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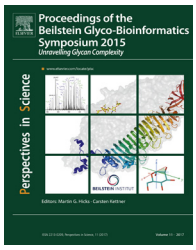

Glycan arrays and other tools produced by automated glycan assembly☆

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Summary Carbohydrates are the dominant biopolymer on earth and play important roles ranging from building material for plants to function in many biological systems. Glycans remain poorly studied due to a lack of synthetic tools. The goal of my laboratory has been to develop a general method for the automated assembly of glycans. The general protocols we developed resulted in the commercialisation of the Glyconeer 2.1™ synthesizer as well as the building blocks and all reagents. Oligosaccharides as long as 50-mers are now accessible within days. Rapid access to defined oligosaccharides has been the foundation to many applications including synthetic tools such as glycan microarrays, glycan nanoparticles and anti-glycan antibodies. The platform technology is helping to address real-life problems by the creation of new vaccines and diagnostics. After addressing mainly mammalian glycobiology earlier, material science and plant biology are benefitting increasingly from synthetic glycans.

Automated glycan assembly from concept to commercialisation

Automated access to peptides and oligonucleotides fundamentally changed the way structure-function studies could be addressed. While the synthesis of oligopeptides (Merrifield and Stewart, 1965) or oligonucleotides (Caruthers, 1985) was a tremendous challenge until the 1970s, solid-phase synthesis methods in concert with improved methods of separation provided access to defined oligomers. Synthetic oligonucleotides were the basis for amplification by polymerase chain reaction (PCR). Analogs of

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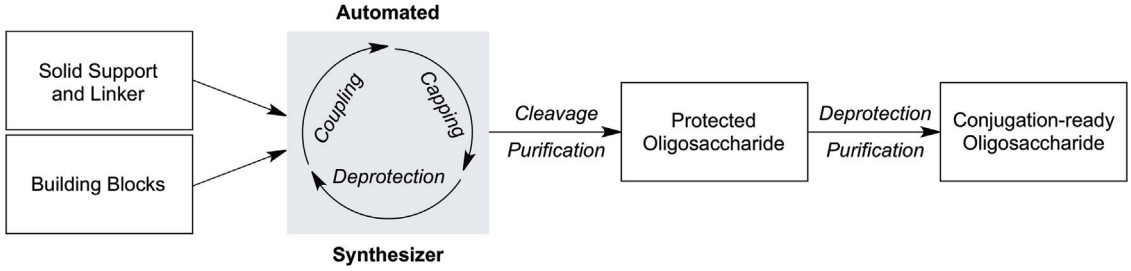


Fig. 1 The automated glycan assembly process. (Reprinted from Seeberger, 2015).

oligopeptides and oligonucleotides proved useful for applications in the medical chemistry and materials applications.

Establishing structure—function relationships in the glycosciences is virtually impossible without pure glycans. Access to pure glycans has been extremely difficult since no amplification methods exist while purification is always challenging and sometimes impossible. Molecular tools are required to advance the fundamental glycosciences and milligram quantities of such glycans have to be accessed by chemical synthesis.

Inspired by the concepts of solid-phase peptide and oligonucleotide synthesis, the automated glycan assembly process relies on a solid support equipped with a linker that is used to install one building block after another using an automated synthesizer (Fig. 1) (Plante et al., 2001; Seeberger 2015). Under the solid-phase synthesis paradigm excess building block is used for mass action to drive reactions to completion. Excess reagents can be readily removed by washing the resin with solvent. High coupling yields and stereoselectivity are important to be able to access pure oligosaccharides using only one purifucation step at the end of the synthesis.

The center-piece of automated glycan assembly is the instrument where the entire assembly process is performed under computer control. Since 2014, a commercial system, the Glyconeer 2.1™ is available and has been installed in several locations world-wide (Fig. 2) (www.glycouniverse.de).

