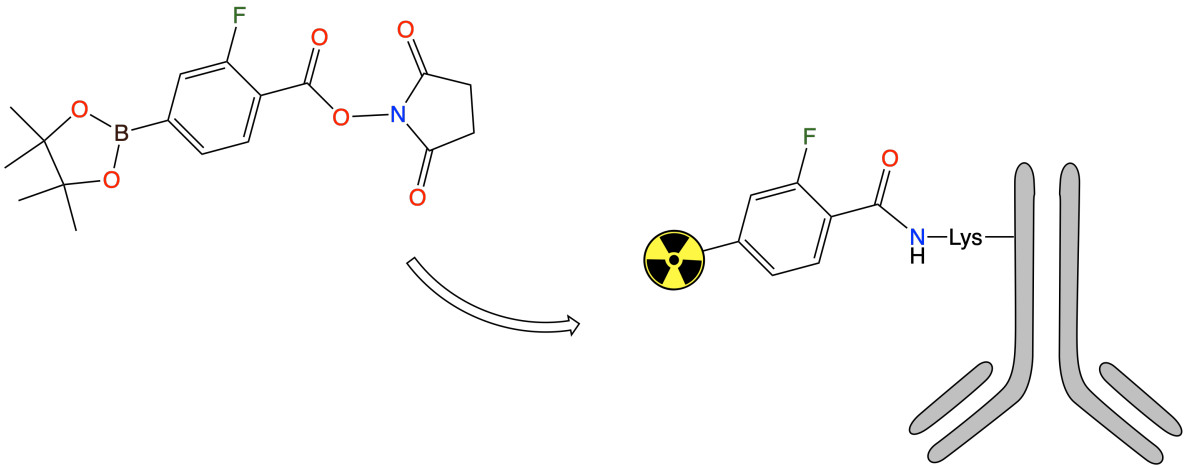

Radiolabeling of Aryl Boronic Ester Derivatives for Cu Catalyzed At-211 Astatination of Biomolecules

Master's thesis in Materials Chemistry, MPMCN

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NUCLEAR CHEMISTRY

CHALMERS UNIVERSITY OF TECHNOLOGY
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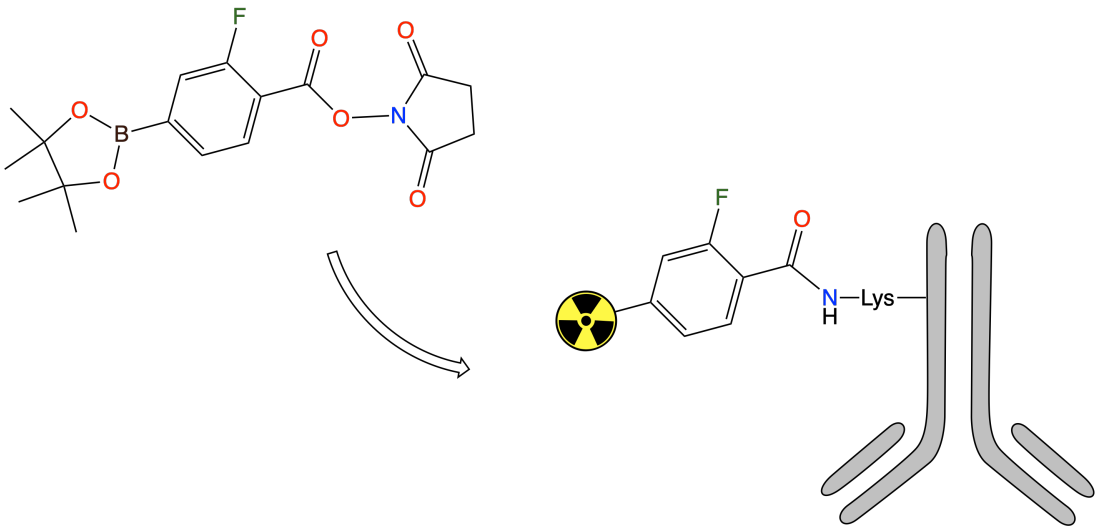

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ABSTRACT

Targeted alpha therapy is an area of research for treatment of spread microscopic cancer. Among the radioactive nuclides that emit alpha-radiation and are eligible for use in targeted alpha therapy, the astatine isotope ^{211}At has been seen as a promising candidate. A common method of attaching the astatine to a targeting biomolecule has used a type of alkyltin reagent as a linking molecule. Since alkyltins are very toxic their use is not preferred. In 2016, an alternative copper catalyzed method using an aryl boronic acid derivative as a linking molecule was presented. In 2018, the same method was applied on aryl boronic ester derivatives as well. In these previous studies of the copper catalyzed method, the radioactive nuclide was attached to the linking molecule which could then be conjugated to a targeting biomolecule. In order to minimize radiation dose, the preferred method of attachment would be to conjugate the linking molecule to a targeting biomolecule first and then attach the radioactive nuclide. The possibility of using the copper catalyzed method for attachment to an already conjugated biomolecule was investigated by examining reaction conditions which could make a transition from a reaction in organic solution to a reaction in aqueous solution possible. As a reaction in aqueous solution proved possible with good yields, the reaction was investigated for attaching the nuclide to a conjugated biomolecule. After optimizing the reaction conditions, a yield of 57.8% was achieved which indicated that the copper catalyzed method could be used to attach a radionuclide to a conjugated biomolecule. By further optimizing the reaction, it seems plausible that the copper catalyzed method can replace the alkyltin requiring method.

Keywords: Iodination, astatination, radiopharmaceuticals, targeted alpha therapy, aryl boronic ester derivative

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1 Introduction

1.1 Background

Radiopharmaceutical chemistry is a field of chemistry where radioactive nuclides are used for diagnostic or therapeutic nuclear medicine applications. There are several nuclides which can be used as an internal radiation source for diagnosis or treatment of cancer. The most common internal radiopharmaceutical used for treatment is a biomolecule on which a radioactive nuclide has been attached. The process is called radiolabeling and creates a tissue specific pharmaceutical that targets a certain type of cells depending on the biomolecule. The biomolecule attaches to receptors on the cancer cells and when the nuclide decays, the emitted radiation is what treats the cancer.[1–3]

Depending on the type of radiation the nuclide emits, the range of affected area is different. Both α and β-radiation are made up of particles which can damage and destroy tissue. β-radiation has a range of a couple of millimeters in tissue and releases its energy along this path. α-radiation has a shorter range in tissue but also a high release of energy in a smaller area. This means that α-radiation is good for treating smaller cancer tumors when attached to the affected cells since the surrounding area is not exposed to as much radiation.[4,5]

One α-emitting nuclide which has been seen as a promising candidate for treatment of spread microscopic cancer tumors is the astatine isotope ^{211}At.[6] However, one difficulty with ^{211}At is that it generally forms weak bonds with biomolecules and is therefore difficult to be stably attached to a biomolecule.[7] Previous methods have commonly used alkyltin reagents which because of their toxicity[8] is not preferred. In 2016, a copper/ligand-catalyzed process using boronic acid precursors was outlined which showed good results of iodinating biomolecules.[9] Two years later, the same copper catalyzed method was used on boronic ester precursors for both iodination and astatination with good results.[6]

1.2 Purpose and Aim

The purpose of this project is to investigate this alternative copper catalyzed method to see if it can replace the common method that requires the toxic alkyltin reagents. Previous studies of the copper catalyzed method have shown good results radiolabeling a linking molecule which can then be attached to the biomolecule. The preferred procedure would be if the linking molecule could be attached to the biomolecule first and then radiolabeled as this would lessen the exposure time of radiation to both personnel and the molecule.

The aim is therefore to investigate if the radiolabeling process using the copper catalyzed reaction on aryl boronic ester derivatives can be performed in aqueous solvents as this is a must if the labeling will be done on biomolecules with the linking molecule attached to it. Then, the aryl boronic ester derivative will be attached to a biomolecule and the radiolabeling process will be investigated and evaluated.

2 Theory

2.1 Radioactive Decay

Radioactive nuclides (radionuclides) are unstable isotopes of an element with excess nuclear energy which decay after a period of time. The nuclei send out the excess energy in form of ionizing radiation and thereby relax to a lower energy state. If the first decay product is unstable, a series of decays can follow until a stable product is formed. The three most common decay modes are α, β and γ. α-decay is a type of particle radiation where helium nuclei, consisting of two protons and two neutrons, are emitted. β-decay is also a type of particle radiation but instead, a positron or electron is emitted together with a neutrino or antineutrino particle respectively. Electron capture (EC) is also counted as a β-decay because it is similar to β^+-decay since a neutrino is sent out and the decay product will be the same. The difference is that when a nucleus decays by EC, an electron from an inner shell of the atom is captured and no positron is emitted as it is in β^+-decay. γ-radiation contains no particles but is made up of high energy photons. The unit for activity is Becquerel (Bq) and is defined as one disintegration of the radioactive material per second. A general equation for simple radioactive decay can be written as in equation 2.1 where N is the number of atoms at the current time, N_0 is the number of atoms there was at time zero, λ is the decay constant, a proportionality constant for the decay of a certain nuclide, and t is the time that has passed from time zero until the current time.[10]

$$N = N_0 \times e^{-\lambda t} \tag{2.1}$$

The time it takes for a radioactive substance to decay to half the original amount is called physical half-life. Biological half-life is the time it takes for the concentration of a substance in the body to be reduced to half. Combining these two gives the effective half-life which is a measure of how long it takes until only half the amount of radioactive substance remains in the body.[10]

A radionuclide is commonly written as the mass number of the nuclide, the total number of protons and neutrons in its nucleus, in superscript in front of the chemical abbreviation for the element.[10,11] For example, astatine (At) with mass number 211 is written as ^{211}At and iodine (I) with mass number 125 is written as ^{125}I.

2.2 Targeted Alpha Therapy

Targeted alpha therapy is an area of medical research where α-emitting nuclides are used to treat spread microscopic cancer. By attaching the α-emitter to biomolecules, for example antibodies, it can target and treat certain cancer tissues. A shorter range and a high transfer of energy to the tissue are desired for targeted treatment of cancer since this means that the targeted area is affected while the surrounding tissue is less affected.[4] α-particles suit this description well since they have a high linear energy transfer (LET), meaning that the high energy particles deposit their energy over a short distance, and the range of α-particles in tissue is short, 50-100 μm.[4,5] The high LET of the α-particles causes tissue damage and can kill cells by generating DNA breaks in the targeted cell. The DNA can be broken in several ways but the most damaging is when both strands of the DNA break, a double strand break, since it is more difficult for the cell to repair successfully.[4,12]

2.2.1 Radiopharmaceuticals

A radiopharmaceutical can be described as a molecule containing a radioactive compound or element that is used for diagnosing or treating a disease.[3] In the case of targeted alpha therapy, the nuclide is an α-emitting nuclide. There are many nuclides that emit α-radiation but not all of them are suitable for use in nuclear medicine. One of the reasons for this can be because of a too short or too long physical half-life. It needs to be long enough such that preparation and administration can be performed but not too long such that it increases risk of toxicity in the patient. Another criterion for a suitable radionuclide is that the decay series should preferably not be long or have any long-lived decay products so that the formation of a safe, stable isotope is quick.[3,4] Another important consideration is the stability of the bond between the radionuclide and a biomolecule. If the bond is weak, there is a risk of detachment which would result in random distribution of the radioactivity in the body instead of transport to the targeted area. The availability of the nuclide is also an important factor for choosing a suitable radionuclide. Producing the radionuclide should preferably be easy using materials that are not in deficiency or rare.[3]

2.3 ^{211}At and ^{125}I

Two radionuclides that have applications in nuclear medicine research are ^{211}At and ^{125}I. The decay series of ^{125}I and ^{211}At are shown in equations 2.2 - 2.4. 100% of ^{125}I decays by EC to stable ^{125}Te (tellurium) with a half-life of 59.4 days. ^{211}At however has a branched decay where there is a 41.78% chance of decay by α-decay to ^{207}Bi (bismuth) with a half-life of 7.2 hours which then decays by β^+-decay to stable ^{207}Pb (lead) with a half-life of 32.9 years, see equation 2.3, and a 58.22% chance of decay by EC to ^{211}Po (polonium) which then decays by α-decay to ^{207}Pb with a half-life of 0.5 seconds, see equation 2.4.[13]

$$^{125}\mathrm{I} \xrightarrow[59.4\ \mathrm{d}]{\mathrm{EC}} {}^{125}\mathrm{Te\ (stable)} \tag{2.2}$$

$$^{211}\mathrm{At} \xrightarrow[7.2\ \mathrm{h}]{\alpha} {}^{207}\mathrm{Bi} \xrightarrow[32.9\ \mathrm{a}]{\beta^+} {}^{207}\mathrm{Pb\ (stable)} \tag{2.3}$$

$$^{211}\mathrm{At} \xrightarrow[7.2\ \mathrm{h}]{\mathrm{EC}} {}^{211}\mathrm{Po} \xrightarrow[0.5\ \mathrm{s}]{\alpha} {}^{207}\mathrm{Pb\ (stable)} \tag{2.4}$$

