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Experimental design to optimize an *Haemophilus influenzae* type b conjugate vaccine made with hydrazide-derivatized tetanus toxoid

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Abstract The introduction of type b *Haemophilus influenzae* conjugate vaccines into routine vaccination schedules has significantly reduced the burden of this disease; however, widespread use in developing countries is constrained by vaccine costs, and there is a need for a simple and high-yielding manufacturing process. The vaccine is composed of purified capsular polysaccharide conjugated to an immunogenic carrier protein. To improve the yield and rate of the reductive amination conjugation reaction used to make this vaccine, some of the carboxyl groups of the carrier protein, tetanus toxoid, were modified to hydrazides, which are more reactive than the ε -amine of lysine. Other reaction parameters, including the ratio of the reactants, the size of the polysaccharide, the temperature and the salt concentration, were also investigated. Experimental design was used to minimize the number of experiments required to optimize all these parameters to obtain conjugate in high yield with target characteristics. It was found that increasing the reactant ratio and decreasing the size of the polysaccharide increased the polysaccharide:protein mass ratio in the product. Temperature and salt concentration did not improve this ratio. These results are consistent with a diffusion controlled rate limiting step in the conjugation reaction. Excessive modification of tetanus toxoid with hydrazide was correlated with reduced yield and lower free polysaccharide. This was attributed to a greater tendency for precipitation, possibly due to changes in the isoelectric point. Experimental design and multiple regression helped identify key parameters to control and thereby optimize this conjugation reaction.

Keywords *Haemophilus influenzae* type b · Hib · Conjugate vaccine · Experimental design · Tetanus toxoid · Hydrazide · Diffusion controlled reaction

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Introduction

Haemophilus influenzae type b (Hib) is an encapsulated bacterium that can cause pneumonia, sepsis and meningitis in infants less than 1 year age [1]. The introduction of Hib conjugate vaccines into routine vaccination schedules has significantly reduced the burden of Hib-related disease, however, widespread use in developing countries is constrained by vaccine costs and there is a need for a simple and high-yielding Hib manufacturing process. Despite the GAVI Alliance and the Hib Initiative resulting in improved vaccination, only 45% of the world's children were fully immunized with Hib vaccine by the end of 2009 [2].

The Hib capsule is composed of repeating units of polyribosylribitol phosphate (PRP) and antibodies to it are protective by bactericidal and opsonic mechanisms. The

vaccine is made by conjugating PRP to a protein carrier that increases the antibody response by bringing in T-cell help to B-cells which respond only partially to polysaccharides [1]. The PRP in the vaccine is either native polysaccharide or size-reduced fragments generated by acid or periodate depolymerization [1].

Several different conjugation chemistries have been employed to manufacture commercial Hib vaccines. These include reactions using carbodiimides and cyanogen bromide [3], nucleophilic substitution of a bromide by a thiol [4], addition of a thiol to maleimide [5], and reductive amination [6]. The reductive amination method is based upon the reduction of the imine that forms between an aldehyde (in a periodate-oxidized polysaccharide) and an amine (of lysines in the protein carrier). Periodate oxidation was employed in this process as it achieves both depolymerization and activation of PRP in a single step. Even though imine formation is a slow reaction with unfavorable equilibrium, this method has gained widespread use, being the method of choice for a Hib vaccine, two meningococcal C vaccines and a pneumococcal conjugate vaccine.

To improve this reaction, a variation was developed by derivatizing carboxyl groups in the protein carrier to acid hydrazides. These react with aldehydes more rapidly and completely than the ε-amines of lysine [7–10]. This method was chosen to develop a new Hib conjugate vaccine at The Biovac Institute in South Africa.

Several physico-chemical properties of the conjugate vaccine must be controlled to ensure optimal immunogenicity. The most important properties for Hib conjugates are the ratio of polysaccharide to protein and the amount of non-conjugated, or free, polysaccharide (Free-PS).

The mass ratio of PRP to protein has been as low as 0.06:1 with acceptable immunogenicity [1, 3]. On the other hand, Anderson *et al*. reported that the polysaccharide to protein mass ratio had a greater influence on immunogenicity than polysaccharide size [11]. The reported ratio for the commercial Hib vaccine made by reductive amination is 0.4 [1], and this was chosen as a target for manufacturing purposes using the hydrazide-derivatized protein.