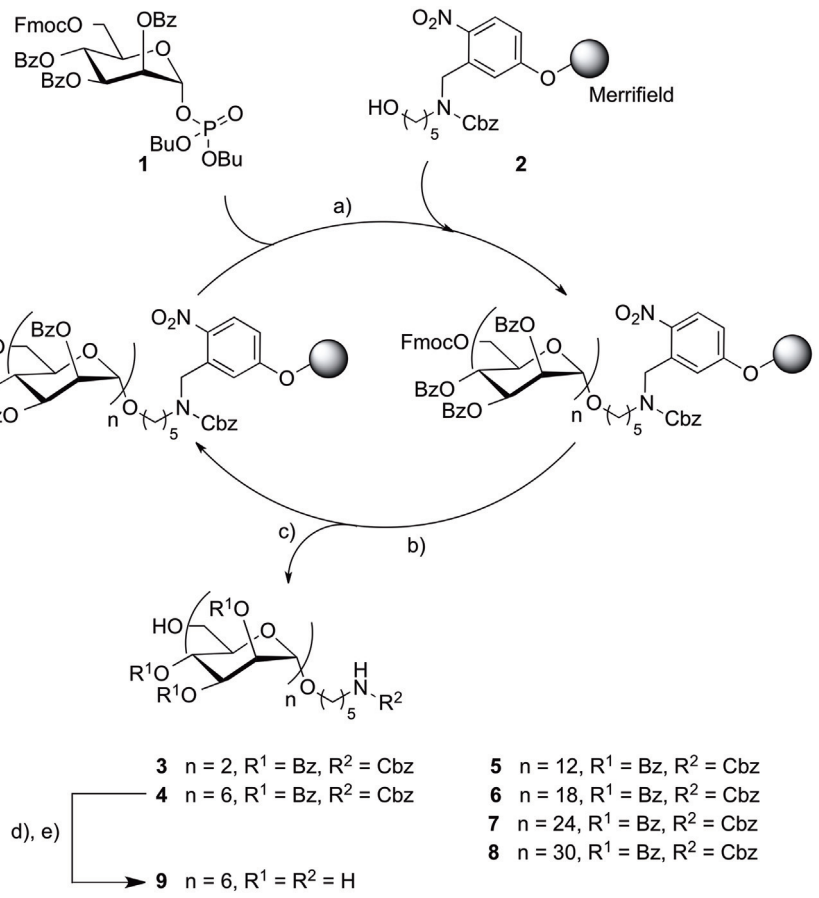
Controlled by a computer program that executes modules of commands the liquid handling in the Glyconeer 2.1™ is managed by valves through which the reagents and solvents flow driven by inert gas pressure. The building blocks are stored in a carousel whereas other reagents reside in reservoirs. The jacketed glass reaction vessel contains the polymeric resin on top of a glass frit. Reaction temperatures can be adjusted from −50 to 50 °C. The effluent following glycosylations can be collected to recycle unreacted building blocks. The reaction efficiency of glycosylations is monitored after removal of Fmoc protecting groups using a UV sensor that is also used in peptide synthesizers. One monosaccharide building block after another is added to the polymer-bound chain using coupling cycles that consist of glycosylation, capping and removal of the temporary protecting group steps.

Automated glycan assembly can provide access to long carbohydrate chains as demonstrated for a 30mer α-(1,6)-oligomannoside as a proof-of-principle (Calin et al., 2013). Mannosyl phosphate building block **1** carries permanent

Fig. 2 The Glyconeer 2.1™—the first dedicated automated oligosaccharide synthesizer.

benzoyl protecting groups that ensure the formation of trans-glycosidic linkages and can be readily removed with base. The temporary C6 fluorenylmethoxycarbonyl protection of the hydroxyl group is readily removed by piperidine. Merrifield resin equipped with photolabile *o*-nitrobenzyl alcohol linker served as solid support for the automated syntheses. α-(1,6)-Oligomannosides ranging in length from disaccharide **3** to 30mer **8** were prepared using this automated method (Scheme 1).

