U.V.S

Uppsala Vancomycin Sensors

Team Results Document

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1. Summary

We are a team of students originating from different countries, specialized in different fields and have one goal; Design a biosensor that makes lives of patients easier. We have developed an inexpensive, rapid, and simple assay for measuring the concentration of vancomycin in blood. We have designed a microfluidic device in the form of a T-sensor, where our Diffusion Immunoassay (DIA) takes place. DIA is classified as a competitive interaction and works by exploiting the difference in diffusion coefficient between antibodies and vancomycin (labelled and unlabeled) to achieve quantification. We have developed a custom 3D-printed platform which holds dedicated sample delivery (micropumps) and introduction (reservoirs) systems. We were successful in manually labelling vancomycin with fluorescein, thus allowing it to be visualized using our inexpensive optical system. Nevertheless, while we have designed our own optical system to work with the assay, we aim to market the device as an "add-on" to commercially-available microscopes in hospitals. We envision that this strategy will keep costs down and performance up, while also expanding the use of these microscopes to include quantitative analysis of biomolecules. Currently, our results are promising and show potential for the success of the technology after more research and optimization.

1.1 Device Overview

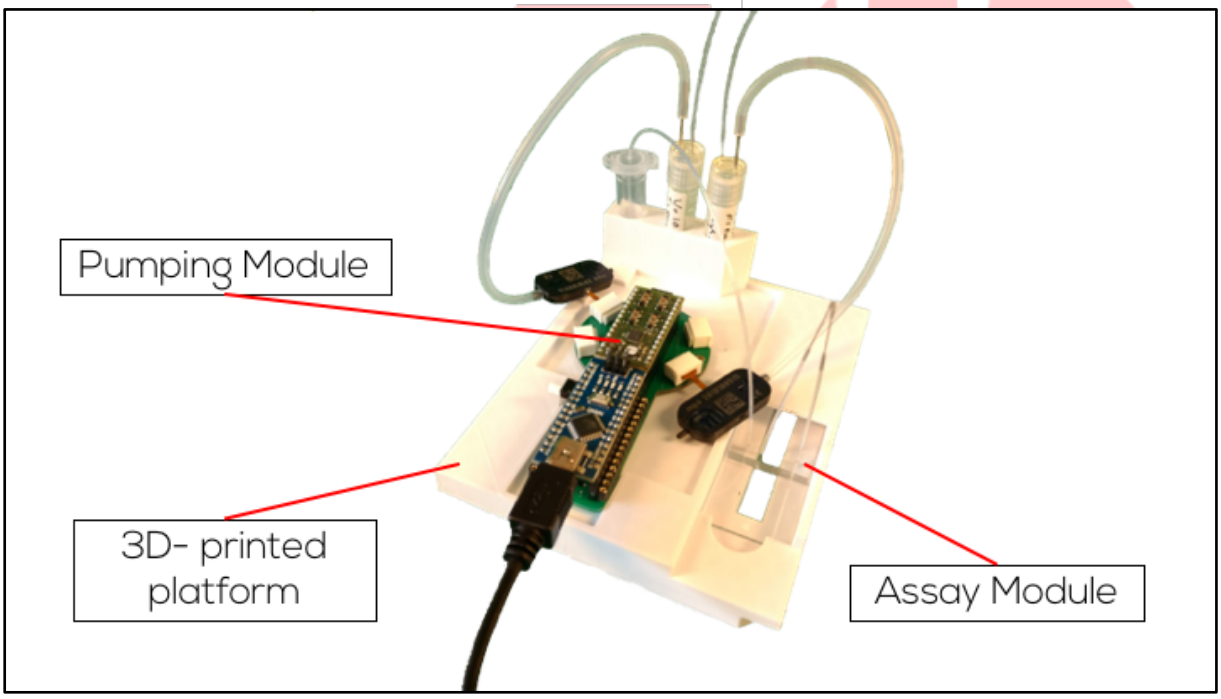

Fig 1. Overview of the device illustrating the main components

2. Biosensor System and Assay

The Biosensor system we have developed is based on the Diffusion Immunoassay (DIA) concept, where a difference in diffusion coefficient between the antibody-bound and unbound vancomycin is exploited to achieve quantification of vancomycin (Fig. 2). In addition, this assay is classified as competitive, where the labelled and unlabeled vancomycin will have to compete for binding sites to the antibody.