2.3.1 Production of ^{211}At and ^{125}I

Radionuclides can be produced in different ways. One of these is the production using a particle accelerator, such as a cyclotron, to irradiate a target. This is how the ^{211}At used in this project is produced. An aluminium target plate is covered with a thin layer of ^{209}Bi and thereafter covered with another thin layer of aluminium. The final aluminium layer is to minimize the loss of ^{211}At as it can be volatilized during the irradiation. Volatilization is further stopped by target cooling using a gas flow on the front and a water flow on the back of the target. Cooling is also important because bismuth has a low melting point as well as a poor thermal conductivity. The target is irradiated with accelerated α-particles which upon collision with the bismuth undergo a nuclear reaction which results in the production of ^{211}At and the release of two neutrons,[14] see reaction in equation 2.5.

$$^{209}\mathrm{Bi}(\alpha,2\mathrm{n})^{211}\mathrm{At} \tag{2.5}$$

An important factor in the production process of ^{211}At is the energy of the incident α-particle beam. This is because there is another reaction that can occur where ^{210}At is produced instead. ^{210}At decays to ^{210}Po which is toxic since it is prone to accumulate in bone and can therefore damage the bone marrow. See reaction[14] and following decay[15] in equation 2.6.

$$^{209}\text{Bi}(\alpha,3n)^{210}\text{At} \xrightarrow[8.1\ \text{h}]{\beta^+} {}^{210}\text{Po} \xrightarrow[138.4\ \text{d}]{\alpha} {}^{206}\text{Pb (stable)} \tag{2.6}$$

In order to minimize the production of ^{210}At, the energy of the incident α-particle beam is set to below or just slightly above the energy threshold for the ^{210}At reaction.[4,14] As the threshold is at approximately 28.4 MeV, the energy of the α-beam is typically in the range of 28-29 MeV.[14,16–18]

After production, there are two common ways of isolating the astatine from the aluminium and unreacted ^{209}Bi: solvent extraction or dry distillation. The solvent extraction method, also called wet extraction, is done by removing the bismuth and astatine from the aluminium plate by dissolving them using concentrated nitric acid. The concentrated acid is evaporated and the sample is again dissolved in nitric acid but in a less concentrated solution. The astatine then is extracted into an organic phase, for example diisopropyl ether.[4,14,16,19] The dry distillation method involves heating up the target, or the scraped off top layers of the target, to temperatures ranging from 650-800 °C in a quartz glass oven to volatilize the astatine but not the aluminium or unreacted ^{209}Bi. A reduced pressure and a nitrogen flow are used to push the astatine vapor into a trap, either a capillary tubing cryotrap, bubbler trap or a silica column, to detain the astatine vapor and to let it solidify again. The astatine can then be eluted from the trap using an organic solvent.[14,17]

^{125}I is produced through neutron irradiation of the stable xenon isotope ^{124}Xe to produce ^{125}Xe which decays to ^{125}I, see reaction and following decay in equation 2.7.[20]

$$^{124}\text{Xe}(n,\gamma)^{125}\text{Xe} \xrightarrow{\beta^+} {}^{125}\text{I} \tag{2.7}$$

2.4 Radiolabeling and Conjugation

Radiolabeling (henceforth referred to as labeling) is the term for the process of attaching a radionuclide to a molecule, one way of creating radiopharmaceuticals. There are several different approaches to labeling depending on what nuclide and what targeting biomolecule is used. For some nuclides, the atom can be attached directly to the desired targeting biomolecule while others require indirect labeling in the form of using a linking molecule in order to create a stable bond.[1] The attachment of the linking molecule to the target molecule is called conjugation. If the linking molecule is conjugated with the target molecule first and the nuclide is attached last it is called post-labeling and if the nuclide is attached to the linking molecule first before conjugating with the target molecule, it is called pre-labeling.[2]

One of the most used linking reagents for conjugation to amines is N-hydroxysuccinimide ester derivatives (NHS-ester derivatives). An NHS-ester derivative reacts with a nucleophile, in this case an amine, and forms an acylated product and releases an NHS molecule,[1] see reaction in figure 2.1.

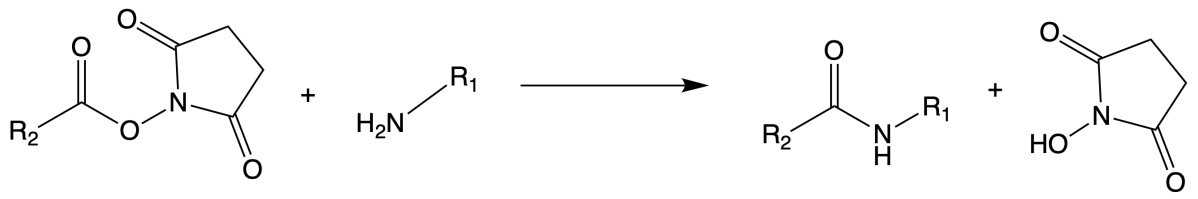

Figure 2.1. General conjugation reaction of an NHS-ester derivative with a primary amine.

In an antibody, the NHS-ester mainly reacts with the ε-amine found in the side chain of the amino acid lysine but can also react with α-amines at the N terminal of an amino acid chain, [1] see figure 2.2.

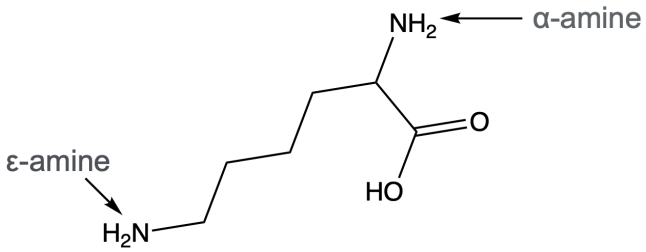

Figure 2.2. The amino acid lysine with arrows pointing to the α and ε-amines.

One of the advantages of using the indirect labeling approach by using a linking molecule is that the labeling can be done on any antibody unlike the direct approach. This is because in the direct approach the labeling can only be done on residues of the amino acids tyrosine or histidine. If the antibody does not contain these amino acids, it cannot be labeled directly. Since all antibodies and other biomolecules made up of amino acids has at least one N-terminal, the indirect method can be utilized. [1]

Another advantage of using the indirect method is that some antibodies are sensitive to the oxidative environment required for direct labeling and could thereby lose their biological activity if the reaction time is too long. The biological activity of an antibody can also be lost if too much conjugation is done since it raises the risk of a linking molecule binding to the antigen binding site, see simple structure of one type of antibody with arrows pointing to the antigen binding sites in figure 2.3. If there is a linking molecule at the binding site, it can block or change the conformation of the site rendering it incapable of interacting with an antigen molecule. Since the distribution of amines in an antibody is nearly uniform, it is important to keep the molar ratio of the linking molecule low to reduce the risk of conjugating to the antigen binding site. [1]

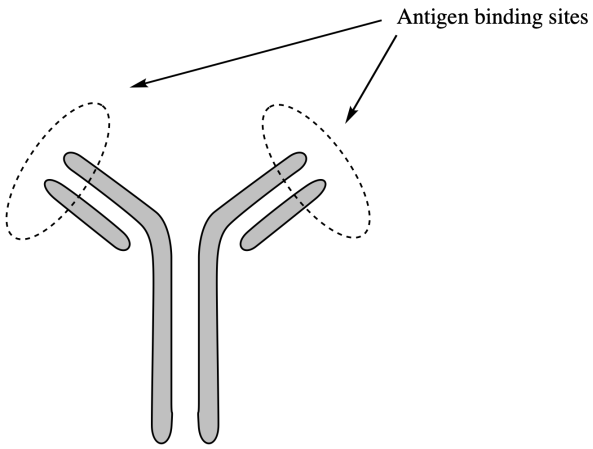

Figure 2.3. Simplified structure of one type of antibody molecule with arrows pointing to the antigen binding sites.

2.4.1 Halogenation

The other part of the linking molecule contains a functional group that facilitates the attachment of a radionuclide. If the radionuclide is a halogen, the process is called radiohalogenation. One radionuclide that has been seen as a good candidate for targeted alpha therapy is the halogen ^{211}At. With its half-life of 7.2 h, it provides enough time for preparation and radiolabeling. An additional advantage is the possibility for external imaging of biodistribution tests due to the X-rays that the decay product ^{211}Po emits.[4,5] One problem with ^{211}At however, is its weak bond to biomolecules.[7] The process for astatination (halogenation with astatine) was developed based on methods for iodination (halogenation with iodine), for example ^{125}I, because of their similarities as halogens since the chemistry of astatine is still not fully known.[7,21] However, a direct attachment of ^{211}At to a biomolecule is not possible since it leads to detachment but it can be connected using a linking molecule where the ^{211}At is bound to an aryl group.[4]

Iodination and astatination have commonly been done via an electrophilic substitution reaction.[6,9] One type of reagent used for the electrophilic methods is a linking molecule containing alkyltin, see example of a reaction in figure 2.4 where the alkyltin reagent is first conjugated and then labeled with astatine.[22]

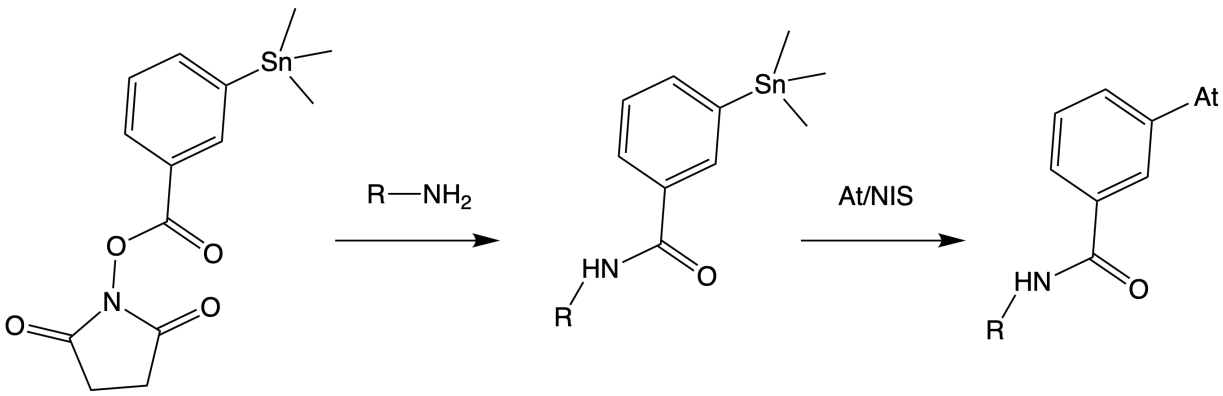

Figure 2.4. Example of an astatination reaction using an alkyltin reagent.

Because the alkyltin reagents and their residues after reaction are toxic,[8] an alternative method where these reagents are not needed would be preferred. In 2016, a nucleophilic reaction using aryl boronic acids was reported by Zhang and colleagues.[9] The reaction was done using a copper catalyst

and a ligand was used to improve the efficiency. In 2018, Reilly and colleagues reported a similar procedure but achieved quicker reaction times using aryl boronic esters and no additional ligand.[6] An example of the reaction with an aryl boronic ester derivative can be seen in figure 2.5 where *R* is a suitable group for conjugation with a biomolecule and *X* is the halogen that is used.

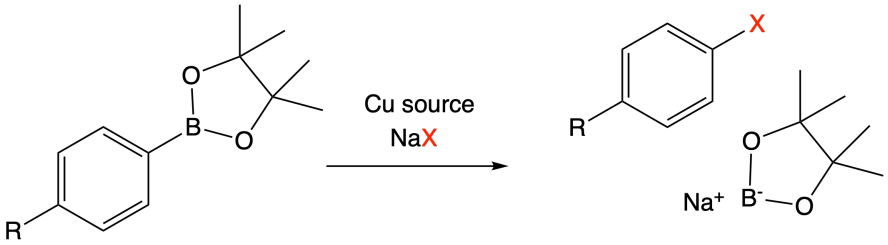

Figure 2.5. Example of a halogenation reaction using the copper catalyzed process.

