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Shelf life of ε-Lysyl-3-(Trimethylstannyl) Benzamide Immunoconjugates and Poly-L-Lysine Conjugates

Master's thesis in the Master Degree Program, Nuclear Engineering

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Abstract

Micrometastatic disease is the most common cause of death from cancer. Even if the visible tumour bulk is macroscopically removed and, repeatedly, additional systemic adjuvant is given as medical treatment, relapse is frequently occurring.

Intraperitoneal tumours could arise from disseminated carcinomas, particularly those originating from the ovaries. The ovarian cancer is in most cases advanced when the disease is diagnosed. The most common treatment for ovarian cancer is surgery followed by chemotherapy. Despite a high initial favourable response, most tumours relapse and a 5 year survival rate is not higher than 30%, and therefore new more effective treatments are needed. Radioimmunotherapy is one such new treatment. Among the radionuclides used ^{211}At, an α-emitting radionuclide, has been recognized as one of the most promising candidate for endoradiotherapeutic treatment of disseminated microtumours. Even though the technique and the potential benefit of using ^{211}At for targeted radiotherapy has been recognized for over 25 years, the radiochemical route for producing astatinated antibodies has recently been developed to enable clinical use.

In this study, the shelf life of different conjugates was examined. ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugates was produced through a reaction between an antibody and a labelling reagent. The immunoconjugate were labelled with ^{211}At and stored at different time interval prior to labelling.

Poly-L-lysine conjugates was produced through a reaction between poly-L-lysine and the same labelling reagent as for producing the antibody immunoconjugates. The poly-L-lysine conjugates was labelled with ^{125}I. Due to the similar behaviour of ^{125}I compared with ^{211}At and a longer half life it facilitate the evaluation of shelf life of the ^{125}I poly-L-lysine conjugates.

The ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugates was proven to be unaffected by the storage up to nine days as determined by radiochemical purity and a radiochemical yield of the labelled product. After nine days storage the radiochemical yield gradually declined with time. Cell binding test shows a good bonding ability for most examined samples, even for those stored longer than nine days.

However, for poly-L-lysine conjugates the yield started to decline earlier than for the immunoconjugates. The radiochemical yield was above 95% for all samples but the radiochemical yield dropped below 60% after only four days.

The shelf-life of ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugates can be set to nine days and for the poly-L-lysine conjugates four days.

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List of Abbreviations

DMSO	Dimethyl Sulfoxide
m-MeATE	N-succinimidyl-3-(Trimethylstannyl)Benzoate
NBS	N-Bromosuccinimide
NIS	N-Iodosuccinimide
PBS	Phosphate Buffered Saline
PBS/1%BSA	Phosphate Buffered Saline /1% Bovine Serum Albumin
RCP	Radiochemical purity
RCY	Radiochemical yield
RIT	Radioimmunotherapy
TCA	Trichloracetic

1 Introduction

The majority of deaths from cancer originates from micrometastatic disease, even if the visible tumour bulk is macroscopically removed and, repeatedly, additional systemic adjuvant chemothearpy is given relapse is frequently occurring. (Andersson et al., 2003)

Intraperitoneal tumours may arise from disseminated carcinomas, particularly those originating from the ovaries. When the patients are diagnosed with ovarian cancer the disease is in most cases at an advanced stage. Surgery followed by chemotherapy is the most common treatment for ovarian cancer. Despite a high initial favourable response, most tumours relapse and a 5 year survival rate is not higher than 30%. (Epenetos et al., 1987, Molthoff et al., 1992) Therefore new more effective treatments are needed.

Since the ovarian carcinoma is radiosensitive radioimmunotherapy, RIT, have been investigated and among those studies both beta and alpha emitting radionuclides have been used. (Lindegren et al., 2002, Andersson et al., 2003, Epenetos et al., 1987)

The α-emitting radionuclide ^{211}At, the heaviest element in the halogen group, has frequently been recognized as one of the most promising candidate for endoradiotherapeutic treatment of disseminated microtumours. (Zalutsky et al., 1994) The potential benefit of using ^{211}At for targeted radiotherapy has been recognized for over 25 years. (Zalutsky et al., 2001)

In the decay of ^{211}At, the α-particles emitted have a short range and high linear energy transfer. This provides an opportunity to devote highly focal and cytotoxic radiation on malignant cell populations and even single tumour cells while leaving the adjacent normal tissue intact. Over the years has a variety of strategies to selectively delivering ^{211}At to tumours been developed. Among other colloids, drugs, thymidine uptake analogs, carrier substrate, biotin analogs bisphosphonate complexes, melanin precursors, and monoclonal antibodies which are examined in this study. (Zalutsky et al., 2001)

The general route for synthesising astatine labelled antibodies has been in two radiochemical steps, first labelling of the reagent and then conjugation of the labelled reagent to antibody. However, using this strategy often gives problems with low yields and deleterious affects the final quality at high activity condition, this being due to radiolytic effects in the reacting solvent. (Lindegren et al., 2008, Zalutsky and Narula, 1988)

Recently a different chemical route has been developed for producing astatinated antibodies. This method is based on conjugation of the antibodies before labelling which means that only one radiochemical step is needed for the radiochemistry reaction. This enables very fast production of astatinated antibodies and therefore no detrimental absorbed doses arise in the reacting solvent not even at high activity levels. Radiochemical yields and quality has been shown to be very good and the labelling system has the potential to be used for clinical preparations of astatinated antibodies. (Wilbur, 2001, Lindegren et al., 2008)

1.1 Aim

The aim of this study have been to examine the shelf life of ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugates and poly-L-lysine conjugate, i.e. ε-lysyl-3-(trimethylstannyl)benzamide conjugated and succinilated poly-L-lysine, i.e. study the effect on the quality after different storage time . The quality being refereed to as the capacity to maintain a good radiochemical yield and good binding properties.