In essence, two streams (one containing an antibody to vancomycin and the other containing a mix of the sample and labelled vancomycin) enter a microfluidic feature known as the T-sensor. Due to the low Reynold number conditions, the two streams do not instantaneously mix, but instead flow side-by-side through the channel. Nevertheless, diffusion still does occur between the two streams in both directions. The rate of this diffusion is highly dependent on the characteristics of molecules in the streams.

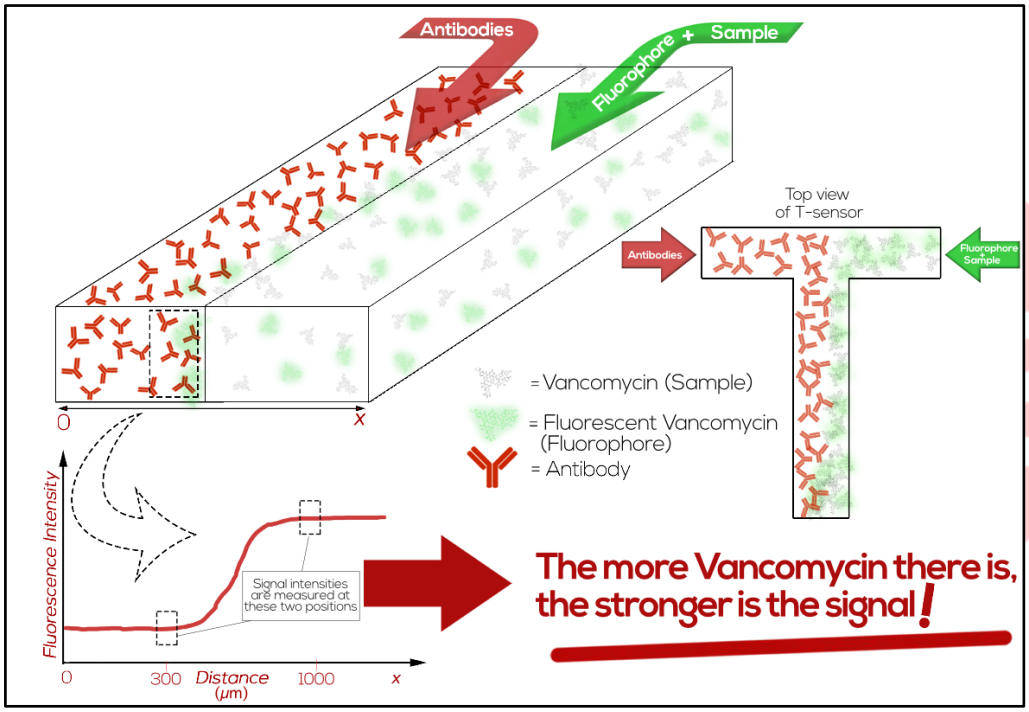

Fig 2. Outline of the Diffusion Immunoassay system. Note the movement of the labelled vancomycin into the opposing stream in a greater extent as compared to the antibody.

In this particular case, there will be two major diffusion profiles: one of the antibodies diffusing into the sample stream and the antigen (labeled and unlabeled vancomycin) diffusing into the antibody stream. As the antibody has a significantly lower diffusion coefficient ($4.4 \times 10^{-7} cm^2 s^{-1}$, Saltzman et al., 1994) compared to vancomycin ($2.8 \times 10^{-6} cm^2 s^{-1}$, Stewart, 1996), vancomycin is able to diffuse relatively deeper into the antibody stream as opposed to the antibody diffusing into the sample stream. This causes a build-up of the antibody-antigen complex towards the center of the channel, which is measurable as a bump in the fluorescence intensity. It is important to note that the diffusion coefficients of labeled and unlabeled vancomycin have been assumed to be the same. Finally, as long as there is at least an order of magnitude in difference between the diffusion coefficients of the antigen and its respective antibody, DIA is possible. This allows our

technique to be effortlessly adapted to other antibiotics, biomarkers and various molecules of interest, thus greatly increasing its scope of use.