The presence of Free-PS in the conjugate vaccine has been shown to decrease the antibody response in animal models [12, unpublished observations made by CL]; however, it is unclear if this is true in human immunization. The WHO Technical Report Series #897 does not specify limits for the Free-PS content for Hib vaccines as one vaccine had up to 40% Free-PS [13], but this vaccine was never licensed for use in children under age 2 since it failed to demonstrate significant protection against invasive disease in a large efficacy study in Alaska [1]. It is generally believed that Free-PS limits should be below 15–20% and remain within this range during the entire shelf life of the product.

Size has also been considered an important factor in immunogenicity of some polysaccharide conjugates [11, 14, 15]. PRP conjugates have been prepared from low molar-mass polysaccharide fragments as these have narrow, reproducible molar mass distributions [16]. It was shown that conjugates made with 20 repeat units (RU) were more immunogenic than 8 RU in infants [15]. The 8 RU PRP had a greater number of sugar chains per protein molecule (3.3 *vs* 2.1), but a lower relative mass ratio (0.16 *vs* 0.24). Later studies indicated that the mass ratio was the major factor controlling immunogenicity [11].

In order to achieve the desired characteristics, the chemical conjugation requires the optimization of many variables, such as concentrations of the reagents, temperature, pH, time, and degree of activation of the polysaccharide and protein. A statistical analysis of all the data can help to identify trends not apparent with multiple variables.

We decided to use experimental design and regression analysis to identify the important factors in optimizing this conjugation chemistry.

Materials and methods

All experiments were performed at The Biovac Institute, Cape Town, South Africa. Bulk tetanus toxoid (TT) with antigen content 2,000 Lf/ml and ≥1,500 Lf/mg PN (flocculating units per mg protein nitrogen) was purchased from BioFarma, Indonesia. MES hydrate (99.5%) was purchased from Sigma-Aldrich, and EDC (*N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride, 99.0%) from Fluka. All other reagents were purchased from SAARCHEM. Diafiltration was performed using an Amicon Ultra-15 Centrifugal Filter Device equipped with an Ultracel 10 kDa MWCO membrane (Millipore Corporation, Bedford, MA). Size exclusion chromatography (SEC-HPLC) was performed on an Agilent 1200 Series HPLC with Waters Ultrahydrogel 1,000 and 500 columns using 0.2 M $NaNO_3$+ 0.01 M Na_2HPO_4 pH 7.0 mobile phase. Signals were detected with Agilent Refractive Index Monitor and Diode Array Detector. The system was calibrated using Pullulan Standards (Shodex P-82, Anatech), and sodium azide (Sigma Aldrich) for the total volume.

Proper safety procedures were followed when handling toxic compounds.

PRP preparation

PRP was purified from fermentation supernatant. Briefly, clinical isolates of Hib were sourced from National Institute of Communicable Diseases, Johannesburg, South Africa, and evaluated for PRP production in Frantz and CY media. Fermentation was performed at a 20 L scale using modified

soy-peptone and yeast-extract (MP) medium [17] at 37°C, pH 7.2 with 15 g/L glucose addition, aeration (1.8 L/min) and stirring (300 rpm). After 15 h the OD_{550} was in the range of 8–9, and PRP concentration was 800 to 1,200 mg/L. Purification using a single step CTAB precipitation with Celite 545 was performed as described by Livyens [18]. PRP purity was demonstrated by NMR, SEC and UV for protein and DNA content. Ribose content was determined by Bial method for pentose [19], and the phosphorus content by Chen's method [20].

De-polymerization and activation of PRP

PRP was depolymerized by oxidation with periodate, which simultaneously introduced terminal aldehydes (see Fig. 1 for an overview of the conjugation chemistry). PRP was dissolved in water (5 mg/ml) and cooled to 4°C with an ice bath. Sodium periodate (2.5 mM aqueous solution) was added and allowed to react in the dark for 20 min. The desired molecular weight of PRP was controlled by the amount of periodate added since the reaction is quantitative. The reaction was quenched with ethylene glycol, then diafiltered against 10 mM NaCl, then water with a Pellicon XL 10 K filter. Ion-exchange chromatography was used to demonstrate that only PRP oligomers of <10 repeat units passed through this membrane. The molecular weight distribution was determined by SEC-HPLC. Aldehyde content was determined by micro BCA test calibrated with ribose [16], and this was used to estimate the average number of repeat units (ARU).