1.2 Approach

In this study, two different approaches to study shelf life of benzamid conjugates has been applied, one for ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugates, and one for poly-L-lysine conjugates.

Since it is possible to easily attach ^{125}I, a radionuclide with characteristics similar to ^{211}At, to poly-L-lysine conjugates, it was used as complement to astatine labelled immunoconjugates. In this way storage condition could be examined without being dependent on access to ^{211}At.

To be able to attach a radionuclide, ^{211}At or ^{125}I, to the immunoconjugate or to the poly-L-lysine conjugates respectively, a intermediate labelling reagent was required. The reagent was attach by a synthesis after which a radionuclide can be labelled to the compound.

1.2.1 ε-Lysyl-3-(Trimethylstannyl)Benzamide Immunoconjugates

ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugates are produced through a reaction between the antibody, Trastuzumab also called Herceptin, and the intermediate labelling reagent N-succinimidyl-3-(trimethylstannyl)benzoate, m-MeATE. Figure 1 shows a schematic figure of the synthesis.

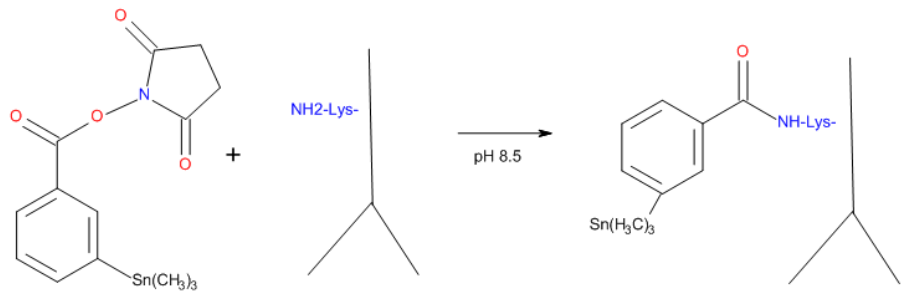

Figure 1: Reaction scheme for synthesis of ε-lysyl-3-(trimethylstannyl) benzamide immunoconjugates.

The produced ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugates was refrigerated at different time intervals between zero days and thirty-one days prior to labelling.

The shelf life of the stored immunoconjugates was examined by substituting the tin group for ^{211}At on trimethylstannyl benzamide residue. A schematic figure of the astatination is shown in Figure 2.

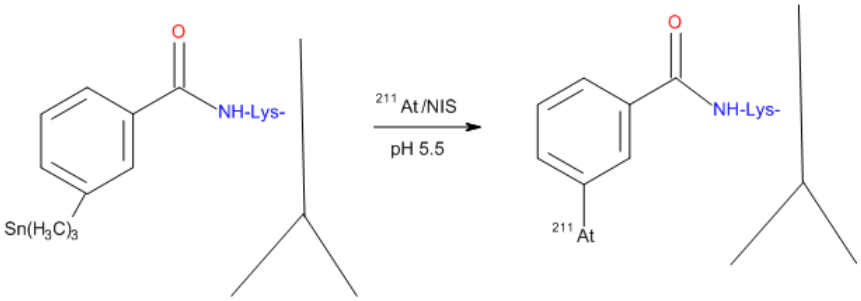

Figure 2: Reaction scheme for astatination of ε-lysyl-3-(trimethylstannyl) benzamide immunoconjugates.

The storage shelf life is based on the ratio between the radiochemical yield of the stored immunoconjugates and the radiochemical yield of a freshly prepared immunoconjugate.

The labelled immunoconjugates are examined for radiochemical purity using methanol precipitation and for immunoreactivity by binding to living tumour cells. (Lindegren et al., 2008)

1.2.2 Poly-L-Lysine Conjugates

The process to synthesize poly-L-lysine conjugates and ε-lysyl-3-(trimethylstannyl benzamide immunoconjugate are similar. Poly-L-lysine has to be modified with the intermediate labelling reagent m-MeATE in order to be labelled with a radionuclide. Figure 3 shows a schematic picture of poly-L-lysine.

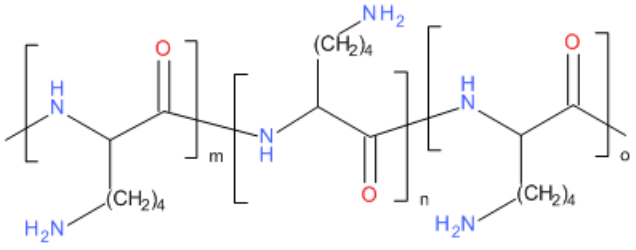

Figure 3: Schematic picture of poly-L-lysine.

The poly-L-lysine must also be charge modified with solid succinic anhydride. Figure 4 shows a schematic picture of succinic anhydride.

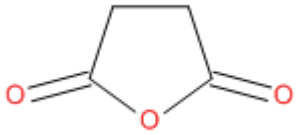

Figure 4: Schematic picture of succinic anhydride.

Succinic anhydride converts the remaining unsubstituted amino groups to carboxylic residues. Figure 5 shows a schematic picture of poly-L-lysine conjugates.

