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# roland pieters

## old concepts and new methods detect galectins in complex biological samples

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Interfering with protein-carbohydrate interactions has great potential in medicine. Especially galectins are of interest because of their role as a biomarker for cancer. The design of a probe to detect galectins is a crucial step towards the diagnosis and prognosis of malignant tumors. The notoriously weak interaction between the galectin and the probe is a problem. Several timely concepts and some revisited old concepts were brought together to overcome this hurdle.

Carbohydrate recognition is of great importance in biological processes. Most cells are covered by carbohydrates that are well-positioned for their involvement in cell-cell recognition phenomena as e.g. in the immune system, leukocyte recruitment and inflammation, cancer and metastasis, bacterial adhesion and infection, blood coagulation and cell differentiation. The proteins that are involved in these biological processes are therefore important players. These could be proteins that bear the carbohydrate structures, for example the glycosylated proteins, or they could be proteins that recognise these structures, such as lectins. Among the lectins the galectins (see Figure 1) are a group with particular relevance as biological marker for, amongst others, cancer. In many laboratories worldwide research on the biological roles of the fourteen galectins is the subject of intense study. Their involvement in the immune system and in cancer, however, is clear.

The goal of this work is to create an easy method that allows the detection of the levels of the various galectins in a complex biological sample with great sensitivity and accuracy. Such a method would have applications e.g. in the diagnosis and prognosis of malignant tissue.

Molecular probes are being designed, synthesised and tested to achieve this goal. These probes are multifunctional molecules that are capable of specifically binding the galectins and covalently attaching themselves to them. Once labelled, the other end of the molecule features functionality that allows the visualisation of the labelled proteins. Our group has developed expertise in the interference with protein-carbohydrate interactions in general and the interference with galectins in particular. The ability to synthesise a variety of carbohydrate-containing candidate probes and test their efficacy in gels is crucial for this work. Mass spectrometry is also an indispensable tool to verify the covalent attachment of the probe to the desired proteins.

**Capture** The galectins all share a binding specificity for the carbohydrate galactose at the end of carbohydrate chains and are linked in a so-called  $\beta$ -fashion to the adjacent sugar. To take advantage of this recognition we set out to design a probe that contained this motif.

Capturing the protein by creating a covalent bond between probe and bound protein was the biggest challenge. The

What this research is about:

## Click chemistry appears to be a useful tool for galectin quantification in plants

Galectins are a special class of protein that can recognise carbohydrates in cells. As protein-carbohydrate interactions play a role in cell-cell recognitions, galectins are particularly important as biological markers for cancer. Detection of galectins in tissue therefore has potential in diagnosis and prognosis of cancer. Dr. Roland Pieters has designed a probe molecule that binds to galectins and facilitates detection of this protein in tissue.

For detection, a probe molecule should be covalently attached to the target. Galectins do not bind covalently to carbohydrates, therefore a trick was needed for the probe to attach to the galectin. Pieters explains: "We designed a probe molecule with an attached photo-affinity label which does attach itself covalently to the galectin. After the protein was captured the opposite end of the probe could be functionalised with a fluorescent label to visualise the galectins in a mixture with other proteins."

Pieters and his group developed the required chemistry to synthesise the probes using new techniques and modifying old methods. The new use of microwave irradiation, which highly accelerated important reactions, was crucial for the selective addition of the photo-affinity label. The attachment of the fluorescent label was possible because of the application of a recently discovered copper-catalysed cycloaddition reaction called 'click chemistry'. The work underscores the usefulness of organic synthesis in the field of proteomics since the type of probes described in this article will facilitate the study of important subsections of the proteome.

Research Theme NPC3: Protein post-translational modification

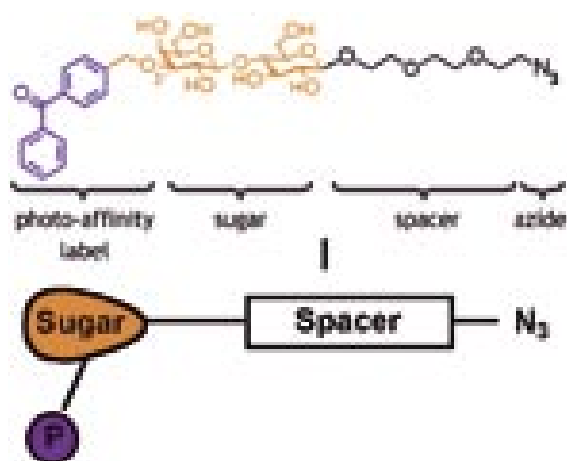


Figure 1 | Crystal structure of galectin-1

galectins only bind to their target sugar by non-covalent interactions and do not interact via a covalent mechanism like enzymes do. In order to capture enzymes, advantage can sometimes be taken of the enzymatic mechanism [1]. In these

