ABSTRACT

Development of TLR7 Agonist-Antibody Conjugate

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The search for better, more effective vaccines has been an intriguing subject of research since the days of Jenner and the first smallpox vaccine. We have proposed a method of overcoming the weaknesses of current vaccines by taking advantage of a specific pathway of the adaptive immune response. This pathway uses the stimulation of an internalized toll-like receptor, which is found in dendritic cells, to promote an antiviral response to a specific pathogen. For this method to work, we must attach our toll-like receptor agonist and a target antigen to an antibody for delivery to the dendritic cell. This thesis includes both the development of the synthesis of the agonist, as well as the chemistry used to conjugate the agonist to the antibody.

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DEVELOPMENT OF TLR7 AGONIST-ANTIBODY CONJUGATE

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In Partial Fulfillment of the Requirements for the

Honors Program

By

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CHAPTER ONE

Introduction and Background

Vaccines have been used for more than a century to prepare a response of the adaptive immune system against a specific pathogen.[1] There can be many pathways for the body to provide this immune response, either through the production of cytokines or production of antibodies. This response is most commonly achieved through the introduction of a viral agent that is usually in the form of a weakened or dead microbe, but can also be a toxin or surface protein from a bacteria or other pathogen.[2] The body is able to identify this agent and prepare an appropriate response should the immune system come in contact with the active virus. This agent, known as an antigen, is any substance that may serve as a target for immunological receptors. For most viruses this antigen is a portion of the exterior protein coat, but for bacteria this could be a toxin or even a unique lipid.[2]

The first vaccine came in the form of the *Variole vaccinae,* which is a form of the smallpox virus that is found in cows. Edward Jenner used this virus in 1789 to immunize a group of people against the deadly smallpox virus.[1] Jenner's vaccine worked because exposure to the "cowpox" virus stimulated an immune response without causing a life-threatening infection to the patient. After exposure to the non-lethal virus, the body had prepared an immune response to the smallpox family of viruses so that an infection by the normal virus could be fought and defeated. This classical form of vaccine is known as prophylactic and is meant to prevent or reduce the effects of future infection.[1] However,

there are complications with prophylactic vaccines, including highly mutable forms of some viruses, such as HIV or other sexually transmitted diseases, which are resistant to attempts to design a vaccine.[3] This is a challenge because of the ability of the virus to rapidly evolve in response to selective pressure from the immune system. A vaccine would need to cover every form of the virus to prevent it from developing resistance to the vaccine.

A second type of vaccine, which is currently an exciting and active topic of research, is the therapeutic vaccine. Rather than prevent an infection from occurring, these vaccines treat an existing disease that is already within the patient. This type is of particular interest in the field of cancer research, where therapeutic vaccines are in the clinical stages.[4] For these vaccines to work they must target an antigen which is specific to the tumor but not found on healthy cells. Unfortunately, identifying and targeting antigens that are only found on tumor cells is somewhat difficult to accomplish. Tumor cells also present many other kinds of cell-surface proteins, some of which send signals to immune cells that suppress an immune response

Besides the antigen, there is another key component of the vaccine that is known as the adjuvant. This is any substance that can be used to accelerate, prolong, or enhance the immune response to an antigen. Common forms of adjuvants are aluminium salts or organic compounds such as squalene.[5] The most frequently used of these types are the aluminium salts, specifically the inorganic salt known as alum, which is the hydrated potassium aluminium sulfate. The exact mechanism of this adjuvant has not been determined, but it has been shown that it does not function with certain antigens such as malaria and tuberculosis. Other, less frequently used adjuvants work by mimicking

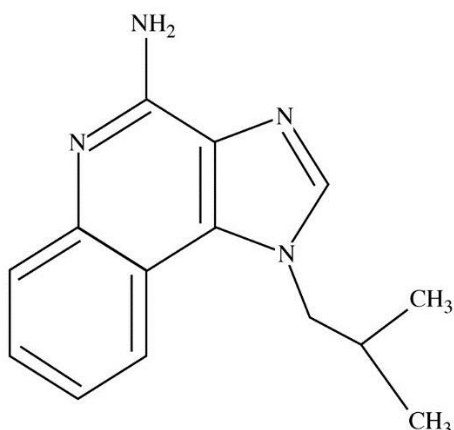
antigenic moieties which the immune system has been specifically programed to recognize.

Another adjuvant that is commonly used in research applications is known as Freund's adjuvant, particularly Freund's complete adjuvant (FCA). FCA is a solution of inactivated mycobacteria in mineral oil that is believed to work by stimulating production of tumor necrosis factor.[6] While this adjuvant does show promise in research applications and animal trials, it is generally considered too toxic for use in humans. However, there is another class of adjuvant that may be even more effective and has already been used as immune stimulating compounds in humans. These compounds that may serve as adjuvants are known as toll-like receptor agonists.

Recent research has shown that a particular immune system response may be triggered through the stimulation of specific cell receptors known as toll-like receptors (TLRs).[4] There are ten characterized TLRs that have been characterized in humans. A special type of immune cells known as dendritic cells, which present these TLR receptors, are typically found in tissues which are related with the immune system, such as the spleen, and areas which are exposed to the external environment, such as the lungs and gastrointestinal tract.[7] The receptors are found in either the exterior membrane of the cell or in the interior endosome. Each type of TLR is specific to one particular type of pathogen-associated molecule. For example, some TLRs are specific to flagella or lipopolysaccharides, while others are specific to double-stranded RNA or single-stranded DNA. All of these antigens are unique to foreign threats such as bacteria or viruses.

The particular TLR that is the subject of this research is TLR7. This receptor is a critical part of the anti-viral immune response, recognizing single-stranded RNA which is

most commonly found in viruses.[9] TLR7 is an endosomal receptor, meaning that any response by this protein must be caused by the presence of ss-RNA within the interior vacuoles of the dendritic cell. Activation of dendritic cells by the TLR7 pathway has been shown to result in increased secretion of the cytokines interferon-a, interleukin-6, and tumor necrosis factor-a via the MyD88 pathway. Imiquimod is the most commonly known activator of the TLR7 pathway.[10] This molecule has been used mainly as a patient-applied cream to treat certain forms of skin cancer and genital warts. Imiquimod's specific pathway has also been shown to activate a specific kind of dendritic cells known as Langerhans cells. These cells are a mobile form of dendritic cell, allowing them to gather and process antigen in the skin and travel to secondary lymphoid tissue, where they can then interact with naïve T-cells.[11,12] This allows for these cells to act as fully functional antigen presenting cells and to stimulate T-cell differentiation into Cytotoxic T-cells and Helper T-cells. Another well-known TLR7 activator is resiquimod, which is a modified form of Imiquimod with an added ethoxymethyl and methyl group.[13] The basic application and mechanisms of these two compounds has been found to be very similar.