In regards to the **biosensor system** as a whole, it has been specifically designed with modularity in mind in order to ensure maximum flexibility and convenience for the end-user. In essence, the biosensor system has been split into three main components which fit together in a 3D-printed platform: The assay component, the pumping component and the detection component. The assay component consists of the aforementioned re-usable DIA chip, as well as custom-built reservoirs which allow rapid loading of sample into the system. The assay component also comes with standardized aliquots of Reagent A (I.e. 500 nM of custom-made fluorescent vancomycin) and Reagent B (I.e. 2 µM of vancomycin antibody). It is important to keep in mind that Reagent A & B can be adapted to other molecules and can still be used with the original DIA chip. In other words, kits of different reagent A & B can be made available to suit the varying demands and needs of consumers.

Moving on, the **pumping component** consists of two mp6 pressure-driven micropumps (Bartels Mikrotechnik, Dortmund, Germany) which pump both reagent A and B into the DIA chip at combined flow rate of 8 µl/min (4 µl/min at each inlet). These pumps are quite small in size (l = 30 mm, w = 15 mm, h = 4 mm) and work via pumping air into a pressurized reservoir containing the reagents using a peristaltic piezoelectric effect. While these pumps were designed for high flow rate function (~ml/min), it was possible to run them at low flow rates (~ 4 µl/min) via adjusting the resistance of the tubing. The use of this pump with our device has been extensively tested and it produces a high level of reproducibility which satisfactorily meets our set standards. Nevertheless, the customer is able to swap out these pumps with a commercially-available syringe pump if they already have one available, as our tests have shown that that would work as well.

Finally, the **detection system** would consist of a strong illumination source able to satisfactorily illuminate our dye, a complementary filter and camera system to capture the resultant image. The development of this component was quite arduous and we have only partly succeeded in developing our own cost-effective optical system which lacks the resolution and power needed to fully capture the diffusion of the labelled antigen at the needed concentration. Theoretically, it might be possible to use our custom-made system using higher concentrations of reagent A & B, but this avenue was eventually abandoned as it would substantially drive up the cost per test of our system. As such, we have devised an alternative strategy whereby the device is designed as an add-on to complement and fit commercially-available fluorescent microscopes. This is made possible due to the small form-factor of the platform, which resembles well plates in shape and size (l = 13 cm, w = 9 cm). Furthermore, as these microscopes are generally available in healthcare facilities and laboratories, our device can be used to expand their functionality at a relatively cost. Using our device as an add-on in this manner also helps to keep reproducibility high and the cost per test down. Nevertheless, during the competition, we would be using our own “homemade” fluorescence microscope system. This would consist of a high-power 470 nm LED (LUXEON Rebel, Schiphol, Netherlands), a 490 nm excitation filter (Omega Optical, Vermont, USA), a 535 nm excitation filter (Omega Optical, Vermont, USA) and a Dino-lite digital USB microscope (AnMo electronics, Taipei, Taiwan). This configuration would require higher concentrations of reagent A & B, but should still be satisfactorily operational.

3. Analytical Performance

To perform a routine measurement using our competition-configured system, the sample has to first be mixed with reagent A at a ratio of Sample:Reagent A = 1:5 (V:V) in its respective reservoir. Theoretically, it is possible to use sample volumes down to 2 µl, however it is recommended to use at least 10 µl of sample to avoid pumping failure due to low operating volumes. The other reservoir is filled with reagent B (should at least equal the volume of liquid in the reagent A reservoir). The pump is then switched on and allowed to run according to its pre-uploaded configuration for 4 mins (to allow the sample to reach the chip). Once 4 mins have passed, the high-power LEDs are switched ON and an image is taken using the digital microscope. Due to the positioning of the chip and microscope in the 3D-printed platform, the image is always taken in the same spot of the chip, thus ensuring reproducibility in the measurements. Using a python-based script, the intensity line profile of the image is automatically extracted, normalized and subsequently plotted as a graph of fluorescence intensity against position along the width of the channel (fig 3A).

