

The workhorse of biology is the protein. But a protein is more than a bunch of amino acids; a point that is often overlooked, is that almost always carbohydrates (or sugars) are attached to it. Hans Kamerling heads the ABC 'Expertise Center for Carbohydrate Analysis and Synthesis'. He knows all about the making and analysis of carbohydrates, an astonishingly complicated process that receives more and more attention since the increasing knowledge of protein structure which leads inevitably to the question of glycosylation. Kamerling expects proteomics, structural biology, and biotechnology to give carbohydrate research (glycomics) an enormous boost.

THE INDISPENSABLE GOLDEN HANDS IN CARBOHYDRATE RESEARCH

Although astonishingly complicated, glycomics offer many possibilities in fundamental and applied research



Prof. dr. Hans Kamerling

Sponges living on the floors of tropical waters are aggregates of hundreds of sponge cells. When separated into individual cells and in the presence of calcium ions, sponges reassemble. The cells recognize each other, knit together and form a sponge once again. Sponge cells even recognize their former mates. When sponges of different colours – yellow, blue, green – are separated in cells and all these cells are mixed up in a Ca^{2+} -containing medium, all sponges reassemble in their original colours. No colour chimeras arise from the colourful cell soup. The trick? Sugars. Or, to be more precise, carbohydrates: Ca^{2+} -mediated carbohydrate-carbohydrate self-recognition.

Prof. dr. Hans Kamerling is head of the ABC 'Expertise Center for Carbohydrate Analysis and Synthesis'. He tells the story about the sponges to illustrate the role of carbohydrates and to point at the fact that differences in two simple sugar molecules can be of extreme biological importance. A lesson bio-



From left to right: HPLC-profiling of glycoprotein glycans (Dr. Gerrit Gerwig); Carbohydrate synthesis (Dr. Koen Halkes); Biacore interaction studies (Adriana Carvalho de Souza).

medical research acknowledges more and more. "More drugs based on carbohydrates would be a good thing for carbohydrate chemistry. We could use the success and the wave of positive attention that would accompany it. Beside successfully produced recombinant therapeutic human glycoproteins with native-like glycosylation patterns, such as erythropoietin, other recent success stories are the development of a heparine-based synthetic pentasaccharide (Arixtra) as antithrombotic drug, and a synthetic sialic acid mimic (Relenza) as anti-influenza drug. And not to forget the very recent launch of the first synthetic carbohydrate-based vaccine, produced as a protein conjugate, against *Haemophilus influenzae* type b (Quimi-Hib)."

Kamerling makes this statement because he thinks carbohydrate chemistry, or more generally glycoscience, is undervalued. "Carbohydrate research is a lot more complicated than the hyped protein research. For analyzing carbohydrates a series of highly specialistic standard protocols to start with exist. However, you have to combine many different techniques and may have to adjust the chosen approach ad hoc as things turn out not to proceed as expected. This holds also for synthesis. An ask-and-delivery concept is nearly impossible in carbohydrate research."

1056 VARIATIONS

Why carbohydrates are so much more complex, can be easily understood from the perspective of connectivity. The complication of carbohydrate analysis is that two monosaccharides can be linked in many ways. Kamerling puts the implications of this difference into numbers by an example of a dimer consisting of two identical units. Two identical amino acids can only yield one dipeptide, whereas two identical monosaccharides can make up to eleven different disaccharides based on pyranose ringforms only. Three identical amino acids still can build only one tripeptide. Three identical monosaccharides however can make 176 different trisaccharides. To top off this argument think of a trimer consisting of three different units. With amino acids one can still only make six tripeptides but with three different monosaccharides in a trimer an impressive 1056 variations can be synthesized.

Kamerling: "Generally, there are no single machines that can resolve analytical or synthetic problems. A new trisaccharide may have such a complicated structure that several months are necessary to analyze it. Besides applying several methodologies, you need golden hands." He alludes to the fact that the analysis of carbohydrates combines high tech NMR spectroscopy and mass spectrometry with (more traditional) HPLC-techniques and – last but not least – good old fashioned 'wet chemistry'. "NMR isn't always necessary anymore", Kamerling explains, "Because, luckily enough, nature's ways of combining monosaccharides are somewhat restricted by the not unlimited abilities of enzymes. In other words, Nature isn't coupling monosaccharides in every theo-

retically possible way. This is certainly true in the field of glycoprotein glycans, where nowadays modern forms of mass spectrometry, combined with HPLC profiling techniques, play major roles. But in the bacterial and plant polysaccharide field you can not do without NMR."

SEARCH FOR DRUGS

With this array of techniques Kamerling and colleagues have been able to not only conduct their own research, but also assist quite some ABC researchers. For example, they learned a PhD-student from the veterinary virology group of de Groot/Rottier different assays to analyze (O-acetylated) sialic acids in de-O-acetylase experiments. "These PhD-students



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simply come around and join our laboratory. Research questions from the ABC are embedded in our own research since we have no technicians. We cannot transfer the work to them. We teach the visiting people what they need, but we never make predictions about how long they are to stay with us. That is simply too hard to tell. Besides working on analysis, my co-worker Gerwig assisted also in the isolation of sialic acids, and carried out synthetic work on sialic acid substrates”.

