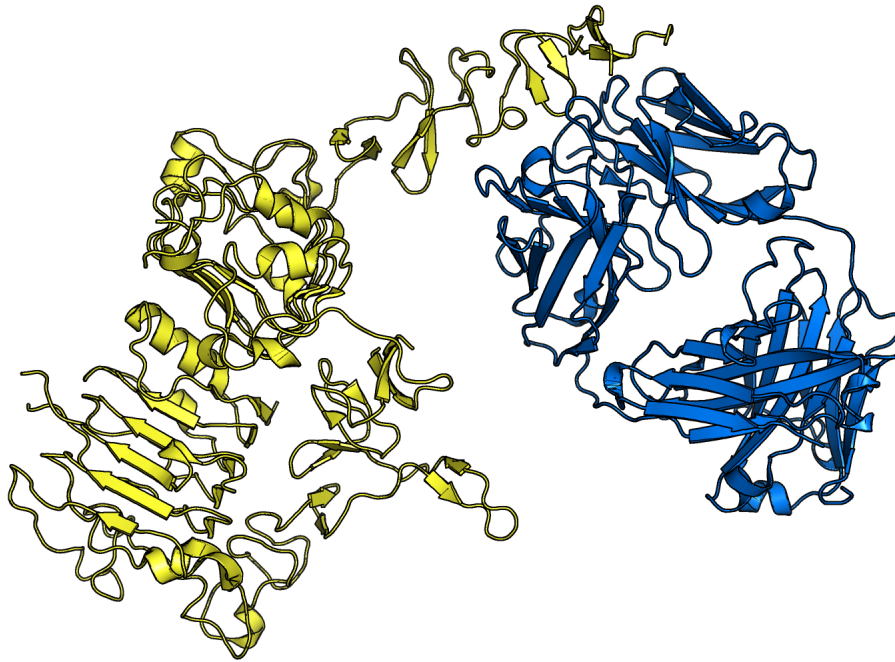




CHALMERS
UNIVERSITY OF TECHNOLOGY



Production of an Avidin Conjugated Antibody Pretargeting Agent

Master's thesis in Nuclear Engineering

JOHN APELMAN

MASTER'S THESIS 2015:05

**Development and Evaluation of
Methods for Producing a
Radioimmunotherapy Pretargeting Agent**

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CHALMERS
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Department of Chemistry and Chemical Engineering
Division of Energy and Materials
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2015

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Development and evaluation of methods for producing a radioimmunotherapy pretargeting agent

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Abstract

Antibodies can be used to target receptors that are over-expressed on certain types of cancer. By using radiolabeled antibodies cytotoxic radiation can be delivered to cancerous cells while minimizing the dose absorbed by healthy tissue. When administering a pretargeting agent and radionuclide separately, radionuclides with shorter half life can be used. In this thesis work the production of the pretargeting agent is investigated. By using the pH dependent interaction between iminobiotin and avidin a possible new production route is examined. The project did not generate a novel working production process but several of the necessary production steps were investigated and can act as a basis for future work.

The iminobiotin-agarose column performed very well outside the manufacturers stated pH interval. This enables some of the pH dependent reaction steps to be performed in the iminobiotin column. The iminobiotin column could advantageously be used to separate avidin conjugated molecules from molecules not containing avidin.

Fast protein liquid chromatography (FPLC) system together with a size exclusion column can be used for identifying and to certain extent isolating the product. This however require that the presence of avidin containing complexes are well separated from the conjugate in terms of molecular weight.

The formation of unwanted products can be minimized by optimizing the reaction parameters. When coupling succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) to form a precursor for protein-protein binding the reaction time should be kept to approximately 30 minutes when using an avidin concentration of 20mg/ml. The molar ratio of SMCC should also be kept under five times the amount of avidin. If an antibody, e.g. trastuzumab, is treated with a reducing agent some of the disulfide bridges are split allowing modification on the sulfhydryl groups. Sulfhydryl groups located on different antibodies can react causing the formation of dimers. Low concentration, short reaction times and quenching prevent this formation. The reaction between the reduced sulfhydryl group and a maleimide is dominant at pH below 8.5. At pH above 8.5 the reaction between the maleimide and primary amines are dominant. This reaction will create unwanted complexes and will also interfere with the intended site specificity of the maleimide-sulfhydryl reaction.

Keywords: PRIT, pretargeting agent, avidin, iminobiotin, FPLC, trastuzumab.

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1

Background

1.1 Introduction

Antibodies are large proteins that the immune system uses to cope with bacteria and virus infections. Antibodies can also be designed to bind to certain tumor specific receptors. HER2 is one of the human epidermal growth factor receptors and is over expressed on the surface of certain types of cancer cells. Antibodies can be used to bind to this HER2 receptor to impede further cancer growth but antibodies can also be used as a tumor specific carrier of a radionuclide to the surface of the cancer cell. When using radiolabeled antibodies for cancer therapy the treatment is referred to as radioimmunotherapy (RIT).

One important factor in RIT is the quota between the dose delivered to the tumor and the dose delivered to healthy tissue. One possibility to increase the dose to cancerous tissue is to let the antibody transport the radionuclide to the tumor and attach to it. If the radionuclide is attached to the tumor a radio nuclide with high linear energy transfer, LET, is favourable since the geometry will lead to a good cancerous to normal tissue dose ratio. This is also a good method when targeting several small tumors. Since the antibodies are dispersed in the entire body the number of tumors that can be affected at the same time are very large. This also enables for treatment of microscopic tumors or single cells which are difficult to treat using surgery or external radiation. [1] [2].

Since antibodies are large molecules they diffuse and transport slowly in the body which is a problem especially when the preferred radio nuclides used in RIT have a short half-life. One method to avoid this is to first administer a pretargeting agent, a modified antibody, and allow them to bind to the tumor cells and later administer the smaller effector molecule containing the radionuclide. Unbound pretargeting agents can be cleared from the circulatory system using a clearing agent prior to the injection of effector molecules. The smaller effector molecule are designed to attach to the pretargeting agent located on the surface of the tumor. Since a smaller molecule allow for faster pharmacokinetics a larger fraction of the dose will be delivered to the tumors and to lesser extent to healthy tissue. The method when administering antibodies and radionuclides separate is called pretargeting or pretargeted radioimmunotherapy, PRIT. [1]

Figure 1.1 below shows the principles of pretargeting. First the pretargeting agent (modified antibody) is injected and is left to accumulate on the surface of the tumor.