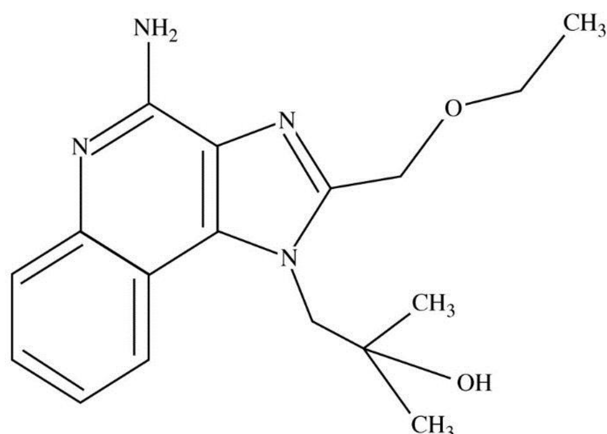


Figure 1. Structure of Imiquimod and resiquimod.[14]

Besides these two commercially available TLR7 agonists, there are many small molecule agonists that are being developed. One popular approach in this field is the design of adenine analogs, which are structurally similar to Imiquimod and resiquimod but have more potential for specific stimulation of TLR7.[16]

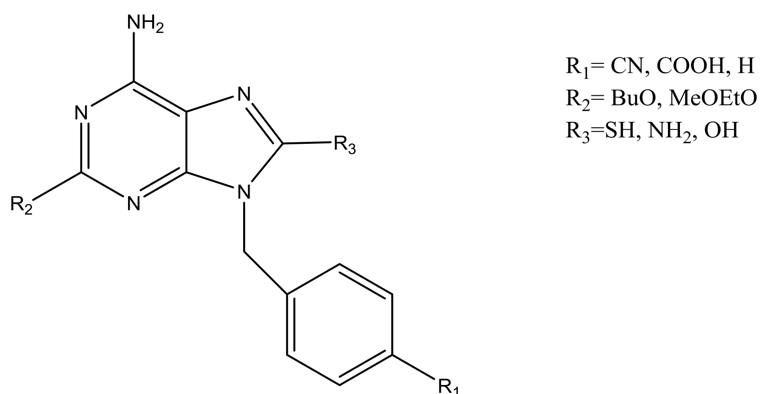


Figure 2. Basic structure of adenine derivatives.

Figure 2 shows the basic structure of the agonists which will be explored in the following chapters. These compounds have particular groups at specific locations on the aromatic structure to allow for the proper interaction with the TLR7.

A challenge in the development of TLR7 agonists is the ability to deliver them to the appropriate cells for stimulation without causing a systemic sensitization. One approach to drug delivery is the antibody-drug conjugate (ADC), which uses an antibody to carry a small molecule to the targeted site where the drug becomes active.[17] In recent research this method has been used to transport cytotoxic compounds to cancer cells, where the drug is then detached from the antibody and proceeds to kill the cancer cells. This method is effective because the antibody that the drug is conjugated to is specifically designed to have an affinity for a tumor marker of the cancer that is being targeted. Once injected into the body, these antibodies will travel through the body until they come in contact with the tumor marker. At this point the antibody will attach itself to the cancer cell and the entire modified structure is taken into the cell and degraded. Upon degradation, the cytotoxin is released in its active form and allowed to disrupt cellular processes in a way which will cause cell death. This method has the benefits of reduced side effects and a wider therapeutic window because of its specificity for its target.[17]

Our approach which is being explored is the possibility of using antibody conjugates to directly stimulate the immune system toward an antigen. This approach uses the antibody to carry a small, immune system-activating compound with the targeted antigen into the dendritic cells. One cell marker that has been considered as a target is Cluster of Differentiation 40 (CD40), which is a costimulatory protein receptor found primarily on antigen presenting cells.[15] Stimulation of this receptor is essential for many

immune and inflammatory responses including macrophage activation and immunoglobulin class switching.[15] On macrophages this protein is primarily activated by the presence of interferon-γ which may trigger microbe ingestion by the macrophage. Because this protein is found on the surface of antigen presenting cells, including dendritic cells, it is a possible candidate for specific targeting via modified antibody.

When attaching the TLR agonist to the antibody it is preferred that a method is used which creates a stable covalent bond. This can be done by conjugation at positions containing free amines, thiols, or carbohydrates. The options for crosslinkers to make this connection varies widely, from cleavable peptides to more permanent thioethers, all with a large variety in sizes and lengths. For the modification of antibody with a TLR7 agonist a more stable crosslinking strategy is desired because the agonist needs to remain attached to the antibody until degradation in the endosome.

N-Hydroxysuccinimide (NHS) esters have been commonly used for protein modification via amine acylation for many years. NHS esters are often stable enough to be purified and stored at low temperatures, making them easy to handle and often commercially available.[18] Crosslinkers with this moiety react under physiologic to slightly alkaline conditions with primary amines in proteins to yield stable amide bonds.

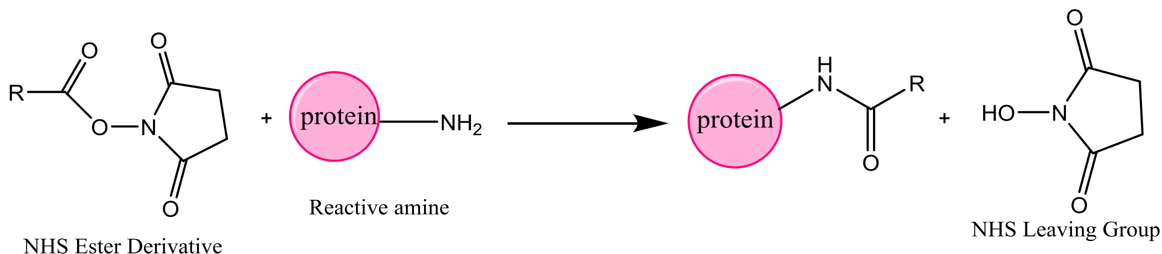


Figure 3. Scheme of NHS ester with amine.

This is a favorable moiety for crosslinking because there are multiple free amines offered by lysine residues on the antibody which may be used for modification.

The reaction that was used for protein conjugation has been attempted with two seperate reactions using three different crosslinkers. The first crosslinker used was NHS-Phosphine and the second was dibenzylcyclooctyne (DBCO)-PEG-NHS. The third crosslinker used was NHS-PEG-Azide, meaning that the modifying molecule must have a DBCO group. For both of these reactions the NHS ester moiety of the crosslinker is meant to be bound to the protein, leaving the other end open for reaction.