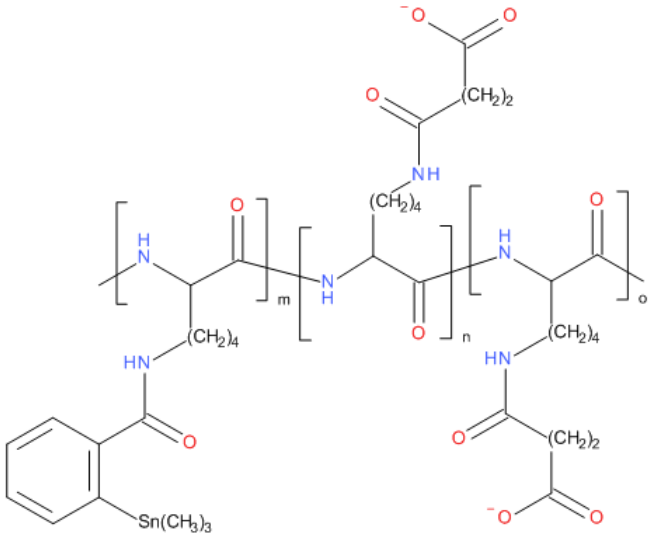

Figure 5: Schematic picture of poly-L-lysine conjugates.

The produced poly-L-lysine conjugates was stored within different time intervals between one and seven days prior to labelling.

To be able to test the storage shelf life the poly-L-lysine conjugate was labelled with ^{125}I. The shelf life is based on the ratio between the radiochemical yield of the stored poly-L-lysine conjugates and the radiochemical yield of a freshly prepared poly-L-lysine conjugates.

The labelled poly-L-lysine conjugate was examined for radiochemical purity using Trichloroacetic acid, TCA, precipitation. (Lindegren et al., 2002)

1.2.3 Radiochemical Yield and Radiochemical Purity

The radiochemical yield, RCY, and radiochemical purity, RCP, was measured to examine the quality of ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugate and poly-L-lysine conjugates.

Radiochemical puriy can be defined as "the proportion of the total radioactivity in the sample which is present as the desired radiolabelled species". (IAEA, 2013)

In radiopharmacy the radiochemical purity is very important. It is the form of the radioactive compound that determines the biodistribution of the radiopharmaceutical. The biodistribution will have different patterns depending on the radiochemical impurities which may conceal the obtained diagnostic images. This can lead to the whole investigation being destroyed. (IAEA, 2013)

For the labelling route used in this study was the radiochemical purity usually above 95%. (Lindegren et al., 2008) All samples have been measured in triplicates for the statistics.

Radiochemical yield is the ration between the amount of purified product and the starting radioactivity. (Keng, 2013)

The radiochemical yield is usually between 60%-80% for the method used in this study (Lindegren et al., 2008).

1.3 Boundaries

In this study has only the shelf life of ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugates and poly-L-lysine conjugates been examined. Cell binding has only been investigated for the immunoconjugate since the step where the polymer binds to the antibody was not included.

Further, the possibility that poly-L-lysine conjugates behave differently labelled with ^{211}At instead of ^{125}I has not been investigated.

2 Theory

When tumours are spreading in patients with ovarian cancer it is primarily in the form of peritoneal micrometastases. For this kind of malignancy ^{211}At may be a suitable choice of radionuclide for adjuvant intraperitoneal radioimmunotherapy, RIT. (Lindegren et al., 2002, Andersson et al., 2009)

Small cell clusters are more effectively irradiated with α-particles since they have a short range and high linear energy transfer. A large part of the emitted energy will be absorbed in the target, because the range of the emitted particles corresponds to the size of the target cluster. (Andersson et al., 2009, Elgqvist et al., 2006)

The short range of the α-particles ensures a significant absorbed dose to small tumours or single cells. (Lindegren et al., 2002, Elgqvist et al., 2006) This makes targeted therapy on occult microtumours possible. Cytotoxicity is mediated by a carrier vector i.e. monoclonal antibodies, labelled with radionuclides which pontentially allows a better way to treat micro tumour since conventional paclitaxel/platinum-based chemotherapy is not specific to tumour cells and which is often ineffective because of cellular resistance mechanisms. (Elgqvist et al., 2005, Andersson et al., 2009, Goldenberg, 2002)

2.1 ε-Lysyl-3-(Trimethylstannyl)Benzamide Immunoconjugates

ε-lysyl-3-(trimethylstannyl)benzamide is an resulting residue following reaction of N-succinimidyl-3-(trimethylstannyl)benzoate of primary aminogroups according to the route shown in Figure 1 and Figure 2. The primary amines, mainly the ε-lysine amines on the antibody, reacts with the stannyl ester, resulting in an residue of ε-lysyl-3-(trimethylstannyl)benzamide. (Lindegren et al., 2008)

With this procedure the labelling reaction is completed almost instantaneously and it is possible to get a radiochemical yields in the range of 60%-80%. This is a great improvement compared to the standard procedure which utilize two radiochemical steps and where the yields are in only in the range of 20%-30% at high activity conditions. (Lindegren et al., 2008)

2.2 Poly-L-Lysine Conjugates

The poly-L-lysine conjugates have been evaluated as an effector carrier for use in pretargeted intraperitoneal tumour therapy. In pretargeted radioimmunotherapy a tagged antibody is preadministrated for tumour targeting. A labelled ligand, effector molecule in this case the labelled poly-L-lysine conjugate is subsequently administrated. As intermediate are avidin and biotin the most common substances because of their high affinity binding characteristics. (Stoldt et al., 1997, Hnatowich et al., 1987) In this study, this step is not performed because it is not necessary for the polymer to bind to the antibody to examining the shelf life.

Furthermore, it has been observed that poly-L-lysine is a good multicarrier of radionuclides and of biotin for pretargeted radioimmunotherapy. (del Rosario and Wahl, 1993, Lindegren et al., 2003)

The main advantages of poly-L-lysine conjugates are that higher specific radioactivity can be obtained and increased avidity for avidin. Additionally, the

available range of molecular weight of poly-L-lysine to allow increased control of biodistribution compared to the mono-derivative of biotin. (Lindegren et al., 2002)

2.3 Astatine-211

^{211}At is a radionuclide decaying by emitting α-particles, i.e. particles with high linear energy transfer radiation, with a range of 50-80 μm in human tissue. Radionuclides emitting α-particles are unstable elements where the atomic nucleus undergoes a transformation upon decay. A schematic picture of α-decay is shown i Figure 6.