2.4.2 Aryl Boronic Ester Derivative

The aryl boronic ester derivative used for the experiments in this project is called 4-(NHS ester)-3-fluorobenzeneboronic acid pinacol ester (henceforth referred to as ABED) and its structure can be seen in figure 2.6.

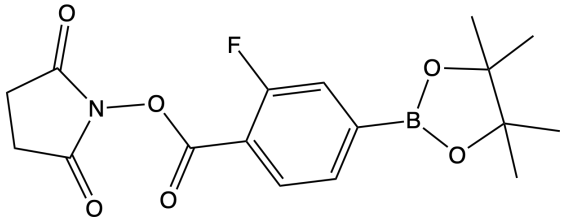

Figure 2.6. Chemical structure of 4-(NHS ester)-3-fluorobenzeneboronic acid pinacol ester.

The group to the left of the benzene ring is the NHS-ester and the group to the right of the benzene ring is the boronic ester. Both the boronic ester and the NHS ester are sensitive to hydrolysis.[1,23] In the case of the NHS-ester, the reaction is similar as to what is shown in figure 2.1 in section 2.4 but the reaction is with water instead of the amine, resulting in the formation of a carboxylic acid instead of the molecule with an amide bond. To minimize the hydrolysis and maximize the conjugation reaction with the amines, the concentration of the targeting biomolecule should be kept high.[1] Hydrolysis of the boronic ester results in the formation of the boronic acid form and a pinacol molecule,[23] see figure 2.7.

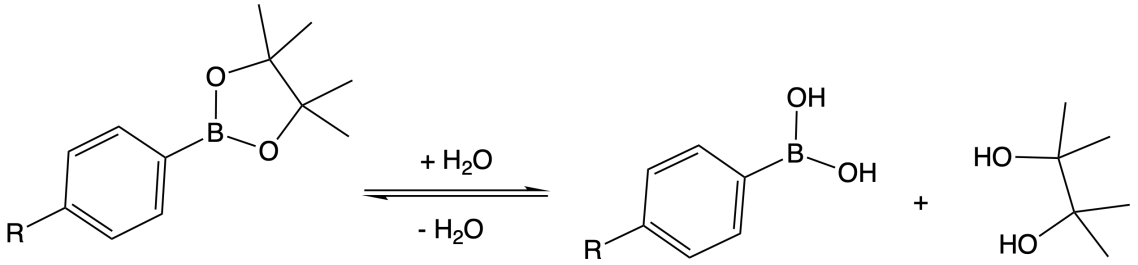

Figure 2.7. General hydrolysis reaction of an aryl boronic acid pinacol ester.

Depending on what the group denoted by R in the picture is, the rate of hydrolysis is different.[23]

2.5 Quality Control

When performing any type of reaction, it is important to be able to analyze the reaction and control the quality of the products that have been formed. There are many types of analytical techniques that can be applied depending on what is to be investigated.

2.5.1 Liquid Chromatography

Liquid chromatography is an analytical method of separation based on different interactions between molecules in a liquid phase that passes by a solid unmoving phase. The molecules of interest are called analytes, the liquid phase is called the mobile phase and the solid phase is called the stationary phase. The mobile phase entering the column is called eluent and the mobile phase exiting the column is called eluate. When the mobile phase passes through the stationary phase, the analytes interact with the stationary phase to different degrees resulting in that analytes with a strong interaction are retarded by the stationary phase while analytes with a weak interaction continue to move with the mobile phase. The analytes are thereby separated based on the amount of interaction they have with the stationary phase. The time it takes for an analyte to pass through the whole system, from injection of sample to detection of the analyte, is called the retention time.[24]

Different types of liquid chromatography can be categorized based on the type of interaction between the analyte and the stationary phase. One type of liquid chromatography is adsorption chromatography where the analytes adsorb onto the surface of the solid particles that make up the stationary phase. One example of adsorption chromatography is thin-layer chromatography (TLC). In this case, the stationary phase is a dry solid media, for example silica coated on a flat plate. The sample is added in a drop near the bottom of the plate and the plate is then placed with the bottom edge of the plate in contact with a solvent. The solvent moves along the plate by capillary action and the media separates the analytes as it travels up the plate.[24]

Another example of adsorption chromatography is high performance liquid chromatography (HPLC) where the mobile phase is forced through a column of small packed particles. The efficiency of separation is higher for smaller particles because of a shorter diffusion distance between them. However, since they are tightly packed, a high pressure is required to force the mobile phase through the column. The most common setup of HPLC is called reversed phase chromatography and utilizes a weakly polar or non-polar stationary phase and a polar mobile phase. An example of a stationary phase used in reverse phase chromatography is silica particles with hydrocarbon chains attached to the surface. The polarity of the mobile phase can be customized by choosing an appropriate solvent or a mixture of solvents. If the same solvent or solvent mixture is used throughout the analysis, it is called isocratic elution. It is also possible to do an analysis where the polarity of the mobile phase is changed during the run to better separate the analytes or attain a quicker analysis. The change in polarity during an analysis is called a gradient. After the column where the analytes have been separated, the sample is led through a detector to analyze the eluate. A common type of detector is an ultraviolet (UV) detector where the absorbance of UV light in the passing liquid is measured. This produces a chromatogram where the peaks of absorbance are plotted against the time of the analysis resulting in that the retention times of the different analytes can be seen.[24]

When analyzing radioactive analytes, a radiation detector can be connected to the HPLC system to measure the activity as well. This is called radio-HPLC. In 2014, Lindegren et al. designed and evaluated a radio-HPLC dual flow cell system for on-line quantification of radioactive samples.[25]

In that system, the sample is not immediately injected into the column but is instead first led into a detector which measures the total activity of the sample before it enters the column. After the column, the sample is led through the detector once more, this time measuring the individual activities of the separated active species in the sample. The sample is then led through the UV detector as usual before it exits the system. A radio-HPLC analysis thereby produces two chromatograms, one showing the UV absorbance peaks versus time and one showing the measured activity versus time.

Another type of liquid chromatography is size exclusion chromatography (SEC) where the analytes are separated based on their size. This is, in an ideal case, not through attractive interaction between the analyte and the stationary phase but rather through a physical interaction. The stationary phase constitutes of porous gel particles with small pores. The larger analytes cannot fit in the pores and pass by the gel particles completely. The smaller analytes can however fit in these pores and are thereby retained in the gel as they have further distance to travel through the column.[24] An example of SEC is gel filtration used in columns for separation of biomolecules.

2.5.2 Radiation Detectors

When working with radioactive materials, it is important to be able to measure the activity of the material. This is done using a radiation detector. There are several types of detectors that can measure radioactivity and one of them is a scintillation detector. The basic principle is that a material absorbs the radiation and an electron is excited by its energy. When deexcitation occurs, the energy is released in form of luminosity, meaning either fluorescence or phosphorescence. These flashes of light can be converted to electrical pulses by a photomultiplier tube. The electrical pulses can then be processed and counted to get the activity of the sample. The scintillating material can be both in solid, liquid or gaseous form. A common solid scintillating material is sodium iodide doped with a small amount of thallium (NaI(Tl)) which is made for detection of γ-rays and is a detector used in gamma counters.[10]

Another type of detector is gas counters such as ion chambers. An ion chamber is a gas filled space with two electrodes in it where the anode has a positive potential of 100-1000 V above that of the cathode. When a radioactive sample is placed in the chamber, the ions and electrons that are formed by the radiation are attracted to the electrodes where they are discharged. The voltage over the two electrodes drops because of the current produced in the chamber. The activity of the sample can then be calculated based on the voltage drop and some factors such as geometric efficiency and energy loss per particle which depend on the type of nuclide and the shape of the sample.[10]

2.5.3 Radiochemical Yield

Radiochemical yield (RCY) is defined as the percentage amount of activity in the product compared to the starting activity that was used. Both the final and initial activities must be from the same radionuclide and they must be decay corrected to the same point in time.[11]

Calculations of RCY can be done in several ways, for example using radio-HPLC. The two measurements of activity in radio-HPLC, before and after the column, gives the total activity in the sample as well as the individual activity of a separated radioactive species. The total activity and the activity of the product can be obtained by integrating the first peak and the desired product peak in the activity chromatogram. The value of the product peak must be decay corrected before it

can be used to calculate the RCY as some decay will occur between the first measurement of total activity and the later measurement of the separated sample. The decay correction is done by using the general equation for simple decay (see equation 2.1 in section 2.1). Since the number of atoms is proportional to the activity,[10] N and N_0 can be replaced by A and A_0, which are the activity values in form of integrated area from the chromatogram. The equation is then solved for A_0 to get the final equation for calculating the decay corrected value, see equation 2.8. λ is the decay constant for the nuclide and t is the time between the two measurements which can be obtained by calculating the difference in retention times for the two peaks.

$$A_0 = A \times e^{\lambda t} \tag{2.8}$$

The ratio between the decay corrected activity of the product peak and the total measured activity gives the RCY of the reaction. An example of an equation for such a calculation can be seen in equation 2.9, where $A_{product}$ is the decay corrected activity of the product peak, A_{total} is the total activity and 8.65 is a factor to correct for the size difference between the two tubes in the detector before and after the column. The whole expression is multiplied by 100 to get the yield in percent.[25]

$$RCY = \frac{A_{product}}{A_{total} \times 8.65} \times 100 \tag{2.9}$$

The RCY can also be calculated using a gamma counter after the sample has been separated, for example using TLC. When performing a TLC, the labeled molecule stays at the point where the sample was added and the activity that is free is carried along with the solution. By cutting the paper in half and analyzing the two halves, the RCY can be calculated by dividing the amount of that activity has been attached to the molecule which is the activity from the bottom half of the TLC plate and the total activity of the whole strip which is the sum of activity from both halves of the TLC plate.

2.5.4 Ultraviolet Spectrophotometry

Other than being used for detection in HPLC, UV has several applications in analytical chemistry. One example is using UV spectrophotometry for determination of sample concentration. The Beer-Lambert law states that the absorbance (A) of a sample is proportional to its concentration (c), the molar absorptivity (ε) of the analyte and the pathlength (b) of the UV-rays in the sample. By rewriting the equation for c instead, an equation for concentration calculation based on measured absorbance is obtained, see equation 2.10.[24]

$$A = \varepsilon bc \implies c = \frac{A}{\varepsilon b} \tag{2.10}$$

3 Method

3.1 List of Chemicals

The chemicals used in this project are listed below. All chemicals were used without further treatment unless otherwise stated.

Methanol (MeOH, $\geq$ 99.8%, Merck), ethanol (EtOH, 96%, Solveco), acetonitrile (ACN, 100.0%, VWR Chemicals), trifluoroacetic acid (TFA, $\geq$ 99%, Merck), dimethylsulfoxide (DMSO, $\geq$ 99.7%, Merck), dimethylformamide (DMF, $\geq$ 99.5%, BDH Chemicals), chloroform ($CHCl_3$, $\geq$99.8%, Merck), sulfuric acid (H_2SO_4, 95-97%, Merck), sodium hydroxide (NaOH, > 99%, VWR Chemicals), sodium iodide (NaI, 99.99%, Merck), sodium azide (NaN_3, > 99.5%, BDH Chemicals), sodium carbonate (Na_2CO_3, anhydrous Suprapur 99.999%, Merck), sodium hydrogen carbonate ($NaHCO_3$, $\geq$ 99.7%, Merck), tetrakis(pyridine)copper(II) triflate ($Cu(pyridine)_4(OTf)_2$, 95%, Merck), 4-(NHS ester)-3-fluorobenzeneboronic acid pinacol ester (97%, SynInnova), 1,10-phenanthroline ($\geq$ 99%, Merck), succinic anhydride (> 96%, anhydrous: > 99.2%, Sigma), phosphate buffered saline (PBS, tablets dissolved in purified water, pH 7.4, Sigma), carbonate buffer (0.1 M $NaHCO_3$ solution in purified water, adjusted with 1 M Na_2CO_3 solution to pH 8.6), borate buffered saline (BBS, tablets dissolved in purified water, pH 8.2, Sigma), tris(hydroxymethyl)aminomethane buffer (TRIS, Merck, 0.5 M solution in purified water, adjusted with sulfuric acid to pH 6), bovine serum albumine (BSA, 1% (wt/vol) solution in PBS with $\sim$0.1% sodium azide as preservative, VWR Chemicals), poly-L-lysine hydrobromide (mol wt 30 000 - 70 000, Sigma), trastuzumab (Herceptin, 150 mg powder dissolved in PBS solution to 15 mg/mL, Roche), $Na^{125}I$ (>350mCi/mL, pH 12-14, Perkin Elmer), ^{211}At (see section 2.3.1 for how it is produced and section 3.3 for how it is prepared).