As another example Kamerling illustrates the importance of carbohydrate-protein interactions in infections by pathogenic bacteria, such as certain *E. coli* strains. As a prelude to infection, bacterial surface proteins called lectins attach to surface carbohydrates on susceptible host cells, e.g. in the intestine. Drugs containing similar carbohydrates could prevent the attachment by binding to the lectins. “However”, Kamerling admits, “this is just a concept. It is far from an actual medicine.” Quite recently, a project within the ABC has been formulated to evaluate the use of synthetic glyco-nanoparticles and glycopeptide libraries (combinatorial synthesis) decorated with different monosaccharides (e.g. mannose, galactose, sialic acid) for such inhibitions.

Mannose binding is highly relevant to atherosclerosis. UMC-researchers Van Dijk and Castro Cabezas hypothesized that people suffering from this disorder might make not enough of intact mannose-binding lectin (MBL). In cooperation with Kamerling’s co-worker Halkes, they set out to randomly synthesize hundreds of thousands of slightly different mannose-containing molecules (‘glycopeptide libraries’) and checked

whether mimics bind to MBL or related lectins or not. Hits will be resynthesized for further evaluation in the UMC-Utrecht complement-lipid-pathway concept.

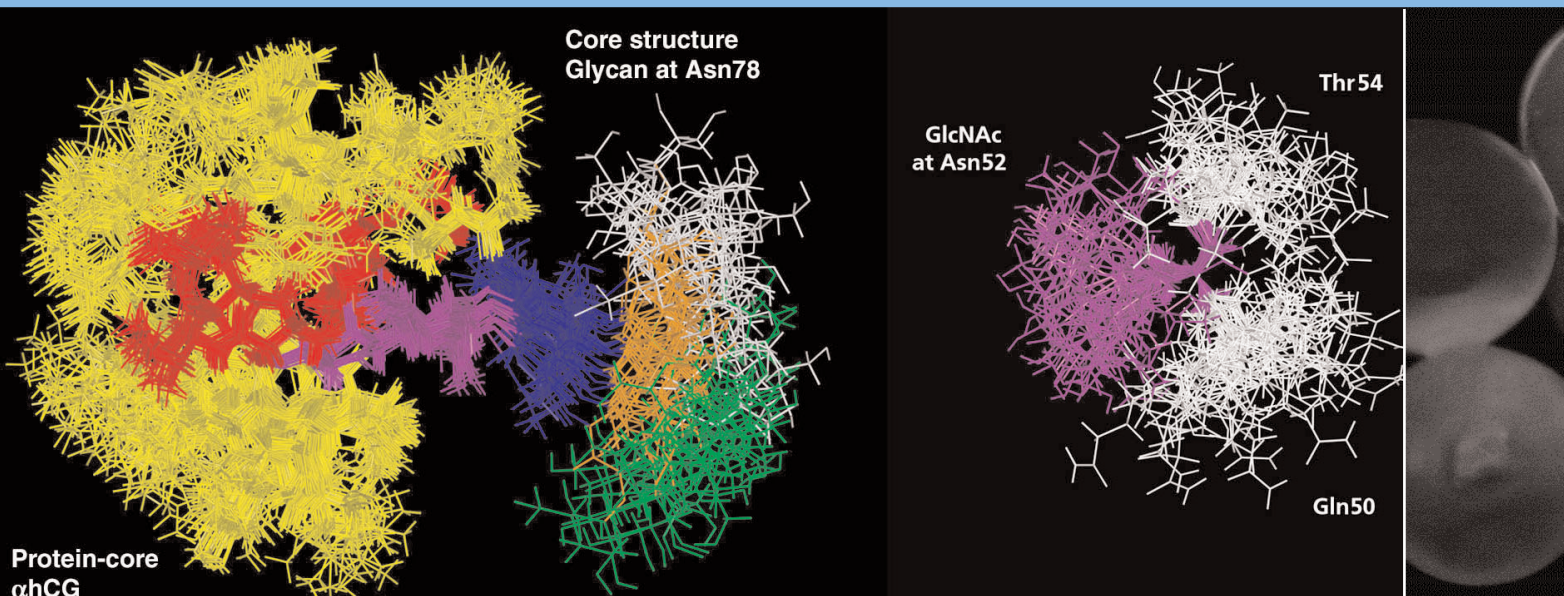
There’s more than just protein-protein recognition, Kamerling tries to tell. In biology interactions between cells and molecules or cells and cells often take place because of adhesive sugar chains. The problem with this type of interactions is that it is a lot weaker than protein-protein interaction and therefore harder to investigate.

Nonetheless Kamerling notices an increasing interest in the Biacore apparatus in his department. This machine analyzes the interaction strength of two molecules, be it sugars, proteins or whatever combination of interest, making use of an optical phenomenon arising in thin metal films under conditions of total internal reflection called surface plasmon resonance. One of the two molecules is attached via a linker system to a gold layer on a chip. The other molecule is, in a liquid current, fed over the chip. When the two molecules bind, the layer attached to the chip gains mass and therefore will have a different light refractive index. An ingenious optical system measures the changes and translates them in a so-called sensorgram.