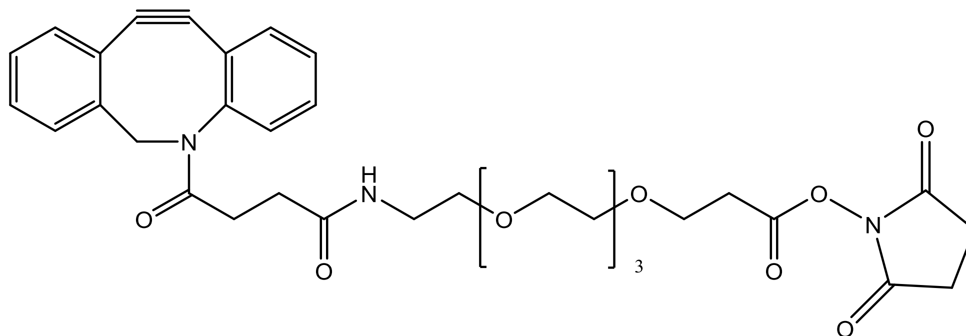


Figure 4. Structure of DBCO-PEG$_4$-NHS[16]

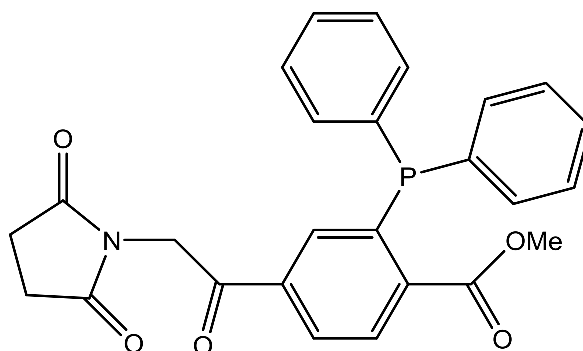


Figure 5. Structure of NHS-Phospine[17]

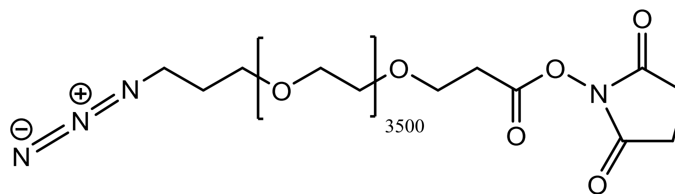


Figure 6. NHS-PEG_{3500}-Azide crosslinker structure.[17]

Dibenzylcyclooctyne (DBCO) is a moiety that reacts with azides through a copper-free click chemistry to form a stable triazole.[19] The reaction is very fast at room temperature and has the benefit of using functional groups that are stable in solution for long terms. The reaction of the DBCO functional group with the azide is promoted by the strain of the triple bond on the 8-member ring.[19] For these experiments, the crosslinkers used contain both the NHS group and either DBCO or azide on either end of a polyethylene glycol (PEG) chain.

For many years the 1,3-dipolar cycloaddition of azide and alkynes was considered a primary example of click chemistry, the quick reaction of joining two smaller fragments of a compound to reliably form a product. This reaction was introduced through the work of Rolf Huisgen in 1963, and improved upon with the introduction of the copper catalyst by Meldal et al. in 2002.[20] In this reaction, an azide reacts as a 1,3-dipole with an alkyne to yield a triazole ring as either a 1,4 or 1,5-adduct.[19]

Reactions of azides with cyclooctynes takes advantage of the strained triple bond to perform the same cycloaddition to form a triazole product without the use of the toxic copper catalyst that is found in most azide-cyclooctyne reactions. The reaction is termed biorthogonal because it may occur within a living system without disrupting natural processes.[21] This method is highly favored in biological systems because the reactants are

small, stable and not naturally occurring, which allows for selectivity without side reactions.[21]

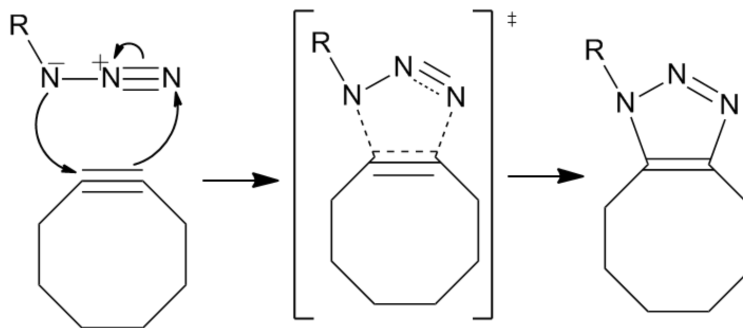


Figure 7. Azide/Cyclooctyne cycloaddition mechanism.

This reaction is an asynchronous, concerted pericyclic shift with little to no regioselectivity.[21] However, the lack of regioselectivity is not a concern because this method is for the purpose of conjugation to a large macromolecule.

The goal of this set of experiments was to develop a modified antibody which could stimulate a desired immune response. To accomplish this goal, an anti-CD40 antibody was modified using an NHS-PEG-Azide crosslinker. This modified antibody was then characterized to ensure that the modification was successful before being tested by a reporter cell assay. This modified antibody is expected to function by first targeting free dendritic cells and being endocytosed. Once inside the endosome of the dendritic cell, the antibody will be broken down and the TLR7 agonist will be free to stimulate its appropriate receptor. This will trigger a viral immune response, eventually generating immunoglobulin and cytotoxic T-cells that will target the antigen that we have chosen.

CHAPTER TWO

TLR7 Agonist Synthesis

Several different forms of adenine derivatives have been synthesized by our lab. Each of these derivatives maintain the aromatic ring structure while modifying the substituents in three key positions.

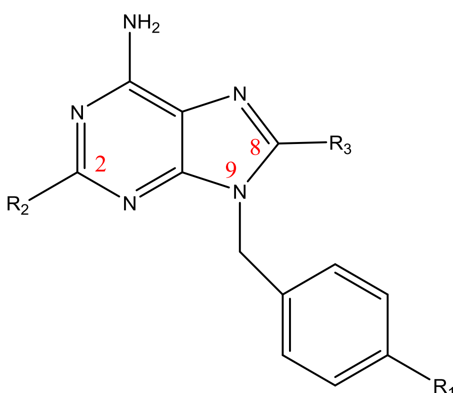


Figure 8. Structure of adenine derivatives.

These locations for modification include the alkyl group at the 2 position between the nitrogens in the six-membered ring, the substituent at the 8 position between the nitrogens in the five-membered ring, and on the para position on the benzyl group at the 9 position of the adenine ring. Substitution at these positions has been optimized to allow for the ideal interaction between the agonist and the TLR.

There is one agonist that has been developed which contains a group at the para position of the benzyl ring that allow for conjugation to proteins. This compound has a

DBCO group, which reacts with and azides, allowing us to use simple crosslinkers to attach our agonist to the antibody of our choosing.

The procedures listed below include some examples of the agonists that I participated in synthesizing as well as a scheme of the synthesis for the agonists containing the DBCO. Mass spectrometry data for the compounds below is provided by Zach Seifert and the final DBCO compound was prepared in our lab by Babatope Akinbobuyi.

The first reaction of this series is an electrophilic aromatic substitution to add a benzyl group to the 9 position of the chloroadenine. A 2.18 g amount of 2-chloroadenine was suspended in 50 mL of DMSO with 5 eq K_2CO_3 and 1.5 eq benzyl bromide. This mixture was allowed to stir overnight at room temperature before being poured into 150 mL of water at 0°C. The mixture was then filtered to give 3.27 g of compound 2 (98% yield) as a white solid. ^{1}H-NMR (DMSO-d_6) δ 8.26 (1H, s), 7.80 (2H, s), 7.32 (5H, m), 5.34 (2H, s). +ESI-HRMS for $[C_{12}H_{10}N_5Cl+H]^+$ calc. 260.0697 m/z; found: 260.0701 m/z (Δppm 1.347).