Purified ultrapure water (henceforth referred to as MilliQ water) was obtained using a Direct-Q® Water Purification System from Merck Millipore.

3.2 Analytical Instruments

The radio-HPLC was a JASCO Prep HPLC system with a C18 YMC column (4.5 $\times$ 150 mm, 5 μm, 120 Å) and a NaI(Tl) detector. The software used for controlling the HPLC system as well as producing and integrating the chromatograms was ChromNAV by JASCO. ACN was used as the organic phase and MilliQ water with 0.1% TFA as the aqueous phase. A very general example of the setup of the gradients that were used was a few minutes isocratic hold at a low organic phase ratio, a ramp up to a high organic phase ratio, isocratic hold for a few minutes, ramp down to starting conditions and finally isocratic hold for a few minutes. More detailed descriptions of the used gradients are presented in section 4. A Veenstra VDC-505 Dose Calibrator was used during the labeling experiments to determine the amount of activity there was in the reactions. A Perkin Elmer WIZARD^2 automatic gamma counter was used to measure the activity of the biomolecules after labeling and separation. To purify the conjugated and labeled biomolecules, size exclusion columns illustra NAP-5 and illustra NAP-10 from GE Healthcare Life Sciences were used. The labeled conjugated biomolecules were also separated using iTLC-SG paper from Agilent. The antibody concentration was measured using a UV-1800 spectrophotometer from SHIMADZU at a wavelength of 280 nm.

3.3 Preparation of Astatine

The ^{211}At used in this project was produced every other week by irradiation in a cyclotron at the PET and Cyclotron Unit at Copenhagen University Hospital, Denmark[26] (see reaction in equation

2.5, section 2.3.1) and then dry distilled at Sahlgrenska University Hospital, Gothenburg. When the irradiated plate arrived at Sahlgrenska, its current activity was first checked. The top layers of the plate were then scraped into a quartz glass container using a custom-made scraping device. The quartz glass container with the radioactive powder was transferred to a glovebox and was then put into a preheated quartz glass oven inside a tube furnace at circa 700 °C. The glass oven was closed with a glass stopper that was connected to a flow of nitrogen gas (N_2) with a capillary. The ^{211}At was then vaporized from the aluminum and unreacted ^{209}Bi under a flow of N_2. The ^{211}At vapor was let through a valve and into a cold trap cooled with liquid N_2 to between -40 and -50 °C. The system was pressure equalized and after a few minutes, the ^{211}At was solid again. It was then eluted with $CHCl_3$ from the capillary in the cold trap into a vial.[17] The vial could then be taken out of the glovebox through the airlock and the ^{211}At could be portioned out and used for subsequent experiments. Before reactions, the $CHCl_3$ was evaporated under a N_2 flow of approximately 200 mL/min.

3.4 HPLC-Analysis of Aryl Boronic Ester Derivative in Different Solvents

Small amounts of ABED were added to 1.5 mL plastic vials, dissolved in ACN, DMSO or DMF and then analyzed on the HPLC. Parts of these stock solutions were mixed with water and also analyzed on the HPLC. Different gradient settings were tried until a suitable gradient was found. The retention times of the analytes were determined by integration of the peaks using the HPLC software.

3.5 Labeling Process of Aryl Boronic Ester Derivative in Organic Solvents

The first experiments on the ABED for the reactions in organic solvents were based on the reaction conditions that Reilly and colleagues had used.[6] Inactive Na^{127}I ($\sim$1.5 mg) or active Na^{125}I ($\sim$1 μL, $\sim$6 MBq) or ^{211}At ($\sim$5 MBq) was added to a reaction vial. 100 μL NaOH-solution (0.1 M in MeOH), 40 μL BE-solution (0.375 M in ACN) and 10 μL Cu(pyridine)$_4$(OTf)$_2$ (Cu catalyst, 0.075 M in MeOH) was then added and the reaction was vortexed for 15 minutes. 50 μL of MilliQ water was added to quench the reaction and the reaction vial was mixed again for about 30 seconds. A portion of the reaction solution was diluted with ACN and vortexed to ensure a homogeneous solution and then analyzed using radio-HPLC. Based on the activity chromatograms, the RCYs of the reactions were calculated as described in section 2.5.3.

Some variations of this method were tried as well. An experiment for time dependence was done as above with ^{211}At but with reaction times of 5, 15, 30 and 60 minutes. Other experiments were done for the effect of NaOH concentration. One experiment with ^{211}At with a NaOH concentration of 0.05 M as well as three experiments using ^{125}I with NaOH concentrations 0.01, 0.001 and 0.0001 M were performed.

3.6 Labeling Process of Aryl Boronic Ester Derivative in Aqueous Solution

Na^{125}I ($\sim$1 μL, $\sim$7 MBq) was added to a 1.5 mL reaction vial. 20 μL NaOH (0.2 mM in MilliQ water), 100 μL MilliQ water, 20 μL BE-solution (0.25 M in DMF or DMSO) and 10 μL Cu catalyst (25 mM in DMF or DMSO) were added and the vial was agitated for 15 minutes. A portion of the reaction solution was diluted with ACN and vortexed to mix it before it was analyzed using radio-HPLC. The reaction was also performed with 1,10-phenanthroline as an additional ligand to enhance the efficiency of the Cu catalyst. 10 μL (50 mM in DMF or DMSO) was added and the added amount of MilliQ water was reduced to 90 μL to get the same total volume in the two reactions.

The reaction was also performed with ^{211}At with DMF as the organic solvent. One reaction was performed as for ^{125}I (organic ratio of reaction mixture was 20%). Two more reactions were done with 10% organic solvents, one using the ligand and one without. $\sim$7 MBq of ^{211}At was added to a reaction vial followed by 20 μL NaOH (0.2 mM), 150 μL MilliQ water, 20 μL ABED (1.25 mM in 50/50 DMF/MilliQ water) and 10 μL Cu catalyst/ligand solution (0.25 mM Cu catalyst, 0.5 mM ligand in DMF) or 10 μL Cu catalyst (0.25 mM in DMF). The vial was agitated for 15 minutes and then a portion of the reaction solution was diluted with ACN and vortexed to mix it before it was analyzed using radio-HPLC.

3.7 Conjugation of Aryl Boronic Ester Derivative to Poly-L-Lysine

The general process of conjugating the ABED to poly-L-lysine (PL) was to dissolve the PL in carbonate buffer in concentrations ranging from 5-10 mg/mL. ABED in DMSO was added in mole excess 5-10 times that of the calculated number of moles of PL in the buffer solution. The mixture was left to react with gentle shaking for 30-60 minutes. A charge modification was then performed to ensure that the PL would pass through a NAP-5 column in order to purify the final product. The approximate number of moles of ε-amines in the reaction mixture was calculated by dividing the mass of PL by the molar mass of lysine. Succinic anhydride was then added in a molar excess of 10-50 to the number of moles of ε-amines in form of crushed flakes and the reaction was run for another 20-30 minutes. 4×20 μL of 1 M Na_2CO_3 solution was added spaced out evenly during the reaction to keep the pH high. After the reaction, the solution was purified on a prepared NAP-5 column equilibrated with the buffer the labeling would be performed in (see appendix A.1 for preparation and usage of NAP columns).

3.8 Labeling Process of Poly-L-Lysine Conjugate

The first tests for labeling of the PL conjugate were performed in single batches using PBS, BBS or TRIS buffer. 90 μL PL conjugate solution ($\sim$1.9 mg/mL), 4 μL additional buffer, 5 μL Cu catalyst (0.04 M in DMSO) and 1 μL ^{125}I ($\sim$10.5 MBq) were added to a 1.5 mL vial and reacted for 30 minutes. After the reaction, the solution was purified on a prepared NAP-5 column equilibrated with the same buffer as the reaction. The activity of the eluate, column and reaction vessel was measured using an ion chamber and the RCYs were calculated by dividing the activity of the eluate with the sum of all activities.

The PL conjugate was then labeled under varying conditions to investigate different factors of the reaction. A series of reactions were performed with ^{125}I using smaller amounts of activity from a diluted solution. The activity was diluted to approximately 0.1 MBq/μL using 0.02 M NaOH solution. The first factor to be investigated was time. In a 1.5 mL plastic vial, 85 μL of conjugate (4 mg/mL), 5 μL DMSO, 5 μL Cu catalyst (0.1 M in DMSO) and 5 μL Na^{125}I ($\sim$0.6 MBq) were added and put in a vortex mixer to agitate it slightly. After five minutes, three samples of 1.5 μL were taken and placed on iTLC-SG plates. The reaction vessel was put back on agitation and the three iTLC-SG plates were placed in MeOH to separate the samples. The procedure was repeated every five minutes until a total reaction time of 60 minutes had passed. All the iTLC-SG plates were removed from the MeOH when the solvent had almost reached the end of the plate, covered in tape in order to minimize losses during handling and then cut in half before they were measured on a gamma counter with a program for ^{125}I with a measuring time of 60 seconds. The later measured samples were decay corrected using equation 2.8 in section 2.5.3 and RCYs were calculated as described in section 2.5.3 for each triplicate and then averaged. The averaged RCYs were plotted against the time to find the optimal reaction

time.

The second factor to be investigated was the concentration of the conjugate. Four reactions were performed as before with 85 μL of conjugate but with different concentrations, 3.5 mg/mL, 3 mg/mL, 2.5 mg/mL and 2 mg/mL respectively. 5 μL DMSO, 5 μL Cu catalyst (0.1 M in DMSO) and 5 μL $Na^{125}I$ (∼0.6 MBq) were added as before and the reaction was run for the time that gave the best RCY in the first experiment. After the reaction, three samples were taken from each reaction vessel and separated and analyzed as for the time experiment. The calculated RCYs were this time plotted against the concentration of conjugate to find which concentration gave the highest RCY.

The third factor to be investigated was the concentration of the Cu catalyst. Two reactions were performed with the optimal reaction time and conjugate concentration with Cu catalyst concentrations of 0.05 M and 0.2 M. Three samples from each reaction vessel were analyzed and the RCYs were calculated as for the preceding PL labeling experiments and then plotted against the Cu catalyst concentration to find which concentration gave the highest RCY.

The last factor to be investigated was the concentration of the ligand. Two reactions were performed with the optimal reaction time, conjugate and Cu catalyst concentrations with ligand concentrations of 0.05 M and 0.1 M to get one reaction with a 1:1 ratio and one reaction with a 2:1 ratio of ligand to Cu catalyst. Again, three samples from each reaction vessel were analyzed as before and the RCYs were calculated and then plotted against the ligand concentration to find which concentration gave the highest RCY.

3.9 Conjugation of Aryl Boronic Ester Derivative to Antibodies

The antibody Herceptin had previously been made into a stock solution of 15 mg/mL in PBS. In order to perform the conjugation, the pH needed to be higher than that of the PBS solution. The buffer solution was changed to a carbonate buffer by running it through a prepared NAP-10 column equilibrated with carbonate buffer which resulted in dilution to a concentration of ∼10 mg/mL. 494 μL of the Herceptin solution (∼10 mg/mL, ∼0.034 μmol) was added to a 1.5 mL plastic vial. ABED was weighed in and dissolved in DMSO to yield a concentration of 10 μg/μL. Based on the number of moles of antibodies in the solution, ABED in DMSO was added in a mole excess of 5 (6.16 μL, ∼0.17 μmol). The mixture was left to react with gentle shaking for 30 minutes after which it was purified on a prepared NAP-5 column equilibrated with TRIS buffer resulting in a final concentration of ∼5 mg/mL. The concentration was verified on a UV-spectrophotometer (see description of method in appendix A.2).