cases the enzyme can perform the first addition step, but, due to the specific features of the modified substrate used, is subsequently incapable of moving forward to complete the catalytic cycle. The amount of captured protein is a measure of the amount of active enzyme, which is more relevant than determining the total amount of enzyme present which could be (partially) inactivated by natural inhibitors.

This strategy, however, could not be used for the galectins because they are not enzymes and merely bind their target sugar sequence. To overcome this we attached photo-affinity labels to the  $\beta$ -galactoside. The photo-affinity label can covalently attach itself to protein residues if it comes within a short distance of protein residues when the  $\beta$ -galactoside is bound in the binding groove and when it is irradiated with light of the proper wavelength. Our first attempt indicated that we placed the photo-affinity label too far away from these residues since the probe failed to capture the galectin, even though binding of the probe was shown to occur. Based on this information we studied the available crystal structures of the galectins to see where photo-affinity label placement would be more productive. The 3'-position of a lactose ligand seemed promising since the area available around this carbohydrate oxygen was

conserved amongst the galectin family.

The next challenge was the synthesis of such a molecule. The carbohydrate lactose has many hydroxyl groups and we only wanted to couple the photo-affinity label to one of them. Synthetic methodology involving tin-acetals was available, but reactions were lengthy and often low yielding. Experimenting with microwave irradiation of the reaction mixture proved useful and the reaction was both faster and yielded more of the desired probe (see Figure 2). The method proved to be general for the addition of all kinds of groups to the 3-OH of a galactose unit [2].

**Click chemistry** The effect of the new placement of the photo-affinity label was evaluated by mass spectrometry of a galectin that was incubated with the probe and irradiated with light. The mass spectrum showed that around 70-80% of the galectin had a probe molecule covalently attached to it. With this promising result we moved ahead and tried to develop the protocol that would selectively visualise the captured galectins in a protein gel (see Figure 3). In order to do this we reacted the other end of the probe molecule, which contained an azido group, with an alkyne-linked fluorescent dye, in a highly selective coupling reaction. This coupling reaction between an alkyne and azide was recently rediscovered [3] and greatly improved by copper catalysis and yields a triazole moiety. Most importantly, the transformation can be performed in the presence of all the functional groups present in biomolecules. This so-called 'click chemistry' reaction was ideally suited for our purposes, since now the fluorescent dye did not have to be introduced into the probe until after it had already captured the protein. This greatly expands the areas

of application of the probe since the bulky dyes often lead to interference with binding, have non-specific interactions or lead to reduced transport properties hampering *in vivo* work.

After some optimisation of the conditions for the 'click chemistry' a fluorescent image on a protein gel could be observed at the proper position when galectin-1 was used. When denatured galectin-1 was used, or competing galactosides were added, no such image was observed, indicating that binding of the probe to the protein was required.

Finally, a sample of four proteins was tested that contained galectin-1 and -3 and two non-galectins. After using the protocol involving probe incubation, irradiation and 'click chemistry' the fluorescent image on the gels showed only the two galectins, while the Coomassie stained gel showed all four proteins, indicating that indeed only the galectins were labelled.