Activation of TT

Bulk TT was diafiltered against MES buffer, pH 6.0 using a 30 K MWCO membrane before being concentrated. Hydrazine was incorporated into the protein by dehydration reaction (see Fig. 1). Hydrazine (5 M, 15,000 mol excess) and EDC (1 M, 700 mol excess) were added and the solution stirred for 4 h at 20°C. After quenching with NaOH (1 M), the solution was diafiltered (x4) against carbonate buffer pH 10. Each filtrate was collected and analyzed for the presence of residual hydrazine and the final retentate was analyzed for protein concentration (determined by UV using the extinction coefficient at 280 nm of 185,210 M^{-1} cm^{-1}) and hydrazide concentration by the selective use of absorbances at different wavelengths

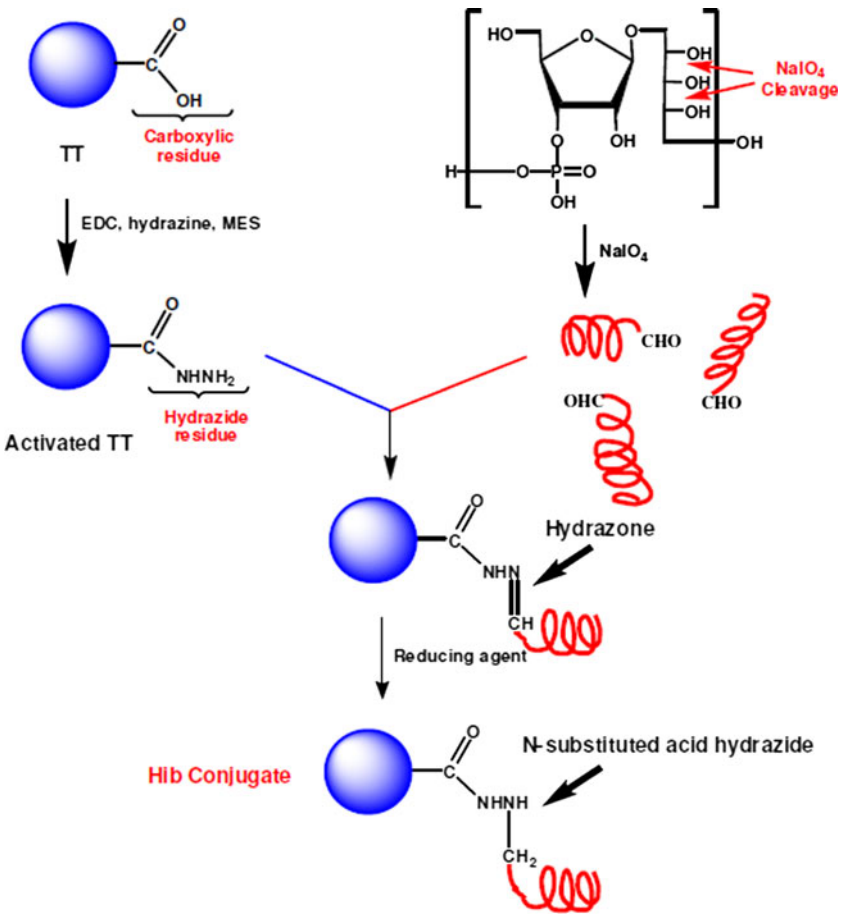

Fig. 1 Chemistry of the conjugation reaction of hydrazide-activated TT with periodate oxidized PRP

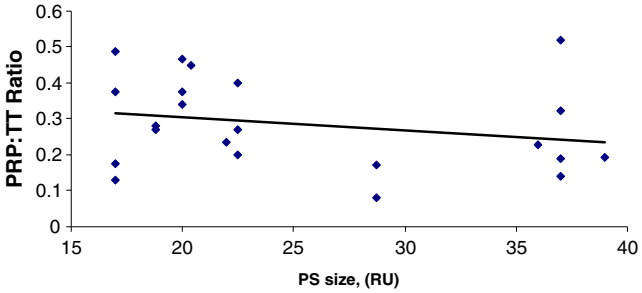

Fig. 2 Mass ratio of PRP to TT in product as a function of the PRP size

for hydrazides and primary amino groups using the TNBS assay [21].The percentage of carboxyl groups modified with hydrazine was calculated (%CS) by dividing the number of hydrazides by 160, the number of carboxyl groups in TT [22]. The degree of modification was controlled by the reaction time. The hydrazine activated TT (TT-H) was stored at pH 10.5 at 2–8°C to prevent precipitation until use.

