Development of a Fluorescence-Based Quantification Method to Determine the Amount of Glycans Immobilized on a Surface

Andrew Acevedo
Biomedical Engineering, Washington University in St. Louis

NNIN REU Site: Nanotechnology Research Center, Georgia Institute of Technology, Atlanta, GA
NNIN REU Principal Investigator: Dr. Julia Babensee, Biomedical Engineering, Georgia Institute of Technology
NNIN REU Mentor: Nathan Hotaling, Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology
Contact: andrew.acevedo@wustl.edu, julia.babensee@bme.gatech.edu, nathan.hotaling@gatech.edu

Abstract:

The current best method for quantifying the amount of glycan immobilized on a surface involves connecting a large fluorescent linker to a carbohydrate and measuring the fluorescent intensity after surface modification to validate immobilization. However, it has been shown that the chemical moiety to which the carbohydrate is attached drastically alters the binding affinity of carbohydrate binding proteins. The purpose of this project is to address this issue. The approach was to attach a smaller, less intrusive azide linker to a glycan and then use this linker to attach the glycan to the surface. Microbead surfaces were chosen for modification because they allow for easy control of surface area and also because they allow carbohydrates to be presented on a scale that is either phagocytosable or non-phagocytosable to cells. The surface reaction is a substitution reaction in which a fluorescent moiety (dansyl chloride) is substituted for an azide-modified glycan. A dansyl-modified surface was first modified with Alexa Fluor 594, an azide-modified fluorophore. Green fluorescence, from the dansyl group, was shown to decrease, and red fluorescence, from the Alexa Fluor 594, increased over the course of the reaction. The final step used an azide-modified glycan and used the change in gross mean fluorescence intensity (GMFI) to quantify the amount of carbohydrate immobilized on the surface. Fluorescent microscopy and thermo x-ray photoelectron spectroscopy (XPS) were used to observe the surface modifications.

Methods:

Dansyl Modification. Amine functionalized silica microbeads, 1 μm and 45 μm diameter, were modified with dansyl chloride (DsCl) according to the procedure in [1]. The 45 μm beads were reacted at 5000:1 mol DsCl: mol amine and the 1 μm beads at 100:1 mol DsCl: mol amine to achieve optimal fluorescence.

Azide Fluorophore Substitution (See Figure 1A). We added 300 μM Alexa Fluor 594 in dimethylformamide (DMF) to 10^6 1 μm dansyl modified beads in a 384-well glass-coated polypropylene plate. The plate was heated to 70°C. Using a Tecan Infinite F500 microplate reader, GMFI was measured at excitation/emission wavelengths of 340/535 nm and 585/617 nm over the course of three hours. The beads were washed between readings.

Azide Glycan Substitution (See Figure 1B). We added 5 mM 1-azido-1-deoxy-β-D-lactopyranoside in DMF to 4.35 × 10^6 1 μm dansyl modified beads in a 384-well glass-coated polypropylene plate. The plate was heated to 70°C. Gross mean fluorescence intensity was measured at excitation/emission wavelength of 340/535 nm over a span of 26 hours. The beads were washed between readings.

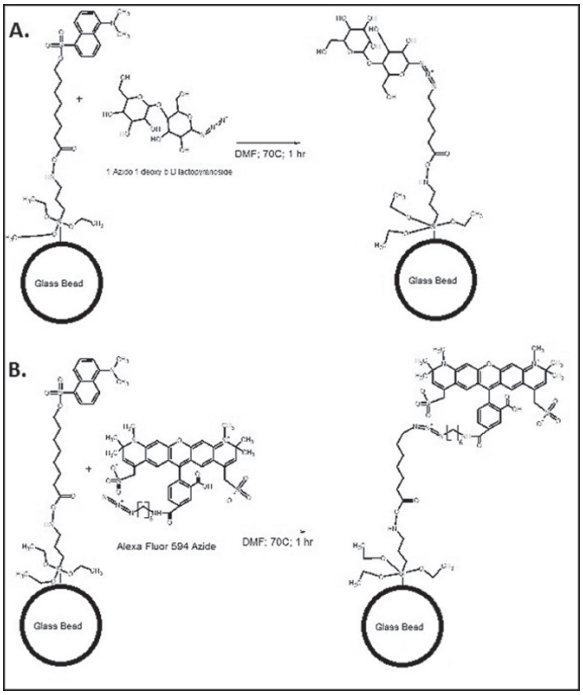

Figure 1: Schematic of the chemistry involved in (A) the azide fluorophore substitution reaction, and (B) the azide linked glycan substitution reaction.

Fluorescent Microscopy and XPS. Using a Nikon TI fluorescent microscope, 45 μm beads, before and after dansyl modification, were observed at excitation/emission wavelengths of 340/535 nm. XPS analysis of unmodified, dansyl modified, and Alexa Fluor 594 modified 1 μm beads was conducted using a Thermo K-alpha XPS.