**Scratching the surface** The identification of the biological and medical importance of the galectins is still only in its infancy. Despite the hundreds of publications on galectins their roles are so varied, even between intracellular processes and extracellular processes, that we are just scratching the surface. It is becoming increasingly clear that galectins play an important role in cancer and particularly in the metastasis of cancer. Improved and simpler methods to get quantitative insights into the presence of galectins in tissues are of growing importance. The work that is described above and that we also recently published [4] showed that it is possible to selectively label and visualise galectins in the presence of other proteins even though protein-carbohydrate interactions

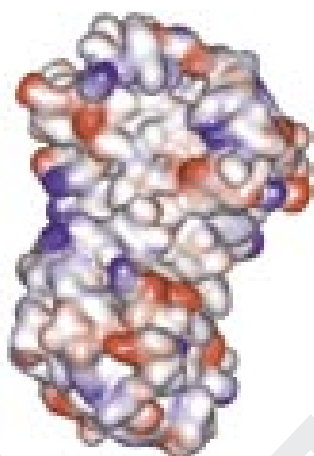
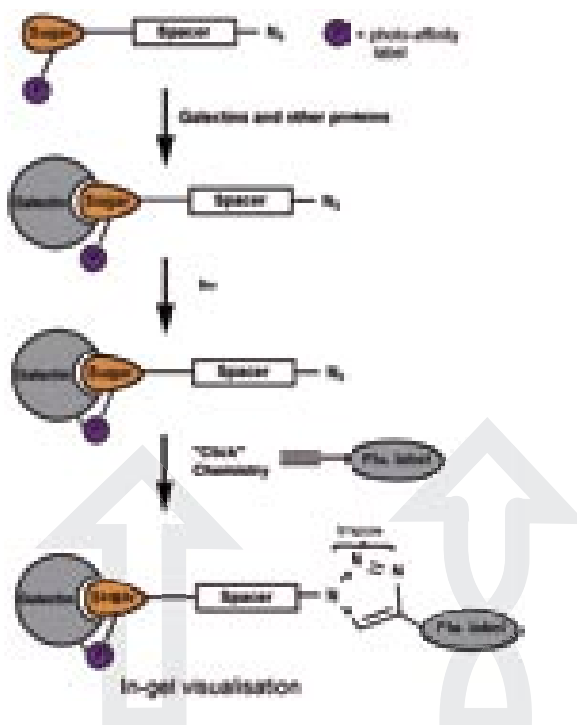


Figure 2 | Probe. Chemical structure and schematic representation of the galectin-selective probe.

Figure 3 | Incubation, irradiation and click chemistry. Protocol for selective visualisation of galectins in a mixture of proteins using the probe as represented in Figure 2.



are notoriously weak and also despite the fact that galectins are not enzymes, thus no chemical mechanism is available to take advantage of.

The present account shows that several timely concepts such as microwave-assisted chemistry and click chemistry, and also revisited old concepts such as photo-affinity labelling had to come together in order to achieve the desired results. Examining crystal structures was also an important factor.

Furthermore, we have shown that in the specific case of the galectins progress can be made in the design and synthesis of probe molecules that selectively capture members of this segment of the proteome. The work generates optimism that optimised reagents of this type may indeed find application for use in diagnosis and prognosis. In order to improve the first generation that is described here, we are currently taking advantage of multivalency, a common method in nature that enhances binding affinities. By linking multiple copies of the sugar ligands together, we have previously shown that enhanced binding to the galectins results [5]. The probes based on this are in development and have already shown promise with respect to sensitivity and selectivity.

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#### Roland Pieters, PhD

Department of Medicinal Chemistry  
Utrecht Institute for Pharmaceutical Sciences  
Utrecht University  
P.O. Box 80082,  
3508 TB Utrecht  
T +31 30 253 69 44  
r.j.pieters@pharm.uu.nl

#### Summary

Galectins are an important class of carbohydrate binding proteins that play roles in the immune system and most prominently in cancer. In our laboratories we are developing synthetic probe molecules as tools that enable the quantification of the galectins in complex protein mixtures such as tissue samples. These tools should be useful for the determination of the diagnosis and prognosis. Successful probes were prepared that contain the carbohydrate ligand with an attached photo-affinity label. The latter was able to covalently attach itself to

the protein. After the protein was captured the opposite end of the probe could be functionalised with a fluorescent label that allowed the selective visualisation of galectins in a mixture with other proteins. The chemistry required to synthesise the probes involved new use of microwave irradiation which highly accelerated important reactions. Furthermore, the attachment of the fluorescent label was possible because of the application of a recently discovered copper-catalysed cycloaddition reaction, now usually referred to as 'click chemistry'. The work underscores the usefulness of organic synthesis in the field of proteomics since the type of probe described in this article will facilitate the study of important subsections of the proteome.

# summary