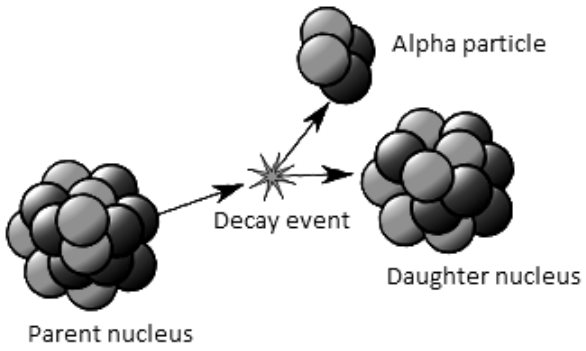

Figure 6: Schematic picture of alpha decay.

The α-particle is initially part of the nucleus from the decayed radionuclide and consists of two protons and two neutrons. This means that the α-particle corresponds to a nucleus of the element helium, $^{4}_{2}$He, but lacks two electrons. This gives that the α-particle is a heavy weight +2 charged particle. (Bäck, 2011)

2.3.1 Decay Chains

When ^{211}At decays with a half-life of 7.21 hours and α-particle emission is associated with each of its decays. (Zalutsky et al., 2001) ^{211}At has two possible decay chains, either to form the daughter nuclide ^{207}Bi (58.3% possibility). This decay chain can be described according to the simplified reaction scheme shown in Equation 1.

$$^{211}_{85}At \rightarrow {}^{207}_{83}Bi + {}^{4}_{2}He\ [\alpha,\ 5.87\ MeV] \tag{1}$$

^{207}Bi decays further with a half-life of 38 years to form ^{207}Pb.

The other decay chain is when ^{211}At decays by electron capture to form the daughter ^{211}Po (42.7% possibility) with a half-life of 0.52 seconds. ^{211}Po decays further to form ^{207}Pb. This decay chain can be described according to

the simplified reaction scheme shown in Equation 2.

$$^{211}_{85}At \rightarrow ^{207}_{84}Po \rightarrow ^{207}_{82}Pb + ^{4}_{2}He\ [\alpha,\ 5.87\ MeV] \quad (2)$$

^{207}Pb, is a stable element and the final product from both decay chains. (Bäck, 2011)

2.3.2 Production

^{211}At is generally produced by the use of a cyclotron. The process is conducted by using the nuclear reaction ^{211}Bi(α,2n)^{211}At. The ^{209}Bi target is bombarded with α-particles that are accelerated to high energies in the cyclotron. (Bäck, 2011, Andersson et al., 2003) The reaction is described in Equation 3.

$$^{4}_{2}He\ [\alpha,\ \sim\ 28\ MeV] + ^{209}_{83}Bi \rightarrow ^{211}_{85}At + 2\ neutrons \quad (3)$$

In the study of this thesis was the used ^{211}At produced at the PET and Cyclotron unit, Rigshospitalet, Copenhagen, Denmark.

2.4 Iodine-125

Since ^{125}I and ^{211}At both are halogens they chair similar characteristics and it was possible to use ^{125}I instead of ^{211}At in this research study.

The ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugate, has several possible sites where the ^{125}I can be positioned, shown in Figure 7, and therefore the immunoconjugate is not suitable for direct labelling with ^{125}I.

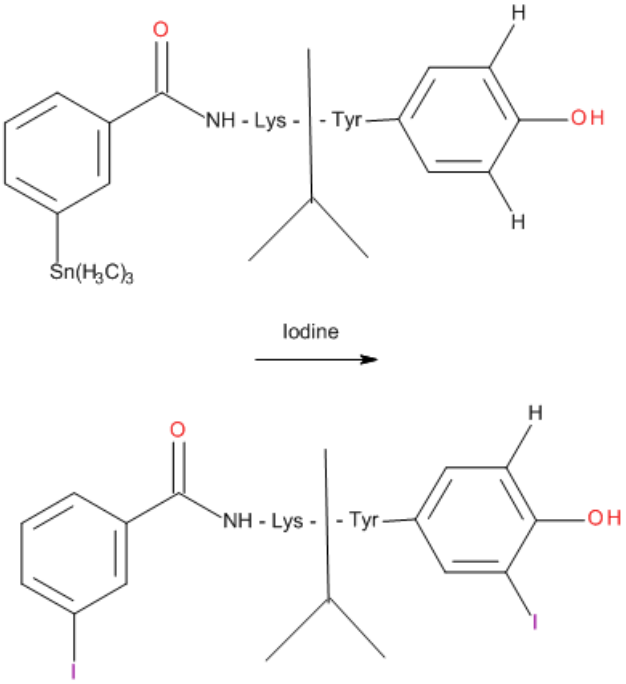

Figure 7: Possible side reaction scheme for iodination of ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugates.

^{125}I has a half-live of 60.14 days and it decays by electron capture and an excited state of ^{125}Te is produced. The produced state is not the metastable ^{125m}Te state, instead it is a lower energy state that decays immediately by gamma decay and X-rays with a maximum energy of 35 keV. (Haffty and Wilson, 2009) The reaction is described in Equation 4.

$$^{125}_{53}I \rightarrow {}^{125}_{52}Te\ [\gamma,\ 35\ keV] \tag{4}$$

^{125}Te is a stable nuclide.

