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Exploring the Potential for B12-based Oral Vaccine Delivery; Cubilin mediated uptake of B12-conjugate systems greater than 160 kDa

Teresa Soldner

Acknowledgements

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1.0 Tetanus

1.1 Disease

Tetanus is one the most preventable causes of death worldwide. Caused by *Clostridium tetani*, bacteria found in soil everywhere, the spores enter the body most often through puncture wounds or unsterile tools or care of umbilical cords. Though patients can survive a tetanus infection, tetanus has a 20-75% mortality rate depending on the quality of medical care and severity at time of diagnosis. The only reliable way to decrease the incidence of the disease is through vaccination of each individual in the whole population. Since it is not a readily communicable disease, herd immunity cannot be attained and thus, even in developed countries, vaccination must continue at 100%. Additionally, prior infection does not confer immunity to an individual. The current vaccine recommendation is a minimum of three doses and if possible, a booster every 10 years.[1]

Tetanus is a major element of any national health initiative, as it is responsible for 13% of vaccine-preventable causes of death in children under 5 (along with measles, influenza, and pertussis).[2] Because the fourth Millennium Development Goal aims to reduce maternal and infant mortality by 75% between the years of 1990 and 2015, much more data is now being collected on vaccination in developing countries. Even with this initiative, 59,000 cases of neonatal tetanus and 2,000 cases of adult tetanus are documented each year. Also,

the world vaccination rate is rising, now at 82% from 20% in 1980, but the cost of such a program is also rising.[3]

1.2 Vaccines

The tetanus vaccine was invented in 1924 by Descombey[4] and is used still today. The tetanus toxin (tetanospasmin) is the most potent toxin known, with a lethal dose LD_{50} of 2.5 ng/kg.[4] This toxin is the basis for the vaccine, where the toxin is isolated and heated in formaldehyde and lysine at 80ºC to create linkages either within the protein itself or to the lysine. This new form, called tetanus toxoid, retains the same general 3D structure as the toxin but has lost functionality due to the cross-linking and is administered through intramuscular injection. The immune system gains enough points of recognition from this locked conformation of the protein and confers immunity to the individual, but this must be repeated every 10 years in order to maintain a strong immune response.

There are two easily identifiable problems with administration of and access to the current vaccine. The first is patient compliance. Because some patients have a fear of needles and because the shot itself is uncomfortable, patients are much less likely to keep their vaccinations up to date because of the discomfort associated with getting them.

The second is that it requires many tools in order to be administered: refrigerated transport and storage, trained nurses to give the shot, sterile needles for administration, and biohazard disposal for the used needles. Because of these requirements, the price of administering the vaccine rises and the ability of the

world's poor to obtain the vaccine decreases. The 61,000 deaths that occur every year due to tetanus are absolutely preventable, but only by increasing the access of the world's poor to the vaccine.[1]

An oral vaccine would address both the patient compliance and price concerns. With no need for trained nurses or needles, the price and logistic difficulty for administering the vaccine are directly erased. The difficulty of taking an intramuscular vaccine and making it oral is how to get it through the stomach without being degraded beyond immune recognition and also how to initiate uptake across the intestinal cell membrane. After that, there is further difficulty in that, once in the bloodstream, the immune system has to still be able to recognize it in its new (oral) form.

1.2 Biochemistry

The tetanus bacterium, *Clostridium tetani,* is an anaerobic bacterium whose spores are found ubiquitously in the soil and in the intestines and feces of horses, sheep, cattle, dogs, cats, rats, guinea pigs, and chickens. When the spores enter the anaerobic environment of the body, usually through a puncture wound, they circulate the body through the blood and lymphatics. During growth, *C. tetani* produces the ectotoxin tetanospasmin which targets neurons, mostly in the central nervous system.[4]

The clinical manifestation of tetanus is unopposed muscle contraction and spasm, which often leads to death through pulmonary or cardiac failure. The incubation time is 3-21 days, usually 8. Muscle spasms last about 3-4 weeks, but

a patient can take months to recover. This muscle rigor is caused by the action of the tetanospasmin within the central nervous system.

Tetanospasmin is a 150 kDa protein consisting of two chains, the heavy chain (~100 kDa) and the light chain (~50 kDa), which are connected through a disulfide bond. The heavy chain has two (~50 kDa) subsections, the carboxyl-terminal and the amino-terminal. The function of the heavy chain is to target the inhibitory neurons in the central nervous system and to trigger endocytosis into these cells. [5] The reducing environment inside the cell breaks the disulfide bond [6] and the light chain is freed to cleave one protein, synaptobrevin,[7] a vesicle-associated membrane protein (VAMP) which disrupts vesicle-membrane fusion.[8] With this process blocked, the neurotransmitters cannot be released into the synapse in order to inhibit muscle contraction.

2.0 Targeted drug delivery

Novel drug delivery systems struggle both with getting the drug across barriers, but also with obtaining the correct concentration at the necessary location in the body. This biodistribution problem has led to an increase in targeted drug delivery systems, where a molecule of known biodistribution is complexed with a drug, usually through direct conjugation or through a ligand linking the two. In these systems, the targeting molecule either facilitates crossing of physiological barriers or delivery of the therapeutic molecule to a specific site. This means that scientists are searching not only for new therapeutic molecules, but also new

delivery molecules to advance the field. Current examples under research are the B vitamin family, peptides, antibodies, organic ligands, and polymers.