3.10 Labeling Process of Antibody Conjugate

The labeling of antibody conjugate was performed as the optimized conditions for PL labeling in terms of time and concentrations but with higher amounts of activity and with DMSO or DMF as the organic solvent. Two reactions were performed with 85 μL of the antibody conjugate solution (∼5 mg/mL), 5 μL Cu catalyst (0.05 M in DMSO or DMF), 5 μL ligand (0.1 M in DMSO or DMF) and 5 μL $Na^{125}I$ (∼1.8 MBq). The reaction was run for 25 minutes and then three samples were taken from each reaction vessel and analysed using iTLC-SG for separation and a gamma counter for activity measurement as for the PL experiments. The RCYs were calculated in the same way as for the PL experiments.

4 Results

4.1 HPLC Gradients

A few different gradients were used for the different experiments. At first, a long program with a slow and high gradient was used on the stock solution of ABED in ACN to see where the ABED had its peak. The program was shortened and the gradient was changed to 10% to 70% for the first reaction with inactive iodine. That gradient started with isocratic hold at 10% organic phase from 0-5 minutes, a ramp up to 70% organic phase from 5-20 minutes, no isocratic hold but an immediate ramp down to 10% organic phase from 20-25 minutes and then isocratic hold at 10% organic phase from 25-30 minutes. Based on the results, the analysis was changed to a slightly longer gradient and an isocratic hold at the high organic ratio was added to get a better separation of the peaks and to flush out the system. An isocratic hold at the end of the gradient was also added which resulted in the gradient used for the rest of the experiments. The final gradient started with isocratic hold at 10% organic phase from 0-5 minutes, a ramp up to 80% organic phase from 5-22 minutes, isocratic hold at 80% organic phase from 22-25 minutes, ramp down to 10% organic phase from 25-30 minutes and then isocratic hold at 10% organic phase from 30-40 minutes. This gradient will be denoted as *gradient A* and the gradient used for the reaction with inactive iodine will be called *gradient B*.

4.2 HPLC-Analysis of the Aryl Boronic Ester Derivative

Analyses of ABED dissolved in ACN, DMSO or DMF all showed similar results with a single peak at a retention time of around 13.9 minutes when analyzed with gradient A. When MilliQ water was added to the stock solutions, another peak appeared at around 9.9 minutes. In figure 4.1, the chromatogram from analysis of ABED dissolved in DMSO mixed with MilliQ water is shown.

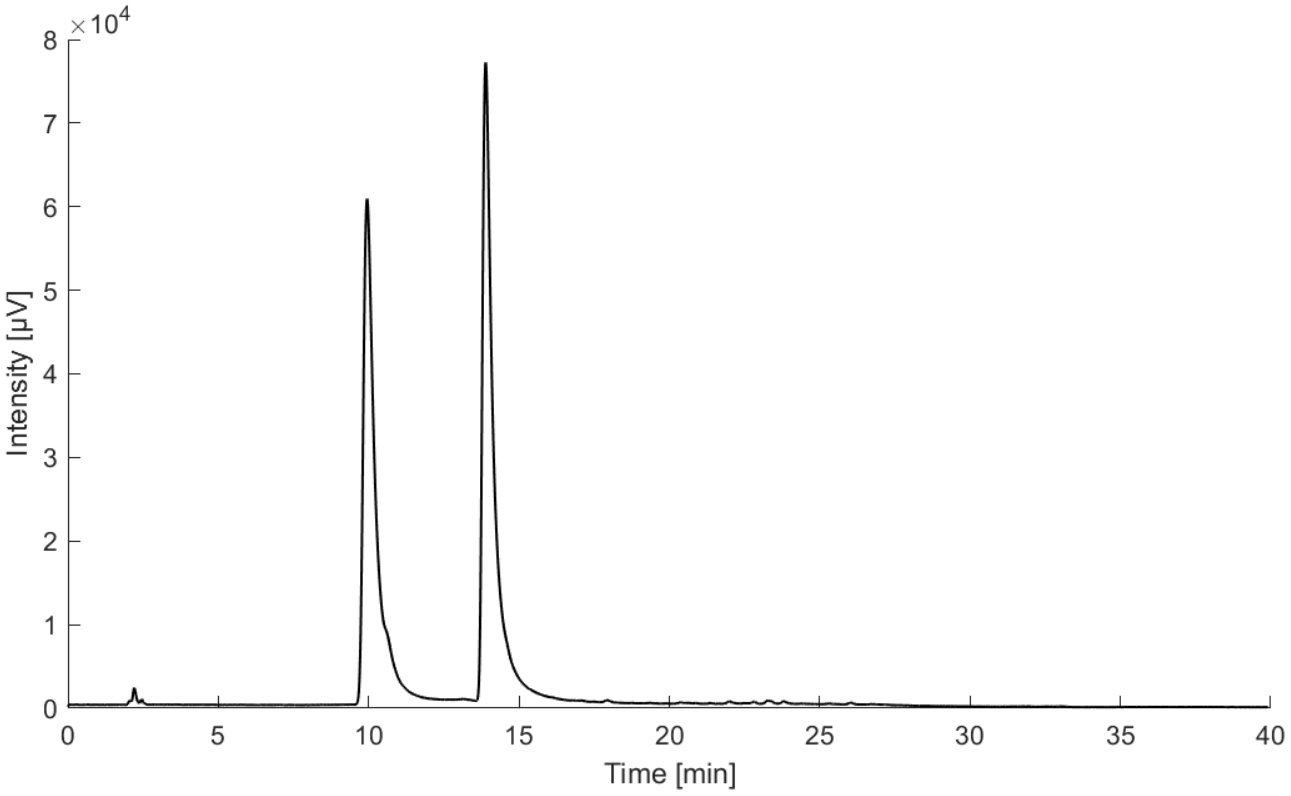

Figure 4.1. UV-chromatogram of ABED dissolved in DMSO and mixed with MilliQ water, analyzed with gradient A.

Partial hydrolysis of the ABED, either the NHS-ester, the boronic ester or both, was suspected to be the cause of the additional peak in the chromatogram but this could not be confirmed.

4.3 Labeling of Aryl Boronic Ester Derivative in Organic Solvents

The labeling reaction of ABED in organic solvents was first performed using inactive iodine to locate reaction product peaks in the UV-chromatogram. A small product peak was found at a retention time of 19.1 minutes as well as the ABED peak at 13.9 minutes with gradient B, see chromatogram in figure 4.2.

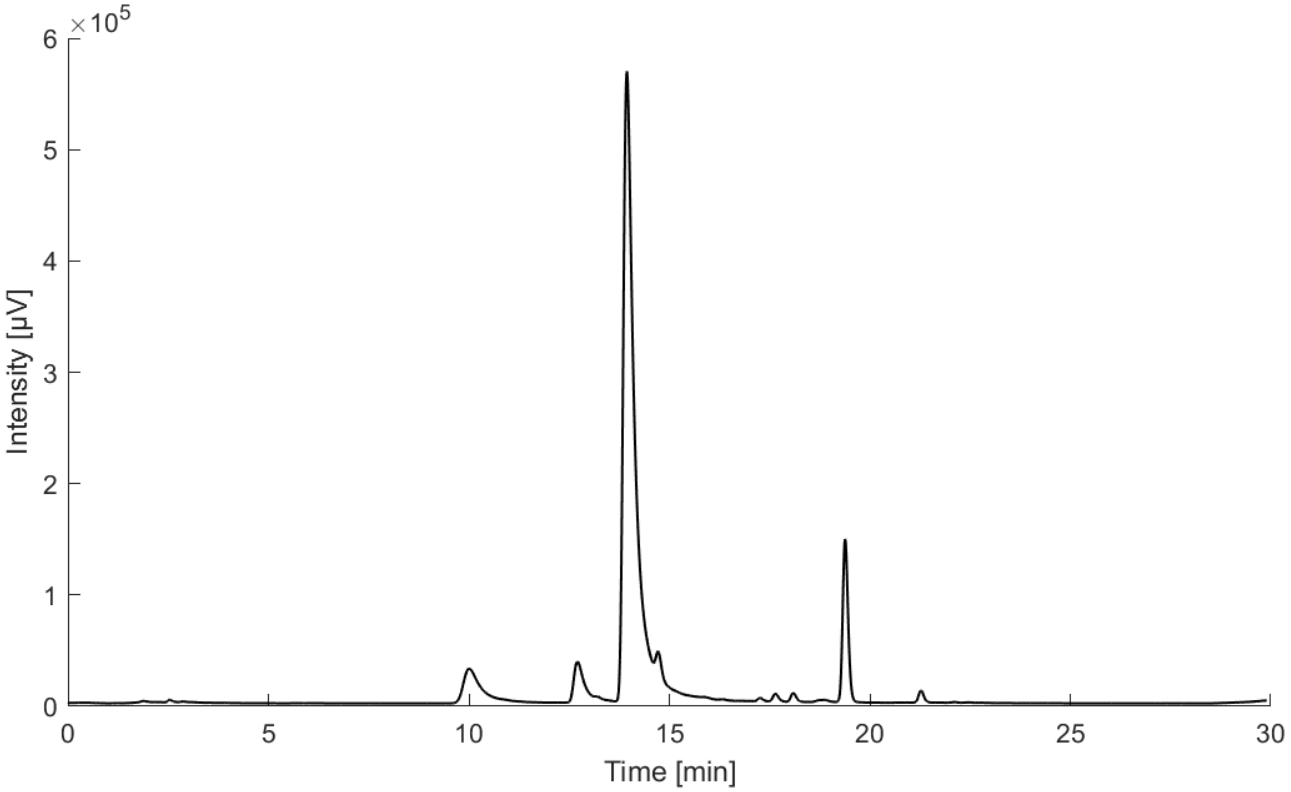

Figure 4.2. UV-chromatogram of ABED labeling reaction with inactive iodine in organic solvents, analyzed with gradient B.

The reaction was then performed with ^{211}At, trying different reaction times of 5, 15, 30 and 60 minutes. The reactions yielded similar peaks to that of inactive iodine but since this analysis was done with gradient A, the retention times later in the analysis differ more. In figure 4.3 and figure 4.4, the UV-chromatogram and activity chromatogram from radio-HPLC analysis of one of the astatination reactions is shown.

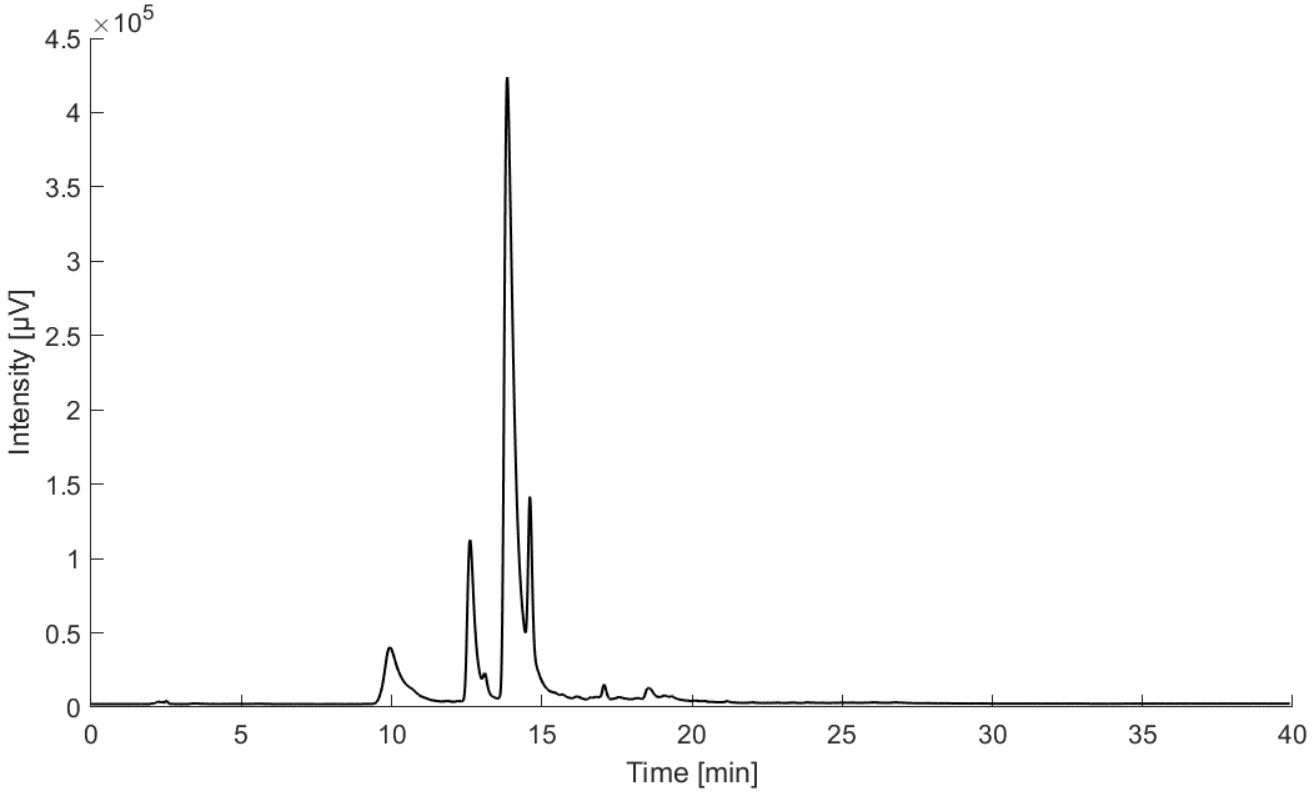

Figure 4.3. UV-chromatogram for astatination of ABED in organic solvents with a reaction time of 15 minutes, analyzed with gradient A.