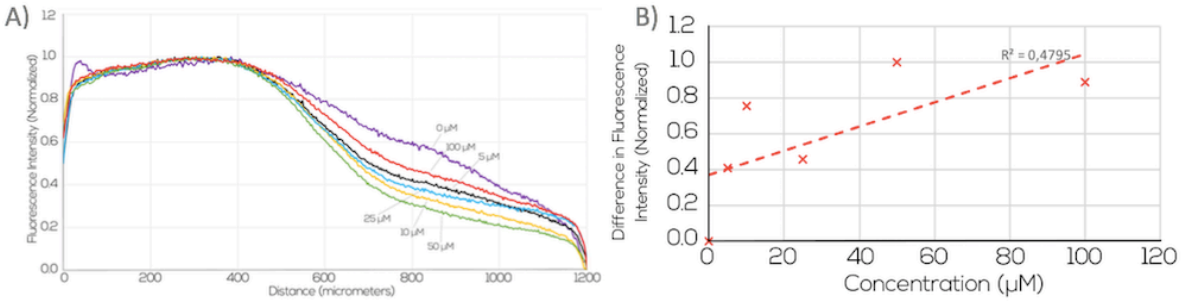

Fig 3. A) Normalized Fluorescence intensity measurements plotted against position along the width of the channel. Note the different profiles generated by each sample vancomycin concentration tested. B) Calibration curve of normalized difference in fluorescence intensity plotted against vancomycin sample concentration. Note the positive correlation and spread of the points sampled.

In order to construct a calibration curve for our device, two fluorescence intensity values are taken at specific points along the graph: One at 300 µm and the other at 1000 µm. The difference between these values is calculated and then plotted against the sample concentration (fig 3B). Through this form of analysis, a positive correlation was obtained with an R^2=0.48 (fig 3B), thus indicating promising potential for this technique as a possible alternative for the quantification of vancomycin.

4. Novelty and Creativity

4.1 Already Available

The Diffusion Immunoassay (DIA) was pioneered by Hatch et al in 2001, with a few follow-ups from other groups which describe **theoretical** improvements to original concept, such as the incorporation of magnetophoresis (Forbes et al., 2013) and optimization of the data analysis stage (Jha and Bahga, 2016). To the best of our knowledge, we have not found any traces of the technology being directly implemented in any existing analytical devices. As such, the novelty in our work is the miniaturization of the DIA concept and the reduction of the overall costs to make it more accessible and easier to use in the point-of-care setting.

4.2 Novel Developments

Our DIA chip employs a similar, yet more compact, microfluidic design to the original. It is constructed using PDMS (Polydimethylsiloxane) instead of glass, which helps to keep production costs low. As a DIA for vancomycin was never previously performed, we were able to determine the most effective antibody and labelled vancomycin concentrations to use through our work. This was mostly done through a combination of trial-and-error and using Hatch et al's (2001) paper as a guide.

Moving on, it was possible to run the mp6 pumping system in a stable and reproducible manner at the required low flow-rates specifically by using our custom-designed reservoirs, which are able to act as both flow restrictors and rapid sample introduction systems. Their usage helps to simplify the assay protocol and greatly reduces the total time needed for the assay to run. In addition, as they are cheap (50 cents per reservoir) and easy to manufacture, they don’t add much to the overall cost of the device.

In regards to the reagents, the newly synthesized and purified labelled vancomycin we produced could be considered novel as it is more powerful (produced a stronger signal at low concentrations) and responsive to the vancomycin antibody than the commercially-available alternative (Thermofischer BODIPY Vancomycin). This dye was produced via direct conjugation to Fluorescein Isothiocyanate, followed by purification via preparative high-performance liquid chromatography (HPLC).