2.5 Cell Binding

It is importance to examine if all the radioactivity is bound to the antibody and that the radiochemical labelling procedure does not destroy the antibodies affinity for the antigen, i.e., the radiochemical labelled antibody retains its immunoreactivity. (Lindmo et al., 1984)

The fraction of immunoreactive antibodies is conventionally determined by how much of the radiolabelled antibody that will bind to the cells under the conditions of an excess of antigen. (Buildera and Segel, 1978, Lindmo et al.,

1984) However, a more reliable determination has been obtained. The cell is diluted in series and measured with decreasing antigen concentrations. This way, the fraction of bound antibody is seen to approach a plateau value and from there the affinity of the labelled antibody can be obtained. (Buildera and Segel, 1978)

In this study was the biologic function of the antibodies investigated by binding to SKOV-3 cells after labelling.

3 Method

The solutions were prepared in two main steps, the first where ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugates and poly-L-lysine conjugate was produced through a synthesis between the antibody, Trastuzumab (Herceptin, Roche Pharma AG), or poly-L-lysine (Sigma-Aldrich Corporation) and the intermediate labelling reagent N-succinimidyl-3-(trimethylstnnyl)benzoate (Toronto Research Chemicals Inc.), and the second where the immunoconjugates and the poly-L-lysine conjugate was radiolabelled.

The conjugates were prepared in various time intervals in relation to the labelling, the conjugates then were stored in a refrigerator.

All calculation can be seen in Appendix A on page 32.

The prepared ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugates and poly-L-lysine conjugates were measured to examine the radiochemical yield and purity. The ability of cell binding was examined for the antibodies attached to the immunoconjugates.

3.1 Purification

The synthesized molecules were purified using NAP 5 (GE Healthcare) columns. First, 200 μL Phosphate Buffered Saline (Sigma-Aldrich Corporation) /1% Bovine Serum Albumin (Sigma-Aldrich Corporation, 99%), PBS/1%BSA, was added to the NAP 5 (GE Healthcare) column to remove any unspecific protein binding sites in the column. Second, the NAP 5 (GE Healthcare) column was rinsed four times, each time with 2.7 mL of a desired buffer with a desired pH to be equilibrated.

In the purification, the sample, reaction mixture from conjugation or labelling, was added to the NAP 5 (GE Healthcare) column. Buffer was added until the total added amount was 500 μL. 800 μL buffer was added to the NAP 5 (GE Healthcare) column and the eluate was collected in a small Eppendorf tube.

3.1.1 ε-Lysyl-3-(Trimethylstannyl)Benzamide Immunoconjugates

The reaction between Trastuzumab (Herceptin, Roche Pharma AG) and the reagent m-MeATE (Toronto Research Chemicals Inc.) requires a high pH, around 8.5. Trastuzumab (Herceptin, Roche Pharma AG) had a lower pH than 8.5 and a change of buffer was therefore required. A NAP 5 (GE Healthcare) column is prepared with 0.2 M carbonate buffer with a pH of 8.5. The eluated Trastuzumab (Herceptin, Roche Pharma AG) was mixed with an excess of 7.5 times more of a stock solution containing the intermediate labelling reagent m-MeATE (Toronto Research Chemicals Inc.). The solution was incubated for 30 minutes.

After conjugation the immunoconjugate was purified from unreacted low molecular weight species using the NAP 5 column as described above. The incubated solution was eluted from the NAP 5 column with 0.1 M citrate buffer. The eluate will only contain the desired immunoconjugates.

3.1.2 Poly-L-Lysine Conjugates

To produce poly-L-lysine conjugates from poly-L-lysine (Sigma-Aldrich Corporation) a NAP 5 (GE Healthcare) column prepared with 0.1 M citrate buffer was required.

Poly-L-lysine (Sigma-Aldrich Corporation) was mixed with an excess of 7.5 times more of a stock solution containing the intermediate labelling regent m-MeATE (Toronto Research Chemicals Inc.). The solution was incubated for 30 minutes.

Solid succinic anhydride (Sigma-Aldrich Corporation, 99%) in form of flake was added to the incubated solution in an excess amount in relation to available amines, to convert the remaining unsubstituted amino groups to carboxylic residues. The pH was adjusted with 1 M carbonate buffer, pH 8.5, during the reaction in order to maintain the amino residues unprotonated. The solution was incubated for 20 minutes and was then added to the NAP 5 (GE Healthcare) column prepared with 0.1 M citrate buffer. The eluate will only contain the desired poly-L-lysine conjugates.

3.2 Radionuclide Labelling

The labelling process was performed in eight different steps depending on the radionuclide used in the labelling step.

The dry astatine sample was adjusted for labelling by reaction with 10μL N-iodosuccinimide, NIS, (Sigma-Aldrich Corporation, 95%) at a concentration of 20 μg/mL in methanol (Merck, 99.8%)/1% acetic acid (Merck, 99.8%) immediately before labelling. 100 μL of the immunoconjugate were added to the dilution and the sample was incubated with approximately 10 MBq of the astatine NIS preparation for 60 seconds. 3 μL of NIS at a concentration of 1 mg/mL in methanol (Merck, 99.8%)/1% acetic acid (Merck, 99.8%) were added to the reaction mixture for reduction of any unreacted astatine. The sample was incubated for another 60 seconds. To quench the sample was 5 μL of L-ascorbic acid (Sigma-Aldrich Corporation, 98%) at a concentration of 50 mg/mL in Milli-Q (Fischer Scientific, Millipore Direct-Q 3 Ultrapure Water Systems) added immediately before purification. To remove unreacted species with a low molecular weight was the sample added to a NAP-5 (GE-Healthcare) column prepared with phosphate-buffered saline, PBS (Sigma-Aldrich Corporation) .

The approach for iodination was the same as for astatination except that the iodine was diluted in 10 μL N-Bromosuccinimide, NBS, (Sigma-Aldrich Corporation, 99%) at a concentration of 20 μg/mL in Milli-Q (Fischer Scientific, Millipore Direct-Q 3 Ultrapure Water Systems).