Glycosaminoglycans (GAGs) are structurally diverse macromolecules that are usually located in the extracellular matrix and are essential for many fundamental cellular processes. These acidic, negatively charged polysaccharides transduce extracellular signals to the interior of the cell. GAGs are highly variable in size, ranging from 20 to 200 disaccharide repeating units, backbone composition, and the degree and pattern of sulfation. Chondroitin sulfate contains *N*-acetyl-β-D-galactosamine and β-D-glucuronic acid and sulfation and acetylation of particular hydroxyl and amino groups vary. Two chondroitin sulfate hexasaccharides served as targets to illustrate that automated glycan assembly can be used to procure this class of molecules quickly. Two differentially-protected galactosamine (GalNAc) and



Scheme 1 Automated synthesis of α-(1,6)-polysaccharides. Reactions and conditions: (a) glycosylation: **1**, TMSOTf, DCM, −15 (45 min) −0 °C (15 min); (b) Fmoc deprotection: piperidine, DMF, 25 °C (5 min); (c) cleavage from solid support: hν, DCM; (d) NaOMe, MeOH; (e) Pd/C, H_2, H_2O. Bu = Butyl, Bz = benzoyl, Cbz = benzyloxycarbonyl, DCM = dichloromethane, DMF = dimethylformamide, Fmoc = fluorenylmethyloxycarbonyl, Me = methyl, TMSOTf = trifluoromethanesulfonic acid trimethylsilylester.
(Reprinted from Calin et al., 2013).

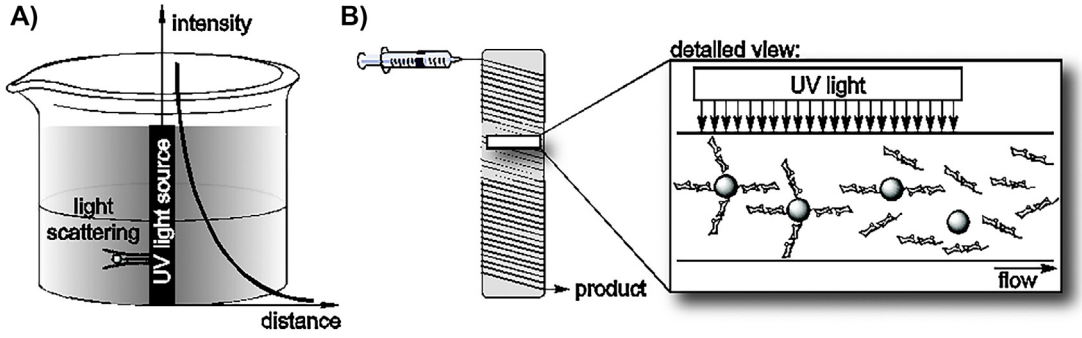
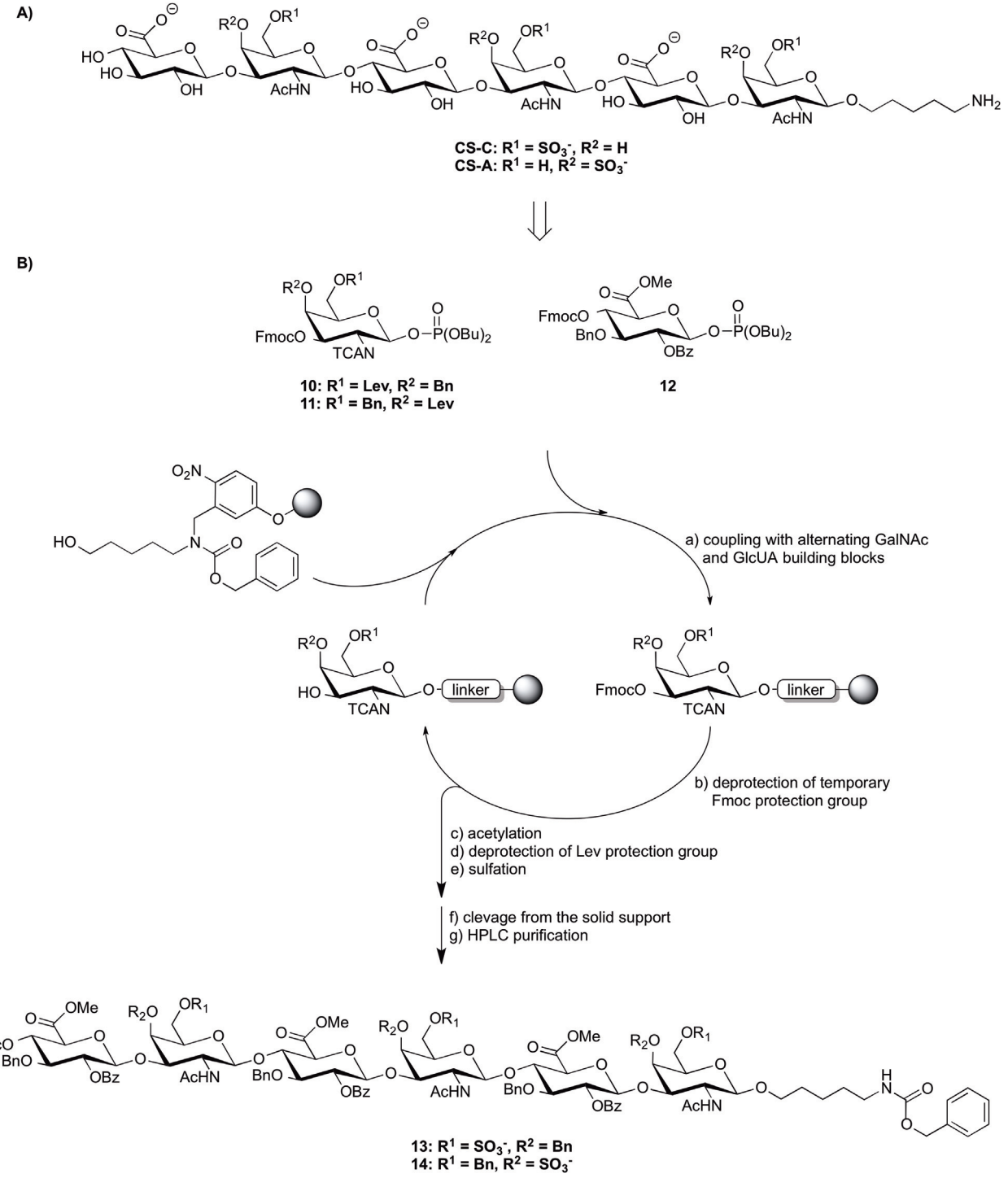


