Carbohydrate-based nanocarriers for targeted drug and siRNA delivery

Tan, Yu Jia

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NANYANG
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**CARBOHYDRATE-BASED NANOCARRIERS FOR
TARGETED DRUG AND siRNA DELIVERY**

TAN YU JIA

**SCHOOL OF PHYSICAL AND MATHEMATICAL
SCIENCES**

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**CARBOHYDRATE-BASED NANOCARRIERS FOR
TARGETED DRUG AND siRNA DELIVERY**

TAN YU JIA

School of Physical and Mathematical Sciences

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Abstract

The specific delivery of chemotherapeutics to target cancer cells is a clinical challenge that warrants research attention. Herein, the design of the delivery systems is fundamentally based on the interactions between boronic acid and carbohydrates, as well as the specific recognition of lectins for sugars. Owing to the wide range of topics integral to the thesis, an introduction to these important concepts and recent developments are first described.

In the next part of the thesis, a multi-component system based on boronic acid-conjugated carbohydrate-functionalised gold nanoparticles is presented. Target-specific uptake is achieved through recognition of the carbohydrates and the cell surface lectins, and cleavage to release the fluorophore and pro-drug occurs under thiol-reducing conditions commonly found within cancer cells. *In vitro* assays demonstrated the cell-type specificity of the delivery vehicle, with significant cytotoxicity observed only in the target cells. A notable feature of the delivery system is the potential to target different cells depending on the cell surface lectins expressed and corresponding carbohydrates conjugated onto the nanoparticles.

The synthesis of a boronic acid-functionalised chitosan polymer as a siRNA delivery vehicle is explored in the third part of the thesis. The boronic acid is hypothesized to act as a targeting ligand through interactions with cell surface sialic acids, while the polymer protects the siRNA against nuclease degradation. Release of the siRNA is designed to be stimuli-responsive, in which the polymer sheds the ligand under acidic condition to expose the thiol-linked siRNA that can be cleaved from the polymer under GSH-rich conditions. The formation of the stable siRNA-polymer complex has been demonstrated *via* gel retardation assay and optimised.

Index of abbreviations

δ	chemical shift
$^{\circ}\text{C}$	degree centigrade
λ_{ex}	excitation wavelength
μL	microliter
μM	micromolar
ASGPR	asialoglycoprotein receptor
bp	base-pairs
CDCl_3	deuterated chloroform
CDM	carboxy dimethylmaleic anhydride
d	doublet
DIPEA	<i>N, N</i> -diisopropylethylamine
DMA	dimethyl acetamide
DMAP	4-dimethylaminopyridine
DMF	<i>N, N</i> -dimethylformamide
DMSO	dimethyl sulfoxide
dsRNA	double-stranded RNA
EPR	enhanced permeability and retention effect
ESI	electrospray ionisation
EtOH	ethanol
FAM	6-carboxyfluorescein
FBS	foetal bovine serum
Gal	galactose
GC	glycol chitosan
Glc	glucose
GNPs	carbohydrate-functionalized gold nanoparticles
GSH	glutathione
HRMS	high-resolution mass spectroscopy
J	coupling constant

K _D	dissociation constant
kDa	kilodaltons
m	multiplet or unresolved
M	mol/L
Man	mannose
MeCN	acetonitrile
mM	milimolar
MW	molecular weight
nm	nanometer
NMR	nuclear magnetic resonance
<i>o</i>	<i>ortho</i>
<i>p</i>	<i>para</i>
PBS	phosphate-buffered saline
Pd	palladium
PEG	polyethylene glycol
RNAi	RNA interference
r.t.	room temperature
s	singlet
SD	standard deviation
SEM	standard error of mean
siRNA	short interfering RNA
t	triplet
t-Bu	<i>tert</i> -butyl
THF	tetrahydrofuran
TLC	thin-layer chromatography

*Dedicated to my grandfather, who inspired me to embark on this Ph.D. journey and
the continuous quest for knowledge.*

Chapter 1. Carbohydrates, lectins, boronic acids and their implications in cancer

1.1 Significance of carbohydrates in nature

Carbohydrates exist as a class of biologically significant biomolecules abundant in nature. The monosaccharides, which comprise of polyhydroxy subunits, can be linked through the anomeric hydroxyl group and any hydroxyl functionality of other monosaccharides. The multitude of glycosidic bonds possible in both the *alpha* and *beta* stereochemistry thus gives rise to a diverse library of polysaccharides.¹ Moreover, carbohydrates are often covalently bound to other biomacromolecules, such as proteins and lipids,² enhancing the complexity and versatility of carbohydrate scaffolds in encoding biological information.³

In cells, carbohydrates form a dense glycocalyx of approximately 10 to 100 nm on the cell surface,⁴ and are involved in many physiological functions such as cell recognition, signaling and host-pathogen recognition.^{5,6} In human immunodeficiency virus (HIV) for instance, the gp120 glycoprotein consists of a high mannose epitope, which interacts with the CD4⁺ receptors on host T cells.⁷ This specific interaction facilitates the attachment and eventual viral entry into the target cell to initiate infection.⁸ Further research has also indicated the role of the heavily mannosylated loop in immunosuppression and inhibition of T cell proliferation,⁹ highlighting the significance of glycans in biological processes.

The level of expression of carbohydrates on cell surfaces is also implicated in cancer progression. Changes in activities of sialyltransferases and sialidases in cancer have been observed to lead to aberrant sialylation, a feature associated with malignant potential of the disease.¹⁰ Abnormal glycosylation also results in the expression of tumor-associated carbohydrate antigens (TACAs) on tumor cell surfaces, which correlates with tumor progression and metastasis.¹¹

1.2 Carbohydrate-lectin interactions

Lectins are carbohydrate-binding proteins that are able to agglutinate cells through the preferential binding of specific carbohydrates present on the cell surface in the form of glycolipids or glycoproteins.^{12,13} In mammals, endogenous lectins have been identified as essential in biological events such as cell adhesion and migration, defence against pathogens and apoptosis.^{14,15}

Carbohydrate-lectin interactions are non-covalent in nature, and are through hydrogen bonds, van der Waals interactions, and may be dependent on the presence of metal ions for coordination.¹⁶ As such, carbohydrate-lectin interactions are considerably weak, with equilibrium dissociation constants (K_D) in the millimolar range.¹⁷ However, lectins often have more than one carbohydrate binding sites, and the interaction between carbohydrate and lectin is not a monomeric but an oligo- or polymeric binding event,¹⁸ which increases the binding affinity by up to 10,000-fold.¹⁹ This multivalency of carbohydrate-lectin binding is known as the glycoside cluster effect and leads to observed K_D values towards the nanomolar range.²⁰ The factors involved in carbohydrate-lectin interactions and the origins of the specificity are briefly discussed below.

Hydrogen bonding

The affinity and specificity of lectins for a particular complex glycan is dependent on the topological presentation of the carbohydrate, which includes factors such as density, conformation and flexibility of the ligand.²¹ Considering the multiple hydroxyl groups present on a carbohydrate scaffold, extensive hydrogen bonding network is predominant and vital in carbohydrate-lectin interactions.²²

Legume lectins, which are plant lectins found mainly in seeds, recognise monosaccharides by binding to the 3-, 4- and 6-OH of sugars. Specificity for mannose arises from the binding of the axial 2-OH, which allows discrimination between D-mannose (Man) and D-glucose (Glc) (Figure 1). In the case of Gal-specific legume lectins, the axial geometry of the 4-OH differs from the equatorial 4-OH in Glc and Man, which accounts for the specificity for galactose (Gal).²³ In addition, these lectins often can accommodate an additional sterically bulky substituent at the C-2 position, such as an acetamido group present in *N*-acetylgalactosamine (GalNAc).

Figure 1. Structures of carbohydrates commonly involved in carbohydrate-lectin interactions.

The ring oxygen can also act as a hydrogen bond acceptor and generally shares the hydrogen bond donor (amino acid residue of the lectin) with another hydroxyl group of the sugar. For GalNAc, *N*-acetylglucosamine (GlcNAc) and *N*-acetylneuraminic acid (Neu5Ac) residues, the acetamido functional group can serve as both a hydrogen bond donor and acceptor through the carboxylate -O and amide -NH moiety in a rigid planar geometry.²³

Hydrophobic stacking

The stereospecific nature of the hydroxyl groups in a pyranose sugar ring results in a net polarity of the α and β face,²⁴ hence, carbohydrates are capable of establishing hydrophobic interactions with the less polar amino acid residues in lectins. The hydrophobic C-H bonds and methyl groups in *N*-acetylated or 6-deoxy glycans can undergo π -stacking with aromatic residues such as Phe, Tyr and Trp in the carbohydrate-lectin binding pocket.²⁵ These C-H $\cdots\pi$ interactions, albeit weak, are entropically driven by desolvation and displacement of water molecules, thus constitute an important component in the stabilization of the carbohydrate ligand in the lectin binding sites.²⁶

Electrostatic interactions

While most of the carbohydrates are neutral, some glycans containing acidic side chains (e.g. sialic acid, glucuronic acid), amino groups, or functionalised phosphorylated and sulfated sugars are charged.²⁴ Hence, these sugars can form ionic interactions with charged amino acid residues, such as arginine (Arg).

Divalent metal cations (Ca^{2+} , Mn^{2+}) are also essential in the binding of certain lectins to the carbohydrate ligands. In plant lectins, the metal ions are responsible for the ensuring a correct spatial orientation of the residues in the binding side and do not participate in the carbohydrate binding.²⁷ On the other hand, in bacterial and mammalian lectins, the cations are usually coordinated to the amino acid residues and the hydroxyl group of carbohydrates concurrently, and are integral to the binding event.²⁸

1.2.1 Asialoglycoprotein receptors (ASGPR)

The first mammalian C-type lectin (Ca^{2+} -dependent) discovered and characterized is the asialoglycoprotein receptor (ASGPR).²⁹ It consists of two polypeptide subunits of 46 kDa and 50 kDa, and has three carbohydrate recognition domains that require the presence of Ca^{2+} for binding to glycoconjugates. In desialylated glycoproteins, the terminal Gal and GalNAc residues are recognized by ASGPR and rapidly cleared from the circulation.³⁰ The expression of ASGPR is the highest in cells of hepatic origin, with minimal existence in the rest of the body. Among hepatic cells, HepG2 (human hepatic carcinoma) expresses a density of more than 76,000 ASGPR per cell, much higher than cells of the same origin.³¹

An important physiological function of the ASGPR is the ability to internalize galactosides. Upon recognition and binding to a Gal-ligand, formation of clathrin-coated pits is triggered and ASGPR internalizes the glycan rapidly *via* receptor-mediated endocytosis, at a rate of 3.4×10^{-8} M and a half-life of 3 minutes.³² The clathrin coat is then shed and in the acidic environment of the lysosomes, the binding affinity to the Ca^{2+} is reduced, prompting the release of the glycan from ASGPR. Thereafter, the ASGPR is sorted to the recycling endosomes in which it is acetylated again and returns to the plasma membrane surface, where it is once again available for the endocytosis of ligands.³³

In terms of binding affinity to Gal ligands, the glycoside cluster effect is apparent in ASGPR binding. Monosaccharides bind to ASGPR with a K_D of 10^{-4} M, which decreases significantly to 9×10^{-9} M in tetra-antennary galactosides.³⁴ The 3-, 4-, 6-hydroxyl groups in Gal are vital in the binding event, as substitution at these positions resulted in steric hindrance and decreased binding affinity was observed.³⁵

1.2.2 Mannose receptors

Another C-type lectin of biological significance is the group of mannose binding receptors. The mannose receptor (MR, CD206) is primarily expressed on dendritic cells and macrophages, and is involved in modulating innate immunity.³⁶

As an endocytic receptor of 175 kDa, the MR consists of an *N*-terminal Cys-rich domain capable of binding to sulfated carbohydrates (SO₄-4-GalNAc, SO₄-3-GalNAc and SO₄-3-Gal) in a Ca²⁺-independent manner.³⁷ This domain is connected, *via* a fibronectin-rich domain, to the C-type lectin-like domains (CTLDs), which specifically recognize Man, fucose and GlcNAc. The binding event requires two Ca²⁺, of which one cation interacts directly with the sugar in the carbohydrate binding domain.^{38,39} The last structural motif associated with the MR is a cytoplasmic tail, which mediate the intracellular targeting of the ligands upon internalization.

1.2.3 Galectins

Galectins consists of a family of 15 Ca²⁺-independent mammalian lectins⁴⁰ that share an affinity for β -galactose (β -Gal), and have significant sequence similarity in the carbohydrate binding site.⁴¹ The galectins can be further classified into three main types: proto-type galectins, which contain two CRDs homodimers that are non-covalently linked and can cross-link cell-surface ligands; chimera-type galectins that possess a C-terminal CRD and Tyr-rich *N*-terminal; and tandem repeat galectins consisting of two distinct CRDs.⁴⁰

Research has indicated strong correlations between galectins and tumor progression in malignant cancers, highlighting the potential of using galectins as cancer biomarkers.^{42,43} In particular, galectin-3, the only chimera-type galectin identified to date, has been implicated in metastatic potential of several cancers^{44,45} with

overexpression of galectin-3 further observed in highly metastatic breast cancer phenotypes.^{46,47} Galectin-3 has also been identified to confer resistance to apoptosis in a variety of cell-types.^{48,49} Apoptosis is a programmed cell-death process mediated by a family of caspases that cleaves proteins at specific Asp sites and tightly regulates cell population. Through the anti-apoptotic effect of galectin-3, tumor cells are able to evade cell death and survive the host immune responses while traversing through the circulatory system.⁵⁰

The anti-apoptotic and angiogenic characteristics of galectin-3 have been attributed to the Asp-Trp-Gly-Arg (NWGR) motif present in the C-terminal domain,⁵¹ which is coincidentally the anti-death motif found to be critical for the anti-apoptotic function in the *Bcl-2* gene family.⁵² Moreover, studies have also demonstrated that interactions between galectin-3 and Thomsen-Friedenreich glycoantigen (TFAg), a galactose β -1,3 *N*-acetylgalactosamine residue present on 90% of cancer cells,⁵³ allow the adhesion of cancer cells to the endothelium, encouraging proliferation into secondary metastatic colonies.^{54,55}

1.3 Introduction to boronic acids

The ability of boronic acids in forming cyclic boronic esters with diols was first discovered in 1954 when a new compound was observed to be formed upon addition of phenylboronic acid to mannitol.⁵⁶ It was confirmed that the conjugate base of boronic acid is in a tetrahedral structure, which differs from the initial trigonal planar structure of the boronic acid due to hybridization of the boron atom from sp^2 to sp^3 .⁵⁷ Upon interaction of the boron atom with a molecule of water, a hydrated proton is released, and the acidity constant is quantified as the K_a (Figure 2).⁵⁸ In general, the pK_a of phenylboronic acids is in the range of 8.7- 8.9.⁵⁹⁻⁶¹

Figure 2. Formation of 5- and 6- membered cyclic boronate esters in the interaction of boronic acid with 1,2- and 1,3-diols respectively.

Subsequent experiments revealed that boronic acids interact reversibly and rapidly with diols,⁵⁷ dicarboxylic acids⁶² and α -hydroxy carboxylic acids⁶³ to form cyclic boronic esters in aqueous media. It became apparent that the kinetics of the ester formation are the fastest when the pH of the media is higher than the pK_a of the boronic acid,⁶⁴ which ensures that the boronic acid is in the anionic tetrahedral structure. In fact, a

rate enhancement of up to 10^4 was observed between boronic acid conjugation in the trigonal and tetrahedral form.⁶⁵

However, the complexation of neutral trigonal boronic acid and the diol is also possible and should be taken into consideration.⁶⁶ The diol boronic acid exists in equilibrium with the tetrahedral boronate ester, and the thermodynamic relationship is represented in Scheme 1, in which the equilibrium constant for diol boronic acid and boronate ester is defined as K_{trig} and K_{tet} respectively. The formation of the tetrahedral boronate ester is more favourable than that of the trigonal diol boronic acid, with $K_{\text{tet}} > K_{\text{trig}}$. Despite this, the boronic ester complex becomes more acidic than the original trigonal boronic acid upon complexation, with the experimental $\text{p}K_{\text{a}} > \text{p}K_{\text{a}}'$. Hence, the equilibrium can be shifted from the neutral boronic acid species to the anionic boronate ester.

Scheme 1. Equilibria for boronate ester formation.

Boronic acids as fluorescence-based sensors

The change in hybridization and ionization associated with the formation of the boronate ester often results in a concomitant change in physical properties, such as solubility or fluorescence characteristics.⁶⁷ The first fluorescence-based carbohydrate sensor was developed in 1992, in which a boronic acid functionality is covalently linked to an anthracene fluorescent moiety.⁶⁸ The anthrylboronic acid has an initial fluorescence profile similar to that of anthracene, with emission maximum centered at 416 nm (Figure 3). With the addition of saccharides, the fluorescence intensity decreased drastically by 30%, a phenomenon postulated to be a result of a diminishment of internal charge transfer (ICT) brought about by the modulation of electronic properties when the hybridization changes.⁶⁷ In ICT systems, a dipole moment is created by an imbalance of electron density in the excited state of the molecule. As a result, the fluorophore interacts with the surrounding solvent *via* favourable dipole-dipole interactions and the fluorescence is sensitive to changes in the chemical environment. This leads to dramatic shifts in fluorescence emission wavelengths, which can be exploited for biosensing applications.

Figure 3. Structures of anthrylboronic acid and 4-(dimethylamino)stilbene-4-boronic acid.

This report subsequently led to the development of other boronic acid-based ICT sensors for carbohydrate recognition. In a related work, the electron-rich amine group in 4-(dimethylamino)stilbene-4-boronic acid is designed to be the donor, while the sp^2 -hybridised trigonal neutral boronic acid acts as the electron acceptor and exhibits resonance with the aromatic ring (Figure 3).⁶⁹ In the excited state, the molecule exhibits ICT, with strong fluorescence emission at 485 nm. Following the addition of fructose, a ligand with strong affinity for boronic acids, a blue shift of 45 nm is observed in the emission spectrum. It is hypothesized that the formation of the anionic boronate ester results in the loss of the ICT excited state, as the sp^3 -hybridised boron centre is no longer able to act as an electron acceptor.

Due to the affinity of boronic acids for diols, the interaction of phenylboronic acid and glucose has been explored in the design of glucose-responsive biosensors for insulin monitoring.⁷⁰ The prerequisite for the development of such biosensors is the selectivity of the probe for glucose over other sugars, which is generally achieved by the installation of two boronic acid receptors in a single molecule (Figure 4).⁷¹⁻⁷³ The spatial orientation of the diboronic acids, as determined by the length of linker and the conformational flexibility that is afforded, dictates the choice of saccharide that the molecule preferentially binds to.⁷¹

In a particular design, the diboronic acid exhibits cooperative saccharide binding as the binding constant, K , is significantly higher than the monoboronic acid compound.⁷⁴ The high selectivity for glucose was attributed to the ability of D-glucose in forming a 1:1 cyclic complex with diboronic acid due to the presence of two pairs of cis-diols. D-fructose, on the other hand, forms acyclic complexes in a 2:1 ratio.^{75,76} The modular design of this diboronic acid forms the basis for the development of an optical fibre

sensor, GlySure™, that has been commercialized for the continuous glucose monitoring in critical care patients.

Figure 4. Structures of diboronic acids incorporated into glucose-responsive biosensors.

Cell capture and release

The reversible nature of the boronate ester allows cells to attach and be released from the heterogeneous surface by simply changing the chemical environment. The boronate esters are generally thermodynamically more stable when formed at pH higher than pK_a (*vide supra*). However, an exception to this is the unique binding modality between sialic acid and phenylboronic acid, which is postulated to involve the glycerol side chain, with additional stabilization provided by the coordination of the amide functionality to the sp^2 -hybridised boron centre.^{77,78} Due to the strong binding affinity, stable complexation is possible even at lower pH with the boronic acid existing in the neutral form. Hence, specific recognition of sialic acid can be achieved under physiological conditions, which forms the basis for the targeting of cancer cells as sialic acids are overexpressed on cancer cells.⁷⁹

As polysialylation and the overexpression of sialic acid on cellular surfaces are implicated in the malignancy of cancer,⁸⁰⁻⁸² the quantification of sialic acid levels may

be indicative of the metastatic potential of tumors. A self-assembled monolayer of boronic acid-linked decane-thiol was coated on a gold electrode, which is subsequently conjugated to a field-effect-transistor that offers monitoring of changes in charge density on the surface of the electrode (Figure 5).⁷⁹

Figure 5. (a) Schematic representation of phenylboronic acid-modified gold electrode for potentiometric sialic acid detection. (b) Electrode response with addition of cells with various degrees of metastasis. Reproduced with permission from ref 79. Copyright 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

The carboxyl anion of sialic acid is expected to result in an increment of threshold voltage (V_T) value, provided that the distance between the acid and electrode

corresponds to the Debye length of a few nanometers.⁸³ As cell surface sialic acid often occurs at the end of a carbohydrate chain, the distance prerequisite is fulfilled.

The hypothesis was tested with the addition of cell suspensions derived from mice tumoral lung specimens with varied extent of metastasis. Cultured murine melanoma B16-F10 and normal pneumocytes were used as the positive (100% metastasis) and negative (0% metastasis) controls respectively. A sharp spike in V_T was observed when B16-F10 cells were subjected to the analysis, which decreased as the degree of metastasis decreased. Notably, the V_T signal from healthy cells was 10-times weaker than that from cells with 100% metastasis, demonstrating the potential of this boronic acid-modified gold electrode in assessing the degree of malignancy in tissue specimens.

Following the success of using boronic acids in detecting extracellular sialic acids, a 3-aminophenylboronic acid-grafted polymer brush was developed to selectively capture MCF-7 cells through binding of the cell surface sialic acids (Figure 6).⁸⁴

Figure 6. Schematic representation of phenylboronic acid-grafted dual-responsive polymer brush for reversible cancer cells capture and release. Reprinted with permission from ref 84. Copyright 2013 American Chemical Society.

At pH 6.8 and in the absence of glucose, the polymer was able to capture the cells at an efficiency of 60% without any apparent toxicity in a non-invasive manner. When the pH is increased to 7.8, the equilibrium shifts to form a higher concentration of anionic tetrahedral boronate ester. Upon addition of excess glucose, competition for binding to the boronic acid results in the release of the cancer cells. Subjecting the cells to a lower pH and glucose-free environment again allows cell surface sialic acids to bind to the polymer, capturing the cancer cells reversibly, a process that was shown to be dual-responsive for at least five cycles.

Delivery agents

Several studies have demonstrated the effectiveness of boronic acids as organic carriers across artificial membranes.^{85,86} As boronic acids are generally compatible with the human physiology⁸⁷ and are non-toxic,⁸⁸ boronic acids have also been explored as intracellular delivery agents.

A phenylboronic acid-functionalised micelle incorporating anti-cancer drug dichloro(1,2-diamino-cyclohexane)platinum (II) (DACHPt) was prepared with the aim of targeting sialic acid-overexpressing B16-F10 murine melanoma cells (Figure 7).⁸⁹ DACHPt was loaded onto the micelle through ionic interactions with the anionic carboxyl-ends on the L-glutamic acid chains of the polymer, forming PBA-DACHPt/m. The sugar binding affinities of the delivery vector with various carbohydrates were first assessed through steady-state fluorescence quenching. As anticipated, sialic acid binding affinity was the strongest among the sugars tested, at both pH 7.4 and 6.5, which is the typical environmental pH in tumor cells.⁹⁰

Figure 7. Preparation of phenylboronic acid polymeric micelle and incorporation of DACHPt drug. Reprinted with permission from ref 89. Copyright 2013 American Chemical Society.

Cellular uptake of fluorescent-labelled micelles with boronic acid conjugation was significantly higher than micelles that were unmodified as observed with confocal laser scanning microscopy. Both the addition of free phenylboronic acid in competitive studies and pre-treatment with sialidase to specifically cleave off sialic acid epitopes on the cell surface led to a drastic decrease in fluorescence intensity. Further *in vivo* studies involving mice with B16-F10 melanoma tumors were conducted to evaluate the anti-tumor activity of PBA-DACHPt/m. While the free drug displayed no anti-tumor effects with respect to the untreated controls, PBA-DACHPt/m demonstrated increased accumulation in the tumor site and suppressed tumor growth. These results indicate that the enhanced uptake is mediated by boronic acid interactions with cell surface sialic acid, hence the viability of using boronic acid ligands for specific targeting to solid tumors.

The application of boronic acid was extended to a carrier of bovine pancreatic ribonuclease (RNase A), a biomacromolecule, into cells (Figure 8).⁹¹ Through the use

of ^1H NMR spectroscopy, it was first confirmed that benzoboroxazole has a greater affinity to sialic acid than phenylboronic acid ($K_a = 43 \text{ M}^{-1}$ vs 28 M^{-1}), a phenomenon that was previously reported by other research groups.^{92,93} RNase A was conjugated to benzoboroxazole *via* amine condensation and delivery was assessed by determining the ability of the boronic acid carrier in allowing RNase A to access the cytosol and activate its cytotoxicity. The concept was tested with Chinese hamster ovary cells Lec-2 and Pro-5, which have low and high levels of sialic acid expression respectively.