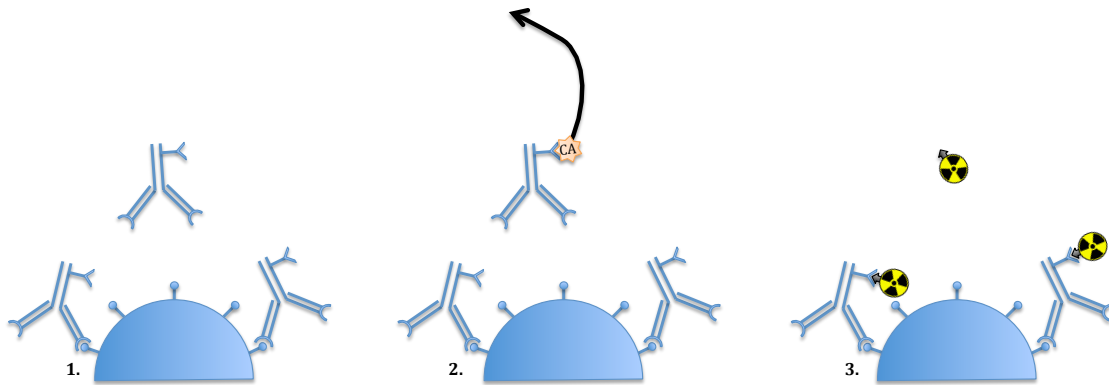


Figure 1.1: Principle of pretargeting shown in three steps. 1, Injection of pretargeting agent. 2, Injection of a clearing agent. 3, Injection of effector molecule containing the radionuclide.

A clearing agent is injected to remove the unbound pretargeting agents. Finally the radionuclide is injected which will attach to the modified antibody and deliver the dose to the tumor.

1.2 Purpose

The purpose of this thesis work is to investigate and optimize the production route of an avidin conjugated antibody that will be used as a pretargeting agent. The method should be possible to scale up to approximately milligram amounts and the yield should be over 30%. When a satisfying yield is reached the pretargeting abilities of that molecule will be investigated *in vitro*.

The yield and purity of the product should be calculated quantitatively. If there is a known and applicable method to perform identification, quantification and isolation of the final product this method will be used. If there is no such method a functional method will be developed to fit the purpose.

There are different methods and strategies when making the pretargeting and effector pair. In this work the pretargeting agent will be based on avidin or streptavidin conjugated antibodies and the effector molecule will be built on a biotinylated polylysine base.

When utilizing the PRIT-method *in vivo* streptavidin is used instead of avidin but due to their chemical similarities and difference in price avidin will be used instead.

2

Theory

2.1 Avidin-biotin bond

Avidin is a protein found in egg-white and exist in a tetrameric and a dimeric form. The bond between avidin and biotin is the strongest non-covalent bond between a ligand and a protein known in nature, $K_a = 10^{15}M^{-1}$. Avidin is most abundant in it's tetrameric form and the molecular weight for the tetramer is approximately 65kDa. Each of the monomers can bind one biotin molecule. It has been shown that when modifying one of three lysine groups on each monomer, the monomer loses the capability to bind biotin at the site of modification. This indicates that the avidin-binding site utilizes all three lysine groups on each monomer to bind one biotin molecule [5].

Just like the bond between biotin and avidin the iminobiotin can be tightly bound to avidin. However the disassociation constant for the avidin-iminobiotin bond is pH dependent. At higher pH, 9.5-11, avidin will bind but when lowering the pH to 4 the avidin-iminobiotin bond will disassociate. Both the binding and the disassociation require a low ionic strength, approximately 50mM, which increases the probability for avidin conjugates to remain intact when being bound and eluted in a column packed with immobilized iminobiotin.

2.2 Fast Protein Liquid Chromatography

Fast Protein Liquid Chromatography, FPLC, is a GE system for analysis and preparation of proteins. By utilizing size exclusion columns it separates proteins and other molecules by size, i.e. molar mass. Smaller molecules are forced to travel a longer path through the column where the larger molecules takes a straighter path and therefore have a higher longitudinal velocity through the column. When entering the column all the molecules are mixed in a solution. When eluting the column the different molecules are separated by size. The absorbance at 280nm is measured directly after the column and this enables molecules with different molar weight to be identified.

In some spectroscopy methods the absorbance of the sample is measured and compared to a blank sample. Apart from the investigated chemical the blank is identical to the tested sample and by calibration the absorbance can be related to a quantitative unit, e.g. mg. When using FPLC the buffer solution containing the

injected chemical might differ from the buffer solution used as a mobile phase in the size exclusion column. When the different buffer solutions mix the extinction coefficient for the background solution changes and make the preparation of a blank sample impossible.

The FPLC does not measure the difference in absorbance between two solutions but how the extinction coefficient of the mobile phase changes over time. Before injecting the sample the entire size exclusion column, tubes, injection valve, on-line filter, pumps and detector have to be equilibrated with the the chosen mobile phase buffer solution. After equilibration the absorbance is set to zero and any differences in absorbance originates from the sample and possibly the injected sample buffer.

2.3 Functional groups

When producing a conjugated protein a cross-linking intermediate reagent is generally required. One suitable cross-linker for antibody-protein binding is Succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, SMCC.

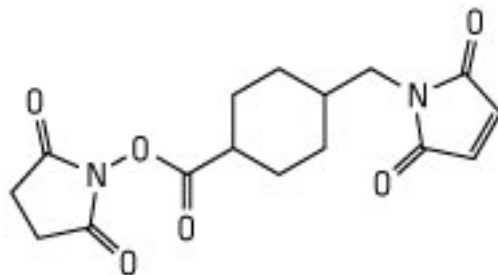


Figure 2.1: SMCC molecule and it's functional groups, the NHS-ester on the left and the maleimide group on the right. [6]

The SMCC consist of a spacer arm connecting a N-hydroxysuccinimide, NHS, ester in one end and a maleimide group in the other end. The NHS-ester is reactive towards amino groups such as primary amines, which for example can be found on the amino acid lysine on the protein. When biotin reacts with avidin it also binds to, or in the proximity of, the lysine groups. This implies that SMCC might compete with biotin when binding to a single binding sites on each monomer. [8]

The maleimide group is reactive towards sulfhydryl groups. By reducing the disulfide bond located at the hinge region of the antibody the reaction between the maleimide and the antibody should in theory become site specific. The maleimide group has affinity towards sulfhydryl groups in the pH range 6.5-7.5 but over pH 8 the reaction between the maleimide and primary amines are favoured. Since avidin contains a substantial amount of lysine which contain a primary amine the maleimide group will be reactive towards the protein and not the antibody in pH exceeding 8. [8]

3

Method

3.1 Material

An Äkta Purifier FPLC System from GE Healthcare Life Sciences was used together with a GE Superdex 200 10/300 GL column. The SMCC was bought from Thermo Scientific. The DL-Dithiothreitol (DTT), the avidin and the 2-aminobiotin-agarose used in the aminobiotin-agarose column was bought from Sigma-Aldrich. The trastuzumab, trade name Herceptin, was obtained from the Swedish medical product agency.

3.2 Conjugation

A new avidin antibody conjugation method based on a previously published report was investigated [3] The general idea of the method was to perform the conjugation in the aminobiotin column. By dispersing and suspending the avidin in the aminobiotin column the pretargeting agent can be built up in steps by attaching one component at a time until the pretargeting agent is obtained and can be eluted from the column.