Fig. 3 Photocleavage of linkers following automated glycan assembly. (a) In suspensions in batch, light intensity decreases with increasing path length and due to light scattering. (b) Photocleavage in a continuous flow reactor ensures high light intensity throughout the reaction solution and efficient cleavage.

glucuronic acid (GlcA) building blocks are required for the synthesis.

Levulinoyl (Lev) esters and Fmoc groups are used as temporary protecting groups to mark the hydroxyl groups for sulfation and extension, respectively. A new orthogonal linker had to be designed that is stable to treatment with acid or base. The photolabile nitrobenzyl ether-based linker can be cleaved selectively with light. To overcome inefficient photocleavage due to decreasing light intensity with increasing distance from the light source due to absorption exacerbated by light scattering by resin beads used as solid support. For cleavage from the solid support a continuous flow reactor was employed where reactions occur in fluorinated ethylene propylene (FEP) tubing with a small



Scheme 2 (a) Retrosynthetic analysis of chondroitin oligosaccharide sequences with different sulfation patterns. (b) Automated synthesis of chondroitin hexasaccharides. Reactions and conditions: (a) 3 × 3 equiv. building block, TMSOTf, DCM, −15 °C (45 min) → 0 °C (15 min); (b) 3 × 20% piperidine in DMF, 25 °C (5 min); (c) 3 × Ac_2O, pyridine 25 °C (30 min); (d) 3 × $H_2NNH_2 \cdot H_2O$, pyridine, AcOH, DCM, 25 °C (60 min); (e) 3 × SO_3·pyridine, pyridine, DMF, 50 °C (3 h); (f) hν, DCM, 25 °C.

diameters to ensure efficient irradiation (Eller et al., 2013) (Fig. 3).