3.0 Vitamin B_{12}

3.1 Structure and Function

Vitamin B_{12}, also called cobalamin, is a highly water-soluble vitamin (10.2 mg/mL) that is produced only by bacteria but is essential for mammalian cell growth and function. [10] As the only organometallic compound found in biology,[11] B_{12} has a six-coordinate cobalt(III) center in a corrin ring, a dimethlybenzimidazole ribonucleotide in the α-axial position, and a variable ligand in the β-axial position. On the ribose is a solvent-exposed hydroxyl group[12] which is the targeted conjugation site in this experiment.

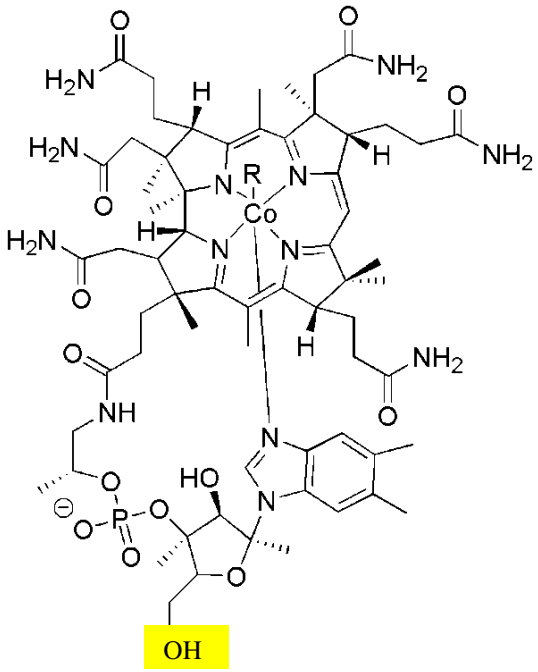


Figure 1. The structure of B_{12} (cobalamin with R group). The target conjugation site is the hydroxyl group at bottom of figure.

B_{12} is a single-carbon transferase essential for cellular function because of its involvement with DNA methylation, fatty acid synthesis, mitochondrial energy production, synthesis of red blood cells, and maintenance of the nervous system. Once in the system, the body modifies the R group to create different derivatives.

The only way to obtain B_{12} is though diet – primarily the consumption of animals or animal products from ruminators.[13] These are animals that are primarily grazers and that have a symbiotic relationship with bacteria that live in their gastrointestinal tract. These bacteria help the host to break down its food and in the process, synthesize B_{12} for the host. In this way, B_{12} is in meat (especially the liver, where it is stored) and milk products. Because absorption from the diet is so essential, a selective system has developed in order to protect and shepherd B_{12} from the mouth to the bloodstream.

3.2 Mechanics of the pathway

Upon ingestion, B_{12} binds to haptocorrin (HC), a binding protein in the mouth,[14] and the HC- B_{12} complex moves through the stomach, the protein protecting the vitamin from the acidic environment (~pH=2). Once through the stomach, HC releases B_{12} in the duodenum (~pH=6) where it is captured by the next binding protein, intrinsic factor (IF). The IF- B_{12} complex makes its way through the intestine to the proximal ileum (~pH=7.5), where it associates with the cubilin receptor (anchored by the transmembrane protein, megalin) which triggers receptor-mediated endocytosis into the enterocyte.[15] Once engulfed, the endosomal pH drops to 5.5, causing proteolytic breakdown of IF and releasing the

B_{12}. When it is released into the bloodstream the third transport protein, transcobalamin II (TCII) binds to the B_{12} and escorts it through the bloodstream for cellular uptake.[16]

3.3 Drug delivery with B_{12}

The B_{12} uptake pathway has been shown to facilitate the delivery of molecules through the stomach, across the intestinal membrane, into the bloodstream, and into cells.[17, 18] Most notably this has been done in this same research group with insulin, a ~5.6 kDa protein.[17]

Questions arise about whether or not a 150 kDa attachment is too large for B_{12} to bring into the cell, as it is three times larger than any other successful uptake, and whether the steric bulk of the protecting groups from the uptake pathway will prevent the immune system from reaching and recognizing the vaccine in the bloodstream. So the experimental question is posed: if we attach TT to vitamin B_{12}, will it be able to be absorbed through cell-mediated endocytosis and still be antigenic enough to confer immunity to the individual?

4.0 Materials

1,1'-carbonyl-di-(1,2,4-triazol) (CDT), vitamin B_{12}, sinapinic acid (SA), dialysis tubing, CypHer 5E, and Alexafluor 405 were purchased from Sigma Aldrich at reagent grade and were used with no additional preparations. The tetanus toxoid (TT) was a gift from the Serum Institute in India, lot number 1170, at a concentration of 2400 L_f/ml. The antibodies were purchased from Abcam in Cambridge, MA. DMSO from Sigma Aldrich was dried using 4Å molecular

sieves (also from Sigma) under dry nitrogen. Water was distilled and deionized to 18.6 MΩ using a Barnstead Diamond RO Reverse Osmosis machine coupled to a Barnstead Nano Diamond ultrapurification machine.