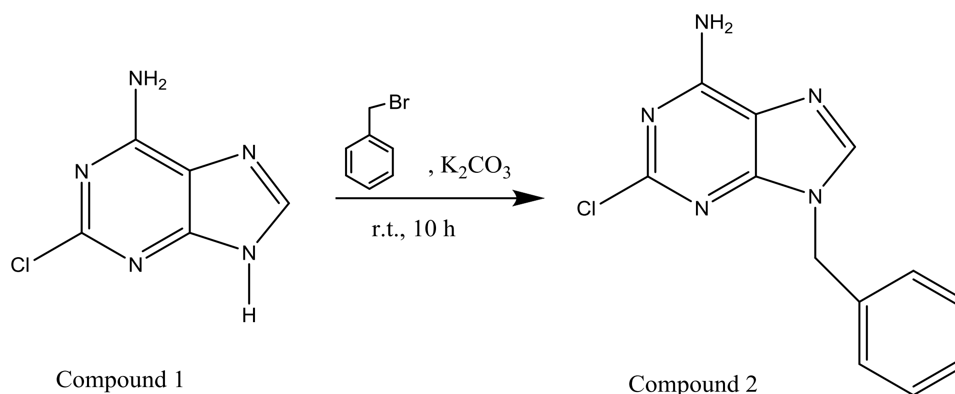


Figure 9. Scheme of reaction 1.

Sodium metal (0.31 g) was added to dry n-butanol (30 mL) under nitrogen. Once the sodium was completely dissolved, Compound 2 (1.98 g) in n-butanol (80 mL) was added and stirred at reflux for 48 hours under nitrogen. The product mixture was then concentrated and filtered to give compound 3 (1.54 g, 68%). ^{1}H-NMR (DMSO-d_6) δ 8.03 (1H, s), 7.32 (5H, m), 7.19 (2H, s), 5.25 (2H, s), 4.20 (2H, t), 1.62 (2H, m), 1.39 (2H, m), 0.91 (3H, t). +ESI-HRMS for $[C_{16}H_{19}N_5O+H]^+$ calc. 298.1662 m/z; found: 298.1675 m/z (Δppm 4.237).

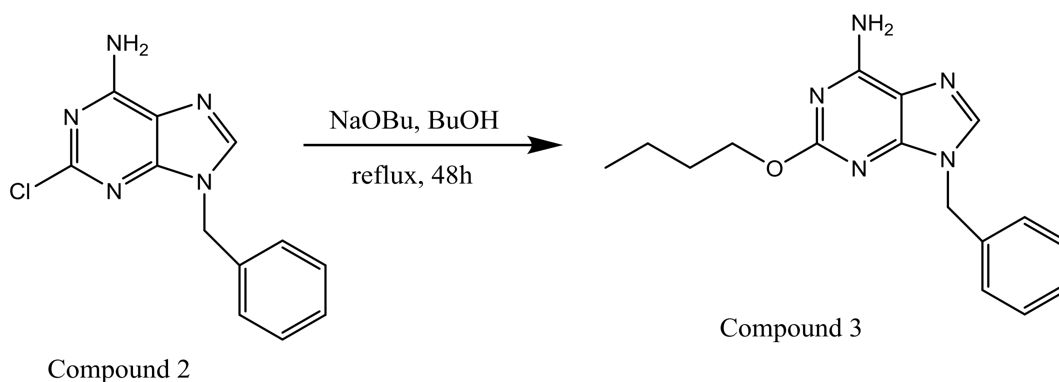


Figure 10. Scheme of reaction 2.

A second electrophilic aromatic substitution was used to add a bromine to the 8 position of the adenine derivative, compound 3. Bromine (8.5 mL) was added to a solution of compound 3 (1.12 g) in DCM (100 mL) and stirred overnight at room temperature. This reaction mixture was then washed with three 200 mL aliquots of 10%$Na_2S_2O_3$ and two 100 mL aliquots of $NaHCO_3$. The DCM was removed in vacuo, yielding compound 4 (1.13 g, 80%) as a yellow powder. ^{1}H-NMR (DMSO-d_6) δ 7.41 (2H, brs), 7.29 (5H, m), 5.25 (2H, s), 4.20 (2H, t), 1.64 (2H, m), 1.38 (2H, m), 0.90 (3H,

t). +ESI-HRMS for $[C_{16}H_{18}N_5Br+H]^+$ calc. 376.0767 m/z; found: 376.0776 m/z (Δppm 2.262).

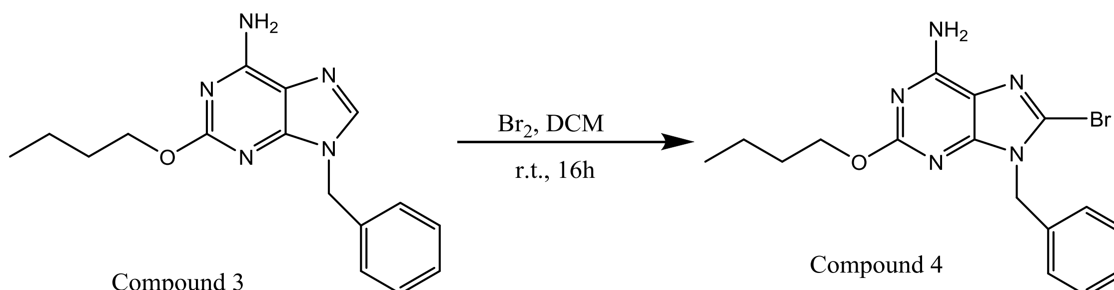


Figure 11. Scheme of reaction 3.

Compound 4 (0.112 g) was added to 8 mL of ethanol with thiourea (0.0.50 g) in a 10 mL microwaveable test tube and heated in a CEM Discover microwave synthesis system at 110 ± 2℃, power of 100 W, and a pressure of 30-40 psi for 30 min. After cooling, the precipitate was filtered and washed with water to give compound 5 (0.075 g, 76%). ^{1}H-NMR (DMSO-d_6) δ 12.19 (1H, s), 7.40-7.23 (5H, m), 6.87 (2H, brs), 5.26 (2H, s), 4.17 (2H, t), 1.66-1.59 (2H, m), 1.41-1.32 (2H, m), 0.90 (3H, t). +ESI-HRMS for $[C_{16}H_{19}N_5OS+H]^+$ calc. 330.1383 m/z; found: 330.1384 m/z (Δppm 0.280).