Figure 8. (Left) Boronation of RNase A and the postulated mechanism. (Right) Uptake efficiency of unmodified and modified RNase A in Lec-2 and Pro-5 cells with and without fructose addition.⁹¹

Internalisation of the RNase A was significantly enhanced with boronation, and this increase was abrogated when excess fructose was added as a competitor for boronic acid binding. The efficiency of boronic acids as carriers is attributed to the ability of boronate groups in forming reversible boronate esters with 1,2- and 1,3-diols of carbohydrates,⁶⁶ which are prevalent in the glycocalyx of cell surface. Complexation of the boronic acid to the cell surface carbohydrate transforms the boronic acid from a neutral trigonal boronate to a tetrahedral boronic ester that is negatively charged and cellular uptake of the boronic acid is increased *via* receptor-mediated endocytosis.⁹¹

In this study, negligible difference in uptake was observed between Lec-2 and Pro-5 cells, suggesting that the endocytosis could be mediated by glucose present in the glycocalyx, even though the initial design involved sialic acid targeting with benzoboroxazole. Further experiments on the biological activity of RNase A confirmed that boronation did not abolish the activity but instead increased the intracellular concentration of RNase A in the cytosol. This is evident in the much higher cytotoxicity of boronated RNase A ($IC_{50} = 4.1 \mu M$) than unmodified RNase A ($IC_{50} > 50 \mu M$).

An interesting research work on gene delivery consists of a boronic acid-(PB) linked 1.8kDa poly(ethyleneimine) (PEI1.8k) and DNA.⁹⁴ The anionic oligonucleotide interacts with the cationic PEI through electrostatic interactions, and stability of the complex is supposedly increased through boronate ester formation with DNA ribose units. Although the polyplex resulted in higher transfection efficiency than naked DNA or non-PB-modified polyplex, the presence of the boronic acid moiety contributed to the observed cytotoxicity. This experimental finding was attributed to the possibility of polymer aggregation on the cell surface⁹⁵ that resulted from the interactions of boronic acid with the carbohydrates in the glycocalyx.

In order to improve the biocompatibility of the delivery vehicle, the authors improvised and included γ -cyclodextrin (γ -CD) in their subsequent design (Figure 9).⁹⁶ The boronic acid forms a reversible covalent boronate ester with the glucose subunits on CD. This CD layer, which comprises of eight glycopyranose subunits, mimics the cell glycocalyx in what is termed a 'surficial saccharide camouflage',^{97,98} allowing the complex to interact with extracellular proteins. Complexation of DNA and anti-cancer drug doxorubicin then proceed *via* condensation with the anionic polymer backbone and hydrophobic 'host-guest chemistry' respectively.^{99,100} The polyplex accumulates

in tumor cells *via* EPR effect and dissociates readily in the tumor microenvironment to release the chemotherapeutic payload.

Figure 9. A representative illustration of PEI-PB-CD formation, complexation with DNA and doxorubicin payload, and intracellular release. Reproduced with permission from ref. 96. Copyright 2013 John Wiley and Sons.

The resultant PEI1.8K-PB-CD/DNA complex was significantly more stable to serum than complexes without cyclodextrin-capping, indicating the serum-shielding effect of CD is essential for enhanced stability. The inclusion of CD also increased the transfection efficiency, which was comparable to that of high molecular weight PEI25K that is generally regarded as an efficient gene carrier. Investigations into the biocompatibility of the vehicle revealed that while the PEI1.8K-PB-CD is more toxic than PEI1.8K alone, the polyplex is overall less toxic than PEI25K, and the presence of CD is able to mitigate some of the toxicity issues related to boronic acid interference with membrane functions.¹⁰¹

While boronic acids are generally biocompatible, the cytotoxicity as observed in the preceding research work demonstrates that boronic acid complexation with extracellular carbohydrates may not be as benign as originally conceived. As such, it would be prudent to consider this possibility and careful optimization of boronic acid ratio in the design of delivery vehicles to minimize these issues is of paramount importance.

1.4 Targeting cancer cells

Cancer is a debilitating disease characterized by the uncontrolled cell division leading to tumor growth and metastasis. In the most recent report published by the World Health Organisation, 8.2 million people die each year due to cancer.¹⁰² Cancer-related deaths accounts for an estimated 13% of all deaths worldwide, a staggering figure that exceeds those caused by malaria, tuberculosis and AIDS combined.¹⁰³ It is also postulated that a 70% increase in new cases of cancer will be observed in the next two decades.

Chemotherapy, which is the most commonly prescribed form of treatment, suffers from drawbacks such as lack of target specificity, leading to severe side effects. Hence, the development of more effective therapies that can circumvent these issues is of primary importance. The delivery of anti-cancer drugs to specific cancer cells can be broadly classified as active or passive targeting, with recent design strategies discussed in the ensuing section.

1.4.1 Passive targeting

The enhanced permeability and retention (EPR) effect refers to the preferential accumulation of macromolecules in tumor cells, a phenomenon first observed 30 years ago.¹⁰⁴ The rapid development of blood vessels from surrounding capillaries to support the growth of tumors results in the formation of blood vessels with fenestrations lacking the proper basal membrane present in normal vascular structures.^{105,106} These fenestrations have typical diameters of 200- 2000 nm and permit the extravasation of small molecules and complexes into the tumor interstitial space, hence the enhanced permeation.¹⁰⁷ In normal tissues, the lymphatic system allows extracellular fluid to flow to the lymphatic vessels and extravasated components are drained and recycled back

into circulation. However, the impaired lymphatic function in tumor tissues results in inefficient uptake of the extracellular interstitial fluid,¹⁰⁸ hence the extravasated compounds cannot be drained effectively through the use of convective forces. While small molecules are capable of escaping by diffusing back into the circulation, larger complexes, such as macromolecules and nanoparticles (> 4 nm or 30 kDa) remain entrapped in the tumor environment, leading to their accumulation in tumor tissues.^{109,110}

The size of the nanoparticles thus plays a crucial role in effective retention in the tumor tissues. In general, the upper limit of the particle size should be less than 300 nm, as nanoparticles larger than this are recognized and cleared rapidly by the reticuloendothelial system.¹¹¹ The optimum size distribution however, appears to be dependent on cancer origin. Research has documented the comparable uptake of nanoparticles in the range of 30- 100 nm in murine colon adenocarcinoma, but in human pancreatic adenocarcinoma, only particles that are smaller than 70 nm were able to accumulate efficiently.¹¹² In another study, the uptake and tumor penetration of 50 nm nanoparticles was significantly higher than nanoparticles of larger sizes.¹¹³ Although promising, drug targeting using EPR effect accounts for only approximately 20- 30 % increase in accumulation in tumor tissues as compared to normal tissues due to tumor heterogeneity and patient variability.¹¹⁴ Passive targeting also suffers from the potential drawback of excessive accumulation in the liver or spleen due to clearance by the reticuloendothelial system, hence, homing of the drug conjugate needs to be optimized for targeting to specific intracellular sites for increased efficacy.¹¹⁵

1.4.2 Active targeting

Active targeting refers to the utilization of ligands with enhanced binding affinity to specific receptors expressed on cells of a target disease model.¹⁰⁷ Through understanding the ligand-receptor binding mode, conjugates can be attached to targeting ligands, leading to accumulation and uptake of the drug in the desired malignant cells.¹¹⁶ This approach increases the ability of the drug in reaching the pharmacological target and concomitantly reduces the potential side effects associated with the drug, since uptake by non-targeted cells is expected to be low.¹¹⁷

In the design of ligand-drug conjugates, the target receptor should ideally be an extracellular receptor expressed on the cell surface to facilitate binding to the ligand.¹¹⁸ Moreover, ligands should possess sufficiently high binding affinity (K_D of approximately 10 nM) to the target receptor such that a lower concentration of drug is needed to reach receptor saturation.¹¹⁹⁻¹²¹ This is however, not an absolute threshold requirement, as the conjugation of several ligands to a single therapeutic agent can increase the avidity of the ligand-receptor binding.^{122,123}

Antibody-based targeting

Antibodies possess high affinity and selectivity to the targeted antigens presented on cell surfaces, as the structure of the antibody is such that there are two epitope binding domains on a single molecule.¹²⁴ Hence, antibodies were one of the first to be explored as potential homing ligands on nanocarriers.¹²⁵

The human epidermal growth factor receptor 2 (HER2 receptor) is an extracellular tyrosine kinase receptor overexpressed in approximately 15- 30% of invasive breast cancers.¹²⁶ In these forms of breast cancer, a 40-100-fold increase in HER2 expression as compared to that in normal breast tissues have been observed, which

results in up to 2 million receptors expressed on the tumor cell surface.¹²⁷ Studies revealed that an amplification of the HER2 gene in breast cancer patients correlates with the staging of the disease, and high overexpression has an implication on shorter survival rates and recurrence of the cancer.^{128,129}

Due to the significance of HER2 in breast cancer, the receptor has been developed as a delivery target, as well as an antigen of prognostic value. Monoclonal antibody (mAb) trastuzumab (Herceptin®), has been designed and developed as a targeting agent in HER2- positive breast cancer treatment modalities.¹³⁰ While the mechanism of action of trastuzumab remains to be fully elucidated, it is hypothesized that one of the mode of actions is the activation of antibody-dependent cellular cytotoxicity (ADCC). Upon binding of trastuzumab to HER2 receptor on the tumor cell, the activation of natural killer cells (NK) is triggered, resulting in the lysis of the cancer cells.^{131,132} HER2 overexpression has also been linked to the secretion of angiogenic growth factors, such as vascular endothelial growth factor (VEGF).¹³⁰ Analysis of murine xenograph models revealed that trastuzumab treatment resulted in normalization of tumor vasculature and is able to inhibit tumor-induced angiogenesis. Overall tumor growth was thus suppressed, resulting in increased survival.¹³³

HER2-antibody was also explored as a targeting ligand for homing paclitaxel and rapamycin loaded magnetic nanoparticles to MCF-7 breast cancer cells.¹³⁴ *In vitro* experiments revealed the increased cellular uptake of the nanoparticles when conjugated to HER2-antibody, and an enhanced anti-proliferative activity of the paclitaxel conjugate (IC₅₀ of 40.6 ng/mL) as compared to the free drug (IC₅₀ of 950 ng/mL), demonstrating the potential of HER2 receptor in active targeting of chemotherapeutic drugs to HER2-positive breast cancer cells.

Folate mediated targeting

The glycosyl phosphatidylinositol-linked folate receptor (FR) is overexpressed in cancers of various origins, and is often associated with malignancy of the disease.^{135,136} The α -isoform is upregulated in epithelial cancers, while the FR- β overexpression is observed in myeloid leukemia and chronic inflammation.¹³⁷ Folate, a natural ligand for FR, is essential for the biosynthesis of pyrimidines and purines and binds to FR with high affinity in the nanomolar range.¹³⁸ Due to the increased expression of FR in cancers, it has been reported that the concentration of folate in cancer cells is 100- 300 times higher than that in normal cells.¹³⁹ Furthermore, the wide availability of folate and the ease of functionalization of the small molecule further enhances the potential of utilizing it as a targeting ligand to FR on cancer cells.

In a related study, PEGylated liposomal doxorubicin derivatised with folate exhibited increased potency in FR-overexpressing KB cancer cells as compared to free doxorubicin and liposomes without folate conjugation.¹⁴⁰ The ligand was introduced by post-synthetic micellar insertion after the formation of the drug-liposome conjugate. It is postulated that the multivalent binding of the folate-coated liposomes to the FR triggers receptor-mediated endocytosis in which lysosomal involvement is probable, though the exact mechanism has yet been fully delineated.¹⁴¹ As approximately 70% of all acute myeloid leukemia cases express increased FR- β , a parallel study developed a FR- β liposomal doxorubicin conjugate for targeted delivery to MV4-11 (human acute myelocytic leukemia).¹⁴² Increased *in vitro* cytotoxicity was observed as compared to the non-targeted K562 (human erythromyeloblastoid leukemia) analogue, expanding the scope of folate targeting to leukemia cells in addition to solid tumors.

In vivo delivery of folate-functionalised poly-lactic acid (PLA) nanoparticles was successfully demonstrated in immunodeficient BALB/c nude mice with FR-overexpressing HeLa xenografts.¹⁴³ The multifunctional nanoparticles exhibited some tumor volume suppression and was able to mediate cardiac toxicity issues often associated with doxorubicin administration.¹⁴⁴ Moreover, a higher rate of tumor cells apoptosis was observed in comparison to free drug or nanoparticles without folate-conjugation. Further incorporation of ¹²³I to evaluate intracellular biodistribution of the conjugate indicated that folate-conjugated complex was localized in the tumors, where drug release could subsequently be triggered by pH decrease. On the contrary, significant accumulation of non-folate-conjugated nanoparticles were found in the liver over tumor cells.

RGD-directed targeting

Integrins are transmembrane receptors involved in the interactions of endothelial cells and the extracellular matrix (ECM).¹⁴⁵ Depending on the non-covalent association of the family of 18 α and 8 β glycoprotein subunits in various combinations, a diverse family of at least 24 heterodimers results, which act as matrix receptors in promoting cell motility and cell-cell adhesion.¹⁴⁶ Integrin clustering also activates intracellular signaling pathways that can regulate gene expression and cell proliferation. In solid tumors, which generally originates from epithelial cells, integrins are capable of mediating the proliferation and migration of cancer cells.¹⁴⁷ Particularly, the expression level of $\alpha_v\beta_3$ integrin is upregulated in angiogenic epithelial cells, an uncommon phenomenon generally not observed in quiescent cells.^{148,149} Research also highlighted the role of $\alpha_v\beta_3$ in promoting tumor progression through cross-talk with the fibroblast growth factor receptor, during which extrinsic apoptosis is inhibited.¹⁵⁰ The $\alpha_v\beta_3$ integrin is thus an attractive target as competitive binding to the receptor would

prevent the natural ligand from attaching, thereby preventing the anti-apoptotic and pro-angiogenesis pathways from contributing to tumor progression.¹⁴⁹

A high affinity ligand that has been discovered to be selective for $\alpha_v\beta_3$ integrin is the family of RGD (arginine-glycine-aspartic acid) peptides. In the investigation of the structure-activity relationships of these peptides, cyclic RGD peptides exhibited 10-fold higher anti-tumor efficacy than the linear analogues.¹⁵¹ Further development led to the discovery of a cyclic pentapeptide bearing a cyclo(-RGDfV-) domain, Cilengitide.¹⁵² As a potent antagonist of $\alpha_v\beta_3$, Cilengitide held great promise for the treatment of several cancers in the pre-clinical phase and entered Phase III clinical trials indicated for patients diagnosed with glioblastoma.¹⁵³ However, combination of Cilengitide with standard chemoradiotherapy failed to improve patient survival outcomes and further development was eventually halted.¹⁵⁴

Despite the unsuccessful development of Cilengitide, the significant role of integrins in cancer continued to motivate researchers in discovering new cRGD analogues with improved potency and activity. A pH-responsive cRGD-polycarboxybetaine methacrylate-*b*-polybenzimidazole methacrylate co-polymer that can encapsulate doxorubicin through π - π stacking with the benzimidazole was recently developed.¹⁵⁵ The monomers self-assemble into stable nanoparticles at physiological pH, but disintegrate to release the drug at low pH due to the protonation of the benzimidazole subunit in the acidic intracellular compartments. The overexpression of $\alpha_v\beta_3$ on HepG2 liver cancer cells was cited as the main reason for the higher cellular uptake of doxorubicin than in NIH3T3 cells with lower levels of $\alpha_v\beta_3$.

Another research group developed a temperature-sensitive liposome consisting of a cRGD-conjugated elastin-like polypeptide (ELP) that shrinks when the local

temperature falls below the critical solution temperature.¹⁵⁶ The polypeptide was incorporated into the rest of the liposome through hydrophobic alkyl chains, with doxorubicin encapsulated in the core of the liposome. Uptake of the cRGD-modified liposome was 7-fold higher than that without cRGD-conjugation in $\alpha_v\beta_3$ integrin-expressing U87MG and HUVEC cells. This distinct enhancement in uptake efficiency was not observed with the KB and B16-F10 negative controls (less than 2-fold increment). It was also found that the liposome was stable at 37 °C and negligible drug was released under physiological condition. However, when the temperature was increased to 42 °C, the mild hyperthermia condition triggered the shrinking of the ELP with concomitant release of the cargo. Hence, the pro-drug can be delivered to the target site and the encapsulated drug released specifically upon thermal induction at the desired site.

Carbohydrate-based targeting

Despite the pre-requisite for multivalent display of carbohydrates in order for sufficient binding affinity to cell surface lectins, carbohydrate-based targeting strategies have been gaining traction with researchers in recent years.¹⁵⁷ This is primarily due to the ease of modification of glycan delivery systems,¹⁵⁸ in which the spatial orientation, density and distribution of the carbohydrate linkers can be tuned to accommodate the receptor binding sites. Moreover, the length and rigidity of the linkers can be varied to further enhance targeting efficiency.

Among the various classes of lectins, research efforts have been mainly focused on C-type lectins, which encompass a large family of Ca^{2+} -dependent lectins with endocytic ability and hence, are potential receptors for cell-type specific delivery.^{159,160} Particularly, targeting of the asialoglycoprotein receptor (ASGPR) allows specific

delivery to the liver due to the exclusive expression on parenchymal hepatocytes, and is a well-established area of research.¹⁶¹

In order to achieve multivalent display of galactosides on a globular surface, galactose ligands of various valency and spatial arrangement were immobilized on magnetic iron nanoparticles (Figure 10).¹⁶²

Figure 10. Structures of glycosylated ligands and synthesis of fluorescent-labelled galactosylated nanoparticles. Reproduced with permission from ref 162. Copyright (2010) WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

The Fe₂O₃ nanoparticles were functionalized with amino groups that can be further modified through reaction with a bifunctional *N*-hydroxysuccinimide ester and labelled with a fluorescent linker. A series of mono- and tri-antennary galactosidic linkers with varied lengths were synthesized and conjugated to the nanoparticles to determine the effect of linker spatial geometry on ASGPR-binding and recognition. As anticipated, nanoparticles with tri-antennary galactosides were more efficiently taken up by HepG2

cells due to the high Gal-content. However, it was also discovered that more rigid and shorter linkers were preferred, as the galactosides are closer in proximity, which decreases the entropy during ASGPR-binding. A maximum distance of 2.5 nm between the galactose residues on the tri-antennary linker was also postulated to be ideal, since it corresponds to the approximate distance between ASGPR binding sites.^{163,164}

Substitution of the mono-antennary Gal for Glc or sialic acid residues resulted in a significant decrease in fluorescence intensities, since these sugars are not recognized by ASGPR and hence, would not be taken up. Further co-localisation confocal studies revealed that the nanoparticles accumulate in the late endosomes/lysosomes, which is indicative of receptor-mediated endocytosis, since phagocytosis would result in accumulation of the nanoparticles in the phagocytic vacuoles of the endosomes instead.¹⁶⁵ This study indicates the potential of using carbohydrate-functionalized metallic nanoparticles in bioimaging and drug delivery, in which multivalency and optimization of ligand spatial orientation may be important.

A polyphosphoester (PPE)-based delivery system was recently developed as a vehicle for doxorubicin delivery to ASGPR-expressing HepG2 cells (Figure 11).¹⁶⁶ The polymer was designed to be biodegradable due to the presence of polyphosphodiesterases in the cell cytoplasm and intracellular compartments.¹⁶⁷ By first preparing a poly(3-caprolactone)-*block*-poly[2-(2-oxo-1,3,2-dioxaphospholoyloxy) ethyl acrylate] (poly(CL-co-OPEA) through ring-opening polymerization (ROP), the authors conjugated a galactosamine residue to the co-polymer, which self-assembled to form micelles in aqueous solution. The micelles consist of a hydrophobic core that can encapsulate doxorubicin, and a hydrophilic polyphosphoester-linked galactosamine surface.

Figure 11. Schematic illustration of poly(CL-co-OPEA-Gal)-encapsulated doxorubicin nanoparticles for hepatocyte delivery. Reproduced from Ref. 166 with permission from The Royal Society of Chemistry.

Stability tests revealed that the micelle was pH-responsive and degraded rapidly to release the drug when the pH was lowered from 7.4 to 5.0. This observation was attributed to the acidic hydrolysis of the polyphosphoester, which resulted in degradation of the micelle.^{167,168} The targeting ability of the micelle was subsequently studied through fluorescence and cytotoxicity tests conducted on HepG2 and HeLa cells, which is ASGPR-deficient. Uptake of doxorubicin appeared to be slower in HeLa cells as compared to HepG2 cells, and the pro-drug was significantly more cytotoxic to the target HepG2 cells. In addition, the pro-drug exhibited higher anti-tumor activity than the doxorubicin-loaded micelle without galactosamine conjugation, indicating the increased uptake in the presence of galactosamine.

Another related study utilized a combination of galactose-functionalised polymeric dithiocarbamate gold nanoparticles and anti-cancer drug, gold(I) triphenylphosphine, for targeted delivery to HepG2 cells (Figure 12).¹⁶⁹ A lactoside-based monomer was

first co-polymerised with glycidyl methacrylate and further modified to introduce two thiol-ends on the resultant polymer.

Figure 12. Synthetic scheme of glycopolymeric nanoparticles preparation and conjugation to Au(I)PPh₃. Reprinted (adapted) with permission from ref. 169. Copyright (2014) American Chemical Society.

By exploiting the favourable binding affinity of gold to sulfur, glycopolymer-decorated Au nanoparticles were successfully synthesized. Thereafter, Au(I)PPh₃ was complexed to the nanoparticles *via* the dithiocarbamate functionality, which stabilizes the complex through strong σ and π -donation and an additional π -back donation.¹⁷⁰ It was demonstrated that the polymeric nanoparticles had the strongest inhibitory effect on HepG2 cells, with an IC₅₀ of 4.13 μ g/mL. In contrast, HeLa and MCF-7 cells, which reportedly have lower ASGPR levels,¹⁷¹ show significantly lower cytotoxic effects when treated with the same complex, with IC₅₀ values being 3- to 4-fold higher. Further competitive experiments with asialofetuin, a natural glycoprotein with lactose ligands that bind ASGPR, resulted in a decrease in cytotoxicity towards HepG2 cells,

presumably due to unavailability of the ASGPR for uptake of the Au(I)PPh₃-loaded nanoparticles.

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Chapter 2. Stimuli-responsive delivery system for targeted drug delivery and cancer cells imaging

Abstract

Targeted delivery of contrast agents for bioimaging and chemotherapeutic drugs to achieve desired therapeutic effects while minimizing the occurrence of side effects are clinical challenges that warrant research attention. Presented herein is a multi-component delivery vehicle based on boronic acid-conjugated gold nanoparticles complexed with a fluorophore (NA-S-BA) and prodrug (CPT-S-BA), both of which are cleavable in the presence of free thiols. The system leverages on active targeting based on carbohydrate-lectin interactions and release of the payload under specific reducing conditions. *In vitro* experiments verified that the high concentration of glutathione (GSH) in cancer cells could cause cleavage of the disulfide bond in NA-S-BA, releasing the free naphthalimide with significant red-shifted fluorescence observed. Cell-type specific delivery of the activatable fluorophore was first examined by confocal imaging, with the target HepG2 cells displaying distinctly brighter fluorescence than HeLa and NIH3T3 cells. The system was further developed into a drug delivery vehicle with camptothecin (CPT) as the model drug. Cell viability assays with the prodrug conjugated system demonstrate that the complex exhibits similar cytotoxicity as free CPT to HepG2 cells and is significantly more cytotoxic to HepG2 cells than control NIH3T3 and normal HDF cells, revealing the high selectivity for the target cancer cells. The delivery vehicle itself exhibits excellent biocompatibility and offers an attractive strategy for cell-type specific delivery depending on the carbohydrates conjugated in the system.

2.1 Introduction

Cancer, which encompasses a complex group of diseases characterized by uncontrolled cell growth and division, has been consistently ranked as one of the medical conditions with the highest fatality rate.¹⁻⁵ While conventional chemotherapy is the mainstay of cancer therapeutics and has proven to be successful clinically, the lack of early diagnostic technology, coupled with the multifaceted nature of cancer, has hampered clinical efforts in curing patients of the disease.⁶⁻⁹ Moreover, the high incidence of severe side effects associated with chemotherapy reduces the overall efficacy of these treatment regimes, and is mainly due to the lack of tumor-targeting specificity of chemotherapeutics.¹⁰⁻¹² Fortunately, each malignant cell type has unique cellular characteristics that allow differentiation between malignant and normal cells.¹³⁻¹⁶ Therefore, targeted fluorescent biomarkers have found great utility in the specific visualization of cancer cells, enabling early stage detection of cancers rather than relying on advanced morphological changes alone.¹⁷⁻²⁰ Targeted treatment modalities can also ideally achieve drug delivery to specific cancer cells and tissues, thereby dramatically improving the selectivity and efficacy of anti-cancer drugs.²¹⁻²⁴ Hence, the development of a targeted system for cancer diagnosis and therapy is highly warranted.

Carbohydrates, which are involved in numerous biological processes such as cell growth, recognition and differentiation, play essential roles in cellular events through interacting with lectins and have thus been widely utilized in receptor-mediated targeting systems.²⁵⁻²⁹ Carbohydrate-functionalized nanoparticles constitutes a class of biomimetic models that can leverage on lectin-mediated endocytotic uptake.³⁰⁻³³ Among the myriad glycoconjugates available, carbohydrate-functionalized gold nanoparticles (GNPs), which were first reported in 2001, are one of the most

extensively studied.³⁴ The colloidal gold provides a globular display of carbohydrates on its surface, mimicking the dense extracellular glycocalyx. Through varying the carbohydrates conjugated and the target lectin expressed on cell surfaces, GNPs have evolved to become valuable tools in the study of carbohydrate-lectin interactions.³⁵⁻⁴¹ To this aim, a 'turn-on/ turn-off' biosensor involving carbohydrate-modified gold nanoparticles conjugated to fluorescein boronic acid was previously developed by our group.⁴² The system exhibits fluorescence in the presence of a carbohydrate-specific lectin that can bind to the GNP and concomitantly displace the fluorescein boronic acid, demonstrating excellent potential in its application in a targeted system.