All reverse-phase size exclusion chromatography was done with an Agilent 1200 HPLC, with a manual injection and an automated fraction collector, which was fitted with a Zorbax GF-450 analytical column (9.4 x 250 mm) in series with a Zorbax GF-250 analytical column (4.6 x 250mm).

Matrix-assisted Laser Desorption/Ionization – Time of Flight/Mass Spectroscopy (MALDI-TOF/MS) was done on an Applied Biosystems Voyager-DE MALDI-TOF Mass Spectrometer at SUNY ESF, Syracuse, New York.

The Quick Start Bradford Assay was purchased from Fischer and was preformed according to the manufacture's instructions (BioRad).

All *in vitro* cell experiments were done in an air-filtered, UV-irradiated Labconco Purifier I laminar flow hood. RPMI 1640 was purchased from the American Type Culture Collection (ATCC). Fetal Bovine Serum and cell stripper were purchased from Mediatech in Manassas, Va. Penicillin-Streptomycin solution with 10,000 units of penicillin and 10 mg per mL streptomycin in 0.9% NaCl was purchased from Sigma. The BeWo choriocarcinoma human cell line (ATCC code CCL-98) was obtained from the American Type Culture Collection (ATCC).

Confocal microscopy experiments were conducted with a Zeiss LSM 4 Pascal Confocal Microscope and Image Analysis and Zeiss LSM 700 Pascal

confocal microscope with Image Analysis software equipped with Ar and HeNe lasers.

5.0 Methods

5.1 Synthesis

5.1.1 B_{12}-Tetanus Toxoid CDT Coupling reaction:

B_{12} was activated with CDT in a 1:1 molecular ratio by placing both to spin in a round-bottomed flask in dimethylsulfoxide (DMSO) at 45°C for one hour or more. The most common scale was to do this reaction with 40mg B_{12}, 5mg CDT, and 3mL of DMSO. One mL of HEPES buffer at pH 8 and 200µL of TT solution were put to stir in another round-bottomed flask. Depending on the desired B_{12} to TT mole ratio, different amounts of the activated B_{12}were added. The reaction was left to stir for 12-24 hours.

The reaction was dialyzed in 50,000 molecular weight (MW 50,000) cutoff tubing in a liter of 0.11 M NaCl solution, exchanging the water multiple times (~6 x 1L) to wash away unreacted B_{12}. This caused a white precipitate to crash out of solution, which refused all attempts to redissolve it. Because a product that will not remain in solution is not helpful to the stated purpose of this experiment, these products only received mild attempts at purification and characterization.

5.1.2 Fluorescent Tagged B_{12}-TT or TT (B_{12}-TT or TT*)*

The B_{12}-TT or TT was added to 200µg/mL carbonate buffer at pH 9.6. 10mg of CypHer 5E was added and the mixture was rotated at room temperature in the dark for 2 hours. This mixture was then dialyzed (MW 50,000) overnight into PBS buffer at pH 7.4 at 4ºC. The samples were kept in 4ºC and away from light until use.

5.1.3 Fluorescent Tagged IF (IF)*

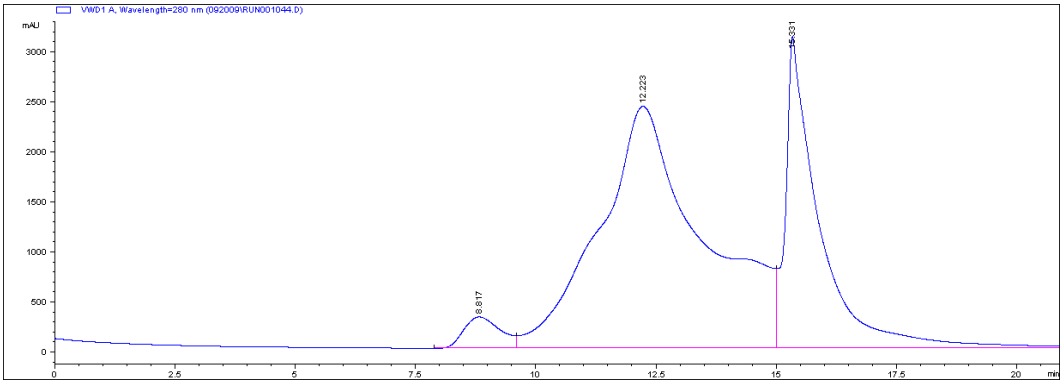
10mg IF was added to 1 mL of 200µg/mL carbonate buffer at pH 9.6. The solution was vortexed and centrifuged to remove undissolved solids. 50 µL of Alexa Fluor 405 (1µg/µL solution in DMSO) was added to the IF solution. The solution was rotated at room temperature and in the dark for one hour, and then dialyzed (MW 25,000) into PBS buffer at pH 7.4 at 4ºC for one day.

5.2 Purification

After dialysis, the purification of the conjugated system *vs.* the unreacted TT was done on the HPLC. Almost all chromatography data looked similar for any ratio of B_{12}:TT that was attempted. This indicates that the TT is the most important factor in determining properties of the compound, making it difficult to distinguish between free TT and the B_{12}-TT compound. Because of this, many different methods were tried before finding a method that be suitable.

The method developed ran the reaction through a reverse-phase size exclusion column and gained better separation of peaks, but not as well as one

could wish. An 0.11 M NaCl solution was used as the mobile phase to dissociate the ionic interactions of the B_{12}-TT with the column. A flow rate of 1.5 mL/min was used to elicit separation on the tandem GF450-250 column.