Finally, the commercialization of the DIA technique as a modular platform with convenient micropump modules and flexibility for usage with different commercially-available microscopes is quite novel. We would even go on to claim that the development of a quantitative assay using microscopes and expands their usage to include quantitative analysis of biomolecules is novel in its own right.

5. Translation Potential

5.1 Stakeholder Desirability

5.1.1 Need in the market:

The first step for entering any market with any device is usually to test this market. This is done by talking to the end users who are going to benefit from our new solution, as well as the decision makers who are going to decide to buy our solution or not. To do so, we have interviewed medical doctors from Sweden (as it is the origin of our market) and USA (as it is one of the biggest markets for medical technology). The feedback we have obtained from the medical doctors can be summarized into two main points:

- Satisfaction with the current centralized solution.
- A much faster solution (less than 15 min) would be convincing for them to invest in as they won't need to wait for 4-5 hours for the lab results.

We additionally shared our idea and solution with the public, mainly consisting of families and potential patients as well as engineers working in the medical field, during our participation in Scifest Uppsala. Their feedback was encouraging and they were interested in how fast and cheap our solution is in comparison with other conventional methods. Finally, we contacted medical sector employees in Europe and North America and inquired regarding the cost of a vancomycin trough in hospitals. The estimates we obtained were in the range of 70 USD in USA, and in the range of 25 EUR in Europe (Jeffres, 2017). Moreover, they stated that patients who are taking vancomycin require longer hospital stays because of the time needed for the measurements to be ready.

5.1.2 Pain and suffering:

Long hospital stays are always an annoyance for patients. Some patients also get anxious of the hospital environment and specifically ask for minimal residency periods. In addition, the procedure for blood sampling can further exacerbate this anxiety, especially when large amounts are taken. As for the hospital itself, the bulkiness and immobility of medical equipment can potentially result in major challenges. As such, smaller and portable equipment able to deliver the same quality of results is always preferred by hospital staff.

5.1.3 Relievers:

We believe that our solution will help patients who are engaged in vancomycin treatment courses shorten their visits to the hospitals dramatically in comparison to current times, as it only takes 5 minutes to perform the measurement with our technology. In addition, as our solution needs low volumes of blood (10 µl) to be functional, it would make the process of taking a blood sample minimally invasive and therefore decrease the potential pain felt during the procedure. Moreover, our solution is smaller in both weight and size when compared with other similar devices. We believe that these advantages will give our device a strong push into the market.

5.2 Technical Feasibility

5.2.1 Our concept:

We believe that the concept we are making use of is highly feasible and that it can be easily deployed to an industrial product both in terms of cost and ease of manufacture. The main challenge at the moment is designing an effective optical system that can replace the microscope currently in-use with our assay.

5.2.2 Cartridge and main parts:

The DIA chips, reservoirs and holder platform are all technically feasible and cheap to mass-produce. Currently, it is possible to produce tens of DIA chips per day in approximately 2-hour batches. The reservoirs are also simple to fabricate and basically involve manual modification of cryofreeze and Eppendorf tubes to make them air-tight and allow the flow of substances out in a steady manner. Furthermore, the holder platform is 3D-printed directly in a single step. The pumping apparatus would be purchased directly on-demand in large quantities from Bartels Mikroteknik.

5.2.3 Our software:

Currently, we are working on a machine learning algorithm (artificial intelligence) which will help increase the accuracy of the measurements taken. We also plan to design an intuitive user interface, to allow easy usage by hospital staff.

5.2.4 Modularity and flexible design:

It is challenging to ensure maximum compatibility with all commercially-available fluorescent microscopes due to differences in their stage constructions and methods of operation. However, it might be possible to attain a favorable degree of compatibility by basing the design of the holder platform on standardized well plates. This is because microscope stages are generally designed with in-built compatibility to well plates and this is a property that we can exploit in our design. Due to the single-step 3D-printing process for the holder platform, different designs could be made to further accommodate small geometrical differences between different microscope manufacturers. It is potentially feasible that the DIA concept can be implemented in a fully portable biosensor but this will require us to design our own optical system or to cooperate with another company specialized in this area of technology.