Conjugation reaction

Conjugation of the aldehyde-containing polysaccharide with TT-H occurs with the subsequent formation of hydrazones upon mixing. This bond can be further stabilized by reduction with sodium borohydride. A summary of the reactions are shown in Fig. 1.

Reactions were performed on a 100 mg scale in a volume of approximately 10 ml. Briefly, activated PRP and TT-H were mixed and the pH was adjusted to 6.8 using 0.1 N HCl. The progress of the reaction was monitored by SEC-HPLC to observe the disappearance of the starting materials and the appearance of high molecular weight conjugate. When the reaction was complete; *i.e.*; no further change in the HPLC profile, usually within 24 h, the mixture was treated with $NaBH_4$ to reduce unreacted aldehydes and to further stabilize the hydrazones. After precipitation with ammonium sulphate at 40–60% saturation, the solid was dissolved in PBS and diafiltered against 7–10 X volumes of the same buffer using a 100 K MWCO regenerated cellulose membrane.

Characterization of the conjugate

Polysaccharide and protein content were determined as described above. Calculations were performed to determine the yield of polysaccharide and protein, and the polysaccharide to protein ratio in the final product (PRP:TT). The amount of Free-PS in the product was determined by SEC-HPLC and the deoxycholate precipitation method [23].

Statistical methods and experimental design

Experimental design was utilized in order to optimize the conjugation reaction, whereby statistical tools are used to minimize the number of experiments required to perform a systematic exploration of the key parameters that influence a process [24, 25]. For a more complete explanation of the mathematical principles of Experimental Design, the reader is referred to reference 24. After preliminary experiments, it was found that four parameters were expected to have an influence on the conjugation reaction: the ratio of polysaccharide to protein in the reaction mixture (PS:Protein), the ARU of the polysaccharide, the temperature and the salt concentration.

The experimental design was created using the software Essential Regression [25], which is an add-on to Microsoft® Excel, and uses menus to select the parameters and statistical methods. Sequential testing of 4 different parameters at 2 levels for each parameter would require a total of 16 experiments. This was reduced to only 8 experiments using a fractional factorial design. Non-linear interactions were not included.

The experimental design is summarized in Table 1. The high and low limits of the parameters to be tested are shown. Two additional centre-points in experiments 5 and 7 are repeats used to determine the variance. The actual values for the parameters in the experiments are reported in Table 2.

In addition to the experimental design, all available experimental data were pooled and analyzed to find the best fit model using the *Autofit* tool of Essential Regression software. This tool automatically tests each independent variable in a *stepwise regression* by adding a new variable

Table 1 Experimental design of the PRP TT-H conjugation reaction

Exp. #	PS size ARU	Reaction mass ratio PS:Pro.	Temp. °C	[NaCl] %
1	15	0.5	40	10
2	40	0.5	40	1
3	40	0.5	20	10
4	15	2	20	10
5	27.5	1.25	30	5.5
6	15	2	40	1
7	27.5	1.25	30	5.5
8	15	0.5	20	1
9	40	2	40	10
10	40	2	20	1

Fractional Factorial Design, Resolution 4,

2 Centerpoints (Experiments 5, 7)

Linear Model with 5 terms,

Response = b0 + b1 × ARU + b2 × PS : Pro + b3 × Temp. + b4× Salt%

Table 2 Actual experimental conditions and results of yield and product composition

Exp. #	PS size ARU	Reaction mass ratio PS:Pro.	Temp. °C	[NaCl] %	TT recovery %	PRP recovery %	Product mass composition PRP:TT ratio
1	17	0.5	40	9.9	80	25	0.16
2	37	0.5	40	1	56	20	0.13
3	36	0.5	20	10	10	10	0.07
4	17	2	20	10	75	13	0.4
5	28.7	1.25	30	5.6	64	8	0.13
6	17	2	40	1	72	16	0.42
7	28.7	1.25	30	5.6	69	5	0.18
8	17	0.5	20	10.3	74	18	0.19
9	36	2	40	10	68	3	0.09
10	37	2	28	1	43	9	0.4

and then reevaluating the variables already in the model using the partial F-statistic. When necessary, one of the variables is then removed and the model is evaluated again by the F-statistic. The software facilitates this for the non-mathematician, but it is important to be able to interpret the statistical analysis. A variety of statistical calculations are available in Essential Regression to assess the goodness of fit [25]. The software tests the linear regression coefficient using a t-Statistic to determine if it differs significantly from 0. The t-Statistic is used to calculate probability and is reported as the p value. The coefficient of multiple determination, R^2 is calculated as an indication of the goodness of fit. $R_{adjusted}^2$ takes into consideration the number of independent variables in the model, and indicates the model may be *overfitted* if it differs greatly from R^2. Finally, graphic representation of the data provides a direct visual way to judge the quality of the fit.