All three peaks came off the column red, indicating the presence of B_{12}. The peak at 15.3 min was found to be mostly B_{12}, with small amounts of protein, and the peaks at 12.2 and 8.8 were found to contain both B_{12} and TT, though the peak at 8.8 had a higher concentration of the protein. This reaffirms the idea stated above, that the different ratios of B_{12}-TT and free TT cannot be separated because their properties are overwhelmingly determined by the TT.

The major peak at 12.2 was collected for characterization and testing. Some of the peak was set aside to that tests could compare their results to the crude reaction as well. The rest of the collected peak was dialyzed in a tinted dialysis container against distilled water, in order to wash the NaCl from the compound. This was then lyophilized and reconstituted in PBS buffer.

Table 1. Summary of the reactions carried out and the different mole ratios (collected from peak at 12.2 min)	
B_{12}:TT reaction mole ratio	Conjugate Designation
295	**1**
60	**2**
15	**3**

5.3 In Vitro *Assays*

5.3.1 Bradford Assay

On a 96-well plate, known concentrations of BSA (2 mg/mL-0.15 mg/mL) were run in order to build a calibration curve and, concurrently, 5 µl of each sample were also plated. All samples and standards were run in triplicate. 250 µL of quick start Bradford dye reagent was added to each well. The plate was allowed to incubate for 10 minutes at room temperature. The absorbance was read at 670 nm and compared to a calibration curve gained from BSA.

5.3.2 ELISA Assay

100 µl of 1µg/mL rabbit antibody solution (ab53829; polyclonal to TT) in 0.05 M carbonate buffer at pH 9.6 was incubated in 96 well plate overnight at 4 °C. The solution was removed and the wells were washed three times with phosphate buffered saline, pH 7.4, 0.05% tween (PBST). After each of the following incubations, the plate was again washed as described. 200 µL of PBST with 2.5% dried milk (PBSTM) was added to the wells and was incubated for one hour at 37 °C. Three wells of 100 µl of the compounds or TT standard dilutions were incubated for two hours at 37 °C. 100 µl (0.8 µg/mL) of mouse antibody

solution (ab26247; monoclonal to TT) in PBSTM were incubated for two hours at 37 °C in all wells. 100 µl (1 µg/mL) of rabbit antibodies (ab6728; polyclonal to mouse IgG) conjugated to horseradish peroxidase in PBSTM was incubated for 1 hour at 37 °C. After the last wash, 100 µl of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) liquid substrate was added to the wells and was allowed to incubate for 15 min at room temperature. The absorbance at 405 nm was then recorded. The control dilutions of TT were plotted and fitted with a linear best fit line which was only considered accurate when the line had an R^2 value greater than 0.9. This calibration curve was used to calculate the concentration of the samples.

5.3.3 MALDI-MS Preparation

Because MALDI is an inherently finicky system, a project was undertaken to search for the best preparation conditions under which to see results from the TT. By varying the kind of matrix, concentration of matrix, and concentration of analyte, a balance of these was found that produced consistent reproducible results. Even though MALDI is usually employed first to know that something worth pursuing has been created, the best course of action was to run a Bradford assay first to determine the concentration of the complex for better MALDI data. This is important because the concentration of the analyte was the major factor in the success of MALDI results.

10 mg of SA was placed in an epindorf tube, along with 30µL of acetonitrile and 70µL of water (both chromatography grade). This mixture was

vortexed to maximize saturation and then centrifuged before use in MADLI plating. The two solutions were plated at 10:1, 5:1 and 1:1 matrix:analyte ratios.

5.3.4 Confocal Methods to Confirm B_{12}-TT Uptake*

The BeWo cells were plated (200,000 cells/dish) on 356x100 mm vented dishes and incubated at 37 °C overnight in RPMI 1640 media. In the morning, each plate was rinsed with PBS buffer (3x1 mL) and then 1 mL of of **B_{12}-TT*** in solution of IF (10 mg/mL) in PBS was added to each plate and incubated for 1 hour. The drug solution was then removed, and the cells were washed with 50 mM PBS (3x1 mL) at a pH of 7.4. The cells were then washed with PBS at 3.5 (3x1 mL) to prevent any noncovalent membrane interaction with the proteins. The plate was then filled with 1mL of PBS buffer at pH 7.4 and viewed under the confocal microscope. A wavelength of 600 nm was used to excite the CypHer 5E, and optical slices were taken by the machine to confirm that the fluorescence was inside the cell instead of attached to the membrane on the outside.

5.3.5 Confocal Methods to Confirm B_{12}-TT and IF* Co-localizaiton*

The BeWo cells were plated (200,000 cells/dish) on 356x100 mm vented dishes and incubated at 37 °C overnight in RPMI 1640 media. In the morning, each plate was rinsed with PBS buffer (3x1 mL) and then 1 mL of **B_{12}-TT*** conjugated to **IF*** in PBS was added to each plate and incubated for 1 hour. The drug solution was then removed, and the cells were washed with 50 mM PBS (3x1 mL) at a pH of 7.4. The cells were then washed with PBS at 3.5 (3x1 mL) to prevent any noncovalent membrane interaction with the proteins. The plate was then filled with 1mL of PBS buffer at pH 7.4 and viewed under the confocal

microscope. A wavelength of 600 nm was used to excite the CypHer 5E and 405 nm to excite the Alexa Fluor 405 dye.