5.2.5 Future work:

We believe that we will need one further year to finalize our research and test our concept. Next, we will need one year to design the final prototype and software and we expect to work on them in parallel. After that, we will need to get the CE approval for sale in the EU territories. Eventually, we will apply for FDA approval for sale in USA depending on the sales of the device in the EU.

5.3 Business Viability

5.3.1 Costs:

The manufacturing cost will be mostly the price of the pump (200 EUR), but we believe this can be markedly reduced through bulk-order. In regards to the optical system, this will depend on if we are going to buy the technology from other companies or develop our own solution. But for our device, which targets the biochemistry labs in hospitals, we will not be selling a microscope with it since they should have at least one in their lab. For the re-usable microfluidic chip, the cost is roughly 40 cents.

5.3.2 Selling price, approach and revenue stream:

Our vision for marketing our solution is the following:

- Pro package: a highly modular apparatus which can be customized to be attached to the existing microscopic equipment in the lab. This will provide faster measurements and will demand lower volumes of blood. The selling price for the device will be 1000 EUR and for a 100-time use cartridge (Reagent A&B aliquots), the selling price will be 80 EUR.
- Stand-alone package: a fully portable biosensor containing an in-built optical system, thus no longer necessitating the use of any external device. The selling price for the biosensor cannot be estimated before finishing the final prototype.

6. Team and Support

6.1 Contributions of Team Members

Team: **U.V.S, Uppsala Vancomycin Sensors**	
Abdul Raouf Atif **Microfluidics** sub-group - Design and manufacture of chips - Design and manufacture of reservoir system - Evaluation and testing of Diffusion immunoassay	Tobias Mages Engineering sub-group - Main programmer - Development of the portable optical system (i,e mini fluorescent microscope).
Robin Söderholm Engineering sub-group - Evaluated and investigated the mathematical diffusion immunoassay model quoted in the original paper	Adnan Al Baba Engineering sub-group - Worked on the entrepreneurial and economical aspects of the project - Development of a cheaper and smaller syringe pump - Development of flexible and modular external housing for the mini-fluorescent microscope
Ken Andersen Brasch Engineering sub-group - Worked on the entrepreneurial and economical aspects of the project - Development of a cheaper and smaller syringe pump	Adena Pepich Biology sub-group - Evaluation and characterization of reagents (i,e Antibodies & labelled vancomycin) using standard techniques (ELISA) - Production of custom-made labelled vancomycin - Production of magnetic dynabead-labelled antibody back-up
Drew Lindsay Biology sub-group - Evaluation and characterization of reagents (i,e Antibodies & labelled vancomycin) using standard techniques (ELISA) - Production of magnetic dynabead-labelled antibody back-up	

6.2 People who have given support

Gemma Mestres **Associate Professor.** Coach of the team	Masood Kamali-Moghaddam **Associate Professor. Supervisor of the team**
Tiscar Graells **PhD Student**. Principal feedback source for biological-related (e.g antibody characterization) matters	Javier Cruz **PhD Student**. Principal feedback source in regards to microfluidics and microfabrication
Frederico Cantoni **PhD Student**. Feedback source in regards to microfluidics, as well as assistance with microfabrication	Ulf Langstrom **Associate Professor.** Greatly aided us with production and purification of labelled vancomycin.
Hanzhao Zhang **Sensus 2017 contestant**	Anna Blasi **Sensus 2017 contestant**
Alireza Azimi **PhD**. Instrumental support on ELISA and assay queries.	Felipe **PhD**. Essential guidance for biology and ELISA/assay queries.