Results

Experimental design

The experimental design used to optimize the PRP to TT ratio of the conjugate (PRP:TT) is shown in Table 1. The overall yield of PRP and TT were also important output measurements for decision making on process. Free-PS was not measured in these experiments, and so it was not a primary endpoint.

The actual reaction conditions and the measured conjugate characteristics are shown in Table 2. Superficial analysis of the data does not reveal any obvious trends; this is because the sequence of experiments is randomized to avoid unintended bias, as might occur in a sequential optimization.

The mathematical model of this data obtained by multiple regression analysis of the experimental design is reported in Table 3.

The ratio PRP:TT in the final product was significantly correlated with the PS:Protein ratio in the reaction mixture (p=0.054) and inversely correlated with the ARU of the polysaccharide (p=0.018). The coefficient of determination was R^2=0.762, indicating that the model was a relatively good fit. To put these coefficients in context, this indicates that a smaller ARU will result in a higher PS:TT ratio. For example, a decrease of the ARU from 37 to 17 ARU would increase the PRP:TT ratio by 0.18. In comparison, doubling the PS:Protein ratio in the reaction mixture from 1:1 to 2:1 would increase the PRP:TT ratio of the product by 0.09.

Surprisingly, the yield of TT was inversely correlated with the ARU. This correlation was borderline significant (p=0.064), and the coefficient of determination indicated this relationship was not strong (R^2=0.409). The yield of

Table 3 Regression statistics of experimental design. Significant correlations were identified from the data in Table 2. Non-significant parameters were eliminated from the equations

Regression equation	R^2	R^2 adjusted	Probability coefficient=0		
			1st Coef.	2nd Coef.	3rd Coef.
$PRP : TT = 0.33 - 0.009 \times ARU + 0.088 \times PS : Pro.$	0.762	0.682	0.013	0.018	0.054
$TT\_\%Recovery = 101.8 - 1.49 \times ARU$	0.409	0.324	<0.001	0.064	

TT was calculated using the UV extinction coefficient described in the Methods without correction for changes due to glycosylation, so there maybe a bias in these results.

The yield of polysaccharide was not significantly correlated with any factor and salt concentration and temperature were not significantly correlated with yield or ratio.

Cumulative analysis

To confirm the results of the experimental design, and to look for any other correlations, the data from 22 previously performed experiments were combined with the above data, and a regression of the output measurements of ratio, Free-PS and yield against all independent variables was undertaken using the *Autofit* tool of Essential Regression. (See Methods for an explanation of *Autofit*). The results are presented in Table 4.

As found above, the ratio of PRP:TT in the product was significantly correlated with the PS:Protein ratio in the reaction mixture, and inversely correlated with the ARU. In addition, the *Autofit* found an inverse correlation with salt concentration. The coefficient of determination for this analysis was R^2=0.527, and the adjusted R^2=0. 448, which indicated a weaker relationship than found above, and the possibility that the model was over-parameterized.

The observation above from the experimental design that TT yield was inversely correlated with ARU was not reproduced using the complete dataset. Since its significance was borderline and not reproducible, we did not consider it to be important.

Graphic representations of the PRP:TT ratio *versus* each of the significant independent variables are shown in Figs. 2, 3 and 4. These graphs show the magnitude of the relationships and the degree of scatter of the experimental points. In contrast to what was observed above, the effect of ARU was as strong as the ratio of the reaction mixture. That is, either decreasing of the ARU from 37 to 17 ARU or doubling the PS:Protein ratio in the reaction would increase the PRP:TT ratio of the conjugate by 0.12.

The appearance of the salt effect might be due to a bias, since many of the previous experiments were performed without salt and so there might not a random distribution as required for correlation analysis. This is especially visible in Fig. 4. On the other hand, a plot of the residuals *versus* the expected values showed only a slight deviation from a straight line (not shown). This indicated that the error was approximately normal and thus the regression method was applicable. In any case, addition of salt did not improve the PRP:TT ratio in the product.