6.0 Testing

6.1 Characterization:

Because of the size of the TT and the fact that complexed and uncomplexed TT were difficult to differentiate, MALDI-MS was the only instrument through which to detect a shift during the experiment. SDS PAGE gels were run, and although red bands in the gel before staining indicated successful conjugation to B_{12}, the poor resolution made it difficult to show a size difference between the free and conjugated TT. The next step was MALDI-MS.

First, the weight of the control (free TT) was established at ~158.5 kDa, (see figure 2), with two MALDI peaks showing both a +1 mass/charge ratio and assumably at +2 mass/charge ratio. This was 8.5 kDa above the known weight of the molecule, but presumably the incorporation of formalin and lysine during the inactivation process has caused an increase in mass. Next, the sample from the first peak from the HPLC separation of **1** was run, showing ~170 kDa, where the mass increase would be roughly equivalent to 8 B_{12} molecules.

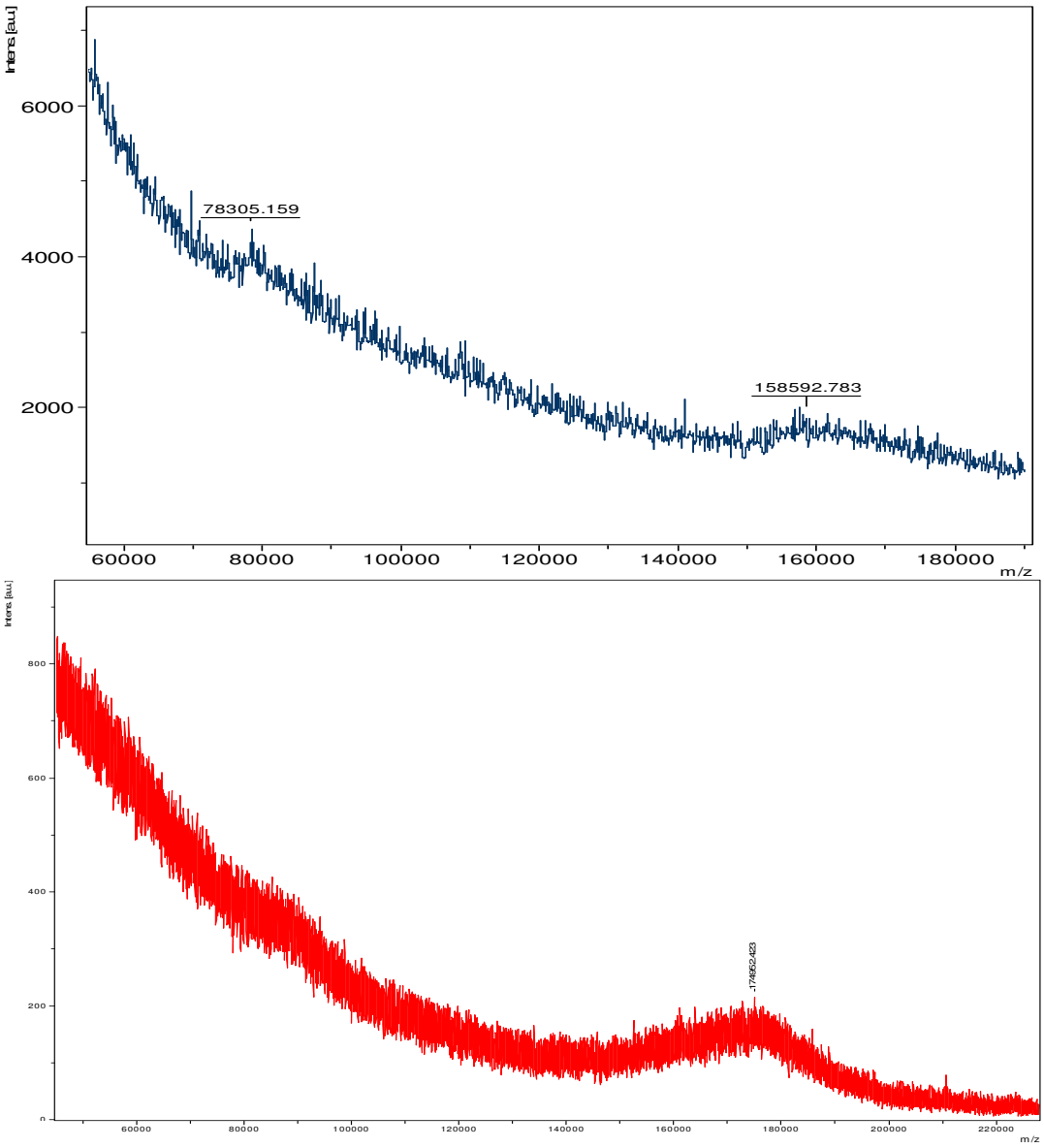


Figure 2. MALDI-TOF/MS of TT (Top) with a weight of ~158 kDa and of B_{12}-TT conjugate (bottom) **1** with a weight of ~ 170 kDa.