The, assembly of chondroitin oligosaccharides **13** and **14** was achieved (Scheme 2a). Chondroitin-6-sulfate hexasaccharide **13** was successfully assembled in 16 steps over three days, with a yield of 13% (88% average yield per step) while chondroitin-4-sulfate hexasaccharide **14** was obtained in 8% yield (86% average yield per step). Partially-protected chondroitin sulfate hexasaccharides **13** and **14** were fully characterised before the final deprotection a hydrogenolysis and saponification liberated pure oligosaccharides.

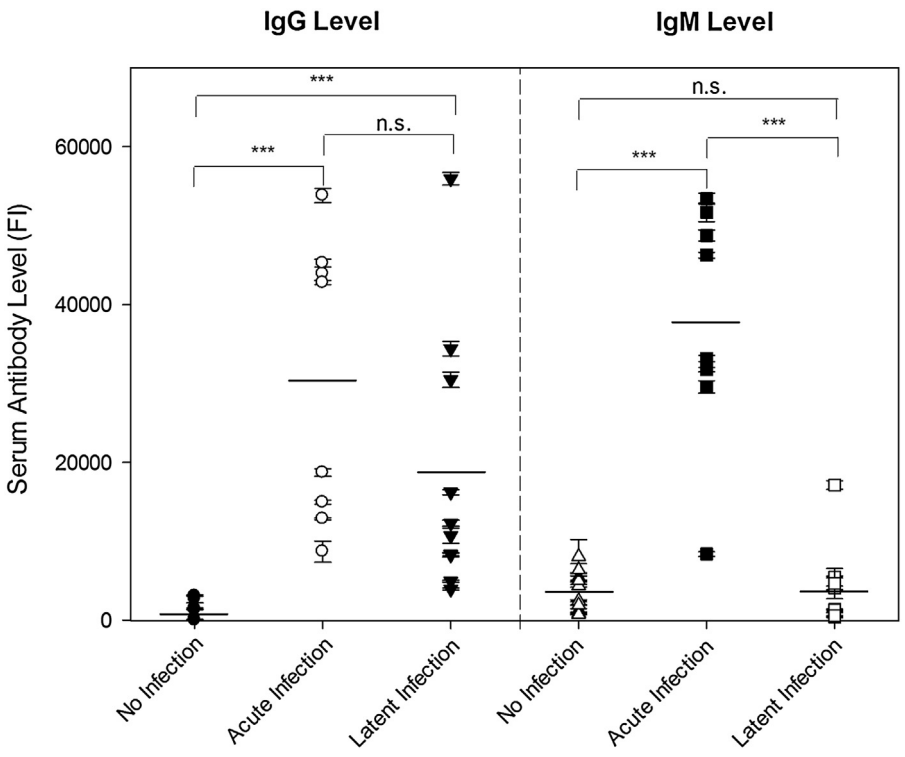


Fig. 4 Antibody levels determined by microarray analysis against a GPI antigen in sera samples from different toxoplasmosis cohorts including standard deviation for every data point (n.s. = not significant; *** = $p < 0.005$). Description of cohorts: no infection ($n = 10$); acute infection ($n = 8$); latent infection ($n = 10$). Black bars represent mean antibody levels. FI = fluorescence intensity. (Reprinted from Götze et al., 2014).

Characterisation and quality control of synthetic oligosaccharides

The inherent structural diversity of glycans due to branched structures and stereogenic centres at each glycosidic linkage renders the analysis of these molecules challenging when anomeric mixtures are obtained. Nuclear magnetic resonance spectroscopy requires milligrams of sample and reaches limits of detecting small amounts of unwanted stereoisomers. Recently, we demonstrated that ion mobility—mass spectrometry (IM—MS) can unambiguously identify glycan regio- and stereoisomers (Hofmann et al., 2015). Using six synthetic carbohydrate isomers that differ in composition, connectivity or configuration we illustrated that IM—MS that separates molecules according to their mass, charge, size, and shape, can detect coexisting glycan isomers at relative concentrations as low as 0.1% of the minor isomer. This fast analysis method requires no derivatisation and very small amounts of sample. We expect IM—MS to become the standard for glycan characterisation.