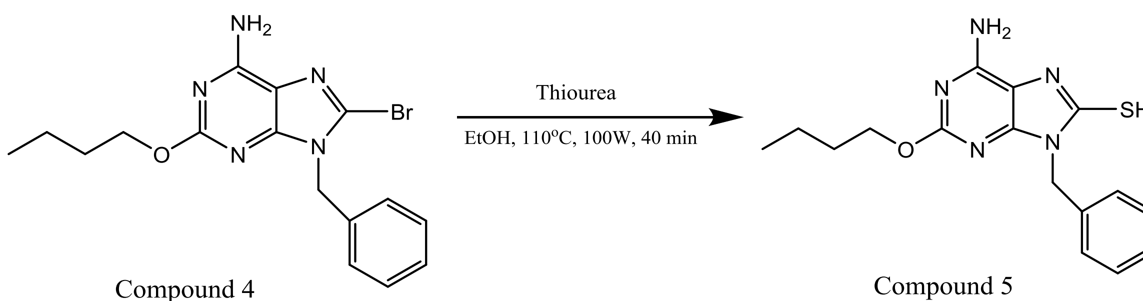


Figure 12. Scheme of reaction 4.

The molecule of special interest for this research contains a DBCO moiety that was attached through the benzyl ring on the 9-position of the adenine. The scheme for the synthesis of this compound is shown below.

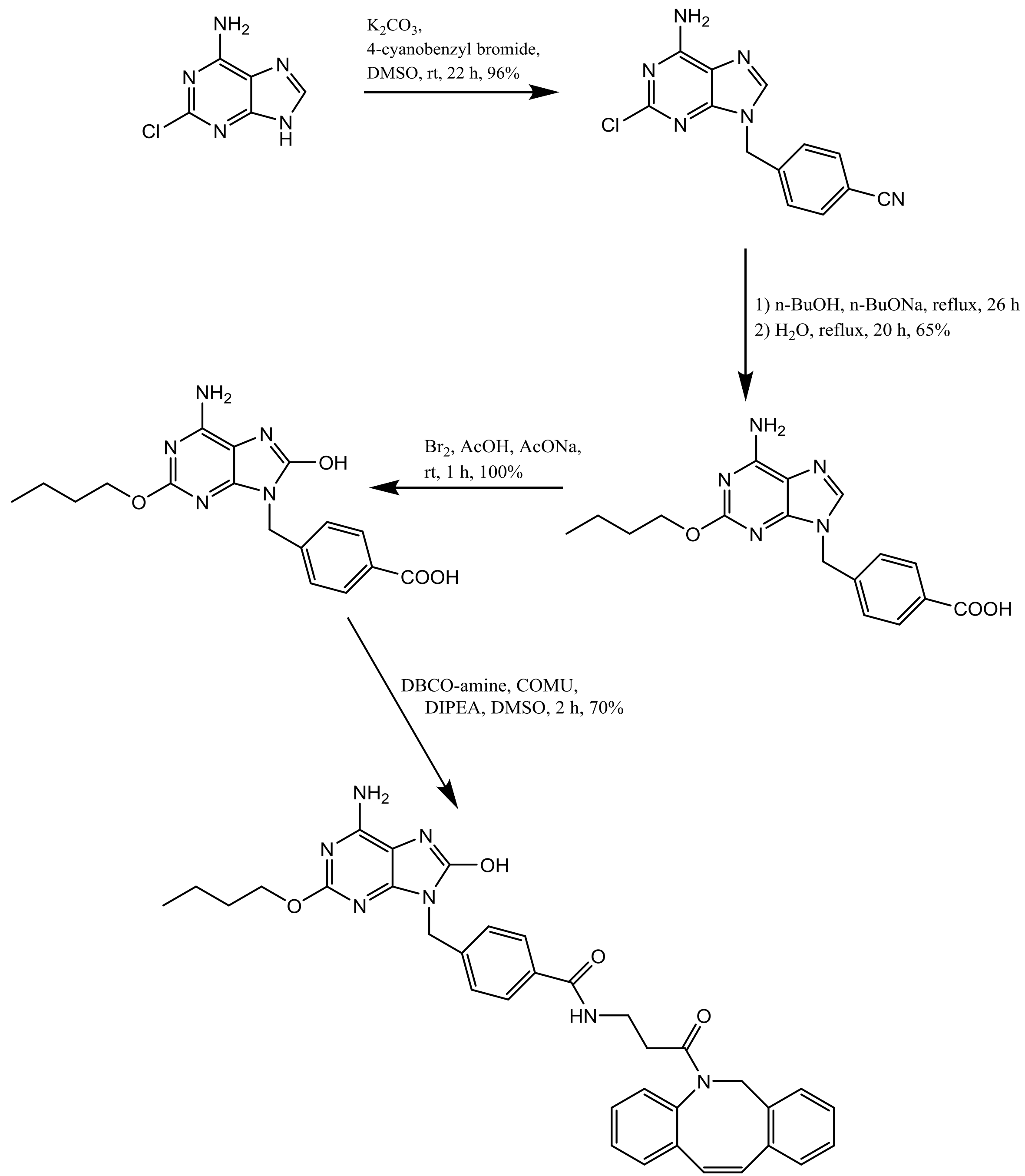


Figure 13. Scheme for the synthesis of adenine derivative with DBCO moiety.

CHAPTER THREE

Protein Modification

For our TLR agonists to be effective stimulators, we needed to develop a method of delivery to the dendritic cells. Our attention was drawn to several IgG forms of antibodies which were specific to surface proteins that are commonly found on dendritic cells. These antibodies include the two that were used in these experiments, anti-MARCO and anti-CD40. After identifying our desired antibody, we then needed to develop a method of conjugation that would insure a stable and efficient bond to the agonist.

To test this conjugation chemistry we first used the small protein RNAse. This protein was chosen because it is inexpensive in large quantities, soluble in aqueous solution and overall easy to handle. This protein contains several lysine residues which can be used for reaction with NHS ester crosslinkers. Unfortunately, this protein does not contain tryptophan, meaning that UV-vis could not be used to reliably analyze the modification of the protein. Instead, the protein was analyzed by coomassie staining and by using a biotin marker for the modification so that the protein could be analyzed by western blotting with streptavidin-horseradish peroxidase.

Experiment One

To determine the necessary conditions for the antibody modification, experimentation was first performed using bovine RNAse A. Modification of RNAse A was done using two different crosslinkers, NHS-PEG-Phosphine and NHS-PEG-DBCO.

Both the phosphine and DBCO groups react with the azide group found on the peptide after the RNAse had been fixed with the crosslinker.

The first synthetic strategy was tested by a time trial of the reaction between the phosphine and the azide. A time trial test was designed to determine the most favorable conditions for the reaction. In order to perform a time trial, we first needed to determine a method of detection. For this we decided that a coomassie stain would be the fastest and easiest way to detect the protein. A simple test was performed using varying amounts of RNAse to determine the amounts of protein and reagents to be used for the time trial.