Next, the first HPLC peaks from reactions **2** and **3** were brought to the MADLI-MS. **2** saw increase of ~10 kDa over the control, equivalent to about 7 molecules of B_{12}, and **3** saw an increase of ~4 kDa, roughly equivalent to 3 B_{12} molecules.

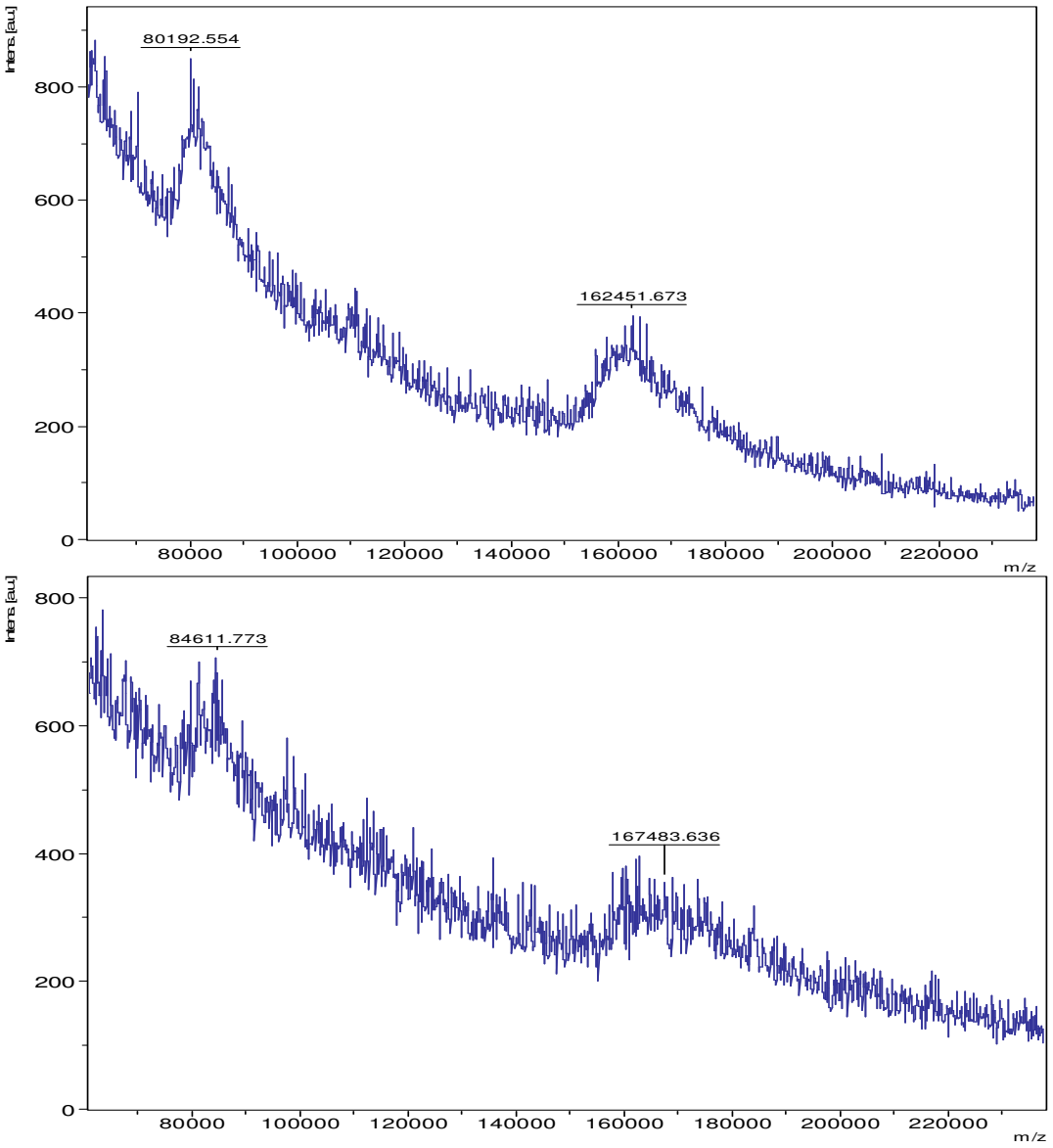


Figure 3. Mass spectrometry of **3** (top) and **2** (bottom) showing a molecular weight of ~ 162 kDa, and ~ 167 kDa respectively.

It appears that the B_{12}-TT load limit is being reached in **1**, since varying the amount of B_{12} from 1, 4, and 20mg causes a ratio increase of only 3, 7, and 8 B_{12} molecules to each TT.

In order to gain a better understanding of the location and number of the B_{12}-TT linkages, a trypsin digest of the conjugate **3** was done and MALDI was

used to obtain the fragment sizes. Using the known TT sequence and known cut sites, the compound was compared to the theoretical, though this comparison was made difficult by the cross-linking of formalin inactivation. All detected peaks that were near to theoretical peaks were examined for a weight increase of theoretical fragment plus B_{12}. Eleven of the peaks collected fit this analysis (see table 2).