3.3 Radiochemical Yield

A small aliquot from the ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugates preparation was mixed with 200 μL PBS (Sigma-Aldrich Corporation) /1%BSA (Sigma-Aldrich Corporation, 99%). 500 μL methanol (Merck, 99.8%) was then added to precipitate the protein. For the polymers was the process the same except 500 μL 10% TCA (Merck, 99.5%) was used for the precipitation instead of methanol (Merck, 99.8%). A protein suspension was formed and was

measured in a NaI(Tl) γ-counter (Wizard 1480, Wallac). After centrifugation the liquid solution was removed with water suction.

The precipitate was measured and the radiochemical purity the ratio between the total activity of the solution and the activity of the precipitate, was calculated according to Equation 5.

$$RCP = \frac{Counts_{precipitate}}{Counts_{sample}} \tag{5}$$

The radiochemical yield is a measurement of the the ratio between the radiolabelled component and the added amount radionuclide multiplied with the radiochemical purity. To calculate the radiochemical yield was Equation 6 used.

$$RCY = \frac{A_{sample} \times RCP}{A_{total}} \tag{6}$$

To avoid errors in the radiochemical purity and the radiochemical yield due to time difference between measurement of the sample and the precipitate was the number of decayed particles, N, calculated according to Equation 7.

$$N = \frac{A_0}{\lambda}(1 - e^{-\lambda t}) \tag{7}$$

Where

$$N_0 = \frac{A_0}{\lambda}$$

and

$$\lambda = \frac{ln(2)}{t_{1/2}}$$

Where N_0 is the amount of radioactive substance in the measurement of the sample and t is the time between the measurements total reaction time.

N was added to the measured counts of the precipitate to erase the time difference between the two measurements.

3.4 Cell Binding

Cell binding was only tested for the immunoconjugate since this report does not include the step where the polymer binds to the antibody.

Since the antibody used in this study was Trastuzumab, the human tumour cell line SKOV-3 [5×10^6 cells/mL] was used for the cell binding evaluation.

The cells were serially diluted with the concentration of 1:2. A constant amount of 5 ng labelled immunoconjugates was added to each dilution. The cells and the immunoconjugates where allowed to react for 3 hours at room temperature during gentle agitation.

The samples were centrifuged for 5 minute, washed with PBS and centrifuged for 5 more minutes. The liquid part of the sample was removed with water suction. The samples were measured in a NaI(Tl) γ-counter (Wizard 1480, Wallac) to be able to determine the bound fraction.

Two dilution series was made for each labelled sample of astatinated immunoconjugate.

4 Result

To evaluate the shelf life and quality of ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugates and poly-L-lysine conjugates some different tests were performed.

The presented result shows if the immunoconjugates or the poly-L-lysine conjugates have a high enough radiochemical purity to not be harmful in vivo trials. A high enough radiochemical yield on the immunoconjugates or on the modified poly-L-lysine for the procedure to be profitable and how well the antibodies binds to the living cells.

4.1 ε-Lysyl-3-(Trimethylstannyl)Benzamide Immunoconjugates

ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugates had a high radiochemical purity of 95% or higher for all samples stored for shorter period then fifteen days. For the samples stored longer times than that decreases in the radiochemical purity to a minimum of 83% for the sample stored the longest time, thirty-one days were seen.

4.1.1 Radiochemical Yield

The samples stored up to nine days have a high radiochemical yield, between 60%-80%. The differences in the yield for the samples in the time frame zero to nine days are significant and within this storage time the radiochemical yields are consistently high. Figure 8 shows the radiochemical yield for each sample according to age.

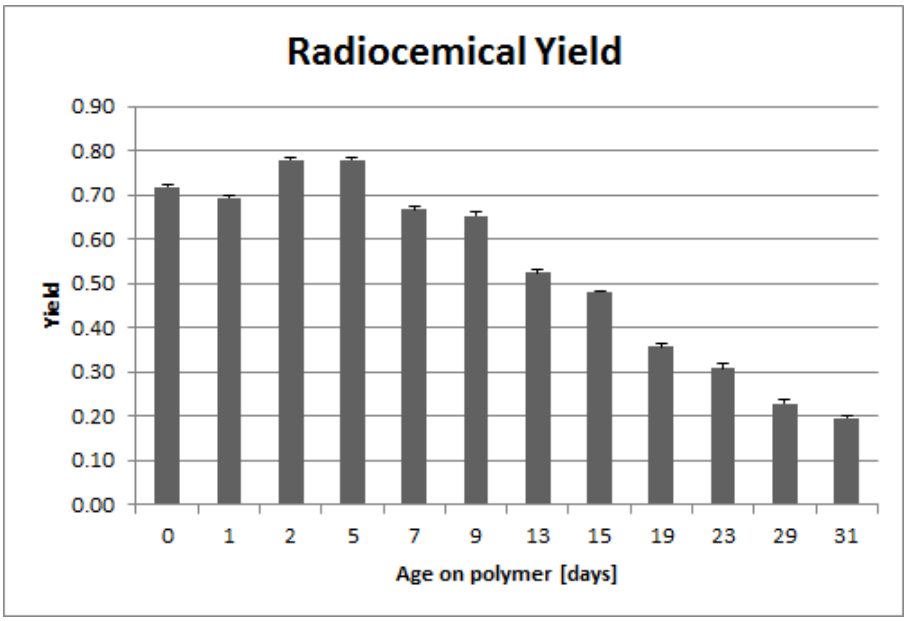

Figure 8: Radiochemical yield after different storage time on the immunoconjugates labelled with astatine.

At thirteen days a decrease in the radiochemical yield was observed. The radiochemical yield after thirteen days continues to decrease.

4.1.2 Cell Binding

The ratio of specific bound immunoconjugates to the total amount is plotted as a function of increasing cell concentration. If the preparation contains some free isotopes or if some of the radionuclide labelled immunoconjugates is not immunoreactive the bound fraction will approach a plateau with a value of less then 1.0.