Currently, stimuli-responsive systems that can be triggered by the unique tumor microenvironment to release a fluorescent payload or active drug have also been regarded as one of the most promising strategies to enhance cancer cell selectivity.^{10,43} Considering the much higher intracellular concentration of glutathione (GSH) in cancer cells as compared to normal cells, a variety of GSH-responsive systems with cleavable disulfide linkages have been developed for targeted cell imaging and drug release.⁴⁴⁻⁴⁶

In view of these considerations, it is envisioned that a dual-channel targeting system can be established based on the synergistic effect of active targeting in the form of carbohydrate-functionalized gold nanoparticles and a GSH-responsive disulfide scaffold (Figure 1).

Figure 1. Schematic representation of the proposed targeted stimuli-responsive vehicle for bioimaging and drug delivery.

Galactose was first selected as the carbohydrate ligand for the surface modification of gold nanoparticles, such that specific recognition by the asialoglycoprotein receptors (ASGPR) expressed on hepatocellular carcinoma cells (HepG2) can be achieved.^{47,48} Thereafter, boronic acid, which can form reversible cyclic boronic esters with the hydroxyl groups of the carbohydrates coated on GNPs, was conjugated to naphthalimide (a widely used fluorophore for bioimaging) and camptothecin (CPT, an inhibitor of topoisomerase I for cancer chemotherapy) *via* cleavable disulfide linkers, which are termed **NA-S-BA** and **CPT-S-BA**, respectively.⁴⁹⁻⁵⁴

It is demonstrated that the fluorophore conjugated nanoparticles (Scheme 1) display significantly enhanced fluorescence at around 550 nm, which is induced by the GSH-mediated cleavage of the disulfide linkage. Stronger fluorescence was observed in ASGPR-expressing HepG2 cells than ASGPR-deficient cancer cells (HeLa cells) or ASGPR-deficient cells with low GSH levels (NIH3T3 cells).^{55,56}

Scheme 1. Chemical structures of activatable fluorophore NA-S-BA and prodrug CPT-S-BA and the schematic diagram of their conjugation with gold nanoparticles.

Accordingly, the pro-drug conjugated nanoparticles exhibit distinct selectivity for cancer cells over normal cells, without compromising the chemotherapeutic efficacy of the native CPT drug. A noteworthy advantage is that by simply changing the carbohydrate moiety on the GNP, this system possesses the potential to target a variety of cancer cells based on the lectin expressed on the cell surface. To the best of our knowledge, this is the first combination of the carbohydrate-modified gold nanoparticles with activatable disulfide delivery system.

2.2 Results and discussion

Design and synthesis

The synthetic routes for NA-S-BA and CPT-S-BA are depicted in Scheme S1 (Methods and materials). The compound NA-NH₂, a key intermediate for the synthesis of NA-S-BA, was synthesized according to established procedures with some minor modifications.⁵⁷ In brief, a disulfide linker 2,2'-dithiodiethanol was conjugated with NA-NH₂ and CPT to form NA-S and CPT-S by utilizing triphosgene, 4-dimethylaminopyridine (DMAP) and *N,N*-diisopropylethylamine (DIPEA) as the base (Scheme 2). Subsequently, the boronic acid unit was introduced by reacting 3-aminophenylboronic acid pinacol ester with NA-S or CPT-S in the presence of triphosgene and DMAP. The target molecules NA-S-BA and CPT-S-BA were obtained by the deprotection of the boronic acid pinacol moiety in a binary solvent system of acetone/water and THF/water, respectively. Meanwhile, a control compound NA-C-BA, with an uncleavable linker ("C-C"), was also synthesized using the same method as with NA-S-BA (Scheme S1).

Scheme 2. Representative synthesis of NA-S-BA (a) i) triphosgene, DIPEA, toluene, reflux, 3 h, ii) 2,2'-dithiodiethanol, r.t., 12 h, 40%; (b) 3-aminophenylboronic acid pinacol ester, triphosgene, DMAP, CHCl₃, r.t., 12 h, 59%; (c) NaIO₄, NH₄OAc, acetone/H₂O (1:1), r.t., 12 h, *quant.*

Thiol-modified β -galactoside was synthesized and conjugated onto citrate-gold nanoparticles with diameter of approximately 15 nm (Figure S1), forming stable Au-Gal nanoparticles. The boronic acid-conjugated fluorophore and pro-drugs were further incubated with Au-Gal to form Au-Gal-BA and Au-Gal-BA(CPT) respectively.

GSH-activatable properties

The spectroscopic properties of the activatable fluorophore NA-S-BA were first tested to investigate the efficiency of the GSH-mediated cleavage of the disulfide linker. Due to the typical donor- π bridge-acceptor (D- π -A) structure, NA-S-BA exhibits broad absorption and fluorescence peaks centered at 374 nm and 472 nm in a binary solvent system of DMSO/PBS (1:1, v/v, pH 7.4) respectively, and appears as a pale yellow solution with blue fluorescence (Figures 2A & 2B). On the other hand, under the same condition, the key intermediate NA-NH₂ possesses distinctly different spectra with absorption at 437 nm and strong green fluorescence at 535 nm (Figure S2). The apparent difference between NA-S-BA and NA-NH₂ can be ascribed to the carbamate structure in the former, which masks the electron-donating ability of the nitrogen atom.

Figure 2. Absorption and emission changes of (A, B) NA-S-BA (10 μ M) and (C, D) control NA-C-BA (10 μ M) in the presence of GSH in DMSO/PBS solution (1:1, v/v, pH = 7.4, 10 mM). Inset (A) and (C): Color changes observed in NA-S-BA and NA-C-BA solutions upon addition of GSH. Inset (B) and (D): Visible fluorescence changes in NA-S-BA and NA-C-BA upon addition of GSH. Each point was recorded after exposure to GSH for 1 h at 37 $^{\circ}$ C, λ_{ex} = 405 nm is chosen as the excitation wavelength in order for an accurate comparison of the fluorescence intensity before and after GSH-induced cleavage of the disulfide bond.

Subsequently, the responsiveness to GSH was investigated *via* the change in the spectral properties of NA-S-BA upon the addition of GSH. As shown in Figures 2A & 2B, after the reaction with GSH in the DMSO/PBS system at 37 $^{\circ}$ C, a red-shift of 63 nm was observed simultaneously in the absorption and fluorescence spectra of NA-S-BA, with concurrent change in color of the solution (from almost colorless to yellow) and fluorescence when irradiated with light (blue to green). Moreover, the isobestic point in the absorption spectra at around 405 nm indicates the generation of a new

compound after interaction with GSH. Meanwhile, the control NA-C-BA, which comprises of stable alkane bonds instead of disulfide bond in the linker, reveals negligible change in the photophysical properties even in the presence of excess GSH (Figures 2C and 2D).

Figure 3. (A) Changes in fluorescence intensity at 535 nm for NA-S-BA and NA-C-BA (10 μ M) in DMSO/PBS solution (1:1, v/v, pH = 7.4, 10 mM) in the presence (black and blue) and absence (red and purple) of GSH (5.0 mM) over time. λ_{ex} = 405 nm. Data was recorded every 0.5 s. (B) Fluorescence response of NA-S-BA (10 μ M) upon addition of various amino acids including Ala, Leu, Ile, Val, Pro, Phe, Met, Trp, Gly, Ser, Gln, Thr, Asn, Tyr, Asp, Glu, Lys, Arg, His (5.0 mM). Each spectrum was recorded after exposure to GSH for 1 h at 37 $^{\circ}$ C, λ_{ex} = 405 nm.

The time-dependent fluorescence spectra of NA-S-BA in the absence of GSH and NA-C-BA under the tested conditions also display similar results (Figure 3A). It is clearly demonstrated that the fluorescence change could only be induced under the simultaneous existence of NA-S-BA and GSH, indicating the key role played by disulfide bond in the GSH-induced fluorescence release.

The spectroscopic data of the GSH-treated NA-S-BA was further compared with NA-NH₂, which is hypothesized to be the final product upon interaction with GSH. As illustrated in Figures S3 and S4, the perfectly identical positions and shapes of the absorption and fluorescence peaks indicate that after the reaction with GSH, NA-NH₂ is generated as the product and accounts for the fluorescence source. Combining the spectroscopic results and the MS analysis results indicating the formation of NA-NH₂ and free CPT upon interaction with GSH (Figures S5 and S6), it can be confirmed that after cleavage of the disulfide bond by thiol-containing GSH, a tandem intramolecular cyclization occurs as shown in Scheme 3.⁵⁸ Notably, the extremely large stokes shift of 98 nm of NA-NH₂, resulting from the intramolecular charge transfer from amino (donor) to naphthalimide unit (acceptor), is desirable for high quality optical imaging due to the enhancement in signal fidelity.^{59,60}

fluorescence release could be achieved under physiological conditions without potential biological interference.

Cellular uptake

Having established the favourable spectroscopic properties of NA-S-BA and CPT-S-BA, *in vitro* studies were conducted to assess the potential applicability of the dual stimuli-responsive system as a bioimaging and drug delivery model. To confirm the role of carbohydrate-lectin binding in targeting to the desired cell-type, HepG2 was first selected for the study. Literature precedence has reported the over-expression of asialoglycoprotein receptors (ASGPR) on hepatic cells,⁴⁸ with minimal existence in the rest of the body. The cellular uptake of Au-Gal-BA was examined by incubating HepG2 cells with increasing concentrations of Au-Gal-BA (Figure 4). The fluorescence intensities displayed represent the relative amount of Au-Gal-BA that has been internalized by each cell after an incubation time of 2 h. It is evident that uptake is concentration dependent, with the fluorescence observed increasing proportionately with amount of Au-Gal-BA added.

Figure 4. Concentration-dependent delivery of Au-Gal-BA to HepG2 cells as determined by flow cytometry. (A) Histograms of HepG2 cells with different concentrations of Au-Gal-BA. (B) Relative fluorescence intensities expressed with respect to control cells as mean $\pm$ SD ($n = 3$). Measured using flow cytometry, violet laser (405 nm), AmCyan, BD Horizon V500 (BD 525/50 filter).

Cytotoxicity evaluation and live cell imaging

To determine the cell-type specificity of the Gal-targeting ligands on the Au-Gal-BA complexes, cellular uptake in ASGPR-overexpressing HepG2 was compared with HeLa and NIH3T3 cells. Preceding literatures have reported that cervical carcinoma HeLa cells and mouse fibroblast NIH3T3 cells have negligible expression of ASGPR.^{55,56,65} As evident in Figure 5, the fluorescence intensity corresponding to uptake and cleavage of Au-Gal-BA was the highest in HepG2 (Figure 5A), due to the high concentration of GSH and overexpression of ASGPR. A discernable difference in

fluorescence intensity is observed in HeLa cells (Figure 5B), which despite having lower ASGPR-expression, is also capable of disulfide-cleavage due to the presence of high GSH-level.

Figure 5. Confocal microscopy images of (A) HepG2, (B) HeLa and (C) NIH3T3 cells incubated with Au-Gal-BA. Cells were treated with the complexes for 2 h and the cytoskeleton was stained with Alexa Fluor 633 phalloidin. Cell images were acquired using an excitation wavelength of 488 nm and 633 nm, and emission filter in the range of 501- 602 nm and 638- 747 nm for the imaging of Au-Gal-BA (green) and phalloidin (red) respectively. The last panel shows the overlay of both channels.

The contrast is the most significant in NIH3T3 cells, which express neither ASGPR nor high GSH levels. It is apparent that the fluorescence signal originating from the cleavage product of Au-Gal-BA is the weakest in NIH3T3. This is primarily due to the fact that the absence of ASGPR results in low cellular uptake, and cleavage of Au-Gal-BA to form the fluorescent NA-NH₂ is hampered by the low concentration of GSH in the cells. Beside cell-type selectivity, another important parameter that determines the practical utility of a bioimaging system is the inherent cytotoxicity. As evident in Figure S10, Au-Gal-BA is non-toxic to all three cell lines across the range of concentrations tested. Thus, the fluorescent payload in Au-Gal-BA can be taken up efficiently by the target cells, and yet is well-tolerated and exhibits excellent biocompatibility.

In order to determine the intracellular localization fate of the fluorescent payload upon cellular uptake, imaging experiments were conducted with lysosome, mitochondria and endoplasmic reticulum (ER)-specific staining reagents. As evident in Figure 6, no co-localization was observed with the Lyso- or Mito-tracker. Diffuse fluorescence of the payload can be seen within the cytosolic environment of the cells, indicating the ability of the compound in escaping from the lysosomes, a key consideration in the delivery of anti-cancer drugs. On the contrary, the fluorescence co-localized well with the ER-tracker, with overlapping signals from the red fluorescence of the ER-tracker and green fluorescence in the Au-Gal-BA channel (Figure 6C). This observation is postulated to be due to the cleavage of the S-S bond in the ER, resulting in the release of the fluorescent payload in the ER-compartment.⁴⁶

Figure 6. Intracellular tracking of Au-Gal-BA in HepG2 cells. Fluorescence images of HepG2 cells incubated with (A) Lyso-, (B) Mito- or (C) ER-tracker (labelled red); Au-Gal-BA (labelled green); merged images.

The potential applicability of the Au-Gal-BA model as a targeted delivery system was further investigated by conjugating a potent chemotherapeutic prodrug, CPT-S-BA, to the delivery vehicle, forming the Au-Gal-BA(CPT) complex. Spectroscopic analysis of Au-Gal-BA(CPT) confirmed the successful conjugation of the drug onto the Au-Gal nanoparticles (Figure S11). When increasing concentrations of Au-Gal-BA(CPT) were incubated with HepG2 cells, a significant decrease in cell viability was observed (Figure 7A). This decrease is noted to be dose-dependent, with less than 15% of the cells viable after a 72 h incubation with 10 μ M Au-Gal-BA(CPT).

Figure 7. (A) Comparative viability of HepG2, NIH3T3 and HDF cells incubated with increasing concentration of Au-Gal-BA(CPT) complexes for 72 h. Comparison of cell viability in (B) HepG2, (C) NIH3T3 and (D) HDF cells with increasing concentration of CPT, CPT-S-BA, Au-Gal and Au-Gal-BA(CPT). Measured using WST-1 assay, with absorbance quantified at 450 nm (reference 650 nm). Data is represented as mean $\pm$ SEM ($n \geq 3$).

On the contrary, when NIH3T3 cells, which do not express ASGPR, were incubated with the complexes, no notable cytotoxicity was observed. More than 80% of NIH3T3 cells remained viable even when the concentration was increased to the micromolar range. Human Dermal Fibroblasts cells (HDF), a model primary human adult somatic cells, were incubated with Au-Gal-BA(CPT) as a control to investigate the effect of the

pro-drug on normal human cells. Negligible cytotoxicity was observed even at high concentrations, which is consistent with the experimental hypothesis that the vehicle is capable of cell-type specific drug delivery.

It is postulated that in ASGPR-expressing HepG2 cells, recognition and binding of the galactose-appended Au-Gal nanoparticles to ASGPR results in the concomitant release of the CPT-S-BA payload. The boronic acid moiety acts as a delivery agent which allows the pro-drug to be delivered across the cell membrane.^{66,67} Upon cellular entry, the high concentration of GSH in the cytoplasmic environment of HepG2 cancer cells leads to the cleavage of the disulfide linkage in the ER, thus releasing the CPT chemotherapeutic drug. Thereafter, CPT diffuses into the nucleus, where it is able to bind to DNA topoisomerase I and inhibit DNA replication, leading to cell death.⁶⁸ In ASGPR-deficient NIH3T3 and HDF cells, the lack of galactose-binding receptors results in an inability to take up Au-Gal-BA(CPT) efficiently. Moreover, as normal cells express much lower concentrations of GSH than malignant cells, the cleavage of the S-S bond in CPT-S-BA is expected to be slower in NIH3T3 and HDF cells as compared to HepG2. Hence, the overall uptake of Au-Gal-BA(CPT) and intracellular release of CPT are considerably less efficient in NIH3T3 and HDF cells, leading to the much lower cytotoxicity observed.

In order to investigate the biocompatibility of the Au-Gal delivery vehicle, the cells were incubated with increasing concentrations of Au-Gal (Figures 7B – 7D). The model demonstrates excellent biocompatibility, and no cytotoxicity was observed in all three cell lines tested, highlighting the potential for the system to be extended to translational applications. More notably, the boronic acid moiety exhibits no observable cytotoxicity, which is congruent with the reported biocompatibility of boronic acid with the human physiology.^{69,70} The cytotoxicity profiles of CPT-S-BA and CPT in HepG2, NIH3T3 and

HDF are identical (Figures 7B – 7D), indicating that while the boronic acid component alone is capable of intracellular entry, the toxicity is ascribable to the chemotherapeutic effect of CPT and does not arise from the boronic acid functionality.

Figure 8. Annexin V/ PI assay of HepG2 with and without drug addition. Fluorescence was analysed *via* flow cytometry (PE-CF594 and FITC channel). Inserted numbers indicate percentage of cells in each area.

Annexin V/ PI was used to quantify the percentages of live and apoptotic cells when incubated with Au-Gal-BA(CPT) (Figure 8). In the absence of any chemotherapeutic agent, most of the cells are viable. As anticipated, the addition of Au-Gal-BA(CPT) complex was able to induce apoptosis in HepG2 cells, and the total apoptotic cell population increased significantly to 88.8%, a drastic increase as compared to the control cells. Cells treated with CPT were analysed as a reference, and it is evident that the results obtained with Au-Gal-BA(CPT) are comparable with that of CPT, with the total apoptotic population accounting for 82.6% of the cell population in the latter.

2.3 Conclusion

In summary, we have developed a stimuli-responsive model for both bioimaging and delivery of chemotherapeutic drugs to target cancer cells. The carbohydrates coated on the GNP act as the targeting ligand by binding to cell surface lectins, concomitantly releasing the boronic acid-linked payload, which is internalized into the cells. High intracellular GSH levels result in disulfide bond cleavage, which triggers an intramolecular cyclization that leads to the release of the fluorophore and a red-shifted fluorescence enhancement. The uptake of the Au-Gal-BA was further studied through flow cytometry and confocal microscopy. When CPT was incorporated as a model pro-drug, selective targeting of HepG2 cells over control NIH3T3 and normal HDF cells was achieved, with significant cytotoxicity observed only in the target HepG2 cells. The delivery vehicle itself is non-toxic and biocompatible, indicating the potential of developing it into a useful bioimaging tool, as well as targeted drug delivery system in translational research. The most prominent advantage of this system is the ability to target different cells based on the extracellular lectins expressed on the cell surface and the corresponding carbohydrates coated on the GNP.

2.4 Methods and materials

All reagents and solvents were purchased from commercial sources and are of analytical grade. ^1H and ^{13}C NMR in CDCl_3 or $\text{DMSO}-d_6$ were recorded on a Bruker AV 300MHz NMR instrument with tetramethylsilane (TMS) as internal standard. Data for ^1H NMR spectra is reported as follows: chemical shift (ppm) and multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet). Data for ^{13}C NMR spectra is reported in ppm. High Resolution Mass Spectroscopy (HRMS) spectra were recorded on a Waters Q-ToF premierTM mass Spectrometer. UV/Vis spectra were measured with a Varian Cary 100 spectrophotometer (1-cm quartz cell). Emission spectra were measured with Varian Cary Eclipse (1-cm quartz cell). The time dependence of fluorescence of compounds was induced *in situ* and measured with a Varian Cary Eclipse (1-cm quartz cell) at 37°C. Purification by flash column chromatography was carried out using silica gel 60 (0.010–0.063 mm) with eluents as noted in experimental data section for respective compounds. Deionized water was used in the preparation of all samples.

The intermediate compound NA-NH_2 was synthesized by the established procedures.⁷¹

Scheme S1. Synthetic route of NA-S-BA, NA-C-BA and CPT-S-BA.

Synthesis of NA-S: To a mixture of NA-NH₂ (240 mg, 0.88 mmol), triphosgene (296 mg, 1 mmol) and dry toluene (20 mL) was added DIPEA (412 mg, 3.2 mmol) dropwise under an argon atmosphere at room temperature. The resulting solution was refluxed under argon protection for 3 h. After removal of unreacted phosgene gas by flushing with argon gas, a solution of 2,2'-dithiodiethanol (904 mg, 90%, 5.28 mmol) in CH₂Cl₂/THF (1:1, 10 mL) was added to the mixture and the reaction mixture was stirred overnight at room temperature. After removing the solvent under reduced pressure, the crude product was purified by silica gel chromatography using ethyl acetate/PE (v/v, 1:1) as the eluent to afford Na-S as a yellow solid (160 mg): Yield 40%. Melting point: 142-145°C. ¹H NMR (300 MHz, CDCl₃, ppm): δ 8.63 (t, *J* = 8.7 Hz, 1H, Ph-H), 8.38 (d, *J* = 8.4 Hz, 1H, Ph-H), 8.25 (d, *J* = 8.7 Hz, 1H, Ph-H), 7.80 (t, *J* = 8.1 Hz, 1H, Ph-H), 7.69 (s, 1H, Ph-H), 4.86 (t, *J* = 6.0 Hz, 2H, N-CH₂), 4.20 (t, *J* = 7.5 Hz, 2H, -O-CH₂), 3.98 (d, *J* = 8.7 Hz, 2H, OH-CH₂), 3.10 (t, *J* = 6.0 Hz, 2H, -CH₂), 2.98 (t, *J* = 5.7 Hz, 2H, -CH₂), 2.21 (s, 1H, OH), 1.74 (tt, *J* = 10.8, 7.5 Hz, 2H, N-CH₂-CH₂-), 1.48 (m, 2H, CH₃-CH₂), 1.00 (t, *J* = 7.5 Hz, 3H, -CH₃). ¹³C NMR (75 MHz, CDCl₃,

ppm): δ 164.1, 163.6, 153.0, 138.8, 132.4, 131.2, 128.9, 126.6, 126.1, 123.4, 123.1, 118.0, 117.0, 63.9, 60.6, 41.6, 40.2, 37.5, 30.2, 20.4, 13.8. HRMS (ESI-MS, m/z): $[M + H]^+$ calcd. for $C_{21}H_{25}N_2O_5S_2$, 449.1205; found: 449.1194.

Synthesis of NA-S-BAP: A mixture of triphosgene (96 mg, 0.32 mmol), DMAP (336 mg, 2.75 mmol) and 4-aminophenylboronic acid pinacol ester (200 mg, 0.92 mmol) in 10 mL of anhydrous chloroform was stirred under argon protection for 0.5 h at room temperature. Then a solution of NA-S (330 mg, 0.74 mmol) in anhydrous chloroform was added. The resulting mixture was stirred overnight at room temperature. After the removal of the solvent under reduced pressure, the crude product was purified by flash silica gel chromatography using ethyl acetate/dichloromethane (v/v, 1:6) as the eluent to afford NA-S-BAP as a pale yellow solid (300 mg): Yield 58.6%. Melting point: 100-105°C. 1H NMR (300 MHz, $CDCl_3$, ppm): δ 8.61 (dd, J = 8.1, 6.6 Hz, 2H, Ph-H), 8.32 (t, J = 7.5 Hz, 2H, Ph-H), 7.98 (s, 1H, NH), 7.65 (m, 2H, Ph-H), 7.55 (d, J = 8.1 Hz, 1H, Ph-H), 7.47 (d, J = 7.2 Hz, 1H, Ph-H), 7.19 (t, J = 7.5 Hz, 1H, Ph-H), 6.83 (s, 1H, NH), 4.50-4.61 (m, 4H, $-CH_2-$), 4.19 (t, J = 7.2 Hz, 2H, N- CH_2), 3.04-3.13 (m, 4H, $-CH_2-$), 1.74 (m, 2H, N- CH_2-CH_2), 1.48 (m, 2H, $-CH_2-CH_3$), 1.31 (s, 12H, $-CH_3$), 0.98 (t, J = 7.2 Hz, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$, ppm): δ 164.2, 163.7, 153.5, 153.3, 139.1, 136.9, 132.3, 131.2, 130.2, 128.9, 128.5, 126.7, 126.5, 123.4, 123.2, 118.1, 117.5, 83.9, 63.2, 63.0, 40.2, 37.6, 37.0, 31.6, 30.2, 24.8, 22.6, 20.4, 14.1, 13.8. HRMS (ESI-MS, m/z): $[M + H]^+$ calcd. for $C_{34}H_{41}BN_3O_8S_2$, 694.2429; found: 694.2428.

Synthesis of NA-S-BA: A mixture of NA-S-BAP (30 mg, 0.04 mmol), sodium periodate (46 mg, 0.22 mmol) and ammonia acetate (17 mg, 0.22 mmol) in acetone/water (1:1, v/v, 10 mL) was stirred overnight at room temperature. After the removal of acetone, the precipitate was collected and washed with hexane to afford quantitative yield of NA-S-BA as a pale yellow solid (24 mg). Melting point: 137-140°C.