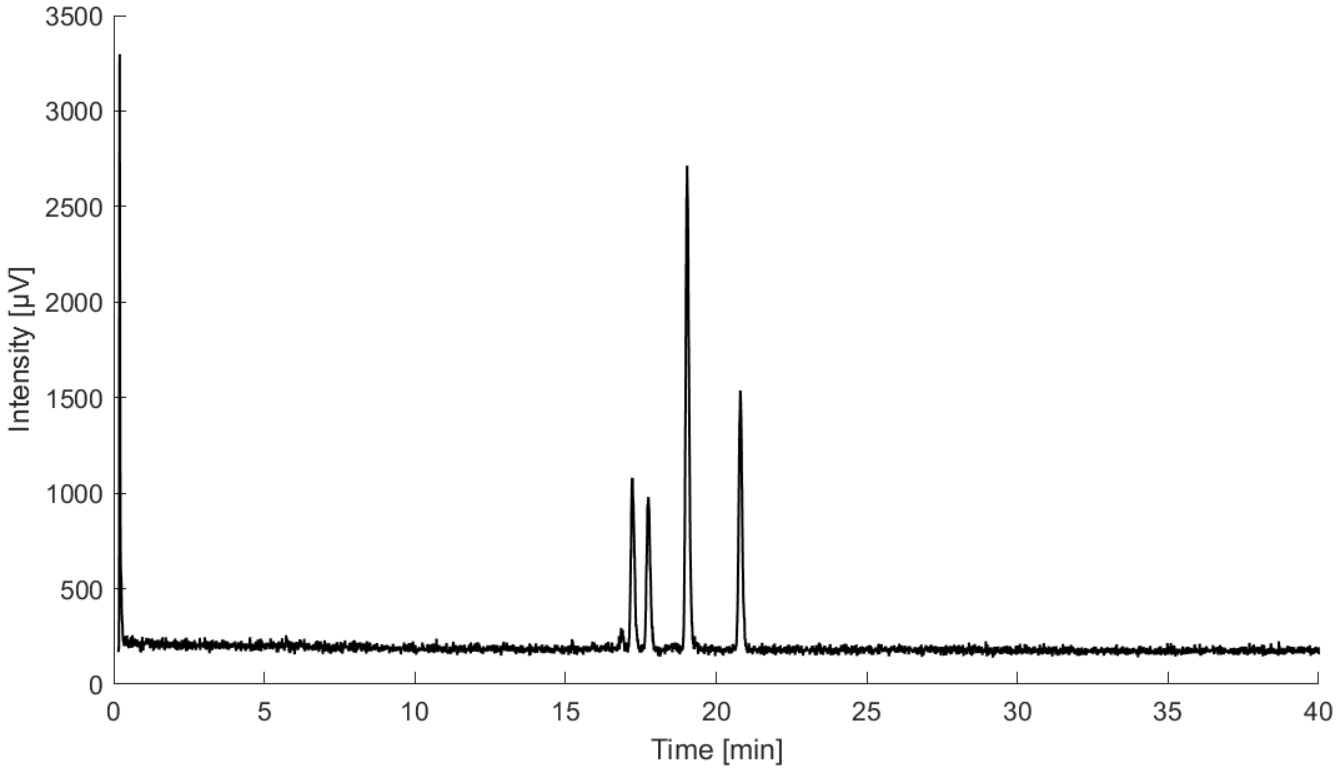

Figure 4.4. Activity chromatogram for astatination of ABED in organic solvents with a reaction time of 15 minutes, analyzed with gradient A.

The activity peak found at 18.9 minutes was deemed to be the same product as in the reaction with inactive iodine as a slightly earlier retention time was expected with the new gradient. RCYs were

calculated for the peaks at 18.9 minutes for the four reactions and the highest RCY was for the reaction time of 15 minutes at 40.3%. Because of a color change of the Cu catalyst upon addition to the reaction solution from blue to green, one reaction was performed with a lower NaOH concentration with a reaction time of 15 minutes. The RCY from that reaction was calculated to be 61.4%.

Further investigations on lower NaOH concentrations were performed using ^{125}I and analyzed with gradient A which resulted in similar retention times to the ones found for ^{211}At. An increase in RCY could be seen for a lowered NaOH concentration, see table 4.1.

Table 4.1. Calculated RCYs for iodination of ABED in organic solvents with varying NaOH concentration.

NaOH concentration	RCY
0.01 M	78.9%
0.001 M	86.9%
0.0001 M	87.7%

4.4 Labeling of Aryl Boronic Ester Derivative in Aqueous Solution

The labeling reaction of ABED in aqueous solution was first attempted using ^{125}I. The reactions were analyzed using radio-HPLC with gradient A. When no ligand was used, the calculated RCYs were high for both DMSO and DMF as the organic solvent. Both DMSO and DMF had lower RCYs when the ligand was used but DMSO had a very low RCY compared to the others, see table 4.2.

Table 4.2. Calculated RCYs for iodination of ABED in aqueous solution using DMSO or DMF, with or without ligand.

Reaction	RCY
DMSO with ligand	27.7%
DMSO without ligand	82.6%
DMF with ligand	76.6%
DMF without ligand	92.0%

The reaction was also done with ^{211}At in DMF without using the ligand as this gave the highest RCY for ^{125}I, as well as with and without the ligand at a lower percentage of organic solvent in the reaction. The previous ratio of organic solvent was 20% and the lowered ratio was 10%. The resulting RCYs are shown in table 4.3.

Table 4.3. Calculated RCYs for astatination of ABED in aqueous solution with different organic solvent percentages.

Reaction	RCY
20% DMF, without ligand	87.4%
10% DMF, with ligand	62.8%
10% DMF, without ligand	78.6%

4.5 Labeling of Poly-L-Lysine Conjugate

The first tests for labeling of PL conjugate with ^{125}I were performed in three different buffers. The reaction did not go well in PBS or BBS, resulting in barely any conversion. While the TRIS buffer gave a very low RCY of 8.2 %, it was higher than for the other two buffers and was therefore used in the following experiments. The first experiment was for reaction time. A graph of how the RCY changed depending on reaction time is shown in figure 4.5.

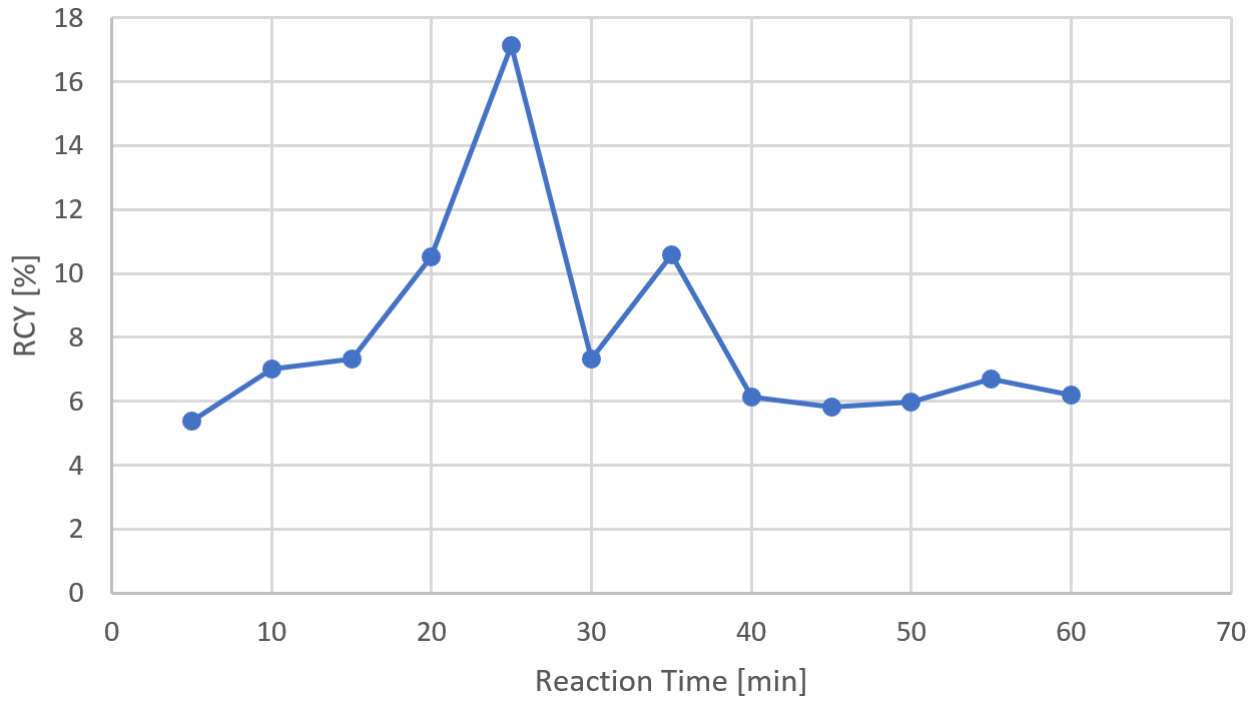

Figure 4.5. Plot of RCY versus reaction time.

The highest RCY of 17.1% was obtained after 25 minutes of reaction. The following experiment was to investigate if a lower concentration of conjugate could be used. A graph of how the RCY changed with conjugate concentration is shown in figure 4.6.

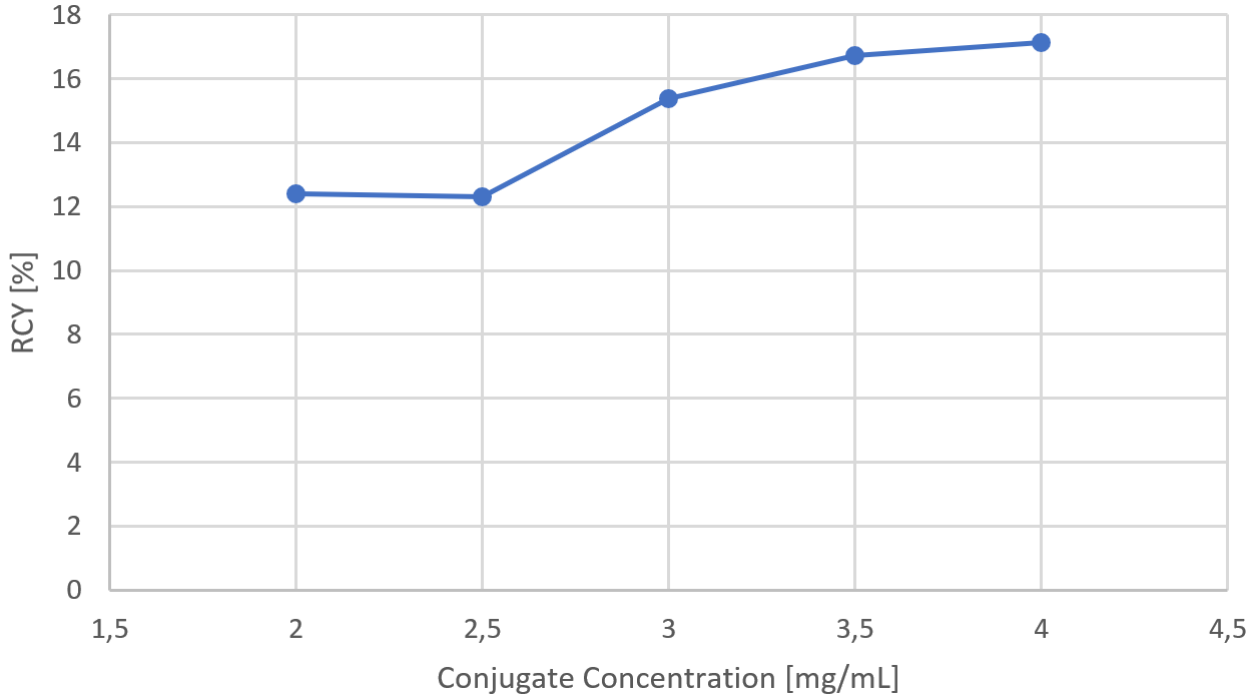

Figure 4.6. Plot of RCY versus conjugate concentration.