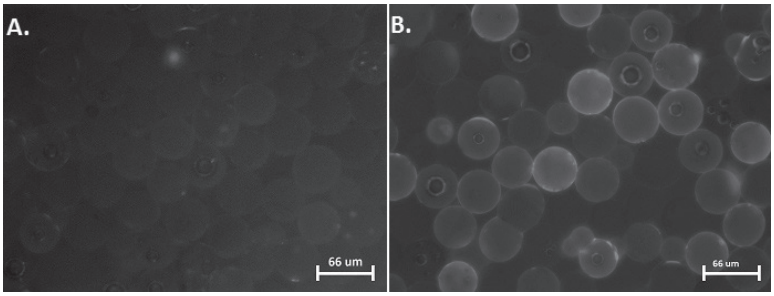

Figure 2: Fluorescent microscopy photos of (A) unmodified, and (B) dansyl modified 45 µm beads. 20x objective.

Results:

To determine the lower threshold of number of beads that could be detected by the microplate reader, dansyl modified beads were serially diluted in a 2:1 ratio. It was found that at least 2000 45 μm beads and 325,000 1 μm beads gave a fluorescent intensity signal above background. Fluorescent microscopy was used as validation that dansyl modification had taken place, as seen in Figure 2, A and B.

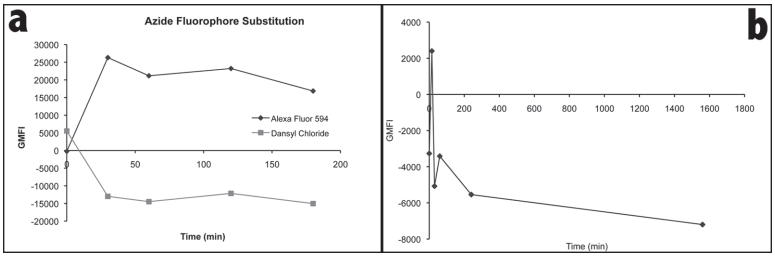

Figure 3: Representative trials of (A) azide fluorophore substitution, and (B) lactopyranoside substitution reactions. 1 µm beads.

The azide fluorophore substitution reaction was performed as verification that our azide linker was capable of substituting off the dansyl group on the beads. Red fluorescence, from the Alexa Fluor 594, and green fluorescence, from the dansyl chloride, were measured over three hours. A representative trial is displayed in Figure 3A. Red fluorescence is shown to reach a maximum and green fluorescence a minimum around 30 minutes after the reaction begins. Red fluorescence then begins to decrease slightly after that; this is suspected to be due to bleaching of the fluorophore after prolonged heat exposure. Figure 4 illustrates the XPS analysis of the nitrogen spectra of dansyl modified 1 μm beads and Alexa Fluor modified 1 μm beads. The Alexa Fluor 594 adds nitrogen with a higher binding energy as seen by the peak at 407eV (A), as opposed the nitrogen peak at 401eV in the spectra of the dansyl modified beads (B).

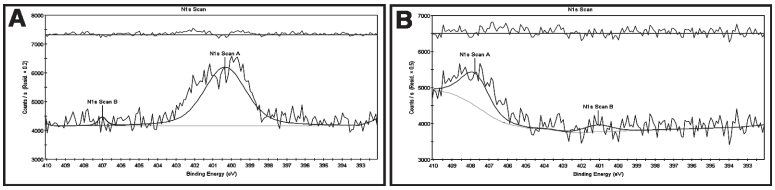

Figure 4: Nitrogen spectra of (A) dansyl modified, and (B) Alexa Fluor 594 modified 1 µm beads.

When the substitution reaction with the lactopyranoside was performed, a decrease in fluorescence greater than that of background was observed over the course of 26 hours. However, this reaction is still being optimized. This can be seen in a representative graph in Figure 3B.

Conclusion and Future Directions:

These experiments have shown that fluorescent modification of a surface is a viable alternative to current quantification methods of surface immobilization. Dansylation of a surface is reproducible and can be used to monitor reaction progress. Azide-linked glycan substitution was shown to occur; however, that reaction is still being optimized.

The next step in this project would be to focus on the dansyl modification of other surfaces. Surfaces of interest include 384-well and 1536-well glass-bottom microplates and 45 μm silica beads. These surfaces would then be used in high throughput quantification assays. The ultimate goal of this project is to use these modified surfaces to present glycans to dendritic cells to invoke controlled immune responses.

Acknowledgements:

I would like to thank the National Science Foundation and the National Nanotechnology Infrastructure Network for supporting this Research Experience for Undergraduates program. I would also like to thank my mentor, Nathan Hotaling, and my Principal Investigator, Dr. Julia Babensee, for hosting me.

References:

[1] "A real time monitoring using fluorescent dansyl group as a solid phase leaving group": Suenaga, T; Schutz, C; Nataka, T; Tetrahedran Letters, 2003, 44, 5799.