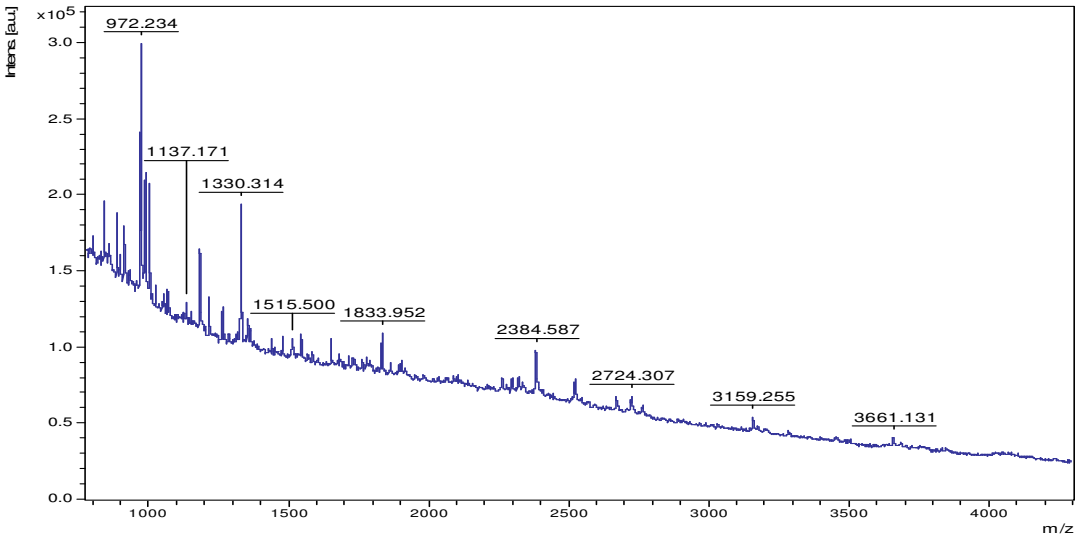


Figure 4. Mass spectrum of a trypsin digest of **3**.

Table 2. Molecular weights, position and sequence of suspected fragments of conjugation. (Weight of peptide only)

Molecule Weight (Da)	Position	Amino Acid Sequence
882	405-411	DLKSEYKGQNMR
882	86-92	FLQTMVK
940	1007-1014	FNAYLANK
1003	721-727	RSYQMYR
1143	99-109	NNVAGEALLDK
1291	1211-1222	VGYNAPGIPLYK
1382	334-345	DSNGQYIVNEDK
1384	97-109	IKNNVAGEALLDK
1777.9	417-433	VNTNAFRNVDGSGLVSK
2069.9	602-619	DIIDDFTNESSQKTTIDK
3007	560-584	ITMTNSVDDALINSTKIYSYFPSVISK

All of the above fragments contain a lysine but for one that contains two arginine residues. Though lysine is a stronger nucleophile and therefore the expected target of CDT coupling, arginine is still a nucleophile for possible binding. The missing possible conjugation sites could be involved in cross liking, or it could be that certain fragments were unable to ionize and were therefore not seen, or that some fragments were not solvent exposed in such a way that enabled binding.

Since **3** has a calculated B_{12}-TT ratio of 3:1, the appearance of 11 binding sites in the tryspin digest indicates that the sample is a collection of polymorphs. This means that there is unavoidable variation in each sample, but also that the B_{12} binding is less likely to block the same antigenic epitopes every time, perhaps making the vaccine more effective.

6.2 *In Vitro* Studies

In order to test cellular uptake, the human placenta choriocarcinoma BeWo cell line was used because past literature suggests that it expresses the cubilin receptor.[19, 20] Confocal microscopy studies showed colocalization of fluorescently tagged **IF*** and **B_{12}-TT*** inside the cell.

First, immunostaining studies were done with fluorescently tagged cubilin antibodies in order to ensure that the cells did indeed express the cubilin receptor. CHO cells, which are known to not express the cubilin receptor, were used as a control. This successfully showed the presence of cubilin receptors on the cell line, enabling B_{12} uptake studies. (see figure 5)

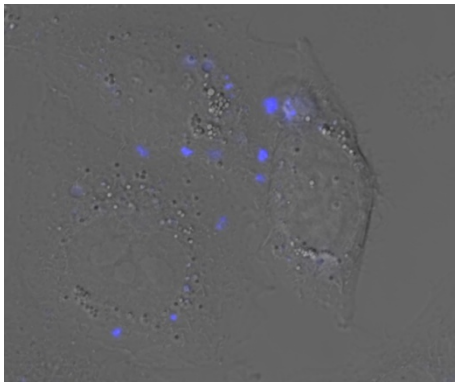


Figure 5. 63X image of BeWo Cells and fluorescently tagged Cub-antibody using confocal microscopy.

Secondly, the **B_{12}-TT*** was incubated with **IF*** for 30 minutes, after which the **B_{12}-TT*-IF*** was incubated with the BeWo cells for 6 hours. The cells were then rinsed with PBS at a pH of 7.4 and then with PBS at a pH of 3.0, in order to remove the compound and break up any nonspecific interactions. The plate was then filled with PBS at pH of 7.4 and taken for confocal. Using optical slices, the two fluorescent signals were found to be colocalized inside the cell. A further study with **B_{12}-TT*-IF** also showed internalization of the compound by itself as well.