A lower concentration of conjugate lowered the RCY so the concentration of conjugate was kept high for the next experiment where Cu catalyst concentration was investigated. The resulting graph of how the RCY changed depending on Cu catalyst concentration is shown in figure 4.7.

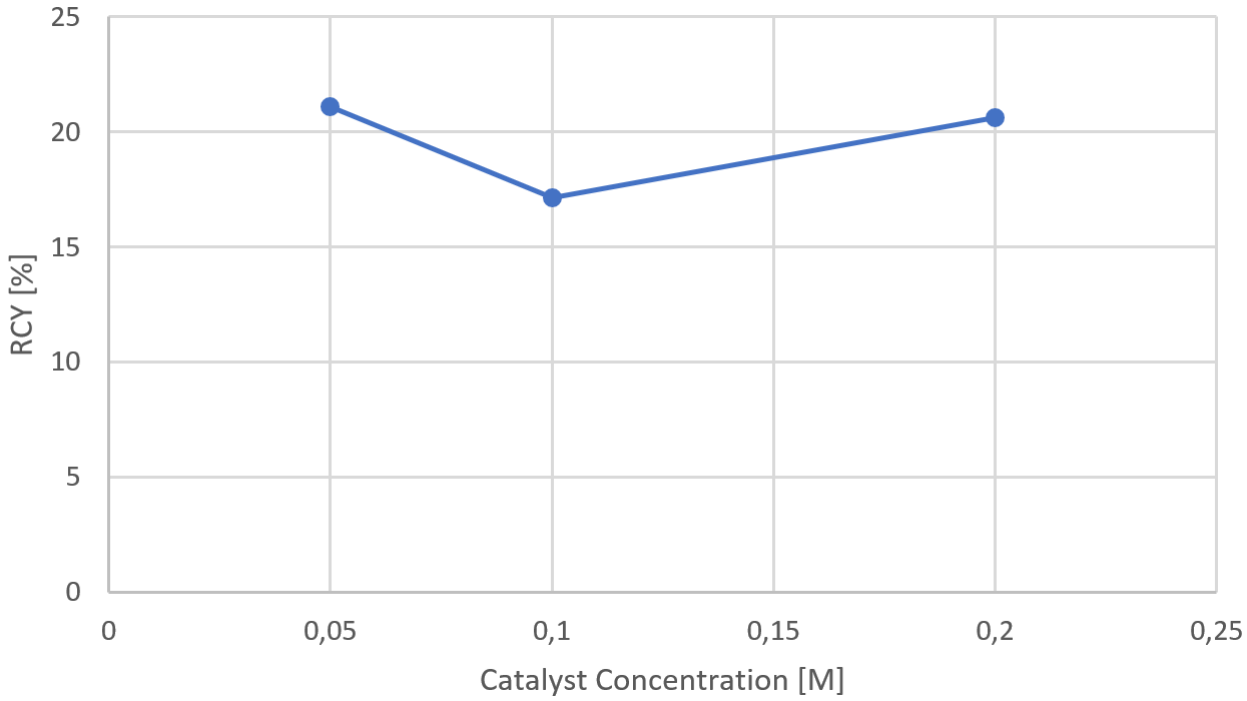

Figure 4.7. Plot of RCY versus Cu catalyst concentration.

The highest RCY of 21.1% was obtained with a Cu catalyst concentration of 0.05 M. The last experiment of how ligand concentration affected the reaction was performed with ratios of 1:1 and 2:1 with regards to the concentration of Cu catalyst. The ligand concentrations were therefore 0.05 and 0.1 M. The RCYs were plotted against the ligand concentration used in the experiment, see graph in figure 4.8.

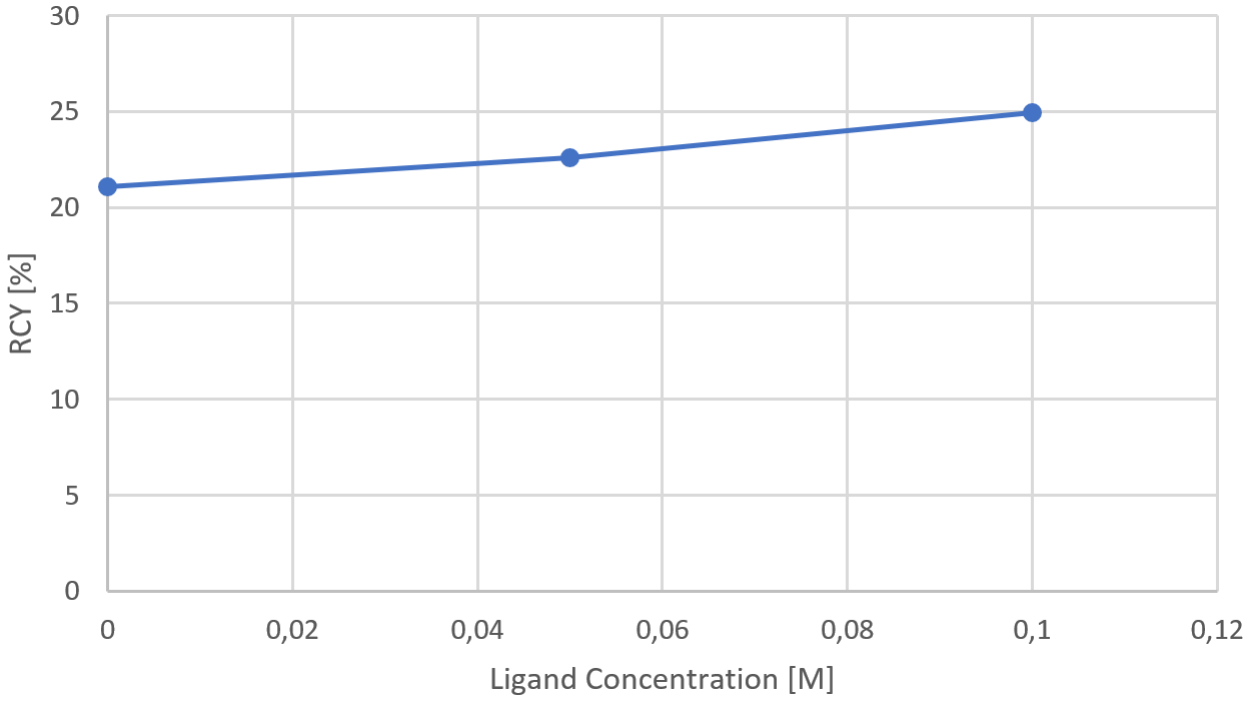

Figure 4.8. Plot of RCY versus ligand concentration.

A ligand concentration of 0.1 M gave the highest RCY of 25.0%.

4.6 Labeling of Antibody Conjugate

The antibody conjugate was labeled with ^{125}I using the reaction conditions that gave the best RCYs for PL labeling with two changes. The antibody conjugate concentration was higher than that of the PL conjugate, measured to 4.8 mg/mL using a UV-spectrophotometer, and a reaction with DMF as the organic solvent for dissolving the Cu catalyst and the ligand were performed as well as one with DMSO. Using DMF as the organic solvent gave an RCY of 17.2% while the reaction with DMSO had an RCY of 57.8%.

5 Discussion

The initial experiments in this study were based on two previous studies of halogenation using a Cu catalyzed reaction, one by Zhang and colleagues in 2016[9] and one by Reilly and colleagues in 2018[6]. Reilly performed the Cu catalyzed labeling reaction with both ^{211}At and ^{125}I on a molecule very similar to the ABED used in this project. The difference between them is that the aryl boronic ester derivative that Reilly uses does not contain a fluorine atom. Reilly's reaction was performed in organic solvents and reported a very high RCY of 100% for both astatination with ^{211}At and iodination with ^{125}I. Zhang performed the iodination with ^{131}I on the boronic acid equivalent of the molecule that Reilly also labeled with a RCY of 99%. The first astatination reaction in this project was performed with ^{211}At with the reaction conditions that Reilly had used, the only difference being the aryl boronic ester derivative (see figure 2.6 for the molecule used in this project). This reaction resulted in a RCY of 40.3%, quite drastically lower than what Reilly had reported. With a reduced NaOH concentration, the astatination RCY was increased to 61.4%. Iodination reactions with ^{125}I at even lower NaOH concentrations were performed and resulted in a maximal RCY of 87.7%. The reaction was then attempted in aqueous solutions with highest RCY of 92.0% for iodination and 87.4% for astatination. The ratio of organic solvents used in the reaction was reduced which lowered the astatination RCY to 78.6%. With this, the iodination reaction was attempted on conjugated poly-L-lysine (PL) in aqueous solutions with varying reaction conditions. The conditions that resulted in the highest RCY were used for an iodination reaction on a conjugated antibody (Herceptin) with a final RCY of 57.8%.

In order to find a reference peak of unreacted product in the reaction, the ABED was dissolved in an organic solvent and analyzed on its own using HPLC. It was also analyzed after the organic solution of ABED had been mixed with water which resulted in a new peak with an earlier retention time. As both the NHS-ester and boronic ester are susceptible to hydrolysis, it was suspected that either one of them or both had been hydrolyzed. When the labeling reaction was then performed, both previously seen peaks were detected but also a couple of new peaks. This could mean that in the first analysis, only one ester had been hydrolyzed but under the reaction conditions there was a combination of different hydrolysis products created, resulting in four possible peaks of unlabeled product, hydrolysis of none, hydrolysis of both or hydrolysis of one of the two esters. In figure 4.3 for example, there are four bigger peaks visible in the chromatogram, which probably correspond to the four different species of unlabeled product.

In the activity chromatogram, see figure 4.4, there are also four peaks. However, the same explanation cannot be used here as a labeled product would only be in two forms, either with the NHS-ester or with the hydrolyzed version. Since the labeling reaction has been performed both with boronic esters[6] and boronic acids[9], hydrolysis of the boronic ester should theoretically not pose a problem for the labeling reaction. This means that it is possible that two of these peaks are side reactions where the labeling is not proceeding as desired. In an effort to verify the identity of the main peak that was assumed to be the product peak, several reactions were run to synthesize enough material for an nuclear magnetic resonance (NMR) analysis and structure identification. Unfortunately, due to problems with the HPLC, this investigation was put on hold until the machine could be serviced. Because the service work of the HPLC took longer than expected, no NMR analysis could be performed.

Instead of doing an NMR analysis, another way of identifying a peak in the chromatogram could have been to purchase one of the suspected products and analyze it on the HPLC to examine what retention time it gives. For example, 2-fluoro-4-iodobenzoate is a possible side product that would form if the labeling is successful but the NHS-ester has been hydrolyzed. However, because of the presence of

a fluorine atom, it was difficult to find an available product. The identity of the main product peak is thus not confirmed and consequently, the RCYs calculated based on the area of that peak may not be the actual RCY for the labeling reaction. It is possible that one of the other peaks is the correct product or that two peaks should be combined as they both are the correctly labeled product but one peak is for the molecules where the NHS-ester has been hydrolyzed.

Before the HPLC needed servicing, some experiments in aqueous solution had been performed as well. As the aim of this project was to label an already conjugated biomolecule, the reaction had to be possible in aqueous solvents with only small amounts of organic solvents in order to not damage the biomolecules. Therefore, for the aqueous experiments, the reaction solvent was changed from methanolic NaOH to aqueous NaOH but with a lower concentration. Since hydrolysis of the ABED was suspected already in the organic reaction, it is very probable that hydrolysis of the boronic ester occurs during the reaction in aqueous solvents. Since the hydrolysis of the boronic ester forms a boronic acid, there should be no problems with the reaction as Zhang utilized boronic acids for their labeling.[9] As Zhang's method involves the use of a ligand (1,10-phenanthroline) during the reaction, it was used in the aqueous experiments as well. DMSO or DMF were used to dissolve the ABED, Cu catalyst and ligand but the concentrations were lower compared to the organic reaction since a higher concentration caused precipitation upon addition to the reaction. The ratio of organic solvent was reduced to 10% since this was deemed to be low enough that it would not affect the biomolecules negatively. With these conditions, the final labeling reaction still had a RCY of 78.6% which was seen as good enough to proceed. This RCY was achieved for the reaction without a ligand. As reactions using the ligand resulted in lower RCYs, the following experiments with biomolecules were performed without the ligand.