6.3 Sponsors

Merck *Free provision of a selection of the needed reagents*	Uppsala University *Provision of a working space and environment*
Thermofischer *Free provision of a selection of the needed reagents*	EIT Health *Access to promote ourselves at local events, such as CHASE 2018*

7. Final Remarks

We have learned a lot from this year long experience in biosensor development, not just in the basic process of how to build a biosensor, but also in a wide-array of other fields. We have assembled knowledge from preparative and analytical chemistry in conjugating our own desired signaling molecule to a dye, used liquid chromatography and HPLC for isolation of our desired molecule, and run mass spectrometry to finally analyze our product and determine its molecular weight. We implemented a variety of ways to detect our biological compounds, using both direct and competitive ELISA, while looking into alternative methods of capture and mixing using conjugated magnetic beads or dynabeads. As a team we have persevered to apply novel techniques into previously used methods for vancomycin detection. While being innovative in the creation of our own signaling molecule, we have also learned techniques in development of pump systems, delved into photo optic system assemblies, and honed in on how to write a unique code that isolates our desired signal parameters. At the same time, we learned about business and entrepreneurship skills such as how to build our own startup company, how to get patents, and how to market our product. We are now empowered to move forward with our newly acquired multifunctional tool set, invest in our project on the private and public market, and optimize it for home and clinical use. By honing in on our sensor's key features (efficiency, ease of use, and affordability) we can serve the patient population and provide an essential tool to healthcare providers for a better future.

8. References

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9. Appendix

9.1 Engineering part:

9.1.1 Illumination system:

To illuminate the sample, we are using Lumiled LXML-PB02 LEDs. Each of them is running at about 2.5W and emits 32 lm/W at 470 nm with a half-width of 20 nm. To avoid interference with the emitted light by the sample, we pre-filter the LED light with the 490AESP optical filter from Omega Optical. It is a short-pass with a sharp cut-off edge of less than 5 nm width between 490-495 nm. The fluorophore has its excitation range between about 450-515nm and its emission range 495-560 nm. The 535QM50 optical bandpass from Omega Optical with transmission range at 515-550 nm to filter the emitted light by the sample. This avoids interference with the LEDs and can also suppress a part of the rest incoming light from the environment in the spectra around.

9.2 Biological Part:

9.2.1 Antibody Binding:

We began by testing the interaction between the antibody and our first iteration of the detection molecule, BODIPY™ FL Vancomycin, by Thermofischer. Our tests began with a direct ELISA using a NUNC black 96 well plate. Following the general ELISA procedure, we coated our plates overnight at +4^{o}C with Anti-Vancomycin antibody (from Invitrogen acquired by Thermofisher) at a concentration of 4 ng/ml to 8 ng/ml diluted in carbonate bicarbonate buffer at pH 9.6 (which we also measured using the pH meter in the lab and adjusted with NaOH basic and HCl acidic solutions. We had problems in the beginning with our antibody due to inadequate storage handling (too many freeze thaws destabilized the antibody, rendering it unusable). Once we realized we were having difficulty due to freeze thawing, we resolved to store and leave the antibody at +4^{o}C for the remainder of the time. We followed the coating steps with washing and blocking step (blocking using varying amounts of BSA, generally between 0.01% to 1% BSA in PBS). We worked with optimizing this assay for several months by changing blocking buffers, plates, plate readers, antibody (reagents) with little to no success. Finally, we realized the background wells with only fluorescently labeled vancomycin were showing significant signal despite the blocking step and other variations to our experiment. At this point we decided to approach our binding assay from another angle. We conjugated magnetic beads (Dynabeads) to our antibody in hopes of providing an alternative mechanism to test binding of the antibody to vancomycin and measure the relative ratio or binding concentration gradient for competition between labeled and unlabeled vancomycin. Using a magnetic capture system, we tested the binding of the antibody to the fluorescently labeled vancomycin through incubation of the Dynabead labeled antibodies with both the labeled and unlabeled vancomycin, following proof of concept for antibody binding. During this time, we switched to using our own custom made fluorescently labeled vancomycin, in both HPLC purified and unpurified forms. We were able to test competition with this method although it was a much more time-consuming process as all the washing was done in individual Eppendorf tubes instead of on a 96 well plate due to concern for unspecific binding. After the washing steps of each vial, 100 µl/well were

pipetted onto the plate from each vial and read using a Tecan plate reader. Due to the time-consuming process of the washing step in this method and the lack of replicate reactions per experimental run we were only able to produce a limited amount of data from this process. There is also still question on the functionality and reproducibility in signal of the plate reader we were lent access to for these reads.