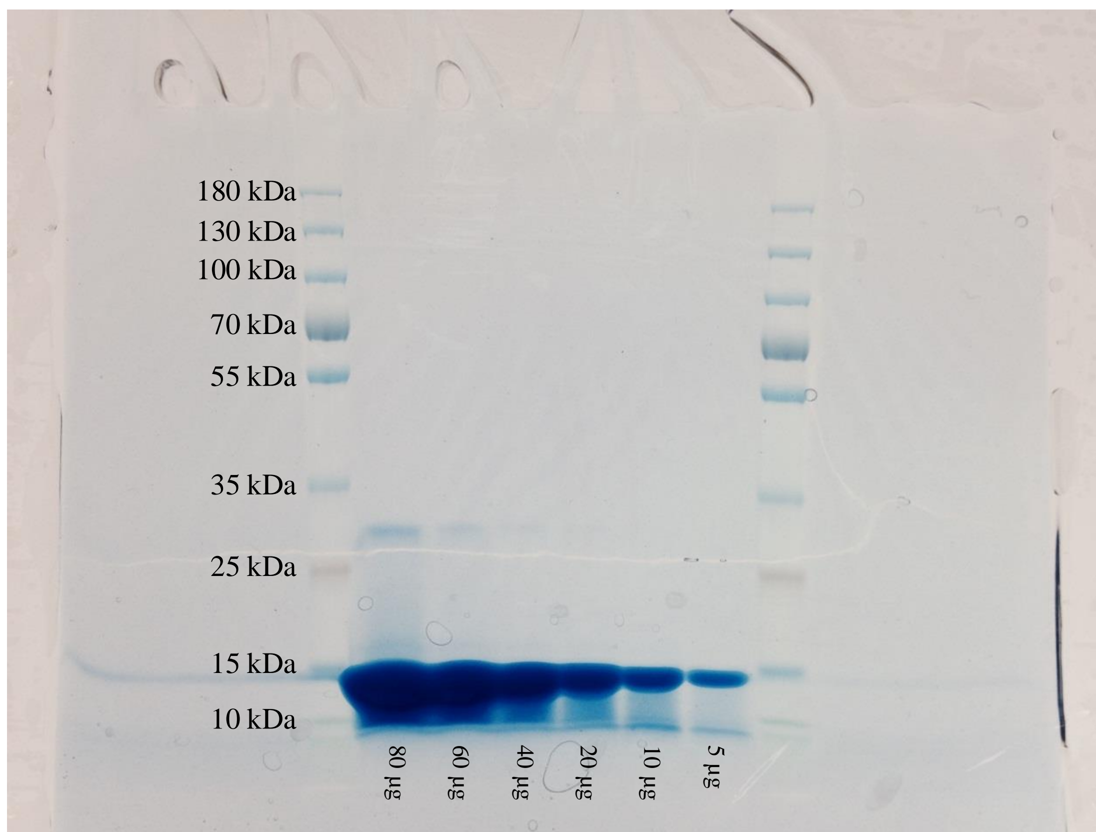


Figure 14. Coomassie blue stain of polyacrylamide gel containing varying amounts of RNAseA

The results shown in this figure come from 5 µL of RNAse solutions loaded into each well with total amounts varying from 80 µg to 5 µg. As seen in this figure, there are bands shown for the monomer of RNAse A at approximately 13.7 kDa. The conclusion from this simple test was that 5 µg of RNAse should be used for coomassie staining.

The next step was to modify the RNAse with a small peptide and see if the coomassie stain would show a shift from the original band. This experiment was conducted using 2 equivalents of peptide for each equivalent of protein. This required volumes of 50 µL stock RNAse solution being reacted first with 50 µL NHS-Phosphine to prepare the protein for reaction with the peptide. This reaction was done at room temperature over a time of one hour before the solution was dialyzed to remove any unreacted NHS-Phosphine. The modified RNAse then had a concentration of 0.146 mM (2 mg/ml), which was reacted with 0.292 mM peptide solution at room temperature for one hour. This gave the appropriate final protein concentration of 0.073 mM (1 mg/ml) for coomassie staining.

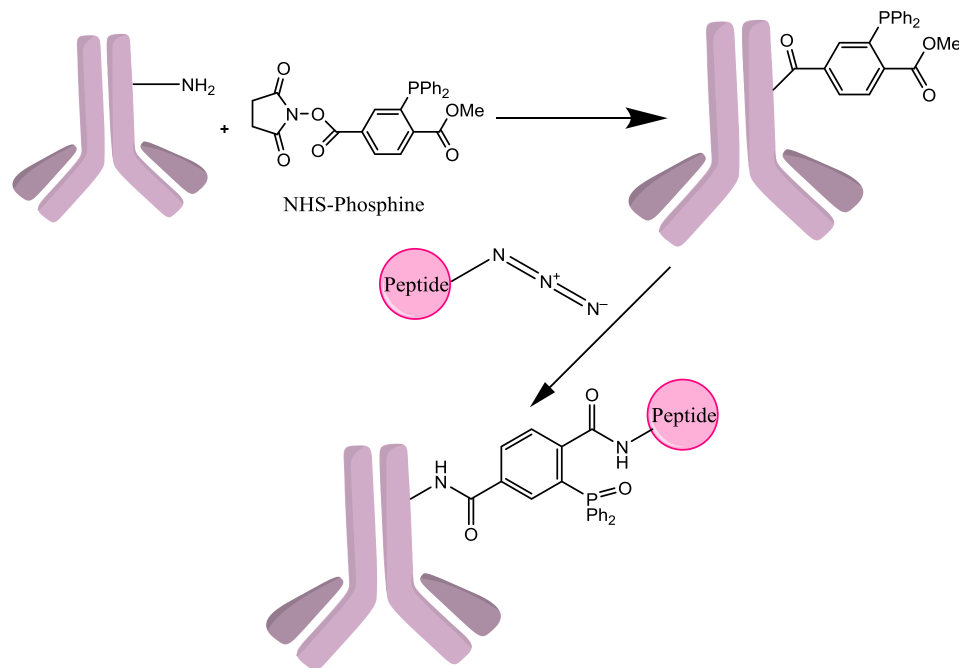


Figure 15. Reaction scheme for first protein modification.

For this time trial experiment, 2 equivalents of peptide were added to the NHS-Phosphine fixed RNAse and allowed to react for various time intervals of 24 hours to 15 minutes. After the desired time, 5 μL of each sample were loaded into the same gel to be analyzed. Unreacted RNAse was used as a control for the modified protein to be compared to. It was expected that the modified protein would be seen to be slightly higher than the unmodified RNAse because the peptide adds 3 kDa. We expected to see a second band to become more evident above the unmodified band as the time increased.