The biomolecule experiments were first performed using PL as this is a simple form of amino acid chain containing only the amino acid lysine which contains the ε-amine on its side chain that is needed for the conjugation reaction. Based on the aqueous ABED experiments, DMF was a slightly better solvent to use for the organic portion. However, since DMF is more dangerous than DMSO, for example in terms of toxicity, and DMSO also had an acceptable RCY without the use of a ligand, DMSO was used for the PL experiments, both to dissolve the ABED for the conjugation and in the labeling reaction to dissolve the Cu catalyst and the ligand. A few different buffers were tried, all with very low RCYs but the TRIS buffer was slightly higher and was therefore used for all future experiments. The concentration of Cu catalyst in the labeling reaction was not reduced to match the previously used ratio for the labeling reactions since a lower catalyst concentration might lead to longer reaction times. The reactions were also performed with lower activity concentrations and thereby also smaller amounts of activity as there was a shortage of material and it was difficult to have more delivered in time. In order to save materials, some experiments were limited and fewer reactions were performed.

Several factors were investigated using the PL conjugate in order to determine which conditions gave a higher RCY. For each factor that was investigated, nothing else was changed in the reaction in order to have comparable results. During the first investigation of reaction time, only one reaction was performed. This was because there was a lack of materials and unlike the other experiments, there was a possibility of analyzing different reaction times using only one reaction mixture. As small aliquoted samples were taken in approximately 5-minute intervals, a continuous investigation on the RCYs dependence on time could be performed. The resulting curve, see figure 4.5, showed a low RCY for the shortest reaction times which then increased after 20 minutes and had a peak at 25 minutes.

The RCYs were then lower for longer reaction times which was not expected. Since the experiment was done on a single reaction, the expected result was a curve with a logarithmic appearance with a maximum RCY after a certain amount of time. Instead, the curve seemed to show that a prolonged reaction would lead to a reduction of labeled products and an increase of free activity. It could be interesting to investigate this further by running another reaction with a longer reaction time without removing any of the volume for sample taking. Because of a lack of materials and since it was clear which reaction time gave the highest RCY, the reaction was not repeated and all subsequent reactions investigating other factors were run for 25 minutes.

The concentrations of the reactants were also investigated and it showed that only a slightly lowered conjugate concentration was possible without lowering the RCY too much. To find the conditions for as high of a RCY as possible, the conjugate concentration was kept high as the concentration of Cu catalyst was investigated. The final experiment was to see if the presence of ligand was needed in the reaction. According to previous results using only ABED, the presence of a ligand only reduced the RCY. Some hydrolysis was suspected for the previous reactions but there were still large amounts of the precursor left in the solution. As the conjugation reaction and purification are performed in aqueous solution, the time in which hydrolysis can occur has been elongated which makes it more likely that the boronic ester has transformed into the boronic acid. The results showed an increase in RCY when the ligand was used and it was highest when the ratio between ligand and Cu catalyst was 2:1. The resulting RCY was low at 25% but as there was a lack of materials, the method was not further investigated using PL. Instead, a reaction with conjugated antibodies with the corresponding conditions as for PL was performed with the exception of antibody conjugate concentration which was slightly higher than that of the PL conjugate. This time, DMF was reintroduced and compared to DMSO to examine if the RCY would increase. The best result was achieved with DMSO, a RCY of 57.8%, which is a large improvement compared to the reaction with the PL conjugate. A reason for this could originate from the additional step that conjugation process for PL requires. The PL is charge modified by converting most of the remaining amines to carboxylic acid in order to eliminate unspecific binding of the product. During this step, succinic anhydride is added to react with the remaining lysine residues which causes a drop in pH. To counteract against this, the pH is adjusted with a $NaCO_3$ solution but there is still likely a large fluctuation in pH which can affect the ABED. One reaction that can happen at highly acidic or basic pH is protodeboronation[27] where a boronic acid is replaced with a proton resulting in a non-reactive compound. If hydrolysis has occurred on some of the boronic esters, it is then possible that these can be protodeboronated. As the conjugation with antibodies is only a reaction with the antibody and the ABED in aqueous solution, it is less likely that something more than hydrolysis of the boronic ester has happened which could explain the higher RCY. It is also possible that the higher RCY is in part due to a higher antibody conjugate concentration since a higher conjugate concentration resulted in a higher RCY, see figure 4.6.

Previously, it was mentioned that the organic ratio in the reaction mixture needed to be kept low in order to preserve the functionality of the biomolecules. This was done by only adding small volumes of organic solvent in which the Cu catalyst and the ligand were dissolved. The Cu catalyst and ligand were added separately during the experiments but it could be interesting to see if it is possible to add them in a mixed solution and still get the same result from the reaction. This would reduce the volume of organic solvent that is added to the reaction mixture, further ensuring that the organic ratio is not too high. Something else to consider is if the boronic ester is hydrolyzed, the organic pinacol molecule is released and it is possible that the presence of pinacol molecules could influence the reaction in some way. Since the amount of pinacol molecules increases with hydrolysis of the boronic

esters, the antibody conjugate should be used directly after purification as this is the time where there are as few pinacol molecules as possible. The purification step on the NAP column separates the molecules based on size and the smaller molecules will not be eluted together with the conjugate. As time passes, more boronic esters can be hydrolyzed which increases the amount of pinacol molecules in the solution. It could therefore be interesting to investigate how a reaction immediately after purification compares to a reaction performed a few hours after the conjugation reaction is done.

The final labeling experiments were performed with only ^{125}I as the ^{211}At production was cancelled starting from the middle of March. It would therefore be interesting to be able to investigate if a similar RCY could be achieved with ^{211}At as this is the desired reaction. Since the ^{211}At is in its atomic form after separation from the target, it needs to be reduced before it can react. This means that the method would not be exactly the same as for what was done with ^{125}I. The difference would be that ^{211}At would be treated with NaOH or another reductant before it could be added to the reaction.

Since the final RCY of 57.8% is lower than what is desired, a continuation of this project could involve optimizing of the reaction by further investigating different condition such as trying other ligands, other Cu catalysts, other buffer solutions, different pH, different organic solvents, different concentrations or a different reductant than NaOH for the reaction with ^{211}At. The optimizing process would be similar to the experiments performed with PL in this project where certain starting conditions are set and one variable at a time is changed and its results are investigated. Maybe the most important factor to optimize is the reaction time. When dealing with such a short-lived and active nuclide as ^{211}At, a shorter preparation time is desired, as this would reduce absorbed dose to the reaction volume as well as to personnel.

Another interesting topic for future investigation could be trying different aryl boronic ester derivatives or an aryl boronic acid derivative. The aryl boronic ester derivative used in this study was a pinacol ester but there are other types that could be investigated, for example an aryl boronic acid neopentyl glycol ester which also gave a high RCY in Reilly's investigations.[6] Since hydrolysis was suspected and Zhang performed the reaction using an aryl boronic acid derivative, it might be interesting to continue an investigation using a boronic acid instead of an ester. It is also interesting to investigate how the position on the aryl ring affects the reaction as well as the stability of the formed bond. In this study, the obtained final product has the radiohalogen in para position to where it is conjugated with the biomolecule. It could therefore be interesting to investigate how a meta position affects the RCY as well as the stability of the labeled product. The aryl boronic ester derivative used in this project also has a fluorine atom on the aryl ring. How the fluorine atom affects the reaction was not investigated. It is possible that it causes negative contribution by introducing possibilities for side reactions which might explain why the first organic reaction has lower RCYs than what was reported in Reilly's article and be the reason for the other peaks found in the radio-HPLC chromatogram. The reason this ABED was chosen was the hope that the fluorine would have a positive effect in terms of stabilizing the carbon-astatine bond and lessen detachment in vivo of the final labeled radiopharmaceutical. Any stability experiments were not performed but could be an interesting topic to investigate further in a future experiment. It could also be interesting to compare what RCY the fluorine-free analogue or another type of boronic ester with a fluorine atom would result in to see what effect the fluorine atom has on the reaction.

With all these possibilities for future investigations, the Cu catalyzed method seems to show promise as an alternative method for astatination of biomolecules. The results achieved in this project are inferior to what the electrophilic substitution reaction using alkyltin reagents achieves

in terms of RCY and a slower reaction time[22] but with further investigations and optimizing the reaction conditions, a shortened reaction time and an increased RCY does not seem impossible. One motivation for investigating another astatination method was due to the toxicity of the alkyltin reagent and its products but it is also important to take into consideration the health hazards of the reactants used in the Cu catalyzed process. The ligand used in this project, 1,10-phenanthroline, is both toxic to humans and is a hazard for the aquatic environment and the Cu catalyst, $Cu(pyridine)_4(OTf)_2$, is corrosive to the skin. It is therefore important to be very careful when handling these substances as they pose a large risk when handled incorrectly. In an effort to minimize one of the risks, DMF was not used for the PL experiments and only used in the last experiment to see if it would give a higher RCY. As it did not, its use is not motivated and DMSO should be used as the organic solvent. Further investigation and optimization of this method could lead to other ways of minimizing hazardous materials, perhaps by finding a less toxic ligand that still helps increase the efficiency and the RCY. Although the ligand used in this project is toxic, it is less toxic than the alkyltin compounds and the Cu catalyzed method is therefore still favorable in terms of toxicity hazards.

6 Conclusion

Based on the final RCY that was achieved using the antibody conjugate, a post-labeling procedure seems to be feasible using the Cu catalyzed reaction. The RCY was lower that what is desired but the method could be optimized further by investigating different reactants and reaction conditions. As there were big differences in yield depending on what the concentrations were and what type of buffer was used during experiments with a PL conjugate, an increase in yield for the antibody conjugate seems possible as well. With some method optimization to also shorten the reaction time, a change from the reaction using alkyltin reagents to the Cu catalyzed method could be possible.

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Appendix

A Other Laboratory Procedures

In this appendix, laboratory procedures not included in the method section are described. This includes preparation and usage of NAP columns as well as determination of antibody conjugate concentration.

A.1 Preparation and Usage of NAP-Columns for Purification of Biomolecules

In some of the reactions involving biomolecules, the reaction solutions were purified using NAP-5 or NAP-10 columns. The columns were prepared by first letting the storage buffer solution drain out of the column. The NAP-5 column was then equilibrated by adding 200 μL BSA solution followed by 4 $\times$ 2.5 mL buffer solution letting it drain through before the next addition. For the NAP-10 columns, 400 μL BSA solution and 4 $\times$ 5 mL buffer solution was added. The sample was added and the solution was left to fully enter the bed material before an additional volume of buffer solution was added if needed to total to 0.5 mL. The additional buffer solution was run through fully before the product was eluted with another volume of buffer solution. Depending on the type of column, different sizes of sample volumes could be separated and consequently, different additional volumes and elution volumes were required, see table A.1.

Table A.1. Sample volumes, required additional volumes and required buffer solution volumes to elute the sample.

Column	Sample Volume [ml]	Added Buffer Volume [ml]	Elution Volume [ml]
NAP-5	0.1	0.4	0.5
	0.25	0.25	0.7
	0.5	0	1.0
NAP-10	0.75	0.25	1.2
	1.0	0	1.5

A.2 Determination of Antibody Conjugate Concentration

To determine the concentration of the antibody conjugate, the solution was measured on a UV-spectrophotometer. To create a baseline, the TRIS buffer solution was measured on its own first and then the machine was zeroed. 50 μL of the antibody conjugate solution was diluted with 950 μL of the TRIS solution and then measured. The measured absorbance was multiplied with the dilution factor to account for the dilution of the sample and then divided by 1.35 which is a compiled constant for the molar absorptivity, pathlength and the molar mass of the antibody to give a concentration in mg/mL, see equation A.1 (based on equation 2.10 in section 2.5.4).

$$c = \frac{A \times DF}{1.35} \tag{A.1}$$

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