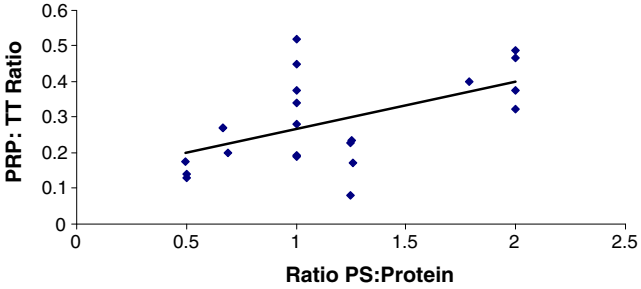

Fig. 3 Mass ratio of PRP to TT in product as a function of the mass ratio of the reactants

It was found that Free-PS was correlated with PS:Protein ratio in the reaction mixture, and inversely correlated with number of hydrazides in the TT (%CS). The coefficient of determination was R^2=0.438, which was less than the regressions described above, but the relationship with %CS was highly significant (p=0.006). This can be seen graphically in Figs. 5 and 6. The Free-PS was measured after purification; thus, additional factors in the purification process could have influenced these results. These correlations followed expected trends.

The yield of conjugate was found to be correlated with the time of the reaction, and inversely correlated to the degree of activation of the protein, as shown in Table 4. The coefficient of determination was R^2=0.527, and the coefficient for %CS was highly significant (p=<0.001). While it is expected that increasing the time of the reaction would increase the yield of conjugate, it was unexpected that the increased activation of TT-H (*i.e.*: increasing the number of hydrazides on TT) would result in lower yields. As shown graphically in Fig. 7, the %CS appeared to be one of the strongest effects for any of the correlations.

The reduced yields at higher %CS suggested a loss of material. The appearance of precipitate was noted frequently

Table 4 Regression statistics from cumulative data including the experimental design and 22 previous experiments. Non-significant parameters were eliminated from the equations

Regression equation	R^2	R^2 adjusted	Probability coefficient=0			
			1st Coef.	2nd Coef.	3rd Coef.	4th Coef.
$PRP : TT = 0.319 + 0.125 \times PS : Pro. - 1.548 \times Salt\% - 0.006 \times ARU$	0.527	0.448	0.002	0.006	0.017	0.044
$\%Free\text{-}PS = 28.62 - 0.875 \times \%CS + 5.913 \times PS : Pro.$	0.438	0.379	0.001	0.006	0.024	
$Yield\_conjugate = 105.07 - 3.276 \times \%CS + 7.196 \times time$	0.527	0.477	<0.001	<0.001	0.020	

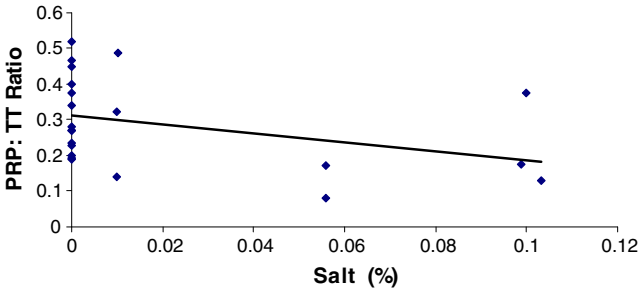

Fig. 4 Mass ratio of PRP to TT in product as a function of salt concentration

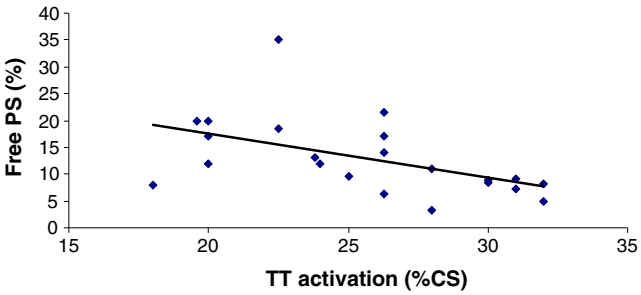

Fig. 6 Unconjugated polysaccharide in the product as a function of the degree of activation of the protein as measured by the number of hydrazides per TT

during the reaction, especially while adjusting pH, and also in some cases during the reaction and work up. At higher %CS, loss of material due to protein precipitation was considered significant and in some cases the conjugates had to be abandoned due to extensive and irreversible precipitation.

Discussion

The objective of this study was to optimize the reaction conditions for the preparation of a Hib conjugate vaccine by a novel conjugation reaction using periodate-oxidized polysaccharide and hydrazide-derivatized protein. The intention was to create a vaccine with a PRP:TT ratio of 0.4 while at the same time maximizing the yield of both PRP and TT and minimizing Free-PS content.