^1H NMR (300 MHz, $\text{DMSO}-d_6$, ppm): δ 9.35 (s, 1H, NH), 8.74 (d, J = 8.7 Hz, 1H, Ph-H), 8.62 (m, 3H, Ph-H), 8.34 (d, J = 8.4 Hz, 1H, Ph-H), 7.95 (s, 1H, Ph-H), 7.84 (t, J = 7.8 Hz, 1H, Ph-H), 7.62 (d, J = 6.9 Hz, 1H, Ph-H), 7.53 (d, J = 7.5 Hz, 1H, Ph-H), 7.25 (t, J = 7.8 Hz, 1H, Ph-H), 7.13 (s, 1H, NH), 4.56 (t, J = 6.3 Hz, 2H, -O-CH₂), 4.44 (t, J = 6.3 Hz, 2H, OH-CH₂), 4.14 (t, J = 7.5 Hz, 2H, N-CH₂), 3.05-3.20 (m, 4H, -CH₂-), 1.71 (m, 2H, N-CH₂-CH₂), 1.47 (m, 2H, -CH₂-CH₃), 0.98 (t, 3H, CH₃). ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$, ppm): δ 163.9, 163.4, 154.4, 153.8, 141.0, 138.5, 132.1, 131.4, 129.8, 128.9, 128.8, 128.1, 126.9, 124.5, 122.7, 119.1, 117.7, 63.4, 62.4, 37.3, 37.3, 30.1, 20.3, 14.2. HRMS (ESI-MS, m/z): $[\text{M} + \text{H}]^+$ calcd. for $\text{C}_{28}\text{H}_{31}\text{BN}_3\text{O}_8\text{S}_2$, 612.1646; found: 612.1654.

Synthesis of NA-C: The compound NA-C was synthesized using the same procedure as in the synthesis of NA-S. NA-C was afforded as a brown solid (120 mg): Yield 45%. Melting point: 156-160°C. ^1H NMR (300 MHz, $\text{DMSO}-d_6$, ppm): δ 10.23 (s, 1H, NH), 8.72 (d, J = 8.7 Hz, 1H, Ph-H), 8.50 (m, 2H, Ph-H), 8.19 (d, J = 8.4 Hz, 1H, Ph-H), 7.85 (t, J = 8.4 Hz, 1H, Ph-H), 4.37 (t, J = 4.8 Hz, 1H, OH), 4.20 (t, J = 6.6 Hz, 2H, O-CH₂-), 4.05 (t, J = 7.5 Hz, 2H, OH-CH₂), 3.40 (m, 2H, CH₂), 1.60-1.73 (m, 4H, CH₂), 1.32-1.48 (m, 8H, CH₂), 0.93 (J = 7.2 Hz, t, 3H, CH₃). ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$, ppm): δ 163.9, 163.3, 154.6, 141.3, 132.1, 131.3, 129.7, 128.7, 126.7, 124.2, 122.6, 118.4, 117.3, 65.5, 61.1, 32.9, 30.1, 29.0, 25.7, 25.7, 20.3, 14.2. HRMS (ESI-MS, m/z): $[\text{M} + \text{H}]^+$ calcd. for $\text{C}_{23}\text{H}_{29}\text{N}_2\text{O}_5$, 413.2076; found: 413.2071.

Synthesis of NA-C-BAP: The compound NA-C-BAP was synthesized using the same procedure as in the synthesis of NA-S-BAP. NA-C-BAP was afforded as a brown solid (180 mg): Yield 62%. Melting point: 138-140°C. ^1H NMR (300 MHz, CDCl_3 , ppm): δ 8.61 (t, 2H, Ph-H & NH), 8.37 (d, J = 8.4 Hz, 1H, Ph-H), 8.25 (d, J = 7.8 Hz, 1H, Ph-H), 7.65 (m, 3H, Ph-H), 7.51 (d, J = 7.2 Hz, 1H, Ph-H), 7.31 (d, J = 7.5 Hz, 1H, Ph-H),

6.72 (s, 1H, NH), 4.29 (dd, $J = 6.6$ Hz, 2H, CH₂), 4.19 (m, 4H, CH₂), 1.68-1.78 (m, 6H, CH₂), 1.49 (m, 6H, CH₂), 1.34 (s, 12H, -CH₃), 0.99 (t, $J = 7.2$ Hz, 3H, CH₃). ¹³C NMR (75 MHz, DMSO-*d*₆, ppm): δ 163.9, 163.4, 154.6, 154.1, 141.3, 139.2, 132.1, 131.3, 129.8, 128.7, 126.8, 124.3, 122.7, 121.7, 118.6, 117.4, 84.1, 65.5, 64.5, 30.1, 28.9, 28.9, 25.5, 25.1, 20.3, 14.2. HRMS (ESI-MS, m/z): $[M + H]^+$ calcd. for C₃₆H₄₅BN₃O₈, 658.3300; found: 658.3301.

Synthesis of NA-C-BA: The compound NA-C-BA was synthesized using the same procedure as in the synthesis of NA-S-BA. NA-C-BA was afforded as a brown solid (22 mg): Yield 98%. Melting point: 125-128°C. ¹H NMR (300 MHz, DMSO-*d*₆, ppm): δ 10.24 (s, 1H, NH), 9.47 (s, 1H), 8.70 (d, $J = 8.4$ Hz, 1H, Ph-H), 8.50 (m, 2H, Ph-H), 8.18 (d, $J = 8.4$ Hz, 1H, Ph-H), 7.95 (m, 2H), 7.74-7.89 (m, 2H, Ph-H), 7.52 (d, $J = 7.8$ Hz, 1H, Ph-H), 7.43 (d, $J = 7.2$ Hz, 1H, Ph-H), 7.23 (m, 1H, Ph-H), 4.22 (t, $J = 6.3$ Hz, 2H, CH₂), 4.07 (m, 4H, CH₂), 1.60-1.74 (m, 6H, CH₂), 1.25-1.50 (m, 6H, CH₂), 0.93 (t, $J = 7.2$ Hz, 3H, CH₃). ¹³C NMR (75 MHz, DMSO-*d*₆, ppm): δ 164.0, 163.4, 154.6, 154.2, 141.3, 138.8, 132.2, 131.4, 129.8, 128.8, 128.7, 128.1, 126.8, 124.3, 122.7, 118.6, 117.4, 65.5, 64.4, 30.1, 29.0, 28.9, 25.6, 20.3, 14.2. HRMS (ESI-MS, m/z): $[M + H]^+$ calcd. for C₃₀H₃₅BN₃O₈, 576.2517; found: 576.2505.

Synthesis of CPT-S: A mixture of triphosgene (40 mg, 0.13 mmol), DMAP (122 mg, 1 mmol) and CPT (100 mg, 0.29 mmol) in 10 mL of anhydrous chloroform was stirred under argon protection for 0.5 h at room temperature. Thereafter, a solution of 2,2'-dithiodiethanol (77 mg, 0.45 mmol) in anhydrous chloroform was added. The resulting mixture was stirred overnight at room temperature. After the removal of the solvent under reduced pressure, the crude product was purified by flash silica gel chromatography using dichloromethane/methanol (v/v, 1:0 to 40:1) as the eluent to afford CPT-S as a white solid (90 mg): Yield 58.8%. Melting point: 168-170°C. ¹H NMR

(300 MHz, CDCl₃, ppm): δ 8.45 (d, J = 9.0 Hz, 1H, Ph-H), 8.24 (d, J = 8.4 Hz, 1H, Ph-H), 7.91 (m, 1H, Ph-H), 7.86 (m, 1H, Ph-H), 7.45-7.74 (m, 2H, Ph-H), 5.75 (m, 1H), 5.30-5.46 (m, 3H), 4.41 (m, 2H), 3.94 (m, 2H), 2.80-3.02 (m, 4H), 2.00-2.40 (m, 1H), 1.90 (m, 1H), 1.04 (m, 3H). ¹³C NMR (75 MHz, CDCl₃, ppm): δ 167.4, 157.3, 153.5, 148.7, 146.3, 145.7, 131.5, 131.0, 129.3, 128.6, 128.2, 120.3, 96.4, 78.1, 67.1, 66.5, 60.5, 60.4, 50.0, 41.3, 41.2, 37.2, 31.9, 29.7, 7.6. HRMS (ESI-MS, m/z): [M + H]⁺ calcd. for C₂₅H₂₅N₂O₇S₂, 529.1103; found: 529.1081.

Synthesis of CPT-S-BAP: The compound CPT-S-BAP was synthesized using the same procedure as in the synthesis of NA-S-BAP. CPT-S-BAP was afforded as a white solid (20 mg): Yield 65%. Melting point: 240-245°C. ¹H NMR (300 MHz, DMSO-*d*₆, ppm): δ 9.62 (s, 1H, NH), 8.68 (s, 1H, Ph-H), 8.18 (d, J = 8.4 Hz, 1H, Ph-H), 8.12 (d, J = 7.5 Hz, 1H, Ph-H), 7.86 (t, J = 7.8 Hz, 2H, Ph-H), 7.71 (t, J = 7.2 Hz, 1H, Ph-H), 7.51 (d, J = 6.9 Hz, 1H, Ph-H), 7.30 (t, J = 7.2 Hz, 2H, Ph-H), 7.10 (s, 1H, Ph-H), 5.53 (s, 2H), 5.323 (s, 2H), 4.36 (t, J = 6.0 Hz, 2H, CH₂), 4.26 (t, J = 6.0 Hz, 2H, CH₂), 3.03 (m, 4H), 2.18 (q, 2H), 1.29 (s, 12H, CH₃), 0.93 (t, 3H, CH₃). ¹³C NMR (75 MHz, DMSO-*d*₆, ppm): δ 167.5, 157.0, 153.6, 153.3, 152.7, 148.4, 146.7, 145.2, 139.0, 132.1, 130.3, 129.5, 129.0, 128.5, 128.2, 124.6, 119.7, 94.8, 84.1, 78.4, 66.9, 66.7, 62.4, 50.8, 37.2, 36.7, 30.8, 25.1, 8.0. HRMS (ESI-MS, m/z): [M + H]⁺ calcd. for C₃₈H₄₁BN₃O₁₀S₂, 774.2326; found: 774.2315.

Synthesis of CPT-S-BA: The compound CPT-S-BA was synthesized using the same procedure as in the synthesis of NA-S-BA, utilizing THF/water (4:1, v/v) as the solvent. CPT-S-BA was afforded as a white solid (16 mg): Yield 80%. Melting point: 225-227°C. ¹H NMR (300 MHz, DMSO-*d*₆, ppm): δ 9.54 (s, 1H, NH), 8.68 (s, 1H, Ph-H), 8.18 (d, J = 8.4 Hz, 1H, Ph-H), 8.13 (d, J = 7.5 Hz, 1H, Ph-H), 7.80-8.08 (m, 3H, Ph-H & B-OH), 7.73 (m, 2H, Ph-H), 7.47 (m, 2H, Ph-H), 7.22 (t, J = 7.8 Hz, 1H, Ph-H), 7.10 (s, 1H,

Ph-H), 5.53 (s, 2H, CH₂), 5.32 (s, 2H, CH₂), 4.35 (t, *J* = 6.0 Hz, 2H, CH₂), 4.25 (t, *J* = 6.0 Hz, 2H, CH₂), 3.02 (m, 4H, CH₂), 2.18 (m, 2H, CH₂), 0.92 (t, 3H, CH₃). ¹³C NMR (75 MHz, DMSO-*d*₆, ppm): δ 167.5, 157.0, 153.7, 153.3, 152.7, 148.4, 146.7, 145.2, 138.6, 132.1, 131.0, 130.3, 129.5, 129.0, 128.8, 128.5, 128.2, 128.1, 119.7, 94.8, 78.4, 66.9, 66.8, 62.3, 50.8, 37.3, 36.7, 30.8, 8.0. HRMS (ESI-MS, *m/z*): [M + H]⁺ calcd. for C₃₂H₃₁BN₃O₁₀S₂, 692.1544; found: 692.1571.

Figure S1. TEM image of gold nanoparticles.

Figure S2. Absorption and emission spectra of NA-NH₂ (10 μM) in DMSO/PBS solution (1:1, v/v, pH = 7.4, 10 mM).

Figure S3. Normalized absorption spectra of NA-S-BA (10 μM), NA-S-BA (10 μM, treated with 50 eq. GSH) and NA-NH₂ (10 μM) in DMSO/PBS solution (1:1, v/v, pH = 7.4, 10 mM). Spectrum of NA-S-BA with GSH was recorded 1 h after exposure at 37 °C.

Figure S4. Normalized emission spectra of NA-S-BA (10 μ M), NA-S-BA (10 μ M, treated with 50 eq. GSH) and NA-NH₂ (10 μ M) in DMSO/PBS solution (1:1, v/v, pH = 7.4, 10 mM). Spectrum of NA-S-BA with GSH was recorded 1 h after exposure at 37 $^{\circ}$ C, λ_{ex} = 405 nm.

Figure S5. MS spectrum of the products from the reaction of NA-S-BA (10 μ M, DMSO/PBS, 1:1, v/v, pH 7.4, 10 mM) with 20 equiv. of GSH. Spectrum was obtained 1 h after exposure at 37 $^{\circ}$ C.

Figure S6. MS spectrum of the products from the reaction of CPT-S-BA (10 μ M, DMSO/PBS, 1:1, v/v, pH 7.4, 10 mM) with 20 equiv. of GSH. Spectrum was obtained 1 h after exposure at 37 $^{\circ}$ C.

Figure S7. Absorption response of NA-S-BA (10 μ M) upon addition of various amino acids including Ala, Leu, Ile, Val, Pro, Phe, Met, Trp, Gly, Ser, Gln, Thr, Asn, Tyr, Asp, Glu, Lys, Arg, His (5.0 mM). Each spectrum was recorded after exposure to GSH for 1 h at 37 $^{\circ}$ C.

Figure S8. Fluorescence response of NA-S-BA (10 μ M) upon addition of thiol-containing compounds including GSH, DTT, Cys and Hcy (5.0 mM). Each spectrum was recorded after exposure to GSH for 1 h at 37 $^{\circ}$ C, λ_{ex} = 405 nm.

Figure S9. Fluorescence intensity at 535 nm of NA-S-BA (10 μ M) as a function of pH value in the absence and presence of GSH (5.0 mM) in DMSO/PBS solution (1:1, v/v, pH = 7.4, 10 mM). Each point was recorded after exposure to GSH for 1 h at 37 $^{\circ}$ C, λ_{ex} = 405 nm.

Figure S10. Cell viability in HepG2, HeLa and NIH3T3 cells with increasing concentrations of Au-Gal-BA. Measured using MTT assay, with absorbance quantified at 540 nm. Data is represented as mean $\pm$ SEM ($n \geq 3$).

Figure S11. The comparison of the absorption spectra of (A) CPT-S-BA with gold nanoparticles conjugated with CPT-S-BA and (B) gold nanoparticles with and without the conjugation of CPT-S-BA.

Preparation of Au-Gal-BA

Spherical citrate-capped gold nanoparticles were first synthesized *via in-situ* reduction of Au(III) to colloidal gold in accordance to established synthetic procedures.⁷² Thiol-modified polyethylene glycol-linked β -galactoside (β -gal-PEG-SH) was synthesized as reported in the literature.⁷³ In order to form β -galactose-coated Au nanoparticles, 40 μ L of citrate-stabilised Au nanoparticles (0.5 mM) was added slowly to 20 μ L of β -gal-PEG-SH (100 mM) and stirred for 72 h. The sample was centrifuged and washed twice with water. Na-S-BA (1.23 mg) was dissolved in 40 μ L DMSO and added to the Au-Gal nanoparticles, and the sample was incubated for 12 h. The complex was centrifuged and washed with DMSO before resuspension in 20 μ L DMSO to form Au-Gal-BA.

Cell culture

HepG2, HeLa and Human Dermal Fibroblast (HDF) cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS), 10,000 U/mL penicillin, 10,000 μ g/mL streptomycin, 2 mM L-glutamine and 0.1 mM non-essential amino acids (NEAA). NIH3T3 cells were grown and maintained in DMEM supplemented with 10% bovine serum, 10,000 U/mL penicillin, 10,000 μ g/mL streptomycin at 37 °C in a humidified chamber with 5% CO₂. Cells were subcultured regularly with 0.025% trypsin-EDTA.

Flow cytometry

HepG2 cells were seeded in 6-well plates at a density of 4×10^5 cells per well one day prior to experiment. The cells were washed once with PBS and DMEM was replenished. The Au-Gal-BA complexes were added at increasing concentration to each well and the cells were allowed to incubate for 2 h. Thereafter, the media was

aspirated and the cells were washed once with PBS. For flow cytometry experiments, the cells were detached with 250 μ L of 0.05% trypsin-EDTA, and the reaction was quenched with the addition of 750 μ L of 10% FBS/ PBS. The cell suspension was transferred to pressure tubes and the fluorescence was analysed on the BD LSRFortessa X-20 flow cytometer (BD Biosciences). The violet laser was used for the excitation of the fluorophore and fluorescence was recorded on the AmCyan channel (BD 525/50 filter), with a total of 10,000 events recorded for each sample.

Cell viability

Cells were seeded in 96-well plates at a density of 1×10^4 cells per well in triplicate one day prior to experiment. After the addition of Au-Gal-BA complexes, the cells were incubated for 2 h. The media was then aspirated and 100 μ L (-)-phenol red DMEM and 10 μ L of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Molecular Probes) were added to each well. The cells were further incubated for another 4 h at 37 °C. Thereafter, 85 μ L of the media was removed and 50 μ L DMSO was added to solubilize the purple formazan crystals. Absorbance was quantified on the Tecan Infinite 200 PRO microplate spectrometer at an absorbance wavelength of 540 nm. Cell viability was expressed as a percentage against the control well of non-treated samples.

Confocal microscopy

Cells were seeded in glass-based confocal dishes (Nunc) one day prior to experiment. 10 μ M Au-Gal-BA complex was added and the cells were incubated for 2 h. The cells were washed twice with PBS and fixed with 4% paraformaldehyde. Thereafter, the cells were stained with Alexa Fluor 633 phalloidin (10 μ L per sample) and incubated at 37 °C for 1 h. Cell images were acquired using an excitation wavelength of 488 nm

and 633 nm, and emission filter in the range of 501- 602 nm and 638- 747 nm for the imaging of Au-Gal-BA and Alexa Fluor 633 phalloidin respectively. For intracellular tracking, the cells were placed in media and incubated with LysoTracker Red DND-99 (0.1 μ M), MitoTracker Deep Red FM (200 nM) or ER-Tracker Red (1 μ M) (Molecular Probes) in accordance to manufacturer's instructions. Fluorescence imaging was conducted on a confocal laser scanning microscope (Zeiss LSM 710).

Preparation of Au-Gal-BA(CPT)

30 μ L of citrate-stabilised Au nanoparticles (0.5 mM) was added slowly to 15 μ L of β -gal-PEG-SH (100 mM) and stirred for 72 h. The sample was centrifuged and washed twice with water. CPT-S-BA (1.04 mg) was dissolved in 30 μ L DMSO and added to the Au-Gal nanoparticles, and the sample was incubated for 12 h. The complex was centrifuged and washed with DMSO before resuspension in 15 μ L DMSO to form Au-Gal-BA(CPT).

Cytotoxicity of Au-Gal-BA(CPT) pro-drug

Cells were seeded in 96-well plates at a density of 1×10^4 cells per well in triplicate one day prior to experiment. Au-Gal-BA(CPT) was added at increasing concentrations and the cells were allowed to incubate at 37 °C for 72 h. After the cells were washed with PBS, DMEM (100 μ L) was replenished and 10 μ L of WST-1 cell proliferation reagent (Roche) was added. The cells were incubated for 2 h and absorbance was quantified on the Tecan Infinite 200 PRO microplate spectrometer at a wavelength of 450 nm (reference wavelength 650 nm). Cell viability was expressed as a percentage against the control well of non-treated samples.

Annexin V/ propidium iodide (PI) assay

HepG2 cells were seeded in 6-well plates at a density of 4×10^5 cells per well one day prior to experiment. The cells were washed once with PBS and DMEM was replenished. Au-Gal-BA(CPT) and CPT were added to each well and the cells were incubated at 37 °C for 72 h. The media was aspirated and the cells were washed once with PBS, then harvested with 0.05 % trypsin-EDTA (250 μ L). The reaction was quenched with the addition of 10% FBS/ PBS (750 μ L) and transferred to pressure tubes. The supernatant formed after centrifugation was discarded, and the cells were resuspended in 1X annexin-binding buffer (100 μ L). Annexin V Alexa Fluor 488 and PI (Invitrogen) were added in accordance to manufacturer's instructions (5 μ L and 1 μ L respectively), and the cells were incubated in the dark for 15 min. Thereafter, the cell suspension was further diluted in 1X annexin-binding buffer (400 μ L) before analysis on the BD LSRFortessa X-20 flow cytometer (BD Biosciences). Fluorescence of Alexa Fluor 488 and PI were measured on the FITC and PE-CF594 channels (488 nm and 561 nm excitation) respectively with a total of 10,000 events recorded for each sample.

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Chapter 3. Dual-responsive chitosan-based boronic acid polymer for targeted siRNA delivery

Abstract

RNA interference (RNAi) *via* short interfering RNA (siRNA) holds great therapeutic promise in the treatment of several diseases, particularly cancer. However, the practical application of RNAi is severely hampered by the lack of a target-specific approach, cell-impermeability and instability of the siRNA in circulation. Herein, a boronic acid-conjugated chitosan polymer was synthesized as an siRNA delivery vehicle. It is proposed that the boronic acid can act as a targeting ligand for cell surface sialic acids, while the chitosan backbone can protect the siRNA from degradation, only releasing it under thiol-reducing conditions in the cell.

3.1 Development of RNAi

RNA interference (RNAi) is a potent strategy of inhibiting specific genes through the use of double-stranded RNA (dsRNA) from endogenous or exogenous sources.¹ When introduced exogenously, the duplexed RNA of approximately 21- 25 base-pairs (bp), termed short-interfering RNA (siRNA), is first recognized by the RNA-induced silencing complex (RISC) (Figure 1).²

Figure 1. RNA interference mechanism. Reprinted by permission from Macmillan Publishers Ltd: Nature Materials,³ copyright 2013.

The double-stranded siRNA binds to Argonaute 2 (Ago2) protein, the catalytic subunit of RISC, and is rapidly unwound in an ATP-dependent manner to yield a sense (passenger) strand and an antisense (guide) strand.⁴ The sense strand is degraded, while the antisense strand guides the activated RISC complex to a complementary

target messenger RNA (mRNA).⁵ Upon recognition of the target mRNA, the endonucleolytic activity of Ago2 results in the degradation of the mRNA, preventing its translation into the corresponding protein.⁶ The siRNA-loaded RISC is concurrently released, and re-enters the catalytic pathway for several more rounds of mRNA cleavage.⁷

The discovery of RNA interference as a therapeutic approach has generated significant research interest as it is potentially useful in the treatment of diseases, in which levels of specific proteins responsible for the disease can be diminished at the post-transcriptional stage.⁸ In particular, cancer therapeutics can be revolutionised such that aberrant genes are silenced prior to the translation into pro-angiogenic or anti-apoptotic proteins.⁹

Despite the promising outlook with regards to RNAi therapeutics, several challenges have to be overcome before application of RNAi in the clinic can become a reality. One of the most pressing issues concerns the efficient delivery of siRNA. Due to its highly hydrophilic and anionic character, siRNA does not cross cell membranes to reach the intracellular site of action easily.¹⁰ Moreover, chemically unmodified siRNA is susceptible to degradation by nucleases in the cytoplasm and hence, is rapidly cleared by the body *via* renal excretion.¹¹

Targeted siRNA delivery to the specific cell-type based on the pathophysiology of the disease is also of paramount importance. By achieving targeted delivery, the dosage of siRNA required for efficient silencing can be reduced and off-target effects can be minimised.¹² In addition, the specific interaction between the ligands and the cell surface receptors can be leveraged to enhance the cellular uptake of the siRNA in receptor-mediated endocytosis.¹² During receptor-mediated endocytosis, the

extracellular ligand is recognized by the specific receptor on the cell surface and both the receptor and ligand are encapsulated in vesicles and internalized.¹³ In the cytosol, the vesicle is uncoated and fuses with endosomes, which become more acidic as they mature. The increase in acidity results in the budding off of transport vesicles that contain the receptor to be recycled. The mature late endosome then fuses with lysosomes that are targeted for degradation. During this process, the ligand has to escape from the endosome or lysosome to reach the target subcellular compartment. To overcome these challenges with regards to siRNA delivery, research efforts in this field have been dedicated to the development of efficient siRNA delivery vehicles with active targeting moieties to the desired cells. Such targeting ligands include peptides,^{14,15} folic acid,^{16,17} antibodies,¹⁸⁻²⁰ cholesterol^{21,22} and aptamers.^{23,24} Among these, folic acid ligands are commonly used²⁵ due to the low cost and high-binding affinity ($K_D \sim 10^{-10}$ M) to folate receptors that have been found to be up-regulated in cancers of various origins.²⁶

3.2 Chitosan-based siRNA delivery

Chitosan is a natural polysaccharide consisting of repeating units of *N*-acetylglucosamine and D-glucosamine in β -1,4-glycosidic bonds, with the latter existing in higher proportion.²⁷ The biocompatibility and low cytotoxicity of chitosan has resulted in its derivatives forming the basis of several research work involving delivery systems.^{28,29} In particular, chitosan is biodegradable and is designated by the FDA as Generally Recognized As Safe (GRAS),³⁰ leading to its application in the food and wound care industry.

At $\text{pH} < \text{pK}_a$ (6.5- 7), the amino groups are protonated and chitosan exists as a polycation,³¹ capable of complexing anionic macromolecules such as siRNA. Under such conditions, siRNA and chitosan spontaneously mix and form polyelectrolyte complexes. Depending on the concentrations and molecular weight ratios, complexes of nanometer size can be formed with high encapsulation efficiency of siRNA.^{32,33} By encapsulating the siRNA within the polymer, steric effects posed by the chitosan prevents nucleases from interacting with the oligonucleotide, thus protecting the siRNA from degradation.³⁴ However, chitosan has limited solubility in aqueous solution at physiological pH and transfection efficiency tends to be low.³⁵ Hence, functional group modifications and optimization of the formulation have to be developed to overcome these challenges.