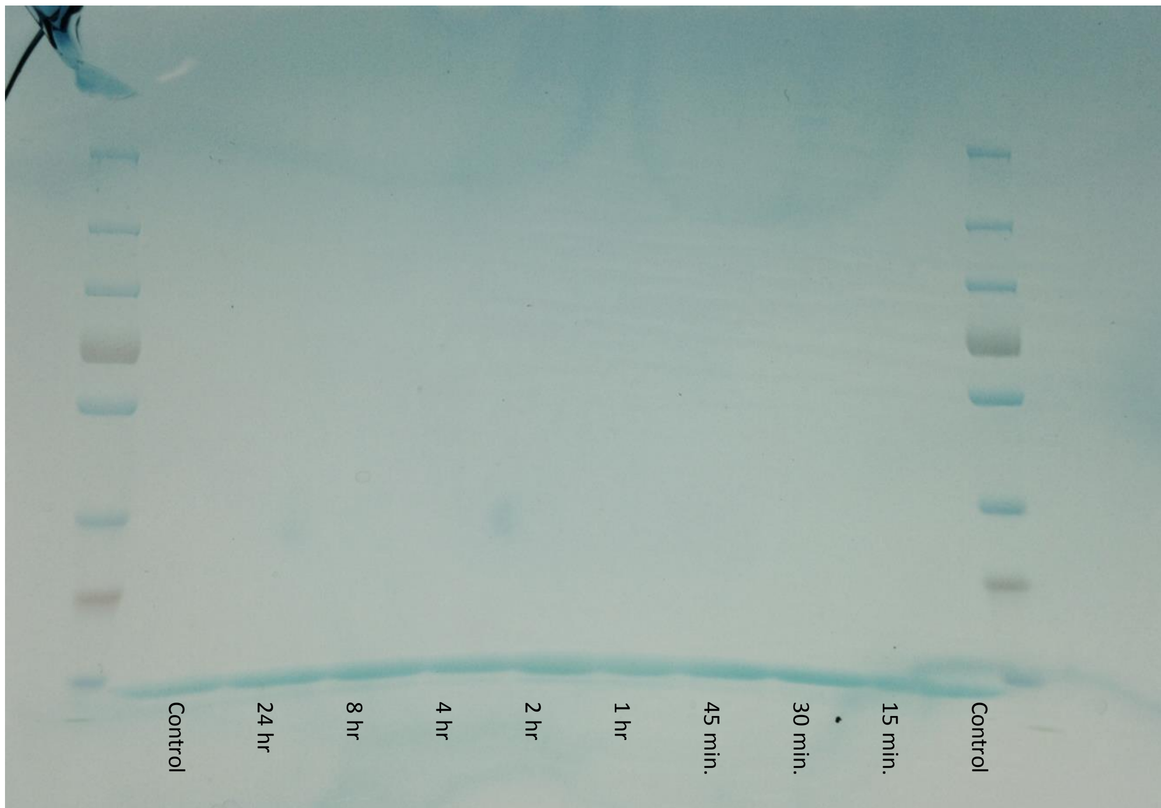


Figure 16. Coomassie Blue stain of first time trial

The results provided above show that there was no clear distinction between the modified and unmodified protein by this detection method. After this trial we decided to move on to a Western blot because this method could give us definite results without depending on slight mass differences. Western blot analysis will allow us to see modified protein while non-modified protein remains unreactive to the agents used for visualization.

For the Western blotting procedure we used the same concentration of RNAse, but because we wanted our intermediate protein to be more stable in water we changed the crosslinker to the DBCO-PEG_4-NHS. The phosphine group could be easily oxidized in aqueous solution, thus making the phosphine unreactive with azide, and it was found to be more convenient to have a stock solution of reactive protein which could be stored and used for multiple trials. For this reason the DBCO crosslinker was used for the following experiment. We began another time trial experiment to be analyzed by Western blotting. Concentrations of all reagents remained the same and again 5 μL of each sample were loaded into the gel. The control for this trial was RNAse reacted with 2 equivalents of biotin-NHS. After completion of the gel electrophoresis, the protein transfer was conducted by a semi-dry transfer. The membrane was blocked using 5% BSA solution and then stained with 1:10,000 streptavidin-horseradish peroxidase. After a quick wash, the membrane was then treated with luminol and viewed under an ImageQuant LAS 4000 chemiluminescent imager.

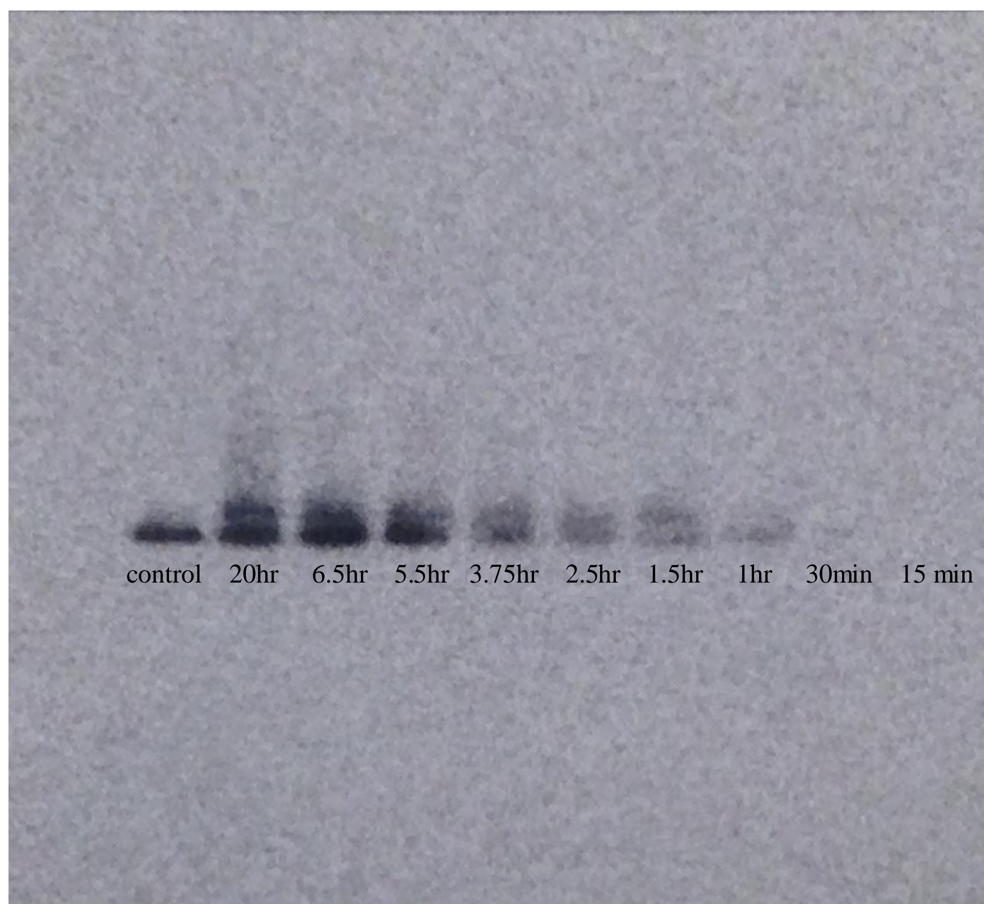


Figure 17**.** Western blot of RNAse modification time trial

The results of the time trial shown above again indicate that there was more than one modification made on the RNAse. From this experiment we determined that the appropriate reaction time for concentration testing was approximately 2 hours. We planned to do a series of reactions at 2 hours to see if we could achieve the same amount of reaction completion with a higher concentration of peptide at 2 hours. The goal was to get the same reaction completion as the six hour reaction with 2 equivalents of peptide.

Experiment Two

The first experiment to test the possibility of using TLR agonist modified antibody was to modify anti-MARCO and anti-CD40 antibodies with an Alexa-fluor 488.