First the avidin and the NHS-ester group on SMCC were reacted in a pH8 borate buffer solution. Unreacted SMCC and NHS groups were removed using a size exclusion column. The disulfide bond on the antibody was reduced using DTT in phosphate buffered saline, PBS. Excess DTT was removed using a size exclusion column. SMCC-avidin was put on the pH 11 borate buffer equilibrated IBC for the avidin and aminobiotin to form a strong bond. The reduced antibody was then put on the column to react the maleimide groups on avidin-SMCC and the reduced sulfhydryl groups on the antibody. After the conjugation excess antibodies was rinsed out using pH 11 borate buffer and the final product could be eluted using pH 4 acetate buffer. By using an excess of antibody the amount of unreacted avidin in the final product can be kept at a minimum and the yield could be fairly good compared to the approximately 30% found in the literature [3].

As the described method did not work adequately a more iterative approach was adopted. When the vital steps in the process were investigated and predictable the actual method of producing the conjugate could be refined. The refinement would continue as long as the time frame allowed.

The iterative method above resulted in the following process. First the avidin was modified with SMCC; 20 μ l of avidin, 20mg/ml, 20 μ l 50mM pH 8 borate buffer and 20 μ l SMCC, 2mg/ml in DMSO and 60 μ l PBS were reacted for 30 minutes under agitation. The molar ratio was 20:1 for SMCC:avidin. Then the antibody was reduced; 200 μ l, 8mg/ml trastuzumab, 4mg DTT and 50 μ l nitrogen sparged PBS were reacted for 30 minutes. The reduced trastuzumab was purified using a NAP-5 size exclusion column to remove the unreacted DTT and the reduced antibody where added to the avidin-SMCC solution. The molar ratio was 1.9:1 for trastuzumab:avidin. The solution was incubated for two days. After incubation the product was isolated using the IBC as described in Section 3.7 and analyzed using the FPLC system.

3.3 Radiolabeling

In this study the Iodogen method was used for radioiodination. The Iodogen is a chlorine based oxidizing agent that oxidizes radioiodine, e.g. $^{125}\text{I}^-$ to $^{125}\text{I}^+$. The $^{125}\text{I}^+$ can substitute the hydrogen atom on the hydroxyl group located on the phenolic group of the tyrosine amino acid [4]. Attempts to radiolabel both proteins i.e. the avidin and antibody were made.

A known amount of ^{125}I was put into a 1,3,4,6-tetrachloro-3a,6a-diphenyl-glycoluril, Iodogen, coated eppendorf tube. Iodogen is an oxidizing reagent that by oxidizing the iodine incorporate the iodine into the amino acid tyrosine found on the proteins. 2,5 μ l, 3-5 MBq was used for the iodination of avidin and for iodination of antibodies 1 μ l, 9.3MBq was used. Iodination buffer was added to the coated tube, 40 μ l for the labeling of avidin and 25 μ l when labeling antibodies. 200 μ l avidin, 2mg/ml, or 200 μ l antibody, 1mg/ml, was added to the tubes and they were agitated for two minutes. After agitation the labelled immunoconjugate was purified on BSA treated and equilibrated NAP-5 columns. After eluting the columns according to specifications the activity of the column and elute was analyzed.

3.4 Iminobiotin column performance

The column was prepared by adding iminobiotin agarose suspension to an empty column, using column filters to confine the agarose and leaving the suspension to settle to a bed. Since the spatial distribution of iminobiotin agarose in the suspension was uneven the exact amount added to the column was not known. Therefore some experiments were conducted to determine the performance of the column.

The column void is the liquid volume of the settled agarose bed. The void represent the volume that needs to be added to completely change the mobile phase in the column, without having any of the added sample leaving the column. The volume of the void was investigated by adding a solution with avidin and antibody to the column. The minimum plausible void was 1ml and the volume of the solution was 1 ml. The solution was added to an empty column and the liquid leaving the column

when the solution was added where 1ml. The sample eluted from the column was analyzed using the FPLC.

After adding the sample and incubating the column for 30 minutes the column was rinsed using pH 11 binding buffer and the rinse liquid was analyzed.

To test the elution performance of the column an experiment using ^{125}I radiolabeled antibodies were performed. Avidin conjugated and radiolabeled antibodies was bound to the column and the unbound molecules were washed out using pH 11 buffer. After rinsing the column was eluted using 2.5ml pH 4 acetate buffer solution.

An experiment to test the iminobiotin column properties at different pH was also performed. The column was equilibrated in pH 9 and 1mg avidin in 250 μl pH 9 buffer was added to the column and allowed to bind to the iminobiotin for 10 minutes. 1ml aliquots were then added to the column and the eluted 1ml fractions were collected and analyzed using the FPLC. The pH of the added buffer was lowered in steps from pH 9, pH 8, pH 7.4 and finally pH 4. For pH 9 and pH 8 borate buffer were used and for pH 7.4 PBS was used. This test was conducted to see if the immobilized iminobiotin could bind the avidin at a pH that was more favourable to the maleimide reaction with the sulfhydryl groups of the antibody. The test would also give an experimental value of the void in the column. The analysis were then made using the FPLC. To reduce the time required for the FPLC to analyze all the samples in this experiment multiple sample injections were made in the same experiment. The flow was set constant to 0.5ml/min and the samples were injected every 4ml. The retention time for avidin is 15ml at that flow rate and the different injections and absorbance responses could be identified and related using the anticipated retention time for avidin.

3.5 Identification of products and reactants

The retention times for the reactants and also the avidin-antibody conjugate had to be determined to be able to use the FPLC for analysis. The antibody, trastuzumab, and the protein, avidin, were put on the column. Either separately or in a mixed sample. By analyzing one sample at a time the retention time for that particular molecule can be determined. By comparing known proteins and their retention times unknown proteins in a sample can be estimated by comparing their relative retention times. To determine the anticipated retention time for the avidin-trastuzumab conjugate a similar conjugate was analyzed. The tested conjugate was a 1F5-streptavidin conjugate and due to it's similarities to the avidin-trastuzumab conjugate the retention times should conform. All experiments were made using the flow rate 0.5ml/min, the mobile phase is PBS and retention times are expressed in the unit [ml].

To determine the resolution of peaks with narrow or similar retention times mathematical calculations were employed. The response in the FPLC UV-detector was modeled using Gaussian curves that super positioned each other forming a

single output containing different components. By constructing these components mathematically and thereby removing uncertainty regarding centroid positioning of each component, a simulated retention time for the overall behaviour of super positioned Gaussian curves were examined.

Retention times for conjugation reaction products were compared to a previously published report [3], and the values were compared to experimentally obtained retention times. The mentioned report used a HPLC size exclusion column to determine the retention times. Since the size exclusion columns used in the FPLC system also utilizes porous beads to separate molecules on size the retention times might be proportional. A comparison between the corresponding retention times will be made to see if they show proportionality by using the quotient

$$\alpha = \frac{\textit{Experimental retention time}}{\textit{Literature value}} \quad (3.1)$$

Avidin, 68kDa, will be compared to streptavidin, 52.8kDa, and trastuzumab, 146kDa, will be compared to a similar antibody, NR-LU-10, 150kDa. Conjugates have the same composition of protein(s) and antibody.