Two overlapping sets of data were analyzed by multiple regression. One set of data was obtained by experimental design. The other set of data was obtained by modifying reaction conditions sequentially.

Unpublished laboratory lore said that decreasing the ARU would increase the PRP:TT mass ratio in the product, although this was considered counter-intuitive since lower molecular weight polymers have lower mass. Therefore, an experimental design was created to address this issue specifically. It was also believed that the negative charge of the PRP phosphates would cause electrostatic repulsion, and thus prevent additional PRP chains from joining to a TT-H molecule once one or several PRP chains were already attached. Increased salt concentration was thought to be able to neutralize some of the negative charge, and thus increase the mass ratio in the product. Finally, both heat and reactant concentration influence bimolecular reaction rates, and would be expected to increase yield and mass ratio of the product.

The experimental design was created using software freely available on the internet. Although this software is not validated, it uses statistical functions from Microsoft® Excel, and was sufficient for our purposes.

The data analysis showed that reactant ratio, ARU and % salt were significant factors in determining the final PRP:TT ratio. As expected from basic principles of organic synthesis, the PS:TT ratio of the product can be increased by increasing the ratio in the reaction mixture. Oddly, this increase does not significantly decrease the yield of PRP (p=0.15), but it is clear that the higher the ratio in the reaction mixture, the lower the yield of PS.

The data indicated that reducing ARU will increase the PS:TT ratio in the product. This was true for both the experimental design and the complete data set. This correlation was stronger in the experimental design than the entire set of data, but with the additional variable of salt concentrating in the latter.

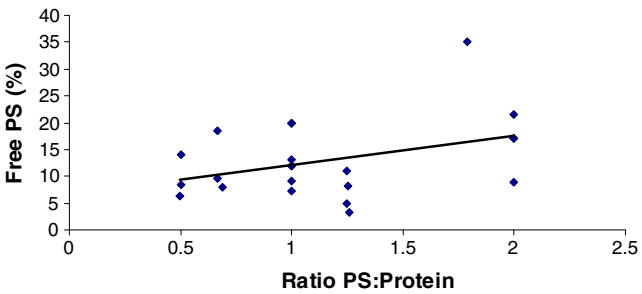

Fig. 5 Unconjugated polysaccharide in the product as a function of the mass ratio of the reactants

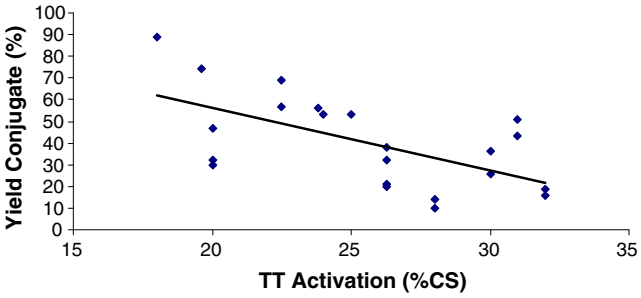

Fig. 7 Yield of the product as a function of the degree of activation of the protein as measured by the percentage of modified carboxyl groups per tetanus toxoid molecule

These relationships are giving important clues about the reaction mechanism and the rate limiting steps. At the atomic scale, we can envision the formation of the hydrazone by organic chemistry mechanism. The nucleophilic hydrazide attacks the carbonyl carbon. After protonation of the resulting alcohol, water must then act as a leaving group to form the hydrazone. Indeed, the reaction must be performed at pH 6.8 to ensure a source of protons. If hydrazone formation were the rate limiting step in the conjugation reaction, we would expect that increasing the reactant concentration and temperature would increase the reaction rate. But that is not the case. Doubling the ratio of PS to Protein in the reaction mixture only increases the ratio in the product by 0.09 or 0.12 (see Results). Furthermore, temperature did not affect any of the measured variables. It appears that the reaction rate is not controlled by the hydrazone formation. To find the rate limiting step we must look at the macromolecular level.

In a review of macromolecular interactions, Berg and von Hippel showed that the probability of encountering another macromolecule in a diffusion controlled process is proportional to the radius of gyration of the macromolecule [26]. In addition, if only one site of a macromolecule is reactive, then the reaction rate is proportional to the fraction of area of the reacting surface compared to the entire surface of the macromolecule. Even in rod-like molecule, such as PRP [27], this will be approximately inversely proportional to the square of the radius of gyration. Taken together with the diffusion rate, the reactivity of a macromolecule with a single reactive site will be proportional to the inverse of the radius.