Intracellular release of siRNA

An important consideration for siRNA delivery involves its protection from serum nucleases so that the siRNA can reach the target cell intact and be released in the cytoplasm. In the biological environment, the presence of serum proteins (e.g. albumin) can result in nanoparticle aggregation, leading to toxicity issues.³⁶ Moreover, negatively charged compounds in the blood can bind competitively to the nanoparticles,³⁷ triggering the premature release of the siRNA in the bloodstream and subsequent renal clearance.³⁸ Even if the complex does not disassemble completely, changes in composition or charge of the complex as a result of protein interactions may significantly alter the biodistribution and cellular uptake.

Upon reaching the intracellular compartment of the target cell, the nanoparticle has to escape the endocytic vesicle in order for the siRNA to be released into the cytoplasm. In general, the endocytosed nanoparticles reside in the early endosomes, which mature gradually into late endosomes.³⁹ This process is characterized by an increase in acidity of the microenvironment, with pH decreasing to 5- 6 due to ATPase proton pump action. The endosome fuses with the lysosome eventually, and the pH dips further to 4- 5. This process is advantageous towards cationic polymers, which comprise of primary amines ($pK_a= 6$) that are protonated in acidic condition. As a result of the acquisition of positive charges, an influx of chloride counter ions into the endosomes occurs, increasing the ionic strength. Osmotic swelling to balance this increment thus leads to endosome rupture, an effect and process commonly described as the proton sponge effect and endosomal escape respectively.⁴⁰ Chitosan alone, however, is unable to promote endosomal escape efficiently, and requires the conjugation of other cationic functional groups for effective membrane destabilization.^{41,42}

Chitosan molecular weight

Chitosan can be classified according to the molecular weight (MW) into the following categories: very low MW (< 10 kDa), low MW (10- 80 kDa), high MW (80- 300 kDa) and very high MW chitosan (> 300 kDa).³⁹ The first report involving chitosan-based nanoparticles in siRNA delivery investigated the use of tripolyphosphate crosslinker and the influence of complexation methods on the siRNA transfection efficiency.⁴³ High molecular weight chitosan ranging from 110- 470 kDa were used and it was discovered that molecular weight has little effect on transfection efficiency in contrast to complexation methods, with entrapment in the form of ionic gelation being the most effective method of siRNA association. The size of chitosan/siRNA complex is however dependent on the chitosan MW. Smaller particles were formed with lower MW chitosan (110 kDa) as compared to higher MW chitosan (270 kDa). In a related work, the authors proposed that considering the molecular weight of siRNA (approximately 13.36 kDa), chitosan molecules of at least 5-10 times the length of siRNA, which corresponds to MW of 64.8- 170 kDa), could stably complex siRNA.⁴⁴ These complexes were further investigated and high knockdown efficiency was observed in human carcinoma H1299 cells. On the contrary, low MW chitosan (10 kDa) was unable to complex siRNA into stable particles, with large aggregates being formed and low silencing efficiency as a result. Taking into consideration the findings of various literature, it is proposed that chitosan MW should generally be in the range of 80- 300 kDa in order for monodisperse nanoparticles of < 300 nm to be formed, and for high *in vitro* knockdown efficiency (> 50%).³⁹

Chitosan deacetylation degree

The percentage of free deacetylated primary amines in chitosan, as represented by the deacetylation degree (DD), influences the ability of chitosan in complexing siRNA as the higher the DD, the higher the chitosan charge density under acidic condition. Due to the high concentration of negative charges in siRNA, a high chitosan DD of more than 80% is necessary for stable complexation and increased siRNA binding.³⁵ Unstable particles were formed when chitosan with low DD was used as siRNA carrier, resulting in low transfection efficiency.⁴⁴ On the other hand, fully deacetylated chitosan demonstrated effective endosomolytic activity, abrogating the need for a crosslinking agent for stable complexation.

Charge ratio

The ratio of chitosan positive charges to siRNA negative charges is represented as the nitrogen/phosphate (N/P) ratio. This figure is used concomitantly with the weight ratio of both components when defining the polyplex, and both have to be optimized during the formulation process.³⁹

In general, the higher the N/P ratio, the better the complexation of the siRNA, which results in smaller particles.⁴⁵ A research study has revealed that nanoparticles in the 100 nm range were formed with high N/P ratio of 50- 100, while larger nanoparticles of 200 nm were formulated when the N/P was reduced to 5- 10.⁴⁶ Other research work also cited the influence of N/P ratios on transfection efficiency of the complex. For instance, an increased silencing efficiency was observed when the nanoparticles were formulated at high N/P of 50- 150 in contrast to low N/P of 2 and 10, with highest efficiency seen at N/P 150.⁴⁴ However, the implication of high N/P ratios on cytotoxicity of the polyplex is apparent as N/P more than 70 resulted in enhanced toxicity.^{44,45} It is

hypothesized that at high N/P, the excess free amines on chitosan can interfere with cellular processes through interactions with the cell membrane, leading to cytotoxicity.⁴⁷

In order to ensure biocompatibility, chitosan/siRNA complexes were formed at low N/P ratios and tested for their gene silencing efficiency. It was discovered that polyplexes with low N/P ratio (4 and 8) formed nanoparticles of 150 nm and could also result in considerable luciferase knockdown in human carcinoma H1299 cells.⁴⁸ Considering the requirement of high N/P for complex stability, a careful balance is thus necessary to negate any toxicity-related issues.

Method of siRNA conjugation

The high number of positive charges on chitosan allow it to interact with negatively charged siRNA through simple complexation involving electrostatic interactions (*vide supra*). However, transfection efficiency tends to be low as the nanoparticles are unstable in transfection media and dissociates prematurely even before cellular uptake can occur.⁴³ The addition of a cross-linker in an ionic gelation method, can enhance the polyplex stability by increasing the strength of siRNA/chitosan interactions. Cross-linking agents physically entrap the oligonucleotide after chitosan/oligo electrostatic interactions are established, thus stabilising the overall complex.^{49,50} This approach was used with much success by a research group which achieved a high luciferase silencing of 82% in Chinese hamster CHO K1 cells by the introduction of sodium polyphosphate (TPP) linker in the design.⁴³ Simple complexation was demonstrated to be less efficient as lower gene silencing was observed without TPP addition. Another cross-linker, thiamine pyrophosphate, was similarly included in a

siRNA/chitosan formulation process that resulted in up to 70% GFP knockdown in human liver carcinoma HepG2 cells.⁵⁰

It should be noted, however, that factors which promote siRNA association with chitosan and stabilize the resultant complex are antithetical to the factors that enhance the dissociation and intracellular release of siRNA from the polyplex.⁵¹ In light of these considerations, using modified stimuli-responsive chitosan that can stably conjugate siRNA and yet, promote its release in the desired intracellular compartment would be highly advantageous.^{52,53}

3.3 Thiolated chitosan/ siRNA delivery

Thiolated siRNA, for tethering to similarly thiol-functionalised polymers, has been developed as a covalent conjugation method that is at the same time, cleavable in the presence of a reducing environment.⁵⁴⁻⁵⁶ The inclusion of a thiol functionality in chitosan-mediated oligonucleotide delivery confers significant advantages over the conventional conjugation method that relies primarily on electrostatic interactions.⁵⁷ With thiolation, the stability of the oligonucleotide is enhanced through the formation of inter- and intramolecular disulfide bonds, offering protection against nuclease degradation. In addition, intracellular release of the oligonucleotide is triggered by the presence of reducing agents in the cytoplasm, such as GSH, which is efficient in promoting dissociation from the chitosan complex.^{58,59} In the case of oral delivery of these macromolecules, the modification of chitosan with thiolated groups, such as cysteine and thioglycolic acid, increases the mucoadhesive strength of the complexes.⁶⁰⁻⁶² This is due to the fact that the intestinal mucosa consists of cysteine-rich glycoproteins that are able to form disulfide linkages with the thiolated chitosan to enhance the bioavailability.⁶³⁻⁶⁵

A self-polymerised siRNA was reported to condense with thiolated glycol chitosan to form stable nanoparticles (Figure 2).⁶⁶ Both 5'-ends of the duplex siRNA were modified with free sulfhydryl groups, allowing poly-siRNA to be formed *via* disulfide bonds under mild oxidative conditions. The poly-siRNA was shown to be more resistant to enzymatic degradation than monomeric siRNA due to the additional steric protection provided. This poly-siRNA was further linked to thiol-functionalised glycol chitosan and stabilised through a combination of disulfide linkages and electrostatic interactions with the amino groups on chitosan. The resultant complex was able to achieve

comparable red fluorescent protein (RFP) gene silencing efficiency as that of commercially available Lipofectamine 2000.

Figure 2. Schematic diagram illustrating a) poly-siRNA formation and b) mechanism for the delivery of poly-siRNA/ thiolated glycol chitosan. Reproduced with permission from ref 66. Copyright 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Further *in vivo* studies revealed that the complex accumulated preferentially in tumor tissues as compared to free poly-siRNA (up to four-fold increase). When pro-angiogenesis gene VEGF was targeted, the treatment resulted in significant reduction of tumor volume by 80%. It was postulated that retention of the complex in tumor tissues is mediated by the EPR effect particularly seen in nanoparticles in the range of 100- 500 nm.⁶⁷ Thereafter, disulfide bond cleavage by the high intracellular concentration of GSH leads to release and dissociation of the poly-siRNA into monomeric siRNA that can enter the RNAi pathway for gene silencing to occur.

The study was extended to grafting of glycol chitosan with a hydrophobic 5 β -cholanolic acid side chain for encapsulation of hydrophobic drugs, such as doxorubicin and paclitaxel.⁶⁸ These DOX-chitosan nanoparticles were combined with the above poly-

siRNA/ thiolated glycol chitosan system to target anti-apoptotic Bcl-2 gene and concurrently administer doxorubicin. This combination delivery platform demonstrated the ability to overcome drug resistance that was apparent when doxorubicin was administered alone. In addition, tumor weight volumes decreased drastically by up to 9.4-fold as compared to the saline-treated control.

More recently, the same research group was able to target multi-drug resistant protein (MDR-1), which is responsible for the efflux of chemotherapeutic drugs and decreases the overall drug concentration in tumor cells.^{69,70} The delivery system exhibits excellent biocompatibility and when it was co-administered with doxorubicin, was able to induce greater tumor suppression than treatment with doxorubicin alone.

3.4 Proposed delivery system

Taking into consideration the key parameters influencing chitosan/siRNA delivery, it is proposed that a dual stimuli-responsive, targeted siRNA delivery system can be designed by incorporating the features of a thiolated siRNA with a boronic acid-linked chitosan polymer (*vide supra* for boronic acid-based targeted delivery systems; Chapter 1). Polymeric siRNA (poly-siRNA) formed from the disulfide linking of thiolated siRNA is included in this design to provide additional nuclease stability as proposed in the literature.⁶⁶

Figure 3. Structure of proposed boronic acid-conjugated chitosan polymer for siRNA delivery.

With reference to Figure 3, a biocompatible glycol chitosan is used as the polymer backbone for tethering of the boronic acid linker and siRNA. Glycol chitosan (GC) is chosen due to the increased water solubility at physiological pH provided by the short ethylene chain, and has been demonstrated to be biocompatible *in vivo*.^{71,72} Boronic acids are widely used as targeting ligands to sialic acid-overexpressing cancer cells,

and have been further incorporated into siRNA delivery systems.⁷³ Hence, it is intended for the polymer to achieve specific targeting of cancer cells through boronic acid binding to sialic acid. In this design, the boronic acid is linked to a carboxy dimethylmaleic anhydride (CDM) linker to facilitate endosomal escape of the siRNA upon cellular internalization. The acid-labile CDM can be cleaved in the acidic environment of the endosomes, revealing positively charged amino groups on the polymer that exhibit the proton sponge effect vital for endosomal escape.^{56,74} Following destabilization of the endosome, the chitosan-linked siRNA complex enters the cytoplasm which consists of high GSH concentration. Hence, GSH-mediated disulfide bond cleavage results in the release of the siRNA from the polymer, and the siRNA can subsequently enter the RNAi pathway for gene silencing.

3.5 Results and discussion

The proposed synthetic plan involves the initial conjugation of the boronic acid linker to the glycol chitosan scaffold under basic condition (Scheme 1). Thereafter, a water-soluble *N*-hydroxysuccinimide ester, sulfo-LC-SPDP, will be attached to the polymer backbone. The attachment of the succinimidyl linker allows the introduction of the 2-pyridyldithio group, which can be displaced by an external amine or sulfhydryl nucleophile, releasing the stable pyridine-2-thione group during the reaction. Hence, the thiolated poly(siRNA) can be covalently linked to the GC-BA-SPDP polymer.

Scheme 1. Synthetic scheme for the synthesis of boronic acid-linked glycol chitosan polymer conjugated to siRNA (GC-BA-SPDP/RNA).

Synthesis of linker

Commercially available tetraethylene glycol **6** was first selectively tosylated on one of the hydroxyl groups and then modified with an azide functional group to form compound **8** (Scheme 2). An esterification reaction was then carried out to conjugate the ethylene glycol to 2-propionic-3-methylmaleic anhydride, forming product **9** as a carboxydimethyl maleic anhydride (CDM) derivative.

Scheme 2. Synthetic route to linker CDM-PEG-BA. (a) TsCl, aq. NaOH, THF, r.t., 5 h, 64% (b) NaN₃, MeCN, reflux, 18 h, 78% (c) 2-propionic-3-methylmaleic anhydride, HATU, DIPEA, THF, r.t., 24 h, 85% (d) 4-ethynylphenylboronic acid pinacol ester, CuI, DIPEA, CH₂Cl₂, r.t., 3 h, 53% (e) NaIO₄, NH₄OAc, acetone/ THF, r.t., 4 h, *quant.*

By using the copper-catalysed azide-alkyne cycloaddition, intermediate **10** and 4-ethynylphenylboronic acid pinacol ester were successfully conjugated through the formation of a triazole ring. A final deprotection resulted in the formation of the desired CDM-PEG-BA linker **1**.

Deprotection and synthesis of poly(siRNA)

The double-stranded siRNA (ds-siRNA) is designed to possess a fluorescent label for tracking in subsequent intracellular delivery, and two thiolated ends to facilitate the formation of poly(siRNA). The disulfide protecting groups on the thiol-ends were first removed with 1,4-dithiothreitol (DTT) to expose the free sulfhydryl groups and the identity of the product was confirmed by ESI-MS (Figure 4). The polymerization of the resultant deprotected ds-siRNA was first attempted with reaction conditions reported in the literature (Table 1).⁶⁶ Using TMAD as a mild oxidant for the disulfide bond formations in ds-siRNA did not result in the detection of poly(siRNA) when analysed on gel electrophoresis (Table 1, Entry 1; Figure S1), while increasing the reaction temperature was also uneventful (Table 1, Entry 2). Further optimization of the reaction was conducted by replacing the oxidant with iodine (Table 1, Entries 3-5).⁷⁵ However, the polymerization did not proceed despite increasing the reaction temperature and prolonging the reaction time. The lack of success with the polymerization can be attributed to the electrostatic repulsion of the negatively charged ds-siRNA strands, which poses a challenge as the thiolated ends have to be brought into close proximity for conjugation to occur.

Figure 4. ESI-MS spectra of (A) ds-siRNA with protected thiol (B) ds-siRNA after DTT treatment to remove the disulfide protecting groups.

Table 1. Optimisation of reaction conditions for poly(siRNA) formation.

Entry	Reaction conditions	Temperature	Time	Poly(siRNA) formation
1	TMAD, HEPES/EDTA (pH 8.0)	r.t.	24 h	N. D.
2	TMAD, HEPES/EDTA (pH 8.0)	37 °C	24 h	N. D.
3	KI/ I ₂ , HEPES (pH 8.5)	r.t.	30 min	N. D.
4	KI/ I ₂ , HEPES (pH 8.5)	r.t.	24 h	N. D.
5	KI/ I ₂ , HEPES (pH 8.5)	37 °C	24 h	N. D.

N.D. indicates not detected, as determined by gel electrophoresis. TMAD= *N,N,N',N'*-Tetramethylazodicarboxamide, HEPES= (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), EDTA= Ethylenediaminetetraacetic acid

While the polymerization of ds-siRNA to form poly(siRNA) of high polymerization ratio was unsuccessful, monomeric ds-siRNA can also be covalently conjugated to the thiolated GC through disulfide bond formation. Monomeric siRNA has also been used in chitosan-based RNA delivery routinely without potential stability issues.^{27,39} Hence, it was concluded that ds-siRNA in this form would be used for conjugation to the GC-polymer in subsequent studies.

Synthesis of GC-BA-SPDP/siRNA

Conjugation of CDM-PEG-BA **1** with the commercially available GC was carried out under basic condition (Methods and materials). The sulfo-LC-SPDP linker was then attached to the GC-BA polymer by reaction of the amino groups on the polymer with the *N*-hydroxysuccinimide ester. The presence of both linkers were confirmed *via* ¹H NMR spectroscopy. Through modifying the ratio of the GC-BA polymer to sulfo-LC-

SPDP linker, a total of three polymers was synthesized (Table 2). It was found that **GC-BA-SPDP-1** was insoluble in organic solvents such as EtOH, aqueous buffers and H₂O, with only slight solubility in DMSO. Considering the aqueous solubility of GC-BA before the conjugation of sulfo-LC-SPDP, the effect of the linker on the solubility was investigated through varying the amount added. At higher sulfo-LC-SPDP ratios, **GC-BA-SPDP-2** exhibited similar solubility issues as **GC-BA-SPDP-1** and remained only slightly soluble in DMSO. By decreasing the amount of sulfo-LC-SPDP added, **GC-BA-SPDP-3** was synthesized and higher solubility of the polymer in DMSO was achieved.

Table 2. Synthesis of GC-BA-SPDP polymers.

Polymer	GC: BA	GC-BA: sulfo-LC-SPDP
GC-BA-SPDP-1	5:1	5:1
GC-BA-SPDP-2	5:1	2:1
GC-BA-SPDP-3	5:1	10:1

The **GC-BA-SPDP** polymers were further complexed to thiol-modified siRNA through disulfide bond formation. The respective thiolated GC-BA-SPDP polymers were added to free siRNA at weight ratios of 20:1, 50:1 and 100:1 and the success of conjugation was evaluated by gel electrophoresis (Figures S2 and 5). The presence of siRNA in the higher molecular weight regions indicate the formation of glycol chitosan-complexed siRNA, since each polymer strand is capable of binding several duplexed siRNA through the multiple thiolated linkers on the polymer. The migration of the resultant GC-BA-SPDP/siRNA is thus retarded due to the high molecular weight of the

complex. No complexation between the **GC-BA-SPDP-1** and siRNA was achieved as noted from the identical migration profiles of the resultant polymer and duplex siRNA control (Figure S2).

On the other hand, **GC-BA-SPDP-3** exhibits the strongest ability to complex the thiolated siRNA, with the most intense smear and gel retardation of the siRNA observed at regions corresponding to molecular weights of more than 300 bp (Figure 5, Lanes 6-8). Interestingly, the proportion of GC-BA-SPDP-3/siRNA is the highest at GC-BA-SPDP: siRNA w/w ratio of 20:1. Above this ratio, the formation of siRNA-complex was comparatively less favourable, with weaker intensity bands observed at the high molecular weight region (Figure 5, Lanes 7 and 8).

Figure 5. Gel electrophoresis of (A) GC-BA-SPDP-2/siRNA and (B) GC-BA-SPDP-3/siRNA at different w/w ratios.

In order to elucidate the optimal ratio for polymer-siRNA complexation, **GC-BA-SPDP-3** was further conjugated to siRNA at ratios of 2:1, 5:1, 10:1 and 20:1 (Figure 6).

However, it is apparent that the conjugation is the highest at 20:1. Further heating to ensure the complete dissolution of the polymer before conjugation resulted in increased binding, with significant amount of the siRNA complexed to the polymer with higher MW (Figure S3). Hence, the optimized polymer-siRNA complex **GC-BA-SPDP-3/siRNA** was formed at ratio of 20:1.

Figure 6. Gel electrophoresis of GC-BA-SPDP-3/siRNA at different w/w ratios.

Cellular uptake

Having confirmed the successful conjugation of the functionalized GC polymer and siRNA, *in vitro* studies were conducted to determine the cellular uptake of **GC-BA-SPDP-3/siRNA**. Metastatic breast cancer MDA-MB-231 cells were selected as the target cells due to the high overexpression of sialic acid on the cell surface.⁷⁶ In this reported study, MDA-MB-231 cells exhibited the strongest binding to *Maackia amurensis* lectin (MAL), which is specific for α 2,3-sialic acid residues. Among the cell lines investigated, the mRNA expression of the α 2,3-sialyltransferase gene was also the highest in MDA-MB-231 cells. In order to investigate the effect of the different

linkers on the glycol chitosan, GC/siRNA (commercially available GC polymer complexed with siRNA) and GC-BA/siRNA (GC-BA complexed with siRNA) polymers were also prepared. As evident in Figure 7, the highest uptake was observed with GC-BA-SPDP/siRNA complexes, in which the fluorescence intensity was more than double that of naked siRNA.

Figure 7. Cellular uptake of naked FAM-siRNA and FAM-siRNA-conjugated polymers in MDA-MB-231 cells. Relative fluorescence intensities are expressed with respect to control cells as mean $\pm$ SD ($n = 3$). *** $P < 0.001$ vs siRNA-treated cells. Measured using flow cytometry, FITC channel (EX 488 nm, EM 530/30).

The uptake of GC/siRNA was the next highest while the fluorescence intensity in cells incubated with GC-BA/siRNA was slightly lower. It is thus hypothesized that GC, which is partially protonated at physiological pH, can complex siRNA through electrostatic interactions. In GC-BA/siRNA, however, the proportion of free amine on the GC polymer is lower due to the conjugation to the CDM-PEG-BA linkers. Hence, in terms of electrostatic interactions, the complexation of GC-BA to siRNA is considerably weaker. By including a thiol linker in GC-BA-SPDP polymer, although the availability

of free amines is significantly decreased, the presence of thiol functionalities on the polymer allows the covalent conjugation of the siRNA onto the polymer, providing additional stabilization to the siRNA.

It is however noted that the uptake of GC-BA-SPDP/siRNA is only slightly more than two times that of naked siRNA, and this level of transfection efficiency might be insufficient in future studies involving knockdown of endogenous genes in the target cells. As the siRNA needs to escape from the endosomes and enter the RNAi machinery, a significantly higher uptake efficiency is necessary to mitigate any possible loss of siRNA in this multi-step process.

Considering the characterisation data of **GC-BA-SPDP-3** and the cellular uptake results, it is hypothesized that while the polymer is able to stably complex siRNA, the low proportion of CDM-PEG-BA linkers on the polymer results in an inability for the boronic acid to sufficiently act as a targeting ligand and mediate the uptake of the polymer. As such, the mechanism for uptake is presumably passive targeting, instead of active targeting. Hence, future strategies to increase the uptake efficiency should predominantly focus on optimizing the GC:BA conjugation ratio, in order to derive a polymer that allows boronic acid to play the role of targeting ligand efficiently. A careful balance should however be made to circumvent any siRNA loading issues, since increasing the CDM-PEG-BA ratio might lead to a corresponding decrease in the sites available for reaction with sulfo-LC-SPDP and the presence of thiol linkers for siRNA conjugation.

3.6 Conclusion

The design and synthesis of a dual-stimuli responsive glycol chitosan polymer for siRNA delivery has been detailed in the preceding sections. The boronic acid-functionalised polymer can ideally result in targeting and enhanced uptake in cancer cells, which exhibit an overexpression of sialic acid on the cell surface. Particularly, the boronic acid is designed to be cleavable in the acidic microenvironment of cancer cells, exposing the free amines on the polymer that can encourage endosomal escape through the proton pump effect. Finally, the thiol-linkages on the polymer are cleaved in the presence of high GSH levels in the cytosol, releasing the duplex siRNA which can enter the RNAi machinery and result in gene silencing. While the siRNA conjugation to the GC-BA-SPDP polymer has been successful, cellular uptake of the polymer/siRNA is only double that of naked siRNA, which requires further optimisation. Strategies for improving the uptake efficiency are discussed, and further experiments towards this end are currently in progress.

3.7 Methods and materials

Unless otherwise indicated, all reagents used in the reactions were obtained from commercial suppliers and used without further purification. Glycol chitosan (> 400 degree of polymerisation) was obtained from Sigma-Aldrich. Sulfosuccinimidyl 6-(3'-(2-pyridyldithio)propionamido)hexanoate (sulfo-LC-SPDP) was purchased from Thermo Scientific. All reactions were conducted in flame-dried glassware under a nitrogen atmosphere. Evaporation of organic solutions was achieved by rotary evaporation with a water bath temperature below 40 °C. Product purification by flash column chromatography was accomplished using silica gel 60 (0.010-0.063 mm) with technical grade solvents distilled before use or analytical reagent grade solvents obtained from commercial suppliers. Chromatograms were visualized by fluorescence quenching with UV light at 254 nm and staining with a basic solution of potassium permanganate. NMR spectra were recorded at room temperature on Bruker AV 300MHz, Bruker AVIII 400MHz and Bruker AV 500MHz NMR spectrometers. The residual solvent signals were taken as the reference (7.26 ppm for ^1H NMR spectra and 77.0 ppm for ^{13}C NMR spectra in CDCl_3). Chemical shift (δ) is reported in ppm with coupling constants (J) given in Hz. The following abbreviations classify the multiplicity: s = singlet, d = doublet, t = triplet, m = multiplet or unresolved. HRMS (ESI) spectra were recorded on a Waters Q-ToF premierTM mass spectrometer.

Duplex FAM-siRNA was obtained from Bioneer Corporation. The sense strand was modified with a 5'-(6-carboxyfluorescein) and a 3'-(C6-dithio)-modifier, while the anti-sense strand was thio-modified at the 3'-position.