3.6 Product quantification

Experiments to relate the arbitrary unit, AU, to a quantitative value was also performed. Avidin and trastuzumab were added in different amounts and the response in AU was measured. The same experiment was made with avidin only due to a faulty FPLC column. By relating the integrated absorbance and the added amount the extinction coefficient can be calculated using the relation

$$k = \frac{m}{AU * ml} \quad (3.2)$$

where k is the extinction coefficient, m is the total mass of the added chemical and AU*ml is the integrated absorbance response.

The extinction coefficient of the avidin-antibody conjugate could be calculated by knowing the extinction coefficients and molar masses of the avidin and antibody respectively.

3.7 Isolation of the avidin-antibody conjugate

Independent on how the conjugation was made, the final product has to be isolated and purified. Different ways to extract the final product was tested.

When isolating the avidin-antibody conjugate from the reaction mixture the FPLC can be used. By running the solution through the column the different sized molecules in the solution get separated and by collecting at specified time intervals one specific type of molecule can be collected and isolated from the solution. This however require that the peaks are fully resolved.

The IBC can also be used in the process of extracting the avidin conjugate. Avidin can be bound to the column where other molecules or conjugates not containing avidin get rinsed out. A qualitative assessment on the usability of this method was made.

Both the product purity and concentration were examined. Concentration comparisons were made based on the minimum amount of dilutant in the final product. A qualitative assessment regarding the product purity was also made.

3.8 Formation of large complexes

When performing the reaction steps described in Section 3.2 there will be unwanted side reaction products. Two experiments were conducted to determine possible side reaction products.

A 200 μ l sample of trastuzumab in PBS, 8mg/ml, was incubated in room temperature for one hour and analyzed to determine the presence of dimers and polymers. The sample was analyzed using FPLC.

A second experiment was made where avidin in PBS, 20mg/ml, and S-SMCC were incubated for two hours. The molar ratio of avidin and S-SMCC was 1:8. This experiment was made to examine the retention times of the formed complexes. The sample was analyzed using FPLC.

4

Results

This section shows the results from the performed experiments.

4.1 Conjugation

When performing the conjugation using the method described in Section 3.2 the chromatogram showed no absorbance peak at retention time 12ml.

When using the iterative method the FPLC analysis showed molecules with the retention time 12ml. As shown in Section 4.4, 12ml conforms with the retention time of the pretargeting agent.

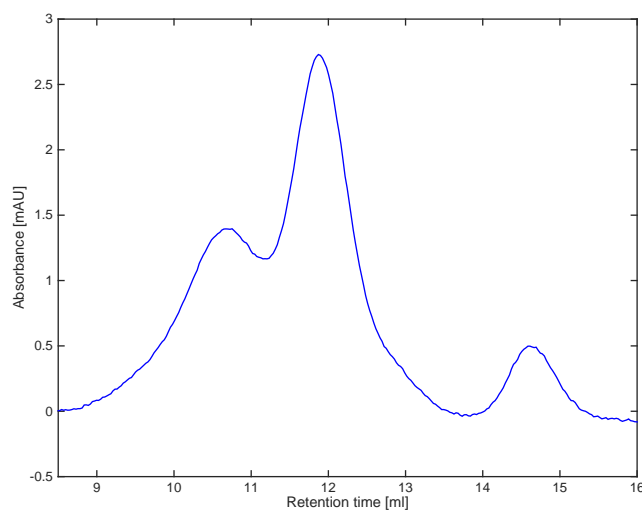


Figure 4.1: Analysis of the crude product mixture using the iterative method described in Section 3.2. The peaks, from left to right, conforms with retention times; heavier complexes, pretargeting agent and unreacted avidin.

The method showed that heavier complexes, which consumes reactant and decreases the yield, were formed but to a lesser extent than the conjugate. As shown in Table 4.2 the retention time for disubstituted conjugates are approximate 10.8ml, which match the predominant peak of the heavier complexes. There are also some unreacted avidin in the sample.

4.2 Radiolabeling

The radiolabeling of the protein avidin did not work by using the iodogen method. This is due to a limited number of tyrosine residues on the protein. Results for labeling of the antibody using iodogen is presented below in Table 4.1.

Table 4.1: Results from radiolabeling trastuzumab with ^{125}I using iodogen.

Sample	Activity [MBq]
Amount of activity used	9.3
Waste, reaction vessel	0
Waste, size exclusion column	4.92
Final product	4.38
Radiochemical yield	47 %
Product specific activity [MBq/ml]	4.87

4.3 Iminobiotin column performance

Figure 4.2 below shows the absorbance plotted versus time for the experiment. Each peak is labelled with the added fluid when the sample were collected. The red circles indicates the position of where the avidin would produce peaks if any avidin was eluted from the column during the experiment. Peaks not coinciding with these positions does not originate from avidin being eluted from the column.

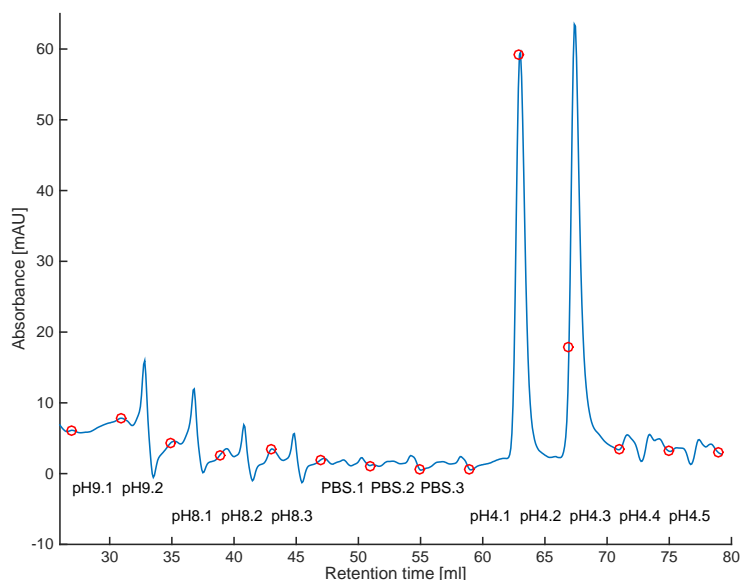


Figure 4.2: Results from column performance experiment. Peaks are labeled with the fluid added to the column. Circles indicates position of the potential avidin peak for each sample.

It should be noted that the retention times are arbitrary in this experiment and was only used to relate the added samples and their respective absorbance responses.

4.4 Identification of products and reactants

This section shows the results from the experiments made to determine the retention time for the investigated molecules. First the experiments are shown and after that a table summarizing the retention times are presented. Finally results from a mathematical experiment is shown, illustrating that the position of the peak can get shifted by super positioning.

Figure 4.3 and Figure 4.4 below shows the results from the experiments made using the FPLC system. All measurements are made using the flow rate of 0.5ml/min and the mobile phase is PBS.