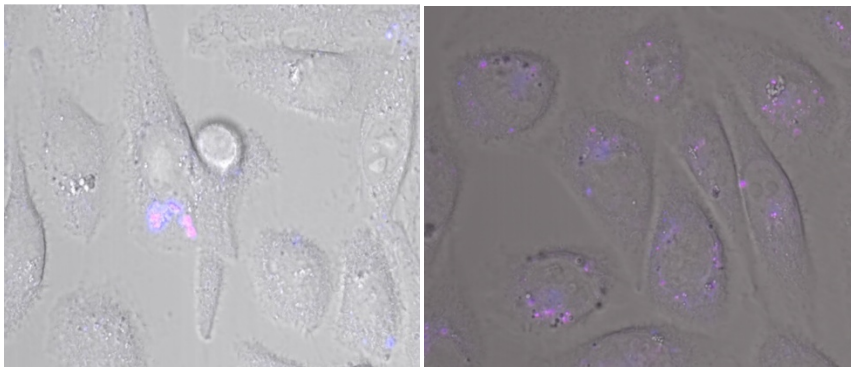


Figure 6. 63X images of BeWo cells and internalized **IF*** and **1*** (left) and **3*** (right).

Colocalization with **1*-IF*** shows that the blue (IF*) surrounds the red (1*) like a halo, consistent with what would be expected from multiple B_{12}s, while the run with **3*** show colocalizaiton but not a halo, which would be expected from the smaller number of B_{12} molecules to attach to the **IF***. This is important because it shows that uptake across the cell membrane occurs even for conjugates with a lower vitamin:vaccine ratio.

As another control, IF and **TT*** were incubated together and then with the cells in the same concentration as the conjugate runs, but showed no uptake. Some external membrane interaction was seen with **TT***, which is as expected, since the heavy chain of the TT seeks the membrane and facilitates uptake.

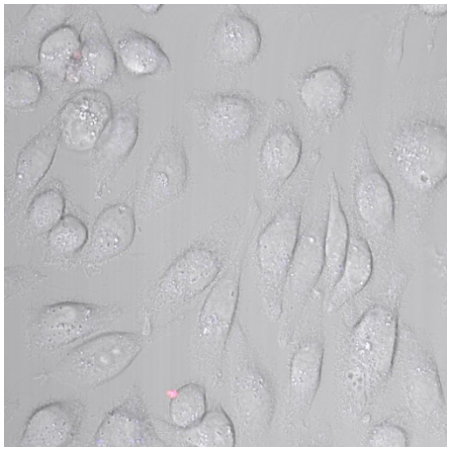


Figure 7. BeWo cells with **TT***. No internalization was observed, only some minor surface interaction, seen in red.

In order to understand the activity of the conjugate, two tests were simultaneously employed to determine the activity and the amount of vaccine in each conjugate, and to relate these two data. Using the TT starting material, a Bradford control test was run, showing a ratio of ~0.32 $L_f/\mu L$. Before ELISA, all

three samples were concentrated or diluted to the same concentration of protein. The stock TT had an activity of 0.32, much higher than the samples, perhaps because the B_{12} blocks activity or because the purification process was not efficient enough.

Table 3. $L_f/\mu g$ ratio as determined by ELISA and Bradford assays.

Compound	Crude (1)	**1**	**2**	**3**
$L_f/\mu g$	0.00022	0.0053	0.0073	0.0031

6.3 *In Vivo* Studies

The *in vivo* studies on guinea pigs were done by Serum Institute in India, as part of the grant provided for this project. Due to this, a shipping method was created: dissolve concentrated solution in a 1% sucrolose and 1% mannose solution and begin lyophilization. Once it is complete, keep refrigerated at all times.

The guinea pigs were put on a low B_{12} diet and then administered PBS, TT, or the compound, either orally or through subcutaneous injection. Blood samples were taken from the animals at 28 days in order to run a titer ELISA for TT IgG antibodies.

Table 4. IgG titer concentrations from guinea pigs, determined by ELISA.			
Experimental group	Dose/animal on day 0	Route of administration	Day 28 Mean IU/mL ± SD
PBS	1 mL	Oral	0.004 ± 0.001
PBS	1 mL	s.c	0.004 ± 0.001
Tetanus	1 L_f	s.c.	2.13 ± 1.4
1	1 L_f	s.c.	0.004 ± 0.001
Tetanus	8 L_f	Oral	0.004 ± 0.001
1	8 L_f	Oral	0.003 ± 0.005

These results suggest that conjugation is causing the TT to lose activity, especially when considering the *in vivo* data that supports the idea that uptake is occurring across the enterocyte. Both the confocal colocalization and the ELISA drop in activity agree with this. The test was also done with **2** and **3**, with similar results.

7.0 Conclusions

Seeing titer increase from the subcutaneous injection of TT but not B_{12}-TT, and knowing the confocal data showed uptake across the *in vitro* cells, the strongest conclusion from this experiment is that there is most likely cellular uptake but not enough antigenicity to confer immunity. The immune system probably cannot reach the TT though all of the steric bulk of the B_{12} and its add-ons.

The remarkable thing about this experiment is that cellular uptake *in vitro* with TT is 5 times larger than the previously known largest molecule carried by B_{12}, EPO (29 kDa).[21]

8.0 Future Work

The future of this project is quite straightforward: attempt to make a 1:1 conjugate in order to address the inability of the B_{12}-TT to confer immunity even as a subcutaneous injection. Also, more reliable and more efficient purification is necessary for any substantial work to be done in the field.

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