These macromolecular considerations explain several of the observations in this study. First, temperature was not significantly correlated with any measured variable. Interestingly, Berg and von Hippel point out that diffusion controlled reactions have weak temperature dependence because diffusion is controlled by solvent viscosity, which tends to have weak temperature dependence [26].

Second, increasing the size of the PRP decreased the PRP:TT mass ratio of the product. If we assume that the radius of gyration will be approximately proportional to the ARU of PRP, then the reaction rate should be inversely proportional to ARU. Indeed, as shown in Table 5, using the inverse of ARU as an independent variable improves the correlation slightly to R^2=0.561 and the significance of the ARU^{-1} coefficient is P=0.02. Salt concentration and PS:protein ratio remain significant variables.

Third, electrostatic repulsion of the negatively charged PRP polymer must also be considered. Roy and Laferriere found that reductive amination conjugation of negatively charged sialic acid monomers to TT was limited to 16 per TT molecule. However, when the negative charge of the carboxyl was eliminated by using the methyl ester, up to 32 monomers per TT could be attached [28]. Attempts to neutralize the electrostatic effect using different salts including $CaCl_2$ were not effective (unpublished observations). Similar results were observed here. Addition of up to 10% NaCl did not improve incorporation of the PRP into the conjugate. In fact, when the entire data set is considered, salt significantly decreased the PRP:TT ratio of the product. If this effect is real, there could be several explanations. The first is a viscosity effect. Increased ionic strength may increase viscosity due to the formation of bridged structures or fibres. However, Hennessey reported that PRP was a rigid rod, and that there was little potential for tertiary structure [27]. Another possibility is that increased salt concentration might create an electrostatic double layer around the negative charges, which could create barriers to polymer diffusion [29].

These characteristics consistently point to a diffusion controlled process.

The degree of activation of the protein was found to be inversely correlated with Free-PS content and yield of conjugate. That is, the greater the number of hydrazide moieties incorporated into TT-H, the lower the Free-PS and the lower the conjugate yield. While we might expect the former result, the latter was more difficult to explain.

Increasing the number of reactive sites on TT should improve reactivity according to the macromolecular considerations discussed above. More reactive sites would increase the probability of an interaction with the terminal aldehyde of activated PRP, and thus decrease Free-PS. We would also expect to find a higher PS:TT ratio, but this was not observed. This led us to suspect that there was another explanation.

The decrease in conjugate yield indicated a loss of protein. Furthermore, we had noticed precipitation in highly

Table 5 Regression statistics using the inverse of the polysaccharide size (ARU^{-1}) as a parameter

Regression equation	R^2	R^2 adjusted	Probability coefficient=0			
			1st Coef.	2nd Coef.	3rd Coef.	4th Coef.
$PRP : TT = -0.015 + 4.480 \times ARU^{-1} - 1.724 \times Salt\% + 0.123 \times PS : Pro.$	0.561	0.488	0.865	0.020	0.009	0.005

activated TT-H. To counteract this, the pH of the activated TT-H had to be kept at 10.5 or higher. This is due to a change in the isoelectric point of TT, which is normally between 6.2 and 6.5. We estimated the isoelectric point increased to approximately 8.6 for 17% CS and 9.1 for 27% CS TT-H based on a simple charge calculation of the amino acid sequence in which the carboxyl containing amino acids were changed to neutral amino acids [30]. At pH 6.8, in which the conjugation reaction occurs, 27% CS TT-H would have approximately +35 charge. This could cause aggregation with the negatively charged PRP, which might result in precipitation, thus reducing both TT yield and Free-PS. However, further experiments have not been performed to confirm or reject this hypothetical mechanism.

In conclusion, we have explored the reaction conditions required to conjugate periodate-oxidized PRP with hydrazide-activated TT. The target was to prepare a conjugate vaccine with a 0.4 ratio of PRP:TT. This could be achieved by increasing the PRP:TT-H ratio of the reaction mixture and by reducing the size of the PRP polysaccharide to <20 repeat units. Temperature did not affect this ratio, and salt was found to be detrimental. These results indicated that diffusion was the rate limiting step in the conjugation reaction. This confirms that the rate limit in reductive amination conjugation is overcome by using the hydrazone conjugation chemistry employed here.

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