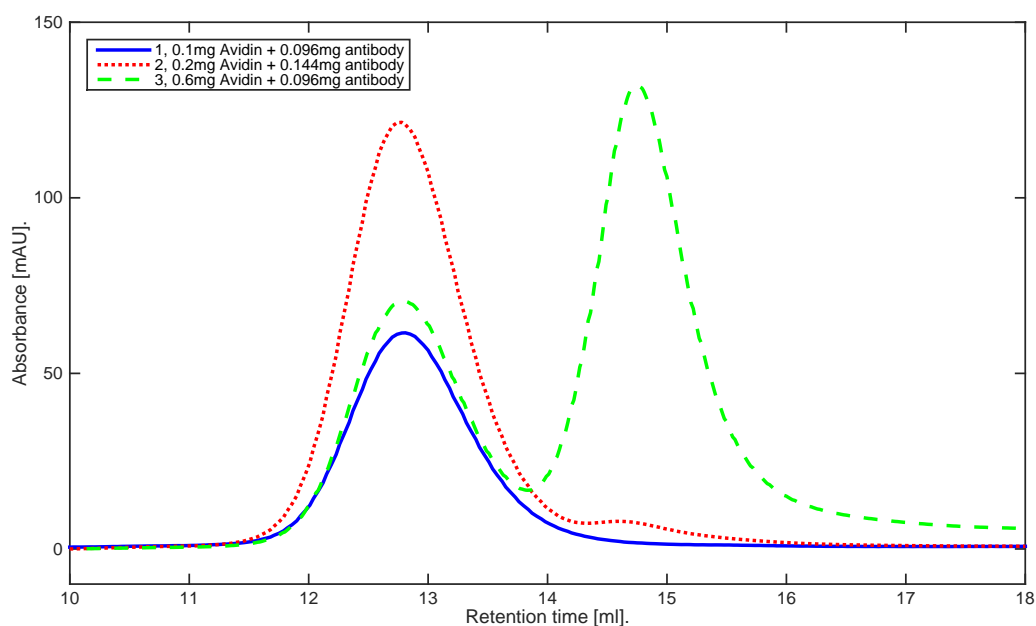


Figure 4.3: FPLC analysis of three samples containing a known composition of avidin and trastuzumab. The peak at 12.8ml contains trastuzumab and the peak at 14.9ml contains avidin.

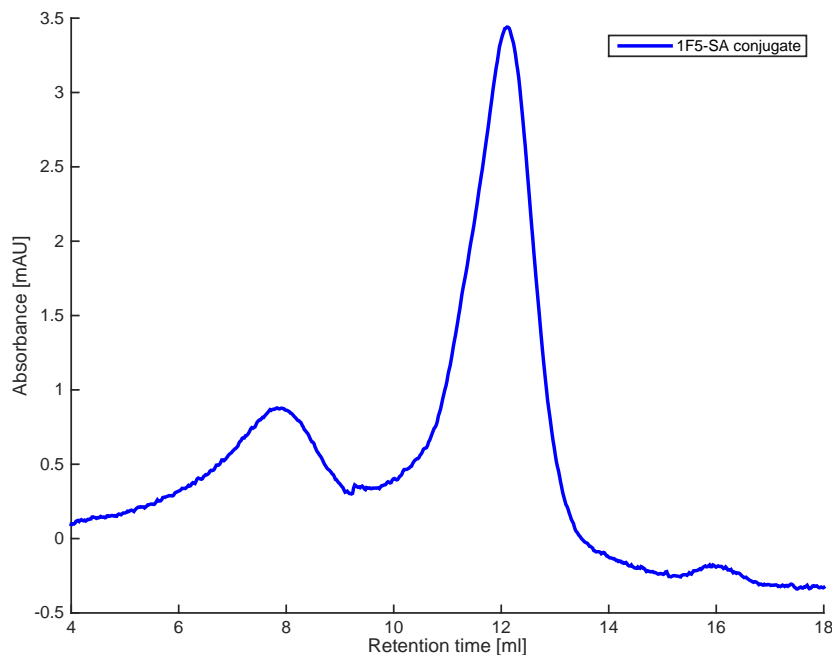


Figure 4.4: FPLC analysis of a 1F5-streptavidin conjugate. The peak at 7.9ml contains large complexes and the peak at 12.0 ml contains the conjugate.

From Figure 4.3 the retention time for avidin and trastuzumab can be determined and from Figure 4.4 the retention time for a conjugate similar to the wanted product is determined. Using this data the remaining SMCC peak was identified by deduction. The identified and confirmed peaks is shown in Table 4.2 below. The retention times might vary depending on the viscosity of the mobile phase and also to uncertainty originating from the variation in flow and pressure. The approximate deviation is ± 0.1 ml.

Table 4.2, column 3, also include retention times from a previously published report [3]. The corresponding retention times are compared to possibly provide approximate retention times for certain molecules using the FPLC system. The comparison is made by using the quotient

$$\alpha = \frac{\textit{Experimental retention time}}{\textit{Literature value}}$$

Since the ratio, α , is nearly constant and lack an obvious trend in the investigated interval the table also include calculated approximate retention times for the FPLC system.

Table 4.2: Confirmed and calculated approximate retention times for investigated molecules using a volumetric flow of 0.5ml/min. Literature values from Hyalarides, Mallett and Meyer, 2001. [3]

Chemical	Retention time [ml]	Literature value	α
Avidin	14.9	10.0	1.49
Antibody (Ab)	12.8	8.4	1.52
Avidin-Ab conjugate	12.0	8.2	1.46
SMCC	20.0	N/A	N/A
Chemical	Aprox ret. time [ml]	Literature value	α
Disubstituted conjugate	10.8	7.6	1.42
Heavier complexes	<10.8	<7.6	1.42

Figure 4.5 shows the results from the mathematical calculations made to determine the effects of overlapping peaks. Component 1 and 2 are simulated absorbance responses and by adding these components the sum is obtained. Y_{max} denotes the peak centroids for Component 1 and the sum.

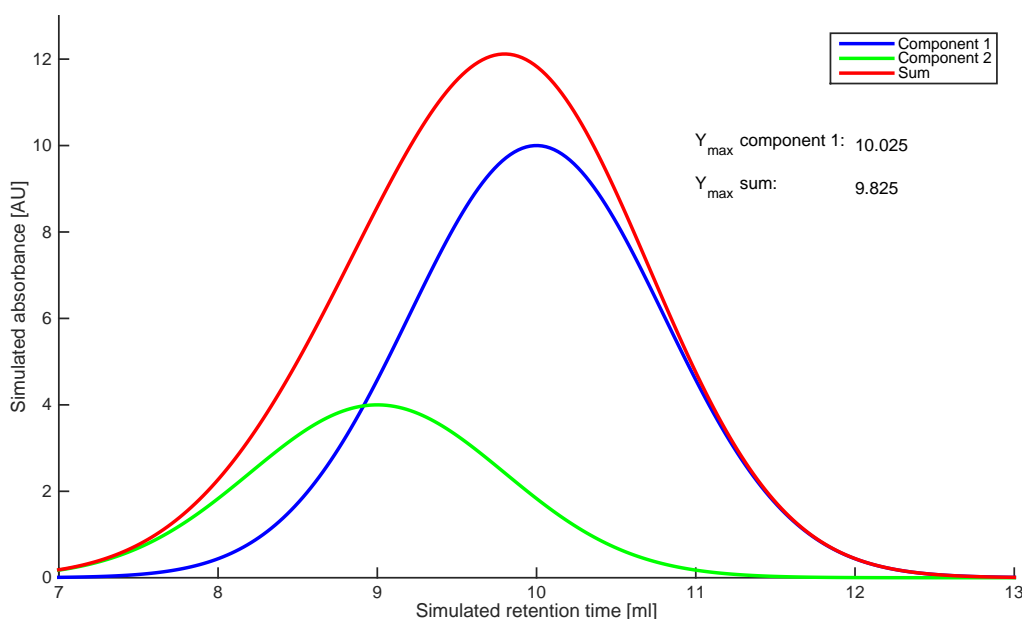


Figure 4.5: Visualization of the effects from not fully resolved peaks.