Figure 9 shows the specific binding of ^{211}At-labelled ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugates to living SKOV-3 cells.

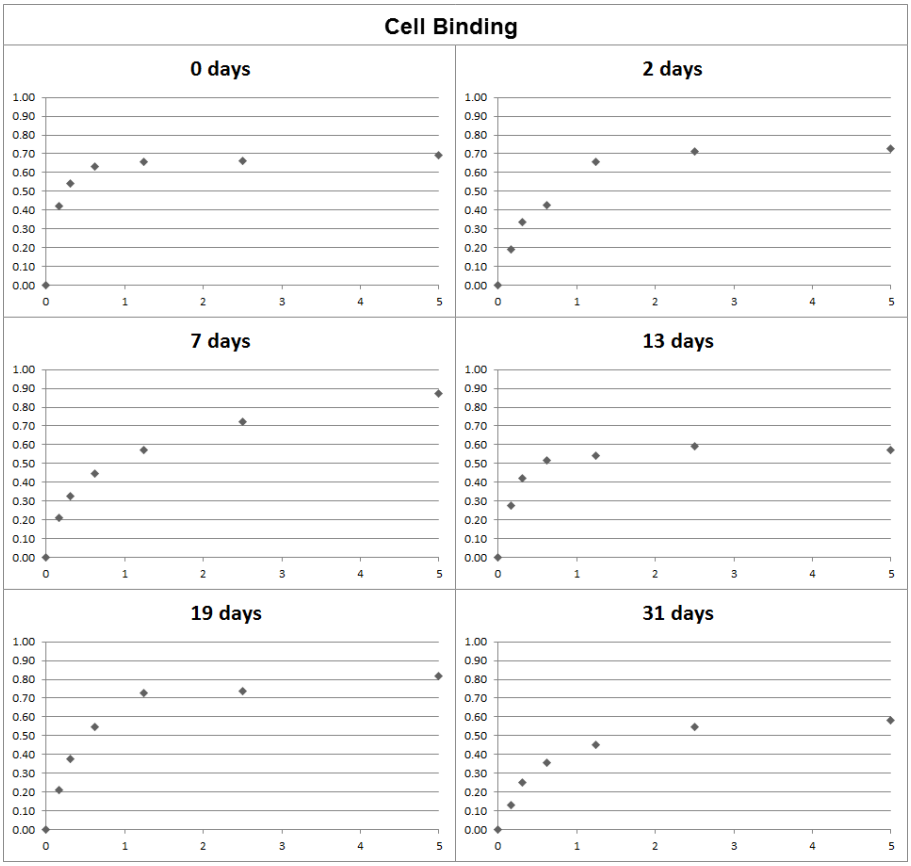

Figure 9: Astatine labelled immunoconjugates, at a few different selected storage times, ability to bind to living cells. The fraction between specific binding and total amount of applied antibodies is plotted on the y-axis and the cell concentration [10^6 cells/mL] on the x-axis.

The cell binding test indicate no specific abnormalities. Some of the graphs does not have the specific shape that cell binding curves with Trastuzumab antibodies usually have. However, a distinct plateau is visible in several of the examined samples.

In Figure 9 are only selected parts of the cell binding result shown. More detailed result is available in Appendix B.1 on page 35.

The specific immunoreactivity fraction was measured to be between 0.69 and 1.01, more specific values on the immunoreactivity fractions is available in Appendix B.2 on page 35. The result is not specific to the age of the ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugates.

4.2 Poly-L-Lysine Conjugates

Poly-L-lysine conjugates had a high radiochemical purity for all samples, 95% or higher. This shows that the quality of the poly-L-lysine conjugates has not

change during the examined time frame.

4.2.1 Radiochemical Yield

The shelf life of poly-L-lysine conjugates was found to be four days. Figure 10 shows the measured yield for each sample at different ages, respectively.

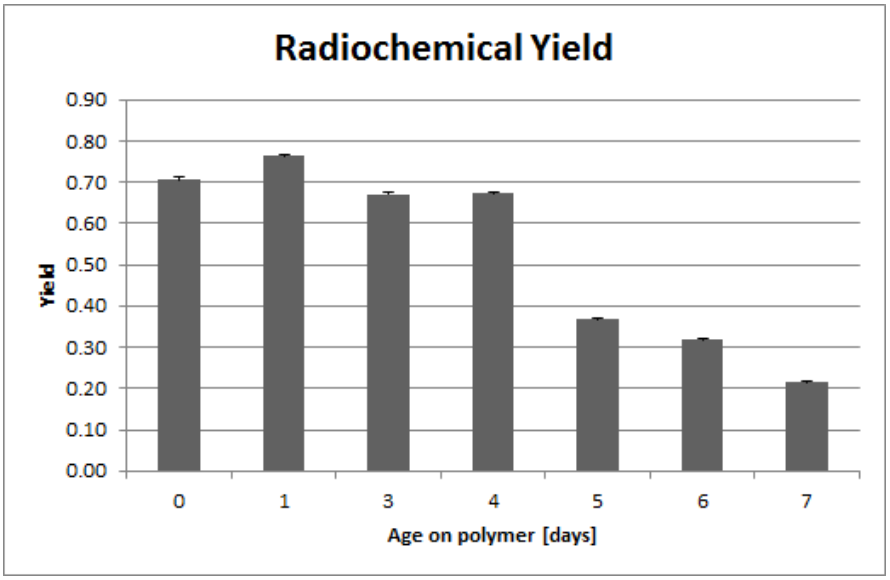

Figure 10: Radiochemical yield after different storage time on poly-L-lysine conjugates labelled with iodine.