9.2.2 Synthesis and Purification of Fluorescent Vancomycin:

In order to increase our signal and keep costs low we decided to develop our own fluorescent vancomycin detection molecule using FITC dye (which has a wider excitation and emission range than BODIPY) and vancomycin. This process involved a one-week sabbatical from the usual laboratory work by changing focus into conjugation of vancomycin to FITC, followed by purification of FITC conjugated Vancomycin and determination of its concentration. Under supervision of the Uppsala University Pharmaceutical Chemistry lab, we investigated various methods of purification using both an elution column setup for S.E. (using PD-10 Desalting Column) and then HPLC. Both columns involved a washing step and equilibrating step with Acetonitrile (ACN) varying from 95% to 30% for washing and then 5% ACN for the equilibrating step. In the elution column setup separation was based on polarity, however most of our product came off in the beginning, perhaps due to weak binding, and did not separate out. Thereafter, we resolved to use preparative HPLC for isolating our product. After washing, equilibrating, and loading the column with 200 ml of our mixed sample (FITC, vancomycin and FITC bound vancomycin), we ran the preparative HPLC machine (collecting roughly 15 ml per fraction for 60 minutes). These fractions were analyzed in an analytical HPLC machine, where we identified the optimal absorption wavelengths for FITC, Vancomycin, and Vancomycin conjugated to FITC in our fractions. This enabled us to identify the compositions of the fractions and which contained only our desired product. The purest samples were combined and lyophilized. During this process the lyophilizer had some malfunctions so the sample had to be re-lyophilized and the pure product was too little to remove from the flask for weighing. We deduced the molecular weight (about 1840 g/mol) and relative concentration (546 nM) of the pure solution through mass spectrometry and comparative analytical HPLC. (fig 4).

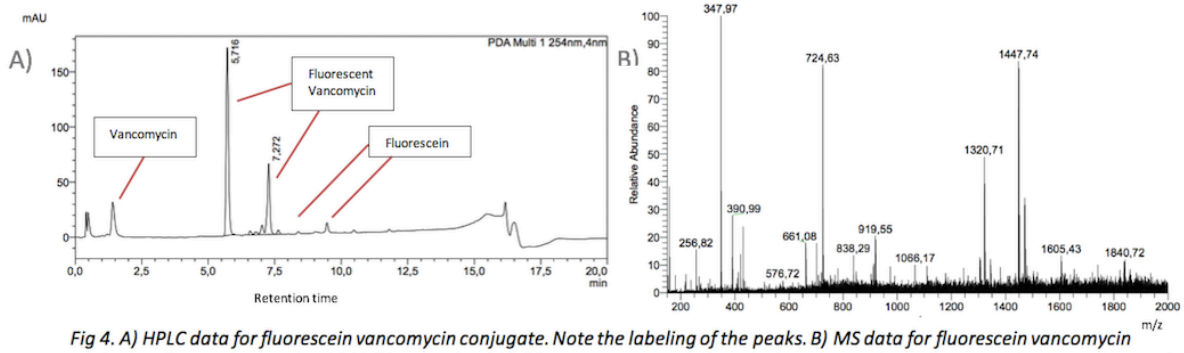

Fig 4. A) HPLC data for fluorescein vancomycin conjugate. Note the labeling of the peaks. B) MS data for fluorescein vancomycin conjugate. The peaks at m/z = 1840.72 and m/z = 919.55 correspond to the fluorescein vancomycin conjugate, while the peaks at m/z = 1447.74 and m/z = 724.63 correspond to vancomycin.