The summation in Figure 4.5 has a lower Y_{max} , i.e. retention time, than the predominant component. The summation contains two components although the general shape of the curve indicates one component.

4.5 Product quantification

In Figure 4.3 the attempt were to relate the extinction coefficients for both avidin and trastuzumab. The area under the trastuzumab peaks were used to calculate the extinction coefficient for the antibody. However, the peaks representing avidin behaved far from expected. The data from that experiment were to inconclusive to act as basis for determining the extinction coefficient for avidin. Data from the experiment shown in Figure 4.6 below was instead used to calculate the extinction coefficient for avidin.

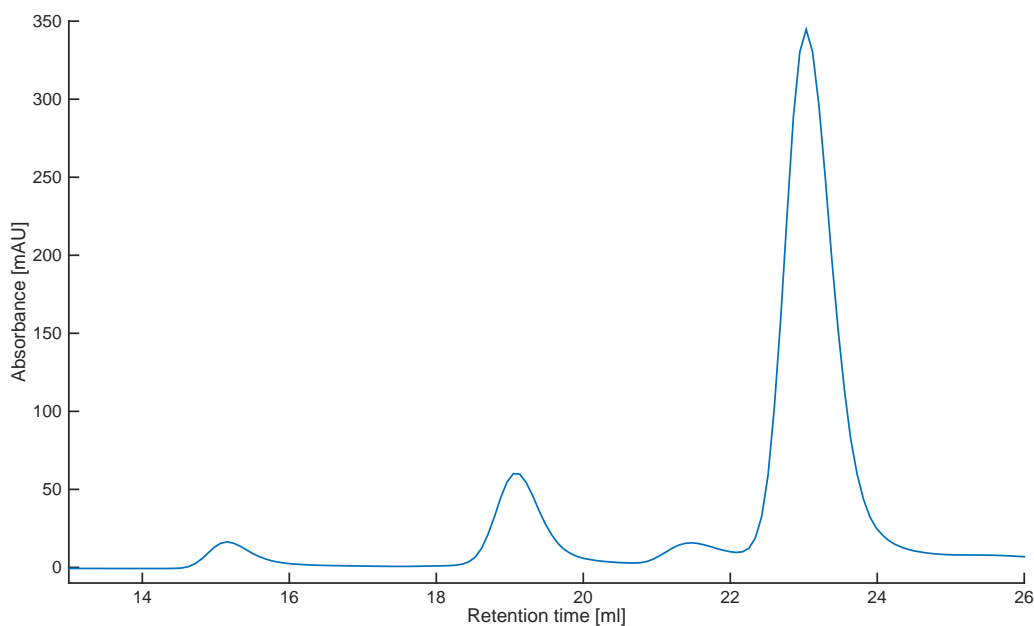


Figure 4.6: Quantification experiment for avidin. From left to right, peaks showing the absorbance response from $40\mu\text{g}$, $100\mu\text{g}$ and $400\mu\text{g}$ avidin.

The coefficients were calculated using Equation 3.2 and are presented in Table 4.3 below.

Table 4.3: Calculated extinction coefficients.

Chemical	Mass [μg]	Area [mAU*ml]	k [$\mu\text{g}/(\text{mAU}*\text{ml})$]
Trastuzumab	96	75.82	1.3
Trastuzumab	96	78.27	1.2
Trastuzumab	144	143.96	1.0
Avidin	40	11.03	3.5
Avidin	100	42.99	2.3
Avidin	400	281.19	1.4

4.6 Isolation of the avidin-antibody conjugate

Regardless of how the conjugation is done the final product have to be isolated from the reaction mixture. By using the experiments of the behaviour of the column together with the void the maximum concentration of the final product is calculated.

The maximum amount of avidin that is possible to attach to the IBC is 1mg/ml of agarose bed. The minimum amount of eluate buffer is equal to the void of the agarose bed and which is approximately 50% of the bed volume. This means that the in the ideal case the maximum concentration of conjugate in the final product will contain 2mg/ml avidin. Using the molar mass for trastuzumab and avidin the monomeric conjugate will have the concentration of

$$\left(\frac{M_{Trastuzumab}}{M_{Avidin}} + 1\right) \cdot m_{Avidin} = 6.55mg/ml.$$

There are however a number of factors that interfere with this ideal case, these will be covered in the discussion section. In Figure 4.1 the sample have undergone the separation method using the IBC. The peak at 12.8ml has virtually disappeared which implies that the unreacted trastuzumab has been removed from the solution. The peak at 12.0ml contains the wanted avidin-antibody conjugate, the peak at 14.7ml is unreacted avidin and the peak at 10.7ml is larger complexes containing avidin and antibody.

Another way to purify the product is to put the solution containing the conjugate on the FPLC column. This method were not tested quantitatively but by using the information of how the absorbance signal in the FPLC is generated together with what the possible products are in the reaction mixture the method can be qualitatively examined. This will also be covered in the discussion section.

4.7 Formation of large complexes

Figure 4.7 below show the formed products. The large peak with centroid at approximately 13ml is the peak form the monomeric antibody. The smaller peak at approximately 10.7ml show an undesired product.

4. Results

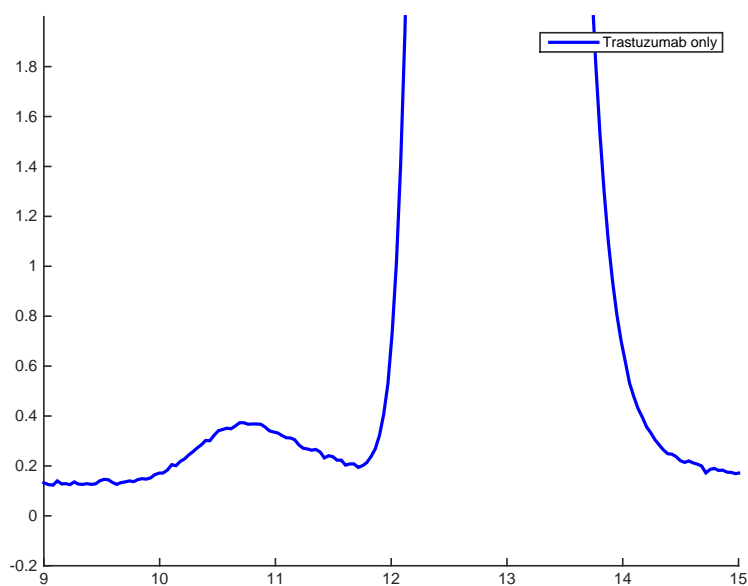


Figure 4.7: Analysis of the reaction where trastuzumab forms dimers. The peak at 13ml contains trastuzumab and the peak at 10.7ml contains large complexes. The curve is cropped to visualize the smaller peak.