The sequence of the ds-siRNA is as follows: (sense) 5'-FAM-GCAAGCUGACCCUGAAGUUCdTdT-SH-3', (anti-sense) 5'-

GAACUUCAGGGUCAGCUUGCdTdT-SH-3'. ESI-MS RNA analysis was conducted by Sangon Biotech (Shanghai). Gel electrophoresis was conducted on 8% polyacrylamide gel and run at 130 V for 30 min in Tris/Borate/EDTA (TBE) buffer. The gel was stained with Gel Red for 30 min and visualized on T:Genius Gel Documentation System using GeneSys.

Synthesis of CDM-PEG-BA linker

The synthesis of compound **8** was previously reported and the characterization data is in accordance with that reported.⁷⁷

2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl 3-(4-methyl-2,5-dioxo-2,5-dihydrofuran-3-yl)propanoate (9)

A mixture of carboxydimethyl maleic anhydride (370 mg, 2.0 mmol), 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (850 mg, 1.1 equiv.) and compound **8** (490 mg, 1.1 equiv.) was dissolved in anhydrous THF (25 mL). The mixture was cooled to 0 °C before the dropwise addition of *N,N*-diisopropylethylamine (0.39 mL, 1.1 equiv.). After 30 min of stirring, the reaction mixture was warmed to room temperature and left to stir for 24 h. The solvent was removed *in-vacuo*, and the crude product was purified by flash column chromatography on silica gel with dichloromethane/ ethyl acetate to obtain the desired product as a yellow oil (85%). ¹H NMR (400 MHz, CDCl₃, ppm) δ 4.21 (dd, *J* = 5.5, 4.0 Hz, 2H), 3.71 – 3.58 (m, 12H), 3.41 – 3.32 (m, 2H), 2.79 – 2.67 (m, 4H), 2.11 (s, 3H); ¹³C NMR (101 MHz, CDCl₃, ppm) δ 171.6, 165.8, 165.6, 142.2, 142.2, 77.3, 77.2, 77.0, 76.7, 70.7, 70.6, 70.6, 70.0, 68.9, 64.1, 50.7, 31.0, 19.9, 9.7; HRMS (ESI): calcd. for C₁₆H₂₄N₃O₈ [M + H]⁺: 386.1563; found: 386.1553.

2-(2-(2-(2-(4-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)-2H-1,2,3-triazol-2-yl)ethoxy)ethoxy)ethoxy)ethyl 3-(4-methyl-2,5-dioxo-2,5-dihydrofuran-3-yl)propanoate (10)

Compound **9** (70.0 mg, 0.18 mmol), 4-ethynylphenylboronic acid pinacol ester (41.4 mg, 1.0 equiv.) and copper (I) iodide (1.70 mg, 0.05 equiv.) were dissolved in anhydrous dichloromethane (0.3 mL). Upon complete dissolution of the copper catalyst, *N,N*-diisopropylethylamine (0.1 mL, 2 equiv.) was added and the reaction mixture was stirred at room temperature for 3 h. Thereafter, the solvent was removed and the crude product was purified by flash column chromatography on silica gel with hexane/ ethyl acetate to yield the product as a yellow oil (53%). ¹H NMR (400 MHz, CDCl₃, ppm) δ 8.01 (s, 1H), 7.83 (dd, *J* = 12.0, 8.0 Hz, 4H), 4.59 (t, *J* = 4.9 Hz, 2H), 4.27 – 4.15 (m, 2H), 3.89 (t, *J* = 4.9 Hz, 2H), 3.65 – 3.54 (m, 10H), 2.75 – 2.61 (m, 4H), 2.06 (s, 3H), 1.34 (s, 12H). ¹³C NMR (101 MHz, CDCl₃, ppm) δ 171.8, 165.8, 165.6, 147.4, 142.2, 142.1, 135.2, 133.1, 124.8, 121.4, 83.8, 77.3, 77.0, 76.7, 70.3, 70.2, 69.4, 69.0, 63.9, 50.2, 30.9, 24.8, 19.7, 9.6; HRMS (ESI): calcd. for C₃₀H₄₁N₃O₁₀B [M + H]⁺: 614.2885; found: 614.2899.

(4-(2-(15-(4-methyl-2,5-dioxo-2,5-dihydrofuran-3-yl)-13-oxo-3,6,9,12-tetraoxapentadecyl)-2H-1,2,3-triazol-4-yl)phenyl)boronic acid (1)

Ammonium acetate (91.7 mg, 5.0 equiv.) was dissolved in H₂O (3 mL) and added to a solution of compound **10** (146 mg, 0.238 mmol) in acetone (3 mL). Sodium periodate (255 mg, 5.0 equiv.) was added and the reaction mixture was stirred at room temperature for 4 h. The solids were then filtered away on celite and the solvents were removed *in-vacuo*. The resultant crude residue was purified by flash column chromatography on silica gel with acetone/ ethyl acetate to yield the product as a yellow oil (*quant.*). ¹H NMR (400 MHz, CDCl₃, ppm) δ 7.84 (s, 1H), 7.61 (d, *J* = 8.6 Hz, 2H), 6.88 (d, *J* = 8.6 Hz, 2H), 4.54 (t, *J* = 4.9 Hz, 2H), 4.20 – 4.12 (m, 2H), 3.88 (t, *J* = 5.0 Hz, 2H), 3.65 – 3.52 (m, 10H), 2.72 – 2.61 (m, 4H), 2.05 (s, 3H); ¹³C NMR (75 MHz, CDCl₃, ppm) δ 171.7, 165.8, 165.6, 156.4, 147.6, 142.2, 142.1, 127.2, 122.8, 120.3, 115.9, 77.4, 77.0, 76.6, 70.5, 70.5, 69.5, 68.9, 64.0, 50.3, 30.9, 19.8, 9.6; HRMS (ESI): calcd. for C₂₄H₃₁N₃O₁₀B [M + H]⁺: 532.2102; found: 532.2088.

Deprotection of disulfide protecting groups in ds-siRNA

Dithio-modified and protected duplex siRNA (0.5 nmol) was dissolved in H₂O (50 μL) before the addition of 5 μL of DTT solution (0.77 mg in 50 μL H₂O). The reaction mixture was briefly vortexed and left to stand at r.t. for 1 h. Thereafter, the excess DTT and reaction by-products were removed with Amicon Ultra-0.5 mL centrifugal filter and

the filtrate was lyophilised. The successful cleavage of the protecting group was confirmed by ESI-MS.

Figure S1. Gel electrophoresis image of selected polymerisation reactions of ds-siRNA.

Figure S2. Polymerisation of GC-BA-SPDP-1 with ds-siRNA. (Right lane)

Synthesis of GC-BA-SPDP-3 polymer

CDM-PEG-BA **1** (2 mg) was dissolved in DMSO (2 mL) and added to glycol chitosan (10 mg). *N, N*-diisopropylethylamine (0.5 mL) was added and the mixture was stirred at 50 °C for 24 h. The yellow mixture was dialysed against deionized water using a dialysis membrane with cut-off molecular weight of 12-14 kDa (Fischerbrand) for 3 days to remove excess reagents and lyophilized to yield GC-BA as a yellow film (16 mg). In order to attach the pyridyldithio linker onto the polymer, GC-BA (5 mg) was first dissolved in DMSO (0.5 mL). Sulfo-LC-SPDP (0.2 mg, w/w 10:1) was dissolved in PBS (pH 7.4, 0.5 mL) and added to the GC-BA solution. The resultant mixture was stirred at r.t. for 24 h and dialysed against deionized water for 2 days. Upon lyophilisation, a white foam corresponding to GC-BA-SPDP-3 was formed. ¹H NMR (400 MHz, CDCl₃, ppm) δ 8.33 (br s, 3H), 8.23 (br s, 1H), 7.82 – 7.74 (m, 4H), 7.67 – 7.63 (m, 2H), 7.25 (br s, 3H), 6.96 – 6.92 (m, 2H), 4.67 (br s, 26H), 4.49 (br s, 15H), 4.13 – 4.10 (d, *J* = 8.6 Hz, 3H), 3.66 (br s, 213H), 3.36 (s, 3H), 3.12 – 2.83 (m, 18H), 2.78 – 2.76 (m, 16H), 2.64 – 2.40 (m, 12H), 2.38 – 2.33 (m, 4H), 2.23 – 2.08 (m, 3H), 2.23 – 2.15 (m, 7H), 2.08 (s, 10H), 1.94 (s, 4H), 1.83 (s, 2H), 1.77 (s, 2H), 1.53 – 1.41 (m, 12H), 1.27 – 1.25 (m, 9H).

Synthesis of GC-BA-SPDP/siRNA

GC-BA-SPDP/siRNA complex was synthesized by incubating GC-BA-SPDP (0.06 mg in 100 µL DMSO) with ds-siRNA (0.329 nmol in 50 µL PBS) at various w/w ratios. The mixtures were vortexed and incubated at r.t. for 24 h. Thereafter, the solvents were removed by lyophilisation and successful conjugation was determined by gel electrophoresis.

Figure S3. Polymerisation of GC-BA-SPDP-3 with ds-siRNA (20:1).

Cell culture

MDA-MB-231 cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS), 10,000 U/mL penicillin, 10,000 µg/mL streptomycin, 2 mM L-glutamine and 0.1 mM non-essential amino acids (NEAA) at 37 °C in a humidified chamber with 5% CO₂. Cells were subcultured regularly with 0.025% trypsin-EDTA.

Flow cytometry

MDA-MB-231 cells were seeded in 6-well plates at a density of 5×10^5 cells per well one day prior to experiment. The cells were washed once with PBS and the media was changed to Opti-MEM. The siRNA and polymer/siRNA complexes were added to each well at a final siRNA concentration of 100 nM and the cells were allowed to incubate for 6 h. Thereafter, the media was aspirated and the cells were washed once with PBS. For flow cytometry experiments, the cells were detached with 250 µL of

0.05% trypsin-EDTA, and the reaction was quenched with the addition of 750 μ L of 10% FBS/ PBS. The cell suspension was transferred to pressure tubes and the fluorescence was analysed on the BD LSRII flow cytometer (BD Bioscience). The blue laser was used for the excitation of the fluorophore and fluorescence was recorded on the FITC channel (BD 530/30 filter), with a total of 10,000 events recorded for each sample.

3.8 References

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**Appendix. Stereoselective synthesis of
C₁-vinylic carbohydrates *via* Pd-
catalyzed decarboxylative allylation
and modified Julia olefination**

Abstract

Glycal-substituted pyridylsulfones were explored as useful intermediates for glycoside C-C bond formations. The advantages of catalytic decarboxylative allylation and high sulfone reactivity were combined to provide an expedient synthetic route to 2,3-unsaturated vinyl glycopyranosides. This molecular scaffold is versatile and can be further functionalised to access natural products with biological significance.

Introduction

Aryl and heterocyclic sulfones are versatile intermediates in synthetic chemistry which enable alkylations of substrates that would otherwise be unachievable using conventional approaches.¹ The sulfone moiety acts as a highly electron-withdrawing functionality to increase the electrophilicity or nucleophilicity of the starting reagent, and the subsequent desulfonation can be carried out with ease,^{2,3} offering access to a multitude of products (Scheme 1).⁴⁻⁶

Scheme 1. Possible transformations of aryl sulfones and β -carbonyl sulfones; electrophiles are represented as E, heterocyclic groups are represented as Het.¹

Catalytic decarboxylative allylation (DcA) is an attractive strategy for C-C bond formations as a variety of nucleophiles can be generated under neutral conditions, and the efficiency is driven by the concomitant release of CO_2 during the reaction. Research contributions in this area by Tunge,⁷⁻¹⁰ Trost,¹¹⁻¹⁴ Stolz¹⁵⁻¹⁷ and others,¹⁸⁻²¹ have resulted in the development of activated scaffolds for efficient decarboxylative allylation. The incorporation of DcA in glycoside synthesis has been further advanced by our group²²⁻²⁵ through the use of palladium catalysts in Tsuji-Trost allylation

reactions involving glycan substrates. The Pd- π -allyl intermediate, formed from the initial decarboxylation reaction, was found to be an excellent electrophile, which can be attacked by a suitable nucleophile in an inter- or intramolecular manner. These methodologies have allowed the access to O- and C-glycosides expediently in one-step from glucal-derived substrates.

The synthesis of C-glycosides continues to be an area of research focus as they constitute a class of compounds which are analogous to the natural O-glycosides with important biological functions and yet, possess enhanced enzymatic stability.²⁶⁻²⁸ Moreover, several naturally occurring C-glycosides have been discovered to exhibit biological activity, such as ambruticin,²⁹ aspergillides³⁰ and cryptopyranmoscatones,³¹ (Figure 1).

Figure 1. Structures of selected C-glycosides with important biological activity.

Particularly, the 2,3-unsaturated C-vinyl glycan structure is a versatile scaffold presenting chiral synthons for subsequent functionalisation and modification, hence is a useful intermediate in the synthesis of C-glycosides. This class of compounds can typically be accessed from glycals through a tandem Lewis acid-mediated Ferrier reaction and nucleophilic addition with alkynyl substrates^{32,33} or glycosyl acceptors with existing alkene.³⁴ The use of vinyl Grignard reagents in a metal-catalysed nucleophilic substitution reaction involving 2,3-unsaturated glycosides has also been reported.³⁵

It is thus hypothesized that the advantages of decarboxylative allylation and sulfone reactivity can be combined and leveraged to develop a strategy for the stereoselective synthesis and further chemical transformations of carbohydrate structures.

Results and discussion

The decarboxylative glycosylation studies commenced with compound **1**, which was reacted with Pd₂dba₃ and dppe as the ligand in toluene at 70 °C. However, no desired product was obtained (Table 1, Entry 1).

Table 1. Initial optimization of decarboxylative glycosylation.

Entry ^a	Catalyst	Ligand	Solvent	Stereoselectivity (β:α) ^b
1	Pd(OAc) ₂	DiPPF	Toluene	N.R.
2	Pd(PPh ₃) ₄	DiPPF	Toluene	N.R.
3	Pd(PPh ₃) ₄	dppb	Toluene	3:1
4	Pd(PPh₃)₄	dppb	CH₃CN	> 25:1
5	Pd(PPh ₃) ₄	DPEphos	CH ₃ CN	4:1
6 ^b	Pd(PPh ₃) ₄	Sphos	CH ₃ CN	4:1
7 ^b	Pd(PPh ₃) ₄	Mephos	CH ₃ CN	7:1
8	Pd(PPh ₃) ₄	BIPHEP	CH ₃ CN	20:1
9	Pd(PPh ₃) ₄	dppb	DMA	5:1
10	Pd(PPh ₃) ₄	dppb	THF	4:1
11	Pd(PPh ₃) ₄	dppb	DMSO	9:1

^a Unless otherwise specified, all reactions were conducted with 0.1 mmol of **1** in the presence of 10% Pd catalyst and 20% ligand in 2 mL of solvent. ^b Ligand loading was increased to 40%. ^c Ratios as determined by ¹H NMR. N. R. denotes no reaction. DiPPF = 1,1'-Bis(di-*i*-propylphosphino)ferrocene; dppe = 1,2-Bis(diphenylphosphino)ethane; dppb = 1,2-Bis(diphenylphosphino)butane; dppf = 1,1'-Ferrocenediyl-bis(diphenylphosphine); DPEphos = Bis[(2-diphenylphosphino)

phenyl] ether; Sphos = 2-Dicyclohexylphosphino-2',6'-dimethoxybiphenyl; Mephos = 2-Dicyclohexylphosphino-2'-methylbiphenyl. BIPHEP = 2,2'-Bis(diphenylphosphino)-1,1'-biphenyl.

Further optimisation revealed that a combination of Pd(PPh₃)₄ and dppb led to the formation of the desired product in a β : α ratio of 3:1 (Table 1, Entry 3). In order to improve the selectivity, the solvent was changed to CH₃CN, which resulted in a significant enhancement of stereoselectivity to > 25:1 (Table 1, Entry 4). Subsequent modification to the ligand used was however uneventful, with lower stereoselectivity observed (Table 1, Entries 5-8). CH₃CN also proved to be the most suitable solvent in this transformation, as other solvents such as DMA, THF and DMSO resulted in lower stereoselectivity (Table 1, Entries 9-11).

Having optimized the reaction conditions for the decarboxylative glycosylation, the incorporation of a nitrogen-containing heterocycle was considered to increase the nucleophilicity of the sulfone functionality. The role of heteroaromatic sulfones in C-C bond formations is particularly prominent in the modified Julia-Kocienski olefination, in which α -metallated sulfones are reacted with aldehydes, and the resultant β -hydroxyl sulfone can undergo a facile Smiles rearrangement to form olefins (Scheme 2).^{36,37}

Table 2. Subsequent optimization of decarboxylative glycosylation. ^{a,b}

Entry ^a	Solvent	Temperature	Yield ^b	Stereoselectivity (β:α) ^c
1	CH ₃ CN	70 °C	60%	6:1
2	DMA	70 °C	51%	17:1
3	THF	70 °C	48%	1:1
4	Pyridine	70 °C	45%	7:1
5	DMF	70 °C	49%	18:1
6	DMSO	70 °C	62%	9:1
7	DMF	80 °C	72%	> 20:1

^a Unless otherwise specified, all reactions were conducted with 0.1 mmol of **3** in the presence of 10% Pd catalyst and 20% ligand in 2 mL of solvent. ^b Isolated yield. ^c Ratios as determined by ¹H NMR.

The study commenced with the coupling of 2-pyridyl sulfonyl acetic acid with 4,6-di-*tert*-butylsilyl-glucal to form starting reagent **3a**, which was used for the optimisation of reaction conditions. It was discovered during initial experiments that the 4,6-*para*-methoxybenzylidene-glucal derived sulfone starting reagent was not stable during the reaction at elevated temperatures, hence the use of a more robust protecting group is preferred. During the optimisation of reaction conditions for the decarboxylative

coupling with pyridyl sulfonyl glycal **3a**, initial reaction with CH₃CN resulted in the formation of desired product **4a** in good yield of 60%, albeit with only a β : α ratio of 6:1 (Table 2, Entry 1). This result is contrary to that obtained with phenyl sulfonyl glycal **1**, in which the decarboxylated product was obtained with high stereocontrol (β : α > 25:1) (Table 1, Entry 4). Changing the solvent to the more polar dimethylacetamide (Table 2, Entry 2) led to a significant improvement in the stereoselectivity of the reaction to β : α > 18:1 with a moderate yield of 51%. The same reaction conducted with THF and pyridine was however, less efficient, as low stereoselectivities of 1:1 and 7:1 were obtained respectively, accompanied by a slight decrease in yield (Table 2, Entries 3, 4). Taking into consideration the higher stereoselectivity obtained with DMA, the reaction was attempted with DMF (Table 2, Entry 5), which gave a high stereoselectivity of 18:1.

The role of solvent on the reaction was further investigated by examining the identity of the side products formed during the reaction. Besides the desired product **4a**, side products **5** and **6** were also observed to be formed (Scheme 2). It is thus hypothesized that product **4a** is able to undergo a subsequent reaction by first deprotonation at the α -sulfone position, followed by intermolecular attack of the C-1 position of the starting reagent **3a**. This side reaction would lead to the displacement of the pyridylsulfonylacetate, which is able to undergo thermal decarboxylation to form 2-(methylsulfonyl)pyridine **6**. Thermal decarboxylation reactions of α -sulfonylacetates have been previously reported.^{7,38}

Scheme 2. Further investigation into the decarboxylative allylation of pyridyl sulfonyl glucal.

Since deprotonation is necessary for the reaction to occur, it is presumed that less basic solvents, such as dimethyl sulfoxide, might be able to suppress the formation of **5** and lead to higher yields. As anticipated, when the reaction was conducted in DMSO (Table 2, Entry 6), the yield of desired product **4a** increased to 62%, however, the stereoselectivity was also affected, with only a β : α ratio of 9:1 observed. Considering the high stereoselectivity obtained with DMF as the solvent, attempts were made to use DMF/ DMSO binary solvent systems in the reaction to reduce the side product formation and yet maintain the stereoselectivity. However, no positive results were obtained (data not shown). Finally, the reaction was conducted at an elevated temperature of 80°C in DMF (Table 2, Entry 7) with the hypothesis that pushing the reaction to completion with higher temperatures may reduce the formation of the undesired side products. This modification proved to be effective, with product **4a** formed in good yield of 72% and in excellent stereoselectivity of > 20:1. The structure of **4a** was further confirmed *via* X-ray crystallography (Table 3).

Table 3. Carbohydrate substrate scope for Pd-catalysed decarboxylative allylation. ^{a,b}

^a Reactions were carried out with 10 mol% $\text{Pd(PPh}_3)_4$ catalyst, 20% dppb. ^b Isolated yield. ^c Yield over two steps; 4,6-protected glucal pyridylsulfone starting material was not purified due to instability on silica gel. ^d Reaction was conducted with L-glucal-derived pyridylsulfone as starting material.

Several 3-pyridylsulfonyl glycal substrates were treated under the optimised reaction conditions (Table 3). It was found that the reactions proceeded smoothly to provide the desired products in good yields and high stereoselectivity. Protecting groups such as cyclohexylidene (**4b**) and isopropylidene (**4c**) are also compatible with the reaction conditions, giving the products in moderate yields albeit in lower selectivities. The 4,6-protecting groups appears to have an implication on the stereoselectivity of the product. Silyl-protected glycals lead to higher stereoselectivities, presumably due to

the higher steric bulk posed by these protecting groups (**4a** and **4d**). When 3-pyridylsulfonyl substituted L-glucal was subjected to the reaction, the desired product (**4e**) was obtained in good yield in high α -selectivity.

Notably, D-galactal-derived 3-pyridylsulfone did not react, indicating the possible role of steric interactions in the approach of the Pd catalyst from the β -face. To further examine the influence of chirality on the glucal scaffold on the stereoselectivity of the reaction, 3,5-*trans* pyridyl sulfonyl glucal starting material was prepared and subjected to the reaction conditions. The desired product (**4f**) was obtained in good yield, with the α -isomer as the major product ($> 20:1$) (Scheme 3).

Scheme 3. Decarboxylative allylation with 3,5-*trans*pyridylsulfonylglucal **3f**.

In a typical Pd-catalysed Tsuji-Trost decarboxylative allylation, the Pd catalyst approaches the glycan from the α -face to minimise steric interactions with the C-3 substituent on the sugar ring (Scheme 4). Pd-mediated ionisation and subsequent decarboxylation generates the phenyl sulfonyl methane carbanion. The incoming nucleophile then attacks the Pd-allylic system on the opposite face, leading to predominantly β -products.

Scheme 4. Proposed mechanism for C-glycosylation in a Pd-catalysed decarboxylative allylation reaction.

Considering that the difference in the two starting reagents is mainly in the aromatic substituent on the sulfonyl group (Tables 1 and 2), it is postulated that the nitrogen on the pyridyl sulfone coordinates to the Pd, resulting in the catalyst approach from the β -face (Scheme 6). This mode of coordination provides an explanation for the unsuccessful reaction with pyridyl sulfonyl galactal, as the sterically bulky 4,6-di-*tert*-butyl silyl protecting groups occupy the β face of the galactal, preventing the approach and coordination of the Pd catalyst to the C-3 pyridyl group. After decarboxylation, 2 competing pathways might be feasible. With the coordination of the pyridine -N to the Pd on the β -face, the nucleophile is stabilised and softened. Considering that the pKa for the methyl phenyl sulfone pronucleophile is approximately 27, it is expected that the pKa for the pyridyl-substituted sulfone is even lower due to the electron-withdrawing effect of the pyridine -N and can be characterised as a soft nucleophile.³⁹ Hence, the pyridyl sulfonyl methyl carbanion attacks the allyl group from the same face, resulting in the formation of the β -isomer (Scheme 5, Path A).

Scheme 5. Proposed mechanism for the Pd-catalysed decarboxylative allylation involving pyridylsulfonyl glycol **3**.

At the same time, the phosphine ligands and solvents may coordinate to the Pd in a ligand substitution reaction, releasing the pyridyl sulfonyl methane carbanion (Scheme 5, Path B). Hence, the π -allyl Pd intermediate **II** is formed and the nucleophile can attack in an intermolecular reaction to form the α -product, which explains the reason behind the decrease in β : α ratio when the sulfonyl substituent was changed from phenyl to pyridyl. However, in polar solvents such as DMF and DMA, the ion-pair in intermediate **I** is stabilized and since the subsequent nucleophile attack occurs in an intramolecular manner, the β -product is formed faster and thus accounts for the major product.

Table 4: Substrate scope for modified Julia olefination. ^{a,b}

^a Reactions were carried out with 0.1 mmol **4**, 3 equiv. aldehyde, 1.2 equiv. KHMDS in 1.7 mL THF. ^b Isolated yield. ^c Reaction was conducted with L-glucal-derived pyridylsulfone as starting material.

To demonstrate the synthetic utility of the pyridylsulfone functionality in C-C bond formations involving glycosides, 2,3-deoxy-pyridylsulfone **4a** was reacted with benzaldehyde in the presence of potassium bis(trimethylsilyl)amide (KHMDs) in a modified Julia-Kocienski reaction (Table 4, Scheme 6). The reaction proceeded smoothly and the desired vinylic glycoside was obtained in a high yield of 81% and *E*-selectivity.

Scheme 6. Brief mechanism of the modified Julia Kocienski olefination.

It was observed that the sequence of reagent addition is crucial to the reaction. Addition of aldehyde to a mixture of base and pyridylsulfonyl glycal (pre-metallation approach) led to a rapid isomerisation at the anomeric centre of the glycan and loss of stereoselectivity of the starting glycan. In this case, the added base is depleted and no olefin product is observed. Hence, inverse addition of base to a solution of pyridylsulfonyl glycal and aldehyde (Barbier approach) is necessary to circumvent the issue of glycan isomerisation.