VACCINES

An area in which carbohydrates traditionally play a pivotal role is vaccine research. Most bacteria wrap themselves in a carbohydrate coating. Vaccines are mostly based on a bacterium specific, harmless carbohydrate, the capsular polysaccharide. Injecting leads to an immune response and to immunological memory. When subsequently infected with a living bacterium, the body will quickly respond with a large scale defensive immune reaction.

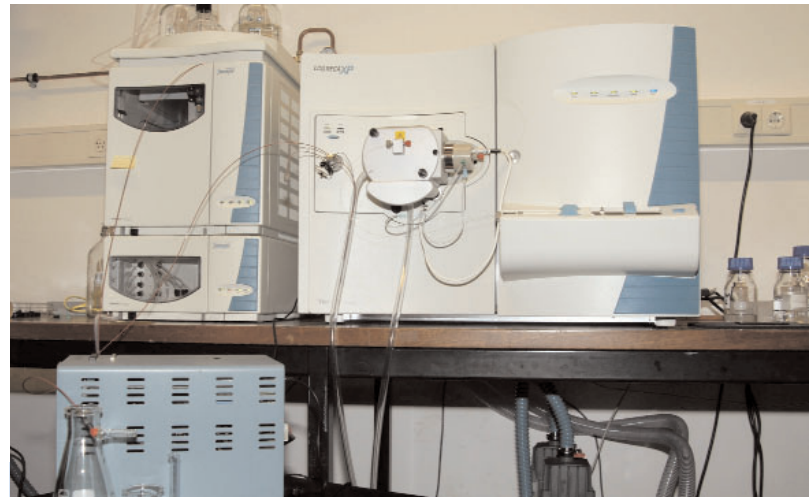
Left: representation of the dynamics of the carbohydrate chains attached to Asn78 and Asn52 of α -human chorionic gonadotropin. Right: Carbohydrate-recognizing proteins, screening for hits with a synthetic glycopeptide library.



It is already known for some time that polysaccharides do not evoke an adequate immune response in children and elderly people. Instead of the effective IgG antigen the less useful IgM was formed. Polysaccharides are T-cell independent antigens, but by coupling them to carrier proteins a switch to T-cell dependent responses can be realized. Kamerling: "One might call them the second generation of carbohydrate-based vaccines. However, coupling of polysaccharide and protein is a random process. A mixture of conjugates will be formed, differing from batch to batch. It is difficult to control the production process of such complex molecules and a clear product identification like for antibiotics or aspirine is impossible."

Already several years ago UMC-Utrecht researchers (group of Snippe) found that when fragments of the capsular polysaccharides are conjugated with carrier proteins, these neo-glycoconjugates were immunogenic and considered as potential vaccine candidates. Enter the carbohydrate know-how of Kamerling and colleagues, which came in handy once again. "So, we discovered that you don't need the entire polysaccharide. Coupling of a small oligosaccharide, one or more repeating units, to the protein seems to be sufficient. This made oligosaccharide synthesis attractive, because such fragments can be coupled very specifically to carrier proteins, yielding well-defined products. We have now a proof of principle for *Streptococcus pneumoniae*. But we are not the only scientists anymore working along these routes." The final hurdle remains: acceptance by the pharmaceutical industry. Synthesis of carbohydrate-based medicines is – compared by any standards – an expensive business. Kamerling recognizes this problem, but he is, however, unwaveringly optimistic.

In the same line of research Kamerling and colleagues also collaborate with the group of Hoepelman from the UMC-



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Utrecht in focusing on polysaccharides of *Cryptococcus neoformans*. "One of his co-workers uses our facility and our experience to do carbohydrate structural work on the mannoproteins, and is doing synthetic carbohydrate work."

Discussing, advising, supporting, facilitating, doing carbohydrate analysis and synthesis on several levels, that is what happens once contacts within the ABC Expertise Center have been initiated. "Recently we helped a veterinary PhD student of the group of Van Putten analysing a bacterial polysaccharide. She had transferred all genetical information necessary for making a capsular polysaccharide from one bacterium in a related species. She needed proof that the carbohydrate was indeed formed in the other bacterium, so she came to us. It appeared she had trouble isolating the polysaccharide, her batches were heavily contaminated for doing structural work. We discussed and controlled several isolation protocols and together we were able to prove that the hoped carbohydrate was formed indeed in the new bacterium."

In the field of commercializing recombinant therapeutic glycoproteins, highly detailed carbohydrate analysis is a must. "Over the years, in our own program we have paid much attention to the glycosylation machinery of CHO cells. To the benefit of several pharmaceutical companies. Now we focus on the glycosylation machinery of the mammary gland cells of transgenic animals, systems that make possible to isolate drugs from the animal's milk. In this context I want to mention that over 50% of the proteins are in fact glycoproteins, meaning that also in proteomics, glycoproteomics have to be considered very seriously. So far, I see no real Dutch activities in this area."

More information on: www.abc.uu.nl <<http://www.abc.uu.nl/>> Expertise Centres > ABC-facilities.

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