In Figure 4.8 three peaks are visible. One at 15ml, one at 13.2ml and one at 12.1ml. The curve is heavily cropped to accentuate the smaller peaks and 12.1ml and 13.2ml.

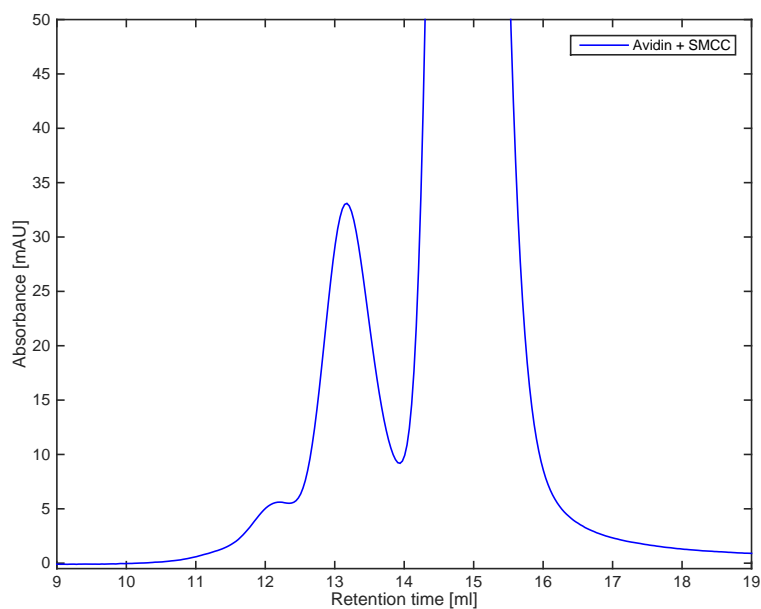


Figure 4.8: Analysis of the reaction between avidin and S-SMCC. The peak at 15ml contains unreacted avidin. The curve is cropped to visualize the smaller peaks.

5

Discussion

5.1 Conjugation

When performing the method described in section 3.2 no conjugate could be detected. As described in Section 2.1 SMCC and iminobiotin might compete for the same binding sites on the protein. When reacting the SMCC and protein prior to binding the protein to the IBC it might bind between zero and four SMCC on each tetramer. The avidin with no bound SMCC will bind to the IBC but will not be able to form a pretargeting agent. The avidin saturated with four SMCC will not be able to bind to the iminobiotin and will be rinsed out of the column. In both these cases the protein is prevented to form a pretargeting agent. The avidin with between one and three SMCC will have the reaction kinetics impaired due to sub-optimal conditions. The proteins that bind easily to the IBC will not be optimal for binding antibodies to form pretargeting agents, and vice versa.

The reaction SMCC-antibody where performed at pH 11. At this pH the maleimide group have affinity against primary amines and not towards the intended sulfhydryl group located on the antibody. This allow for protein-protein bond as well as non site specific reactions between protein and antibody.

When the sulfhydryl groups are introduced to pH 11 in the IBC the reaction rate for the oxidation back to disulfide bonds are increased [9]. When the sulfhydryl groups located on the antibodies are oxidized they become unreactive towards SMCC and can also form dimers when thiol groups on two different antibodies are coupled. Both these reactions will prevent the formation of pretargeting agents.

5.2 Radiolabeling

The iodogen method was used for labeling the antibody and avidin. When labelling the protein with radioiodine using the iodogen method one hydrogen on the phenolic group on tyrosine get substituted with the radioactive iodine ion. However, avidin only contain 0.9% tyrosine and due to its tertiary structure these can in some way be obstructed and not accessible in an water solution. Therefore, attempts to iodinate avidin failed. The radiolabeling of the antibody showed a yield of approximately 47% and considering the reaction time and simplicity the iodogen method is good for labelling trastuzumab.

5.3 Iminobiotin column performance

When comparing the iminobiotin agarose pH properties in the protocol supplied from the manufacturer to the experiments testing the behaviour there was some discrepancies. Avidin was bound and remained bound in a lower pH than specified. Since some of the reactions necessary for the conjugation require a pH that's lower than recommended according to the manufacturers protocol this finding is positive. It should be noted that iminobiotins ability to remain bound is less sensitive to lower pH than the ability to bind to the protein. Consequently the pH can be lowered after binding of avidin. This is particularly important when reacting the maleimide group to the reduced sulfhydryl groups situated in the hinge region on the antibody. The maleimide groups affinity towards primary amine groups increases drastically in the pH increases above approximately pH9 [7].

From Figure 4.2 it can be seen that the void of the iminobiotin column is approximately 1.5ml. Since the avidin started eluate after adding the second ml of pH 4 buffer solution one can determine that the void is more than 1ml. The fact that the entire amount of avidin is eluted in the second and third added ml, and that the amount is almost equally divided between the two aquilotes indicates that the void is approximately 1.5ml. This is under the assumption that the reaction is not instantaneous but fast enough that the reaction kinetics is not a limiting factor.

5.4 Identification and quantification

Quantification of the isolated product was challenging. The plan for quantifying the final product was to first investigate avidin and trastuzumab and relate their area of the absorbance curves to an added amount, as described in Section 3.5. After the conjugation reaction the amount of unreacted avidin and trastuzumab could be measured and the amount of avidin-antibody conjugate could be calculated. The calculation however should be considered as an approximation since some of the formed heavier complexes containing an unknown amount of avidin molecules may be present.

The relation between the arbitrary unit, AU, and the added mass of the specific chemical turned out to not being linear in the investigated range. One way to be able to use the FPLC for quantifications is to determine in what range the measurements are expected and make a linearization in that region with several measurements. This will create an approximate relation but the deviation between the estimated coefficient and the true coefficient can be made very small by doing more measurements in the actual region. By creating more data points in the relevant region the approximated coefficient will converge towards the real value.

When there are more than one type of molecule with similar retention times the absorbances get added, possibly in a not linear way. As can be seen in the example in Figure 4.5 several different proteins can make up a single peak and depending

of the relative amounts of the parts, the visible peak can look very much like a single protein peak. Not only does the single protein peak have ability to hide other structures with similar retention time, the retention time for the peak get shifted up or down depending on the retention time of the interfering structure, e.g. protein, conjugated protein etc. This effect combined with the fact that the FPLC system provides an element of uncertainty can make identification of all contained structures virtually impossible from a single experiment.