Pyridylsulfonyl glycal derived from L-glycal also reacted to form the desired olefin in good yield of 70% (**7b**), while di-isopropyl-di-siloxane-glycal substrate gave moderate

yield of 41% (**7c**). The presence of electron-donating groups on the benzaldehyde, such as *p*-methoxy (**7f**) and *N,N*-dimethyl amino (**7g**) substituents, generally result in good yields of 64% and 54%. Steric hindrance was noted to have a significant influence on the outcome of the reaction. The desired product was obtained in good yield of 69% when *p*-methylbenzaldehyde was used (**7d**), but the yield decreased dramatically to 20% in the case of *o*-methylbenzaldehyde (**7e**). The metallated glycan is presumably unable to approach the aldehyde due to the steric hindrance of the methyl in the *ortho*-position, hence the low yield observed. Aldehydes with electron-withdrawing halogens were also tolerated in the reaction, with the desired chloro- and bromo-substituted olefins obtained in good yields of 74% (**7h**) and 53% (**7i**) respectively. Heteroaromatic aldehydes were also investigated for their compatibility with the optimised reaction conditions. 5-methylpicolinaldehyde (**7j**) gave a lower yield of 49% due to the electron-withdrawing effect of the picoliny -N, while 1-methyl-1*H*-indole-2-carbaldehyde (**7l**) reacted under the optimised conditions and resulted in the formation of the desired product at a low yield of 45%, which could be due to the steric effect of the methyl substituent.

Scheme 7. Postulated mechanism for the modified Julia olefination reaction.

Considering the results obtained from the above studies, a postulated mechanism is presented in Scheme 7. The aldehyde and 2,3-unsaturated pyridyl sulfonyl glycan first react to form *syn*- β -alkoxy-pyridyl sulfone **A**.⁴⁰ The *anti*-diastereomer formation is disfavoured due to the gauche interactions between the aldehyde substituent R, and the glycan. Double coordination of the pyridyl -N and the sulfone -O to the metal sets up the structure for Smiles rearrangement, in which spirocyclisation occurs to form intermediate **B** then rearranges to form **C**. Elimination of the potassium 2-pyridinolate functionality yields zwitterion **D** and extrusion of SO₂ provides the olefin product in *E*-configuration.

Conclusion

Catalytic decarboxylative allylation is an attractive strategy that allows C-C bond formations without the prerequisite for pre-formed organometallics. This advantage, when combined with the high reactivity of sulfones, results in a facile access to 2,3-unsaturated vinyl glycosides, which provides opportunities for further functional group modifications. In the first step of Pd-catalysed decarboxylation, good yields in the range of 50- 70% were obtained, with the desired products formed in high β -selectivity. In the subsequent modified Julia-Kocienski olefination, different aldehydes were used to test the substrate tolerance of the reaction. Moderate to good yields were achieved and the products were obtained as *E*-olefins. Plausible mechanisms leading to the observed products have also been proposed and discussed.

Experimental information

Section A: General information

Unless otherwise indicated, all reagents used in the reactions were obtained from commercial suppliers and used without further purification. The purity of aldehydes used was confirmed *via* ^1H NMR and further purified by distillation or flash column chromatography before use. All reactions were conducted in flame-dried glassware under a nitrogen atmosphere. Evaporation of organic solutions was achieved by rotary evaporation with a water bath temperature below 40 °C. Product purification by flash column chromatography was accomplished using silica gel 60 (0.010-0.063 mm) with technical grade solvents distilled before use or analytical reagent grade solvents obtained from commercial suppliers. TLC chromatograms were visualized by fluorescence quenching with UV light at 254 nm and staining with a basic solution of potassium permanganate. Optical rotations were measured in CHCl_3 on a Schmidt + Haensdch polarimeter with a 1-cm cell (*c* given in g/100 mL). IR spectra were recorded using FTIR Restige-21 (Shimadzu). NMR spectra were recorded at room temperature on Bruker AV 300MHz, Bruker AVIII 400MHz, Bruker AV 500MHz NMR spectrometers. The residual solvent signals were taken as the reference (7.26 ppm for ^1H NMR spectra and 77.0 ppm for ^{13}C NMR spectra in CDCl_3). Chemical shift (δ) is reported in ppm with coupling constants (*J*) given in Hz. The following abbreviations classify the multiplicity: s = singlet, d = doublet, t = triplet, m = multiplet or unresolved. HRMS (ESI) spectra were recorded on a Waters Q-Tof premierTM mass spectrometer. X-ray crystallographic data was collected by using a Bruker X8Apex diffractometer with Mo K/ α radiation (graphite monochromator).

Section B: Procedure for synthesis of substrates

General procedure A for the synthesis of glycal-derived pyridyl sulfones:

A mixture of 2-(pyridin-2-ylsulfonyl)acetic acid (0.88 g, 1.5 equiv.) and 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (2.65 g, 2.0 equiv.) was dissolved in anhydrous tetrahydrofuran (7 mL) under nitrogen atmosphere. The mixture was cooled to 0 °C before the addition of *N,N*-diisopropylethylamine (3.04 mL, 5.0 equiv.). After 5 min of stirring, the 4,6-protected glycal (1.00 g, 3.49 mmol, 1.0 equiv.) in tetrahydrofuran (14.2 mL) was added dropwise. The mixture was warmed to room temperature and stirred for 72 h. The crude compound was concentrated *in-vacuo* before further purification by flash column chromatography on deactivated silica gel (10% H₂O in hexane) with ethyl acetate/hexane.

General procedure B for the decarboxylative coupling:

A mixture of glycal-derived pyridyl sulfone (460 mg, 0.98 mmol, 1.0 equiv.), tetrakis(triphenylphosphine) palladium (0) (56.6 mg, 0.05 equiv.) and bis(diphenylphosphino) butane (37.6 mg, 0.1 equiv.) in anhydrous *N,N*-dimethyl formamide (23.5 mL) was heated at 80 °C for 8 h in a Schlenk tube under nitrogen atmosphere. The mixture was diluted with ethyl acetate, washed with saturated

sodium chloride and the combined organic layer was concentrated under reduced pressure. The crude compound was purified by flash column chromatography on silica gel with ethyl acetate/ hexane.

General procedure C for the modified Julia-olefination:

The respective aldehyde (0.029 mL, 3.0 equiv.) was added to a solution of 2,3-unsaturated pyridyl sulfone glycal (42.4 mg, 1.0 equiv.) in anhydrous tetrahydrofuran (0.5 mL) and the mixture was cooled to $-78\text{ }^{\circ}\text{C}$ and stirred for 5 min. Potassium bis(trimethylsilyl)amide in tetrahydrofuran (1.2 mL, 0.1 M, 1.2 equiv.) was added dropwise to the mixture over a period of 10 min while maintaining the temperature at $-78\text{ }^{\circ}\text{C}$. The mixture was stirred for 4 h and slowly warmed to r.t., then stirred for 24 h. The reaction was quenched with the addition of saturated ammonium chloride at $0\text{ }^{\circ}\text{C}$ then extracted with ethyl acetate. The combined organic layer was concentrated under reduced pressure and purified by flash column chromatography on silica gel using ethyl acetate/ hexane.

Section C: Characterisation of compounds

3-phenylsulfonyl-acetyloxy-1,5-anhydro-2-deoxy-4,6-O-(*p*-methoxybenzylidene)-*D*-arabino-hex-1-enitol (1)

$[\alpha]_{\text{D}}^{22} = -60.9$ ($c = 0.2$, CHCl_3); ^1H NMR (400 MHz, CDCl_3 , ppm) δ 7.89 (dd, $J = 8.4$, 1.1 Hz, 2H), 7.64 – 7.56 (m, 1H), 7.46 – 7.37 (m, 4H), 6.92 – 6.86 (m, 2H), 6.40 (dd, $J = 6.1$, 1.5 Hz, 1H), 5.55 – 5.48 (m, 2H), 4.69 (dd, $J = 6.1$, 2.1 Hz, 1H), 4.40 – 4.31 (m, 1H), 4.13 (s, 2H), 3.99 – 3.91 (m, 2H), 3.80 (s, 3H); ^{13}C NMR (101 MHz, CDCl_3 , ppm) δ 162.0, 160.2, 146.0, 138.3, 134.1, 129.1, 129.0, 128.5, 128.3, 127.5, 113.6, 101.5, 99.3, 77.3, 77.0, 76.7, 76.3, 70.6, 68.6, 67.9, 61.0, 60.6, 55.2; HRMS (ESI): calcd. for $\text{C}_{22}\text{H}_{23}\text{O}_8\text{S}$ $[\text{M} + \text{H}]^+$: 447.1114; found: 447.1118.

1-phenylsulfonylmethyl-4,6-O-(*p*-methoxybenzylidene)- β -*D*-erythro-hex-2-enopyranoside (2)

$[\alpha]_{\text{D}}^{22} = +105.0$ ($c = 0.3$, CHCl_3); ^1H NMR (500 MHz, CDCl_3 , ppm) δ 7.94 (d, $J = 7.6$ Hz, 2H), 7.67 (t, $J = 7.4$ Hz, 1H), 7.56 (t, $J = 7.8$ Hz, 2H), 7.38 (d, $J = 8.6$ Hz, 2H), 6.87 (d, $J = 8.6$ Hz, 2H), 6.02 (d, $J = 10.5$ Hz, 1H), 5.73 (dt, $J = 10.4$, 1.9 Hz, 1H), 5.45 (s, 1H), 4.89 – 4.79 (m, 1H), 4.08 (dd, $J = 9.0$, 3.3 Hz, 1H), 4.06 – 4.01 (m, 1H), 3.79 (s, 3H), 3.54 – 3.42 (m, 2H), 3.34 (ddd, $J = 19.3$, 14.5, 6.1 Hz, 2H); ^{13}C NMR (126 MHz,

CDCl₃, ppm) δ 160.2, 140.1, 133.8, 129.7, 129.0, 128.7, 128.3, 128.1, 127.5, 113.7, 102.0, 77.3, 77.0, 76.8, 74.4, 70.8, 70.7, 68.8, 60.7, 55.3, 30.9; HRMS (ESI): calcd. for C₂₁H₂₃O₆S [M + H]⁺: 403.1215; found: 403.1227.

3-(2-pyridylsulfonyl)-acetyloxy-1,5-anhydro-2-deoxy-4,6-O-[bis(1,1-dimethylethyl)silylene]-D-arabino-hex-1-enitol (3a)

The title compound was obtained *via* general procedure A as a pale yellow oil in 81% yield. $[\alpha]_D^{22} = -22.5$ ($c = 1.8$, CHCl₃); ¹H NMR (500 MHz, CDCl₃, ppm) δ 8.72 (d, $J = 4.4$ Hz, 1H), 8.08 (d, $J = 7.9$ Hz, 1H), 7.96 (td, $J = 7.8$, 1.6 Hz, 1H), 7.55 (ddd, $J = 7.6$, 4.7, 0.9 Hz, 1H), 6.28 (dd, $J = 6.0$, 1.4 Hz, 1H), 5.32 (dt, $J = 7.4$, 1.8 Hz, 1H), 4.59 (dd, $J = 6.1$, 2.0 Hz, 1H), 4.52 (dd, $J = 40.0$, 14.8 Hz, 2H), 4.14 (dd, $J = 10.3$, 4.9 Hz, 1H), 4.00 – 3.88 (m, 2H), 3.82 (td, $J = 10.4$, 4.8 Hz, 1H), 1.02 (s, 9H), 0.92 (s, 9H); ¹³C NMR (126 MHz, CDCl₃, ppm) δ 162.3, 156.7, 150.1, 145.5, 138.1, 127.5, 122.4, 99.2, 77.3, 77.0, 76.8, 74.1, 73.1, 72.7, 65.5, 56.0, 27.3, 26.7, 22.6, 19.7; HRMS (ESI): calcd. for C₂₁H₃₁NO₇SiNa [M + Na]⁺: 492.1488 ; found: 492.1494.

3-(2-pyridylsulfonyl)-acetyloxy-1,5-anhydro-2-deoxy-4,6-O-cyclohexyl-D-arabino-hex-1-enitol (3b)

The title compound was obtained *via* general procedure A as a colourless oil in 33% yield. $[\alpha]_D^{22} = -15.7$ ($c = 1.0$, CHCl_3); ^1H NMR (500 MHz, CDCl_3 , ppm) δ 8.75 (d, $J = 4.5$ Hz, 1H), 8.10 (d, $J = 7.8$ Hz, 1H), 7.98 (td, $J = 7.8, 1.6$ Hz, 1H), 7.62 – 7.54 (m, 1H), 6.32 (d, $J = 6.1$ Hz, 1H), 5.38 – 5.30 (m, 1H), 4.62 (dd, $J = 6.1, 1.9$ Hz, 1H), 4.50 (q, $J = 14.8$ Hz, 2H), 3.97 – 3.86 (m, 2H), 3.86 – 3.69 (m, 2H), 1.88 (d, $J = 52.6$ Hz, 2H), 1.62 – 1.40 (m, 8H). ^{13}C NMR (101 MHz, CDCl_3 , ppm) δ 162.2, 156.6, 150.1, 146.0, 138.1, 127.6, 122.5, 100.0, 99.5, 77.3, 77.0, 76.7, 71.3, 69.8, 68.6, 60.7, 56.0, 37.6, 27.5, 25.5, 22.5, 22.4; HRMS (ESI): calcd. for $\text{C}_{19}\text{H}_{24}\text{NO}_7\text{S}$ $[\text{M} + \text{H}]^+$: 410.1273; found: 410.1275.

3-(2-pyridylsulfonyl)-acetyloxy-1,5-anhydro-2-deoxy-4,6-O-(2,2-dimethyl)-D-arabino-hex-1-enitol (3c)

The title compound was obtained *via* general procedure A as a colourless oil in 20% yield. $[\alpha]_D^{22} = -25.3$ ($c = 1.0$, CHCl_3); ^1H NMR (400 MHz, CDCl_3 , ppm) δ 8.76 (ddd, $J = 4.7, 1.6, 0.9$ Hz, 1H), 8.14 – 8.07 (m, 1H), 7.99 (td, $J = 7.8, 1.7$ Hz, 1H), 7.59 (ddd, $J = 7.6, 4.7, 1.1$ Hz, 1H), 6.33 (dd, $J = 6.1, 1.3$ Hz, 1H), 5.31 (dt, $J = 7.7, 1.8$ Hz, 1H), 4.65 (dd, $J = 6.1, 2.0$ Hz, 1H), 4.49 (dd, $J = 12.5, 3.6$ Hz, 2H), 3.92 (ddd, $J = 18.0, 10.3, 6.4$ Hz, 2H), 3.84 – 3.70 (m, 2H), 1.47 (s, 3H), 1.38 (s, 3H); ^{13}C NMR (101 MHz, CDCl_3 , ppm) δ 162.2, 156.6, 150.1, 145.9, 138.1, 127.6, 122.4, 99.8, 99.5, 77.3, 77.0, 76.7, 71.3, 69.6, 69.3, 61.4, 56.1, 29.6, 28.8, 18.9; HRMS (ESI): calcd. for $\text{C}_{16}\text{H}_{20}\text{NO}_7\text{S}$ $[\text{M} + \text{H}]^+$: 370.0960 ; found: 370.0964.

3-(2-pyridylsulfonyl)-acetyloxy-1,5-anhydro-2-deoxy-4,6-O-[bis(1,1-dimethylethyl)silylene]-L-arabino-hex-1-enitol (3e)

The title compound was obtained *via* general procedure A as a colourless oil in 73% yield. $[\alpha]_D^{22} = +48.7$ ($c = 1.0$, CHCl_3); ^1H NMR (400 MHz, CDCl_3 , ppm) δ 8.74 – 8.64 (m, 1H), 8.05 (d, $J = 7.8$ Hz, 1H), 7.93 (td, $J = 7.8$, 1.6 Hz, 1H), 7.53 (ddd, $J = 7.6$, 4.7, 1.1 Hz, 1H), 6.25 (dd, $J = 6.0$, 1.4 Hz, 1H), 5.30 (dt, $J = 7.4$, 1.8 Hz, 1H), 4.60 – 4.41 (m, 3H), 4.11 (dd, $J = 10.1$, 4.7 Hz, 1H), 3.95 – 3.84 (m, 2H), 3.80 (td, $J = 10.3$, 4.7 Hz, 1H), 0.99 (s, 9H), 0.90 (s, 9H); ^{13}C NMR (101 MHz, CDCl_3 , ppm) δ 162.3, 156.7, 150.1, 145.6, 138.1, 127.6, 122.4, 99.3, 77.3, 77.0, 76.7, 74.2, 73.2, 72.8, 65.5, 56.0, 27.3, 26.8, 22.7, 19.8; HRMS (ESI): calcd. for $\text{C}_{21}\text{H}_{32}\text{NO}_7\text{SiS}$ $[\text{M} + \text{H}]^+$: 470.1669 ; found: 470.1668.

3-(2-pyridylsulfonyl)-acetyloxy-1,5-anhydro-2-deoxy-4,6-O-[bis(1,1-dimethylethyl)silylene]-D-ribo-hex-1-enitol (3f)

The title compound was obtained *via* general procedure A as a pale yellow solid in 43% yield. m.p. 114.2 °C; $[\alpha]_D^{22} = +140.6$ ($c = 0.6$, CHCl_3); ^1H NMR (400 MHz, CD_3CN , ppm) δ 8.74 (d, $J = 4.7$ Hz, 1H), 8.10 – 7.98 (m, 2H), 7.70 – 7.62 (m, 1H), 6.40 (d, $J = 5.9$ Hz, 1H), 5.21 (dd, $J = 5.7$, 4.1 Hz, 1H), 4.76 (t, $J = 6.0$ Hz, 1H), 4.60 (d, $J = 14.6$

Hz, 1H), 4.29 (d, J = 14.6 Hz, 1H), 4.18 – 4.08 (m, 2H), 3.89 (t, J = 10.4 Hz, 1H), 3.51 (td, J = 10.7, 5.1 Hz, 1H), 1.01 (s, 9H), 0.97 (s, 9H). ^{13}C NMR (101 MHz, CD_3CN , ppm) δ 162.8, 157.3, 151.4, 148.4, 139.8, 129.1, 123.5, 118.3, 97.7, 72.9, 69.5, 67.1, 66.4, 57.2, 27.6, 27.4, 23.2, 20.6, 1.9, 1.7, 1.5, 1.3, 1.1, 0.9, 0.7; HRMS (ESI): calcd. for $\text{C}_{21}\text{H}_{32}\text{NO}_7\text{SiS}$ [$\text{M} + \text{H}$] $^+$: 470.1669 ; found: 470.1671.

1-pyridylsulfonylmethyl-4,6-O-[bis(1,1-dimethylethyl)silylene]- β -D-erythro-hex-2-enopyranoside (4a)

The title compound was obtained *via* general procedure B as a pale yellow solid in 72% yield. m.p. 140.9 °C; $[\alpha]_{\text{D}}^{22} = +41.1$ (c = 0.4, CHCl_3); ^1H NMR (400 MHz, CDCl_3 , ppm) δ 8.72 (d, J = 4.3 Hz, 1H), 8.04 (d, J = 7.8 Hz, 1H), 7.92 (td, J = 7.7, 1.3 Hz, 1H), 7.53 (dd, J = 7.0, 4.9 Hz, 1H), 5.91 (d, J = 10.3 Hz, 1H), 5.61 (d, J = 10.4 Hz, 1H), 4.77 (d, J = 8.3 Hz, 1H), 4.20 (d, J = 8.2 Hz, 1H), 3.82 – 3.65 (m, 2H), 3.42 – 3.28 (m, J = 12.1, 7.5, 4.2 Hz, 2H), 3.24 (t, J = 10.1 Hz, 1H), 0.99 (s, 9H), 0.93 (s, 9H); ^{13}C NMR (126 MHz, CDCl_3 , ppm) δ 158.0, 149.9, 137.8, 132.0, 127.1, 126.2, 122.2, 77.3, 77.0, 76.8, 74.0, 70.4, 69.3, 66.4, 56.5, 27.3, 27.0, 22.6, 20.0; HRMS (ESI): calcd. for $\text{C}_{20}\text{H}_{32}\text{NO}_5\text{SiS}$ [$\text{M} + \text{H}$] $^+$: 426.1770 ; found: 426.1773.

1-pyridylsulfonylmethyl-4,6-O-(2,2-dimethyl)- β -D-erythro-hex-2-enopyranoside (4b)

The title compound was obtained *via* general procedure B as a yellow oil in 57% yield. $[\alpha]_D^{22} = +72.8$ ($c = 1.0$, CHCl_3); ^1H NMR (400 MHz, CDCl_3 , ppm) δ 8.74 (ddd, $J = 4.7$, 1.6, 0.8 Hz, 1H), 8.07 (dt, $J = 7.8$, 0.9 Hz, 1H), 7.94 (td, $J = 7.7$, 1.7 Hz, 1H), 7.55 (ddd, $J = 7.6$, 4.7, 1.1 Hz, 1H), 5.90 (d, $J = 10.3$ Hz, 1H), 5.66 (dt, 10.4, 2.4 Hz, 1H), 4.87 – 4.77 (m, 1H), 4.16 – 4.04 (m, 1H), 3.74 (dd, $J = 14.6$, 8.1 Hz, 1H), 3.60 – 3.51 (m, 1H), 3.44 (dd, $J = 14.6$, 4.2 Hz, 1H), 3.37 – 3.22 (m, 2H), 1.92 – 1.73 (m, 2H), 1.58 – 1.37 (m, 8H); ^{13}C NMR (101 MHz, CDCl_3 , ppm) δ 157.9, 149.9, 137.9, 129.7, 127.5, 127.2, 122.1, 99.9, 77.3, 77.0, 76.7, 71.8, 70.7, 65.9, 61.8, 56.4, 37.9, 27.7, 25.6, 22.7, 22.5; HRMS (ESI): calcd. for $\text{C}_{18}\text{H}_{24}\text{NO}_5\text{S}$ $[\text{M} + \text{H}]^+$: 366.1375 ; found: 366.1380.

1-pyridylsulfonylmethyl-4,6-O-cyclohexyl- β -D-erythro-hex-2-enopyranoside (4c)

The title compound was obtained *via* general procedure B as a pale yellow oil in 58% yield. $[\alpha]_D^{22} = +73.2$ ($c = 1.0$, CHCl_3); ^1H NMR (400 MHz, CDCl_3 , ppm) δ 8.74 (ddd, $J = 4.7$, 1.6, 0.9 Hz, 1H), 8.07 (dt, $J = 7.8$, 0.9 Hz, 1H), 7.95 (td, $J = 7.8$, 1.7 Hz, 1H), 7.55 (ddd, $J = 7.6$, 4.7, 1.1 Hz, 1H), 5.88 (d, $J = 10.4$ Hz, 1H), 5.72 – 5.64 (m, 1H), 4.89 – 4.80 (m, 1H), 4.14 – 4.04 (m, 1H), 3.75 (dd, $J = 14.6$, 8.1 Hz, 1H), 3.64 – 3.54 (m, 1H), 3.46 (dd, $J = 14.6$, 4.3 Hz, 1H), 3.37 – 3.19 (m, 2H), 1.43 (s, 3H), 1.38 (s, 3H); ^{13}C NMR (101 MHz, CDCl_3 , ppm) δ 158.0, 149.9, 137.9, 129.5, 127.6, 127.2, 122.1, 99.8, 77.3, 77.0, 76.7, 71.5, 70.6, 66.8, 62.5, 56.3, 29.1, 19.0; HRMS (ESI): calcd. for $\text{C}_{15}\text{H}_{20}\text{NO}_5\text{S}$ $[\text{M} + \text{H}]^+$: 326.1062 ; found: 326.1064.

1-pyridylsulfonylmethyl-4,6-O-[1,1,3,3-tetrakis(1-methylethyl)-1,3-disiloxanediyl]- β -D-erythro-hex-2-enopyranoside (4d)

The title compound was obtained *via* general procedure B as a white solid in 50% yield. m.p. 143.2 °C; $[\alpha]_{\text{D}}^{22} = +89.4$ ($c = 0.4$, CHCl_3); ^1H NMR (400 MHz, CDCl_3 , ppm) δ 8.69 (d, $J = 3.7$ Hz, 1H), 8.06 (d, $J = 7.7$ Hz, 1H), 7.91 (t, $J = 7.6$ Hz, 1H), 7.56 – 7.39 (m, 1H), 5.86 (d, $J = 10.1$ Hz, 1H), 5.69 (d, $J = 10.1$ Hz, 1H), 4.73 (s, 1H), 4.34 (d, $J = 7.6$ Hz, 1H), 3.95 – 3.79 (m, 2H), 3.36 (dd, 2H), 3.12 (d, $J = 8.2$ Hz, 1H), 0.98 (dd, $J = 20.8, 16.7$ Hz, 28H). ^{13}C NMR (101 MHz, CDCl_3 , ppm) δ 158.0, 149.9, 137.6, 132.4, 127.0, 126.9, 122.1, 79.1, 77.3, 77.0, 76.7, 70.5, 61.9, 60.9, 56.6, 17.3, 17.3, 17.2, 17.1, 17.0, 17.0, 13.4, 13.0, 12.6, 12.4; HRMS (ESI): calcd. for $\text{C}_{24}\text{H}_{42}\text{NO}_6\text{Si}_2\text{S}$ $[\text{M} + \text{H}]^+$: 528.2271 ; found: 528.2283.