Glycan arrays as versatile tools for glycomics

Glycan arrays, a.k.a. carbohydrate microarrays, were first introduced in 2003 and have since revolutionised the study of glycan—protein interactions (Adams et al., 2003). Glycans are immobilised on a solid surface that is subsequently probed with the putative interaction partner. This method for high throughput analysis of protein—carbohydrate interactions soon became the standard method in the field. The analysis of biological samples to detect antibodies that bind to glycans has been another main use for glycan microarrays (Geissner and Seeberger, 2016). Following the initial work on malaria (Kamena et al., 2008), several other infectious diseases and allergies were studied.

Here, I use infections with the apicomplexan parasite *Toxoplasma gondii* that induces a variety of medical conditions to illustrate the potential of diagnostic glycan arrays (Götze et al., 2014). Diagnosis of acute toxoplasmosis infections in pregnant women is important since the drugs used to treat *T. gondii* infections are potentially harmful to the unborn child. Inexpensive, fast and reliable diagnostics are needed. Synthetic pathogen-specific glycosylphosphatidylinositol (GPI) glycan antigens were printed and covalently immobilised on glass slides. The resulting microarrays were incubated with reference sera of toxoplasmosis patients as well as seronegative individuals to detect anti-carbohydrate antibodies.

The glycan array screening experiments revealed that all sera from non-infected patients contained undetectable or low levels of IgG and IgM antibodies directed against the printed GPIs or their substructures. All sera from acute toxoplasmosis patients showed high levels of IgG and IgM antibodies recognizing the full GPI glycan. Samples of latently infected patients contained IgG antibodies that bind the GPI glycan while the IgM levels against all printed glycans were considerably reduced.

One GPI glycan was used as diagnostic marker for toxoplasmosis (Fig. 4). IgG as well as IgM serum antibody levels against this GPI are significantly increased during the acute phase of the infection. While the average concentration of IgG in the blood does not considerably decrease after the acute phase, IgM levels drop to values that are in most cases comparable to seronegative individuals.

The GPI antigen is a suitable biomarker for the diagnosis of different stages of toxoplasmosis. The IgG levels can be used to distinguish non-infected from *T. gondii* infected humans whereas the concentration of IgM antibodies binding the same carbohydrate may serve to differentiate latent and acute toxoplasmosis.

Glyconanomaterials as tools

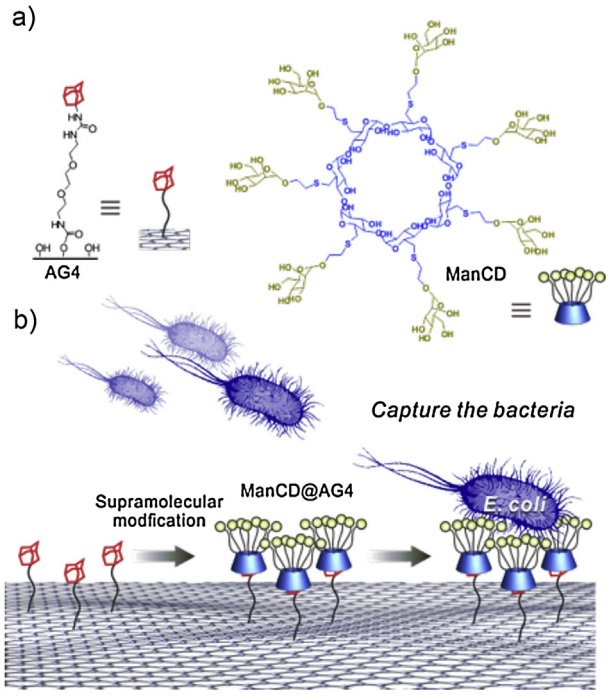
Multivalency plays a pivotal role for many biological processes including adhesion of bacteria or viruses to cell surfaces. The attachment of multiple weak binding ligands on the surface results in significantly stronger adhesion at the interface than those arising from simple monovalent interactions. Multivalent carbohydrate—protein interactions overcome the weak binding of carbohydrate ligands for cell surface recognition. Many synthetic multivalent glycoconjugates with diverse spatial arrangements of ligands have been prepared to interfere with the pathogen adhesion process and serve as antibacterial or antiviral agents (Delbianco et al., 2016).