5.5 Isolation of the avidin-antibody conjugate

The two methods of isolating the final conjugate was to use the IBC and also to use the FPLC for the separation. The method utilizing the IBC had a very high reproducibility and the method is easily scalable to fit the amount. The method however does not separate the conjugates containing avidin. Regardless on how the conjugation is made there will always be some undesired products in the reaction mixture containing avidin. This can be unreacted avidin, dimers containing one antibody and two avidin molecules, molecules containing one avidin and two antibodies or even larger complexes containing one or more avidin molecules. All of these products will bind to the column and will be eluted together with the wanted conjugate, as can be seen in Figure 4.1

If the two methods would be used in sequence the IBC would first remove almost all of the trastuzumab. By using the FPLC and separating by size the sample could be collected at a precise interval, i.e between 11.2ml and 13ml. This method would remove most of the undesired molecules as well as the unreacted avidin and unreacted antibodies. Depending on the product requirements the interval can be altered and the purity can be traded for a higher yield.

5.6 Formation of large complexes

Figure 4.7 shows two distinct peaks. The peak with the retention time 13ml is the unreacted trastuzumab. The smaller peak at 10.8 is something that is substantially heavier than the approximately 150kDa of trastuzumab. It is likely that it is a formed dimer. Using only the oxygen that dissolves in the sample for one hour was enough to form dimers of antibodies. One way to form dimers is to reduce the sulfhydryl groups on the antibodies and form bond between these reduced groups. Although there might be more reaction paths to form heavier complexes this is a very plausible path. The fact that dimers were formed without using any stronger reducing agent than naturally dissolved oxygen indicates that the formation might be much larger when using a reducing agent.

The reaction between avidin and S-SMCC showed three distinct peaks, see Figure 4.8. The peak at 15ml is unreacted avidin but the other two are heavier complexes formed by only avidin and S-SMCC. Since the NHS-ester group on S-SMCC have affinity for primary amines this is the wanted reaction. The maleimide group on S-SMCC however have affinity for sulfhydryl groups but also for primary amine groups.

The affinity for amine groups increases with pH. The fact that these complexes are formed at the relatively low pH 7.4 indicates that in pH above 8.5 the amine directing reaction might be substantial. Another risk with the maleimide group reacting with amine groups is that since the antibody also contain amine groups the reaction is no longer site specific and the pretargeting agent might be ineffective although the molecular weight are consistent with a functional agent. Since the FPLC almost exclusively distinguish depending on molar mass these inactive pretargeting agents cannot be isolated and removed from the solution. The results of this is that the yield of functional pretargeting agents will be over estimated.

6

Conclusions

The method using iodogen coated tubes worked very well for radiolabeling trastuzumab with ^{125}I . The yield at 47% was expected and acceptable. This method did not work for labelling avidin due to the limited number of tyrosine.

The possibility to perform the conjugation inside the iminobiotin column seems possible. More work concerning molar ratios and the reaction kinetics of the avidin-iminobiotin reaction at different pH has to be done. It is proven that the rate of iminobiotin-avidin disassociation is acceptably slow even at pH as low as 7.4. The rate of which the avidin binds to the column is very slow in that pH which implies that the binding of the protein should be made in a much higher pH. The pH can later be lowered to a level that is beneficial for the actual reaction performed. The column performed very predictable and both the binding and elution of avidin worked very well as long as the correct pH buffers were used. The column could be used several times and did not require any maintenance other than using pH neutral storage buffers.

Since biotin might compete with the NHS-ester group on the SMCC a large abundance of SMCC used can inhibit the binding of avidin to the IBC. This complicates the isolation of the final product and will also lower the yield significantly.

The maleimide group is reactive towards primary amines at pH over 8.5 and avidin complexes is formed. This can also interfere with the intended maleimide-sulfhydryl reaction and it's site specificity. This effect is however not significant in lower pH and reasonable reaction times.

The Äkta FPLC system performed well after a proper sample injecting technique had been adopted and a uncontaminated column was used. If the column was properly maintained it performed well and predictable. It should be noted that the width of the peaks grow when the amount increases, however the height grows much faster than the width. This means that when using small amounts the effect from the injection band broadening becomes very significant and the peaks might become indistinguishable. When using larger amounts the peaks separate better and the product purity becomes higher when using the FPLC for product isolation. The purity can be increased since it is mainly the band broadening and not the amount of chemical that determines the width of the peak. If the peak width is maintained and the area under the peak is increased a larger fraction of the mass will be concentrated near the centroid. If the concentration of the injected sample

is increased, and everything else is unchanged the product purity will be increased.

The iminobiotin agarose column was used to remove unreacted antibodies from an avidin antibody reaction mixture. The method works almost perfectly and isolate avidin conjugates from conjugates not containing avidin. By using the iminobiotin column followed by the FPLC most of the reactants and unwanted products can be removed from the solution.

The reaction where trastuzumab forms larger complexes takes place even without a reducing agent. When the sulfhydryl groups on the antibody is reduced the rate of formed complexes should increase. This means that the conjugation reaction must be quenched to prevent conjugates from forming larger complexes.

6.1 Future work

As discussed in the introduction the purpose of the pretargeting molecules is to bind to certain ligands, therefore immunoreactivity of disubstituted and trisubstituted conjugates should be investigated. This information is needed to make accurate assessments regarding the actual requirements on the final product purity. More quantitative experiments concerning the product isolation have to be done. The use of iminobiotin agarose followed by size separation using FPLC is a fast and coarse method to perform the isolation but further analysis on whether the final pretargeting agent is isolated enough to perform as intended has to be done.

The antibody's ability to form dimers and polymers when the sulfhydryl group has been reduced have to be further investigated. If the reaction rate is substantial at the preferred pH interval changes in the conjugation method have to be made. One suggestion is to add the reduced trastuzumab to the IBC in a much lower concentration and in a continuous loop to prevent the sulfhydryl groups to react with each other and form possibly inactive complexes. The possibility to quench the sulfhydryl groups before the pretargeting agent is eluted should also be investigated.

The reaction kinetics governing the avidin-biotin and avidin-NHS-ester should be investigated. In the case that these two reactions compete for the same four sites on the avidin tetramer the avidin have to be bound to the iminobiotin before attaching the SMCC for the highest possible yield. One way to investigate the kinetics is to measure the reaction rate when avidin binds to iminobiotin in the column. By balancing reaction time, pH level and avidin concentration an experiment can be performed where the reaction rate of avidin binding to iminobiotin is the limiting factor. By using pure avidin followed by avidin that has been saturated with SMCC changes of the kinetics can be observed. If the changes are substantial, i.e. they do compete, avidin might have to be bound to the iminobiotin before SMCC is attached to the avidin.

As mentioned in Section 2.2 the FPLC system detects how the absorbance changes over time. When using different buffer solutions this might also impact on the measurements and skew the results. To make the FPLC system more accurate the mobile phase can be changed to the same buffer as the injected sample. If the injected sample is a mixture of different buffer solutions this can also be handled. If the sample is an eluate from the iminobiotin column, i.e. a mixture of borate and acetate buffer, a blank mixture can be prepared by adding and eluting the same amounts of buffer solutions without using any chemicals. When preparing the mobile phase the mixture of acetate and borate buffer only have to have the same extinction coefficient as the blank sample to provide a homogeneous background. This method is fairly time consuming but might offer more exact measurements when needed.

7

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John Apelman, Gothenburg, May 2015.

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