This was done by first preparing the antibody with varying amounts of NHS-PEG_{3500}-Azide and then reacting this with excess DBCO-Alexa-fluor 488. From this we wanted to see two things: a- that a compound containing a DBCO group could be covalently attached using this method, and b- how many equivalents of these compounds could be attached.

For this experiment five samples of 9 mg/ml anti-MARCO and 2.7 mg/ml anti-CD40 were reacted with 0,1,5,10 and 20 equivalents of NHS-PEG_{3500}-Azide. This reaction took place for one hour at 28°C. The samples were then added to spin filters and spun three times with 400 mL of PBS buffer to remove any unreacted crosslinker. Afterwards, these samples were reacted with excess DBCO-fluorophore for one hour at 28°C and again purified through spin filtration. At this point, the final protein concentrations were determined using a nanodrop spectrophotometer. 5µg of each protein were then loaded into a NuSep 4-20% polyacrylamide gel and run in SDS buffer. Pictures of this gel where taken with a Typhoon FLA 9000 imager to determine the fluorescence of each band and on a BioRad Gel Doc™ EZ UV-vis imager to determine the protein amounts present.

Table 1. Volumes and concentrations of each sample and reagent for antibody modification.

Sample Letter	Vol. of MARCO (stock = 9mg/ml)	Vol. of CD40 (stock = 2.7mg/ml)	Vol. of NHS-PEG-Azide (stock = 1mg/ml)	Vol. of PBS buffer added	Final Concentration of Antibody	Eq. of NHS-PEG-Azide: Antibody
A	5.00 μL	-	0.00 μL	45.00 μL	0.9 mg/mL	0
B	5.00 μL	-	1.09 μL	43.91 μL	0.9 mg/mL	1
C	5.00 μL	-	5.75 μL	39.25 μL	0.9 mg/mL	5
D	5.00 μL	-	10.50 μL	34.50 μL	0.9 mg/mL	10
E	5.00 μL	-	21.00 μL	24.00 μL	0.9 mg/mL	20
F	-	16.67 μL	0.00 μL	33.33 μL	0.9 mg/mL	0
G	-	16.67 μL	1.09 μL	32.24 μL	0.9 mg/mL	1
H	-	16.67 μL	5.75 μL	27.58 μL	0.9 mg/mL	5
I	-	16.67 μL	10.50 μL	22.83 μL	0.9 mg/mL	10
J	-	16.67 μL	21.00 μL	12.33 μL	0.9 mg/mL	20

The following images are the results of this experiment:

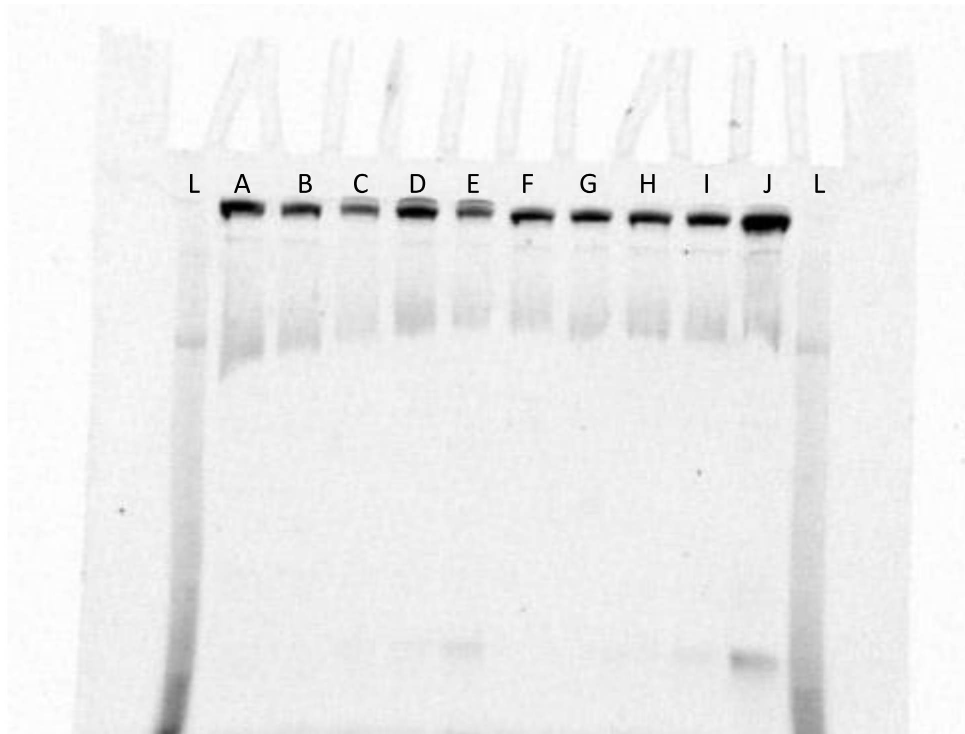


Figure 18. UV-Vis image of the gel containing both modified antibodies.

Figure 19. Typhoon image of the fluorescence for both modified antibodies.

Figures 18 and 19 both show the same gel, with the outermost lanes containing ladder, lanes A and F containing only unreacted antibody, lanes B and G containing 1 equivalents, lanes C and H containing 5 equivalents, lanes D and I containing 10 equivalents, and lanes E and J containing 20 equivalents of the NHS-PEG$_{3500}$-Azide crosslinker. In Figure 19 there is a clear gradient of increased fluorescence across the experimental samples, which would be expected considering that there are multiple points for reaction of the NHS crosslinker.

Results

From this experiment there is significant evidence that the conjugation of the Alexa-fluor 488 was successful. Also there is evidence that the relative amount of crosslinker used does affect the proportion of fluorophore addition. From this information

it should be expected that this chemistry will work for conjugating the TLR7 agonist and that the agonist should be able to be added in a dosage dependent manner.

The fluorescence image shows a strong gradient from low modification to high modification, which can be seen from left to right. Both antibodies show some amount of modification, although the modification appears to be less for the anti-CD40 antibody than for the anti-MARCO. This still means that both anti-MARCO and anti-CD40 could be considered for conjugation for the final antibody-drug conjugate. For future experiments we may consider using higher amount of reagents for reactions with the CD40 antibody to achieve equal modification.

Conclusions

For the next experiment TLR7 agonist will be conjugated to the CD40 antibody using the same reaction scheme as was used for the Alexa-fluor. For the sake of comparison, the resulting antibody conjugate will be run on a gel with comparative Alexa-fluor samples. A shorter crosslinker may also be used so that the conjugated antibodies can be analyzed by mass-spec to ensure that the desired modification is occurring.

For following experiments we will modify antibody that includes a covalently attached antigen. These modified antibodies will be tested with dendritic cell cultures to monitor the production of cytokines which indicate an appropriate immune response. If good results are achieved in these assays, we may move on to large scale production of the antibody for further testing in biological systems. It is important for us to develop multiple methods of conjugation for this purpose. Different antibodies will be delivered

in various buffers which will require different conjugation strategies. By developing these techniques in advance, we will be prepared to adjust to these differences.

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