1-pyridylsulfonylmethyl-4,6-O-[bis(1,1-dimethylethyl)silylene]- β -L-erythro-hex-2-enopyranoside (4e)

The title compound was obtained *via* general procedure B as a white solid in 61% yield. m.p. 140.0 °C; $[\alpha]_{\text{D}}^{22} = -2.5$ ($c = 1.0$, CHCl_3); ^1H NMR (500 MHz, CDCl_3 , ppm) δ 8.76 (d, $J = 4.2$ Hz, 1H), 8.10 (d, $J = 7.9$ Hz, 1H), 7.97 (td, $J = 7.8, 1.6$ Hz, 1H), 7.60 (dd, $J = 7.2, 5.2$ Hz, 1H), 5.95 (d, $J = 10.4$ Hz, 1H), 5.62 (dt, $J = 10.4, 2.4$ Hz, 1H), 4.85 (dd, $J = 7.8, 2.5$ Hz, 1H), 4.34 – 4.27 (m, 1H), 4.21 (d, $J = 8.6$ Hz, 1H), 3.38 (t, $J = 10.1$

Hz, 1H), 3.20 (dd, $J = 15.2, 2.9$ Hz, 1H), 3.17 – 3.12 (m, 1H), 2.83 (dd, $J = 9.9, 4.9$ Hz, 1H), 0.98 (s, 9H), 0.96 (s, 9H); ^{13}C NMR (126 MHz, CDCl_3 , ppm) δ 157.9, 149.8, 137.8, 131.9, 127.1, 126.1, 122.1, 77.3, 77.0, 76.8, 73.9, 70.4, 69.2, 66.4, 56.5, 27.3, 26.9, 22.5, 20.0; HRMS (ESI): calcd. for $\text{C}_{20}\text{H}_{32}\text{NO}_5\text{SiS}$ $[\text{M} + \text{H}]^+$: 426.1770 ; found: 426.1771.

1-pyridylsulfonylmethyl-4,6-O-[bis(1,1-dimethylethyl)silylene]- α -D-erythro-hex-2-enopyranoside (4f)

The title compound was obtained *via* general procedure B as a pale yellow oil in 62% yield. $[\alpha]_{\text{D}}^{22} = +31.1$ ($c = 1.0$, CHCl_3); ^1H NMR (400 MHz, CDCl_3 , ppm) δ 8.75 (d, $J = 4.1$ Hz, 1H), 8.09 (d, $J = 7.9$ Hz, 1H), 7.97 (td, $J = 7.8, 1.6$ Hz, 1H), 7.60 (ddd, $J = 7.6, 4.7, 1.0$ Hz, 1H), 5.95 (d, $J = 10.4$ Hz, 1H), 5.62 (dt, $J = 10.4, 2.5$ Hz, 1H), 4.89 – 4.80 (m, 1H), 4.31 (dd, $J = 15.1, 10.4$ Hz, 1H), 4.23 – 4.16 (m, 1H), 3.38 (t, $J = 10.1$ Hz, 1H), 3.25 – 3.10 (m, 2H), 2.82 (dd, $J = 9.9, 4.9$ Hz, 1H), 0.98 (s, 9H), 0.96 (s, 9H); ^{13}C NMR (101 MHz, CDCl_3 , ppm) δ 158.2, 150.0, 138.2, 132.4, 127.3, 125.4, 121.8, 77.3, 77.0, 76.7, 69.4, 68.8, 68.4, 66.5, 53.0, 27.3, 27.0, 22.6, 20.0; HRMS (ESI): calcd. for $\text{C}_{20}\text{H}_{32}\text{NO}_5\text{SiS}$ $[\text{M} + \text{H}]^+$: 426.1770 ; found: 426.1772.

(E)-styryl-4,6-O-[bis(1,1-dimethylethyl)silylene]- β -D-erythro-hex-2-enopyranoside (7a)

The title compound was obtained *via* general procedure C as a pale yellow oil in 81% yield. $[\alpha]_D^{22} = +42.2$ ($c = 0.2$, CHCl_3); ^1H NMR (500 MHz, CDCl_3 , ppm) δ 7.37 (d, $J = 7.3$ Hz, 2H), 7.30 (t, $J = 7.5$ Hz, 2H), 7.24 (s, 1H), 6.63 (d, $J = 15.8$ Hz, 1H), 6.08 (dd, $J = 15.9, 7.2$ Hz, 1H), 5.93 (d, $J = 10.2$ Hz, 1H), 5.68 (d, $J = 10.3$ Hz, 1H), 4.90 – 4.82 (m, 1H), 4.48 (d, $J = 8.4$ Hz, 1H), 4.23 (dd, $J = 10.0, 5.0$ Hz, 1H), 3.94 (t, $J = 10.3$ Hz, 1H), 3.64 – 3.54 (m, 1H), 1.07 (s, 9H), 1.01 (s, 9H); ^{13}C NMR (101 MHz, CDCl_3) δ 136.4, 132.5, 130.2, 128.5, 128.3, 127.9, 127.7, 126.6, 77.3, 77.0, 76.7, 76.6, 74.6, 70.1, 67.2, 27.5, 27.1, 22.7, 20.1; HRMS (ESI): calcd. for $\text{C}_{22}\text{H}_{33}\text{O}_3\text{Si}$ $[\text{M} + \text{H}]^+$: 373.2199 ; found: 373.2204.

(*E*)-styryl-4,6-*O*-[bis(1,1-dimethylethyl)silylene]- β -L-erythro-hex-2-enopyranoside (7b)

The title compound was obtained *via* general procedure C as a yellow oil in 70% yield. $[\alpha]_D^{22} = -57.8$ ($c = 0.3$, CHCl_3); ^1H NMR (400 MHz, CDCl_3 , ppm) δ 7.37 (dd, $J = 7.2$ Hz, 2H), 7.30 (dd, $J = 7.4$ Hz, 2H), 7.23 (dd, $J = 4.8$ Hz, 1H), 6.63 (d, $J = 15.9$ Hz, 1H), 6.08 (dd, $J = 15.9, 7.2$ Hz, 1H), 5.93 (d, $J = 10.3$ Hz, 1H), 5.68 (dt, $J = 10.3, 1.9$ Hz, 1H), 4.89 – 4.82 (m, 1H), 4.52 – 4.44 (m, 1H), 4.23 (dd, $J = 10.0, 5.1$ Hz, 1H), 3.94 (t, $J = 10.2$ Hz, 1H), 3.60 (ddd, $J = 10.5, 8.5, 5.1$ Hz, 1H), 1.07 (s, 9H), 1.01 (s, 9H); ^{13}C NMR (101 MHz, CDCl_3 , ppm) δ 136.3, 132.5, 130.2, 128.5, 128.3, 127.9, 127.6, 126.6, 77.3, 77.0, 76.7, 76.6, 74.6, 70.1, 67.2, 27.5, 27.1, 22.7, 20.1; HRMS (ESI): calcd. for $\text{C}_{22}\text{H}_{33}\text{O}_3\text{Si}$ $[\text{M} + \text{H}]^+$: 373.2199 ; found: 373.2200.

(*E*)-styryl -4,6-*O*-[1,1,3,3-tetrakis(1-methylethyl)-1,3-disiloxanediyl]- β -D-erythro-hex-2-enopyranoside (7c)

The starting material of the title compound was not purified due to instability on silica gel. The crude product after general procedure B was used directly in general procedure C and the desired product was obtained as a yellow oil (41% yield over 2 steps). $[\alpha]_D^{22} = +16.2$ ($c = 0.5$, CHCl_3); ^1H NMR (500 MHz, CDCl_3 , ppm) δ 7.39 (d, $J = 7.5$ Hz, 2H), 7.31 (t, $J = 7.5$ Hz, 2H), 7.26 – 7.21 (m, $J = 7.3$ Hz, 1H), 6.64 (d, $J = 15.9$ Hz, 1H), 6.12 (dd, $J = 15.9, 7.2$ Hz, 1H), 5.93 (d, $J = 10.3$ Hz, 1H), 5.71 (d, $J = 10.3$ Hz, 1H), 4.82 (d, $J = 7.1$ Hz, 1H), 4.42 (d, $J = 8.5$ Hz, 1H), 4.00 – 3.93 (m, 1H), 3.79 – 3.71 (m, $J = 11.4, 5.7$ Hz, 1H), 3.58 – 3.50 (m, 1H), 2.09 (t, $J = 6.2$ Hz, 1H), 1.07 – 1.01 (m, 24H), 0.98 – 0.85 (m, 4H); ^{13}C NMR (126 MHz, CDCl_3 , ppm) δ 136.4, 132.1, 130.5, 128.9, 128.6, 128.2, 127.9, 126.6, 79.3, 77.3, 77.0, 76.8, 75.9, 64.4, 63.1, 17.4, 17.3, 17.3, 13.6, 13.5, 13.4, 2.1; HRMS (ESI): calcd. for $\text{C}_{26}\text{H}_{42}\text{O}_4\text{Si}_2\text{Na}$ $[\text{M} + \text{Na}]^+ : 497.2519$; found: 497.2513.

[(*E*)-4-methylstyryl]-4,6-*O*-[bis(1,1-dimethylethyl)silylene]- β -D-erythro-hex-2-enopyranoside (7d)

The title compound was obtained *via* general procedure C as a white solid in 68% yield. m.p. 101- 104 °C; $[\alpha]_D^{22} = +120.9$ ($c = 1.0$, CHCl_3); ^1H NMR (400 MHz, CDCl_3 ,

ppm) δ 7.36 (d, J = 7.0 Hz, 2H), 7.20 (d, J = 7.9 Hz, 2H), 6.69 (d, J = 15.9 Hz, 1H), 6.12 (dd, J = 15.9, 7.4 Hz, 1H), 6.01 (d, J = 10.3 Hz, 1H), 5.83 – 5.73 (m, 1H), 4.93 (d, J = 6.1 Hz, 1H), 4.57 (d, J = 8.4 Hz, 1H), 4.33 (dd, J = 10.0, 5.0 Hz, 1H), 4.03 (t, J = 10.3 Hz, 1H), 3.69 (ddd, J = 10.4, 8.6, 5.1 Hz, 1H), 2.48 – 2.39 (m, 3H), 1.16 (s, 9H), 1.10 (s, 9H); ^{13}C NMR (101 MHz, CDCl_3 , ppm) δ 137.8, 133.6, 132.5, 130.1, 129.2, 128.4, 126.5, 77.3, 77.0, 76.7, 74.6, 70.1, 67.2, 27.5, 27.1, 22.7, 21.2, 20.1; HRMS (ESI): calcd. for $\text{C}_{23}\text{H}_{35}\text{O}_3\text{Si}$ $[\text{M} + \text{H}]^+$: 387.2355; found: 387.2349.

[(*E*)-2-methylstyryl]-4,6-*O*-[bis(1,1-dimethylethyl)silylene]- β -D-erythro-hex-2-enopyranoside (7e)

The title compound was obtained *via* general procedure C as a yellow oil in 20% yield. $[\alpha]_{\text{D}}^{22} = +52.5$ (c = 0.4, CHCl_3); ^1H NMR (500 MHz, CDCl_3 , ppm) δ 7.49 – 7.38 (m, 1H), 7.14 (dd, J = 6.0, 3.7 Hz, 3H), 6.84 (d, J = 15.7 Hz, 1H), 6.03 – 5.86 (m, 2H), 5.68 (dd, J = 10.3, 1.7 Hz, 1H), 4.94 – 4.79 (m, 1H), 4.48 (d, J = 8.3 Hz, 1H), 4.24 (dd, J = 9.9, 5.0 Hz, 1H), 3.94 (t, J = 10.2 Hz, 1H), 3.67 – 3.55 (m, 1H), 2.34 (s, 3H), 1.07 (s, 9H), 1.01 (s, 9H). ^{13}C NMR (126 MHz, CDCl_3 , ppm) δ 135.6, 135.4, 130.3, 130.2, 129.0, 128.5, 127.8, 126.0, 125.8, 77.3, 77.0, 76.9, 76.8, 74.6, 70.1, 67.2, 27.5, 27.1, 22.7, 20.1, 19.8; HRMS (ESI): calcd. for $\text{C}_{23}\text{H}_{35}\text{O}_3\text{Si}$ $[\text{M} + \text{H}]^+$: 387.2355 ; found: 387.2364.

[(E)-4-methoxystyryl]-4,6-O-[bis(1,1-dimethylethyl)silylene]- β -D-erythro-hex-2-enopyranoside (7f)

The title compound was obtained *via* general procedure C as a yellow solid in 64% yield. m.p. 110- 113 °C; $[\alpha]_D^{22} = +131.5$ ($c = 0.5$, CHCl_3); ^1H NMR (400 MHz, CDCl_3 , ppm) δ 7.33 – 7.28 (m, 2H), 6.86 – 6.80 (m, 2H), 6.57 (d, $J = 15.8$ Hz, 1H), 5.98 – 5.89 (m, 2H), 5.67 (dt, $J = 10.3, 1.9$ Hz, 1H), 4.85 – 4.80 (m, $J = 3.3, 2.1$ Hz, 1H), 4.51 – 4.44 (m, $J = 5.1, 3.4$ Hz, 1H), 4.23 (dd, $J = 10.0, 5.0$ Hz, 1H), 3.93 (t, $J = 10.2$ Hz, 1H), 3.81 – 3.77 (m, $J = 4.8$ Hz, 3H), 3.59 (ddd, $J = 10.5, 8.5, 5.1$ Hz, 1H), 1.07 (s, 9H), 1.01 (s, 9H); ^{13}C NMR (101 MHz, CDCl_3 , ppm) δ 159.5, 132.2, 130.1, 129.1, 128.5, 127.9, 125.4, 113.9, 77.3, 77.0, 76.8, 76.7, 74.6, 70.1, 67.2, 55.3, 27.5, 27.1; HRMS (ESI): calcd. for $\text{C}_{23}\text{H}_{35}\text{O}_4\text{Si}$ $[\text{M} + \text{H}]^+$: 403.2305 ; found: 403.2303.

[(E)-4-*N,N*-dimethylstyryl]-4,6-O-[bis(1,1-dimethylethyl)silylene]- β -D-erythro-hex-2-enopyranoside (7g)

The title compound was obtained *via* general procedure C as a yellow solid in 54 % yield. m.p. 130- 132 °C; $[\alpha]_D^{22} = 94.8$ ($c = 1.9$, CHCl_3); ^1H NMR (300 MHz, CDCl_3 , ppm) δ 7.25 (d, $J = 5.1, 3.8$ Hz, 2H), 6.64 (d, $J = 8.9$ Hz, 2H), 6.53 (d, $J = 15.8$ Hz, 1H), 5.93 – 5.80 (m, 2H), 5.67 (dt, $J = 10.3, 1.9$ Hz, 1H), 4.81 (ddd, $J = 4.3, 2.0, 0.9$ Hz, 1H), 4.53 – 4.43 (m, 1H), 4.22 (dd, $J = 10.0, 5.0$ Hz, 1H), 3.93 (t, $J = 10.2$ Hz, 1H),

3.59 (ddd, $J = 10.5, 8.5, 5.1$ Hz, 1H), 2.95 (s, $J = 3.6$ Hz, 6H), 1.07 (s, 9H), 1.01 (s, 9H); ^{13}C NMR (101 MHz, CDCl_3 , ppm) δ 150.3, 133.0, 129.8, 128.9, 127.7, 124.7, 123.0, 112.2, 77.3, 77.1, 77.0, 76.7, 74.6, 70.1, 67.3, 40.4, 27.5, 27.1, 22.7, 20.1; HRMS (ESI): calcd. for $\text{C}_{24}\text{H}_{38}\text{NO}_3\text{Si}$ $[\text{M} + \text{H}]^+$: 416.2621 ; found: 416.2611.

[(*E*)-4-chlorostyryl]-4,6-*O*-[bis(1,1-dimethylethyl)silylene]- β -D-*erythro*-hex-2-enopyranoside (7h)

The title compound was obtained *via* general procedure C as a yellow oil in 74% yield. $[\alpha]_{\text{D}}^{22} = +113.6$ ($c = 0.6$, CHCl_3); ^1H NMR (400 MHz, CDCl_3 , ppm) δ 7.36 – 7.26 (m, 4H), 6.58 (d, $J = 15.8$ Hz, 1H), 6.06 (dd, $J = 15.9, 7.1$ Hz, 1H), 5.93 (d, $J = 10.2$ Hz, 1H), 5.66 (dt, $J = 10.3, 1.9$ Hz, 1H), 4.85 (ddd, $J = 3.7, 2.1, 1.0$ Hz, 1H), 4.51 – 4.43 (m, 1H), 4.23 (dd, $J = 10.0, 5.0$ Hz, 1H), 3.93 (t, $J = 10.3$ Hz, 1H), 3.60 (ddd, $J = 10.5, 8.5, 5.1$ Hz, 1H), 1.07 (s, 9H), 1.00 (s, 9H); ^{13}C NMR (101 MHz, CDCl_3 , ppm) δ 134.9, 133.6, 131.1, 130.4, 128.7, 128.3, 128.0, 127.8, 77.3, 77.0, 76.7, 76.3, 74.6, 70.0, 67.2, 29.7, 27.5, 27.1, 22.7, 20.1; HRMS (ESI): calcd. for $\text{C}_{22}\text{H}_{32}\text{O}_3\text{SiCl}$ $[\text{M} + \text{H}]^+$: 407.1809 ; found: 407.1813.

[(*E*)-4-bromostyryl]-4,6-*O*-[bis(1,1-dimethylethyl)silylene]- β -D-*erythro*-hex-2-enopyranoside (7i)

The title compound was obtained *via* general procedure C as a pale yellow oil in 53% yield. $[\alpha]_D^{22} = -38.5$ ($c = 0.1$, CHCl_3); ^1H NMR (400 MHz, CDCl_3 , ppm) δ 7.42 (d, $J = 8.5$ Hz, 1H), 7.23 (d, $J = 8.5$ Hz, 1H), 6.57 (d, $J = 16.1$ Hz, 1H), 6.07 (dd, $J = 15.9$, 7.1 Hz, 1H), 5.93 (d, $J = 10.2$ Hz, 1H), 5.66 (dt, $J = 10.3$, 1.9 Hz, 1H), 4.89 – 4.79 (m, 1H), 4.52 – 4.41 (m, 1H), 4.23 (dd, $J = 9.9$, 5.1 Hz, 1H), 3.93 (t, $J = 10.3$ Hz, 1H), 3.59 (ddd, $J = 10.5$, 8.5, 5.1 Hz, 1H), 1.07 (s, 7H), 1.00 (s, 7H); ^{13}C NMR (101 MHz, CDCl_3 , ppm) δ 135.3, 131.7, 131.5, 131.1, 130.4, 128.5, 128.1, 127.9, 77.3, 77.0, 76.7, 76.3, 74.6, 70.0, 67.2, 27.5, 27.1, 22.7, 20.1; HRMS (ESI): calcd. for $\text{C}_{22}\text{H}_{32}\text{O}_3\text{SiBr}$ $[\text{M} + \text{H}]^+$: 451.1304 ; found: 451.1311.

[(*E*)-5-methylpyridylvinyl]-4,6-*O*-[bis(1,1-dimethylethyl)silylene]- β -D-*erythro*-hex-2-enopyranoside (7j)

The title compound was obtained *via* general procedure C as a pale yellow oil in 49 % yield. $[\alpha]_D^{22} = +32.5$ ($c = 0.3$, CHCl_3); ^1H NMR (300 MHz, CDCl_3 , ppm) δ 8.37 (d, $J = 1.8$ Hz, 1H), 7.42 (dd, $J = 8.0$, 2.0 Hz, 1H), 7.20 (d, $J = 8.0$ Hz, 1H), 6.70 (d, $J = 15.8$ Hz, 1H), 6.52 (dd, $J = 15.8$, 6.4 Hz, 1H), 5.92 (d, $J = 10.3$ Hz, 1H), 5.70 (dt, $J = 10.3$, 1.9 Hz, 1H), 4.97 – 4.86 (m, 1H), 4.46 (ddd, $J = 10.2$, 4.0, 2.6 Hz, 1H), 4.24 (dd, $J = 10.0$, 5.0 Hz, 1H), 3.94 (t, $J = 10.2$ Hz, 1H), 3.61 (ddd, $J = 10.4$, 8.5, 5.1 Hz, 1H), 2.31 (s, 3H), 1.06 (s, 9H), 1.00 (s, 9H); ^{13}C NMR (101 MHz, CDCl_3 , ppm) δ 152.5, 150.0, 136.9, 131.9, 131.4, 131.0, 130.2, 127.9, 121.2, 77.3, 77.2, 77.0, 76.7, 75.9, 74.6, 70.1, 67.2, 27.5, 27.1, 22.7, 20.1, 18.3; HRMS (ESI): calcd. for $\text{C}_{22}\text{H}_{33}\text{NO}_3\text{Si}$ $[\text{M} + \text{H}]^+$: 410.2127 ; found: 410.2110.

**[(*E*)-2-(thieno[2,3-*b*]thiophen-2-yl)vinyl]-4,6-*O*-[bis(1,1-dimethylethyl)silylene]-
β-*D*-erythro-hex-2-enopyranoside (7k)**

The title compound was obtained *via* general procedure C as a yellow solid in 60 % yield. m.p. 130- 133 °C; $[\alpha]_D^{22} = 139.0$ ($c = 0.5$, CHCl₃); ¹H NMR (400 MHz, CDCl₃, ppm) δ 6.92 – 6.76 (m, 2H), 5.93 (d, $J = 10.2$ Hz, 1H), 5.80 (dd, $J = 15.5, 7.2$ Hz, 1H), 5.68 (dt, $J = 10.3, 1.9$ Hz, 1H), 4.91 – 4.76 (m, $J = 6.3$ Hz, 1H), 4.54 – 4.45 (m, $J = 8.4$ Hz, 1H), 4.23 (dd, $J = 10.0, 5.0$ Hz, 1H), 3.95 (t, $J = 10.2$ Hz, 1H), 3.60 (ddd, $J = 10.4, 8.5, 5.1$ Hz, 1H), 2.46 (d, $J = 1.0$ Hz, 3H), 2.44 (s, 3H), 1.07 (d, $J = 2.4$ Hz, 9H), 1.00 (d, $J = 3.0$ Hz, 9H); ¹³C NMR (101 MHz, CDCl₃, ppm) δ 146.8, 137.6, 131.2, 130.3, 129.0, 128.1, 126.2, 124.6, 123.0, 77.3, 77.2, 77.0, 76.7, 76.5, 74.7, 70.0, 67.2, 27.5, 27.1, 22.7, 20.1, 15.6, 12.9; HRMS (ESI): calcd. for C₂₄H₃₅O₃Si₂ [M + H]⁺ : 463.1797 ; found: 463.1777.

**[(*E*)-1-methyl-1*H*-indole vinyl]-4,6-*O*-[bis(1,1-dimethylethyl)silylene]-β-*D*-
erythro-hex-2-enopyranoside (7l)**

The title compound was obtained *via* general procedure C as a yellow oil in 45 % yield. $[\alpha]_D^{22} = +99.4$ ($c = 0.3$, CHCl₃); ¹H NMR (400 MHz, CDCl₃, ppm) δ 7.55 (d, $J = 7.9$ Hz, 1H), 7.28 (s, 1H), 7.18 (t, $J = 7.6$ Hz, 1H), 7.07 (t, $J = 7.3$ Hz, 1H), 6.72 (d, $J = 15.8$ Hz, 1H), 6.65 (s, 1H), 6.17 (dd, $J = 15.7, 7.0$ Hz, 1H), 5.95 (d, $J = 10.4$ Hz, 1H), 5.70

(dt, $J = 10.3$ Hz, 1H), 4.93 – 4.86 (m, 1H), 4.56 – 4.43 (m, 1H), 4.25 (dd, $J = 10.0, 5.0$ Hz, 1H), 3.96 (t, $J = 10.2$ Hz, 1H), 3.74 (s, 3H), 3.67 – 3.58 (m, 1H), 1.08 (s, 9H), 1.01 (s, 9H); ^{13}C NMR (101 MHz, CDCl_3 , ppm) δ 138.0, 137.1, 130.4, 129.9, 127.9, 127.7, 121.8, 121.0, 120.5, 119.8, 109.2, 99.6, 77.3, 77.0, 76.7, 76.5, 74.7, 70.0, 67.2, 29.9, 27.5, 27.1, 22.7, 20.1; HRMS (ESI): calcd. for $\text{C}_{25}\text{H}_{36}\text{NO}_3\text{Si}$ $[\text{M} + \text{H}]^+$: 426.2464 ; found: 426.2462.

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List of publications

- 1) Wu, X.; Tan, Y. J.; Toh, H. T.; Nguyen, L. H.; Chew, S. Y.; Yoon, H. S.; Liu, X.-W. *Stimuli-responsive delivery system for targeted drug delivery and cancer cell imaging*. Manuscript in preparation.
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Conference participation

- Yu Jia Tan, Xue-Wei Liu, Stereoselective synthesis of C1-vinyllic carbohydrates via Pd-catalyzed decarboxylative allylation and modified Julia olefination, 16th Tetrahedron Symposium: Challenges in Bioorganic and Organic Chemistry, Berlin, Germany, June, 2015
- Yu Jia Tan, Bala Kishan Gorityala, Zhiqiang Lu, Xue-Wei Liu, Boronic acid-functionalised carbohydrate complex: An investigation of carbohydrate-lectin interactions and application to cellular delivery, 15th Tetrahedron Symposium (Asia Edition): Challenges in Bioorganic and Organic Medicinal Chemistry, Singapore, October, 2015
- Yu Jia Tan, Min Li Leow, Xue-Wei Liu, Glycosylated Nanomaterials for Probing Carbohydrate-Lectin Interactions, International Conference on Natural Products and Health, Singapore, September, 2013