Scaffolds, such as dendrimers, nanoparticles, calixarenes, and fullerenes, have been functionalised with carbohydrates. Glycans attached to peptides, carbon nanotubes, and self-assembled fibrous nanostructures have been used to investigate cell targeting (Delbianco et al., 2016).

Graphene, a micrometer-scaled material provides unprecedented large-area flexible films and possesses exceptional mechanical, electronic, thermal, and optical properties. Thermally reduced graphene oxide (TRGO), where some carbon atoms of the graphene lattice bear hydroxyl groups on the basal plane of the lattice, retains a similar structure but also has arrays of identical reactive groups that facilitate surface functionalisation.

We developed multivalent carbohydrate-functionalised two-dimensional scaffolds by placing cyclodextrin-based sugar ligands on adamantylated TRGO sheets (Scheme 3). By fixing adamantyl groups on the surface of AG4, the inclusion complex of β-cyclodextrin (β-CD) and adamantyl units reversibly connected the reduced graphene sheet and heptamannosylated β-CD (ManCD) in aqueous medium. The resulting supramolecular carbohydrate-functionalised TRGO derivative agglutinates *Escherichia coli*. Taking advantage of the responsive property of the supramolecular interaction, the captured bacteria were partially released upon addition of a competitive guest. Moreover, owing to their unusual infrared-absorption, these TRGO derivatives exhibit excellent bacteriostatic properties (99% elimination) following near-infrared (NIR) laser irradiation of the graphene-sugar-*E. coli* complexes (Fig. 5) (Qi et al., 2015).

The multivalent sugar-functionalised graphene sheets are better soluble in water and selectively agglutinate *E. coli*. The unique thermal IR-absorption properties of TRGO, enable bacterial killing upon IR-laser irradiation.



Scheme 3 (a) Adamantyl-functionalised graphene derivatives AG4 and heptamannosylated ß-cyclodextrin (ManCD). (b) Schematic representation of supramolecular carbohydrate-functionalised graphene complexes. Binding of bacteria to the complex resulted in a reversible multivalent inhibition of the bacteria.

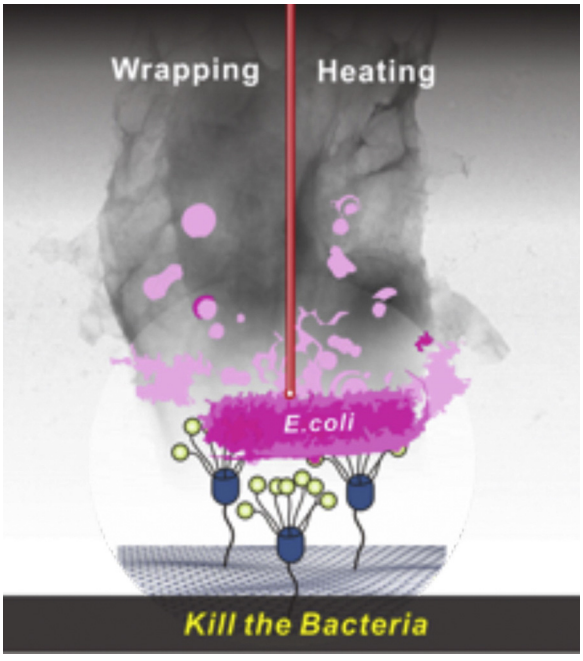


Fig. 5 ManCD@AG4 under NIR irradiation causes the death of captured *E. coli* bacteria. (Reprinted from Qi et al., 2015).

Currently, the multivalent, supramolecular assemblies on graphene are developed as functional materials for filtration in healthcare settings.

Conclusions

The automated glycan assembly process that the Seeberger laboratory has developed over the past 18 years was finally commercialised in 2014. With an increasing number of instruments placed around the world and all essential chemicals and building blocks commercially available, access to complex oligosaccharides has become more facile than ever before. The pure and defined glycans serve now for applications including the development glycan arrays as promising diagnostics and nanomolecular tools such as glycans on graphene sheets that can be used to detect and even destroy bacteria.

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