate reader).

Neutral-red assay. Neutral red was applied in culture medium to each well and incubated for 30 min (hepatocytes) at 37 °C. After fixation of the cells and washing with PBS, the chromophore was solubilised in 100% isopropanol and fluorescence was measured at 530 nm (excitation) and 645 nm (emission) (Millipore Cyto-Fluor 2350 microplate reader).

EROD assay. The enzyme activity was determined in situ. Hepatocyte monolayers were incubated 30 min in 0.5 mL of Hank's buffer solution supplemented with the corresponding substrate (5 μM 5-ethoxyresorufin) and 10 μM dicumarol as described by Wortelboer et al.[50] Fluorescence in the supernatant was measured after 15 and 30 min at Ex/Em: 530 nm (excitation) and 580 nm (emission) (Millipore CytoFluor 2350 microplate reader) and the enzyme activity was calculated.

Incorporation of sialic acid using α-2,6-sialyltransferase

Diazirine **8** was photoimmobilised on ELISA title plates (Nunc). The recombinant α-2,6-sialyltransferase[18] from *Pichia pastoris* was diluted with water (34 μL, except for the no enzyme control in which 44 μL of water was added) and Cacodilate buffer (5 μL). The enzyme substrate [^{14}C]CMP-Neu5Ac (0.925 kBq μL^{-1}, 10.8 GBq $mmol^{-1}$) was added and the samples were incubated for various times at 37 °C. The reaction was stopped with 100 μL of cold water (0 °C). The wells were then sequentially washed four times with water. Radioactivity retained in the PS wells was quantified as described above (triplicate analysis).

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