The sample older than four days has the same high radiochemical purity as the conjugate stored shorter times but since the radiochemical yield drops below 60% is it less efficient to work with the older poly-L-lysine conjugates stored longer times.

5 Discussion

The ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugate has a storage shelf life of at least nine days. There is no harm in using immunoconjugates up to fifteen days, but it is not as profitable.

The cell binding test shows a good cell binding potential. Most samples have a distinct plateau or the beginning of a plateau with an exception for the plot for seven days where the exact plateau value is difficult to determine. An explanation for the divergent behaviour of the plot for seven days could be an excess of immunoconjugate.

Since the cell binding test only examine how well the antibody binds to the cell is it not known how well the actual immunoconjugate is binding.

Poly-L-lysine conjugates has a storage shelf-life of four days. Samples up to seven days could be used without being harmful but the drop in radiochemical yield makes it very unprofitable.

The difference between the shelf life of poly-L-lysine conjugates and ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugate could depend on the different labelling radionuclides, or of the different structure of the carrier molecule, the antibody versus the poly-L-lysine.

6 Conclusions

The result shows that ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugate can easily be stored up to nine days. After nine days decreases the radiochemical yield but the radiochemical purity remains high and is above 95% up to fifteen days and the samples can easily be used with out being toxic. With this long shelf life is it possible to make radiopharmaceutical kits or to send a prepared sample from one facility to another.

The ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugate shows a good cell binding potential. All samples have a high bound fraction for the highest cell concentrations and immunoreactive fractions close to one. There were no indications of any change in the cell binding potential related to the different storage times.

Poly-L-lysine conjugates has a much shorter shelf life then ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugates. It is not clear why there is a difference on the shelf life between the iodinated polylisen and the astatinated antibody. The shelf life of four days may be different if the used radionuclide was ^{211}At instead of ^{125}I which may interact differently with poly-L-lysine conjugates. However, it may also be dependent on the differences of the vector molecule.

Although even with a shorter shelf life of the poly-L-lysine conjugates could it be possible to make radiopharmaceutical kits or to send a prepared sample from one facility to another. Since the radiochemical yield is above 95% up to at least seven days upon storage, the samples can easily be used with out being toxic.

6.1 Future Work

In the future should poly-L-lysine conjugates be examined with ^{211}At and labelled with antibodies for more comprehensive results.

More extensive cell work should also be done, both cell binding and cell survival to be sure that the stored ε-lysyl-3-(trimethylstannyl)benzamide immunoconjugate and poly-L-lysine conjugates is not hazardous to the cells.

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A Calculations

A.1 Solutions

A.1.1 Trastuzumab

A stock solution of trastzumab dissolved in PBS was prepared.

$$m = 150\ kDa$$

Desired mass of Trastuzumab was 1 mg in a concentration of 4 mg/ml [Trastuzumab/ 0.2 M carbonate buffer].

$$\frac{1\ mg}{4\ mg/mL} = 250\ \mu L\ Trastuzumab \tag{8}$$

$$\frac{1\ mg}{150,000\ g/mole} = 6.67\ nmole\ Trastuzumab \tag{9}$$

A.1.2 Poly-L-Lysine

A stock solution of poly-L-lysine dissolved in carbonate buffer was prepared.

$$m = 25,900\ g/mole$$

Desired mass of poly-L-lysine was 0.5 mg in a concentration of 4 mg/ml [poly-L-lysine/ 0.2 M carbonate buffer].

$$\frac{0.5\ mg}{4\ mg/mL} = 125\ \mu L\ poly-L-lysine \tag{10}$$

$$\frac{0.5\ mg}{25,900\ g/mole} = 19.3\ nmole\ poly-L-lysine \tag{11}$$

A.1.3 m-MeATE for ε-Lysyl-3-(Trimethylstannyl)Benzamide Immunoconjugate

A new batch of m-MeATE was prepared for each new immunoconjugate batch.

$$c = 50\ mg/mL\ (in\ chloroform)$$

$$m = 381.7\ g/mole$$

For the m-MeATE solution was a relation of 7.5:1 wanted between the m-MeATE and the immunoconjugate.

$$6.67\ nmole \times 7.5 = 50\ nmole \tag{12}$$

A volume of 2 μL m-MeATE is chosen for the foundation of the solution.

$$\Rightarrow 25\ nmole/\mu L$$

$$2\ \mu L\ \times 50\ mg/mL = 0.1\ mg \tag{13}$$

$$\frac{0.1\ mg}{381.7\ g/mole} = 0.262\ \mu mole \tag{14}$$

$$\frac{0.262\ \mu mole}{25\ nmole/\mu L} = 10.48\ \mu L\ DMSO \tag{15}$$

2 μL m-MeATE was dissolved in 10.48 μL Dimethyl sulfoxide, DMSO (Merck, 99.5%).

A.1.4 m-MeATE for Poly-L-Lysine Conjugates

A new batch of m-MeATE was prepared for each new poly-L-lysine conjugate batch.

For the m-MeATE solution was a relation of 7.5:1 wanted between the m-MeATE and the poly-L-lysine.

$$19.3\ nmole \times 7.5 = 145\ nmole \tag{16}$$

A volume of 2 μL m-MeATE is chosen for the foundation of the solution and corresponds to 0.262 μmole m-MeATE according to Equation 14.

$$\Rightarrow 72.5\ nmole/\mu L$$

$$\frac{0.262\ \mu mole}{72.5\ nmole/\mu L} = 3.61\ \mu L\ DMSO \tag{17}$$

2 μL m-MeATE was disolved in 3.61 μL DMSO (Merck, 99.5%).

B Experimental data

B.1 Cell Binding

Table 1: Experimental data for the yield of cell binding for different ages on the immunoconjugate.

	Concentration of SKOV-3 [10^6 cells/mL]					
Age of Immunoconjugate [Days]	*0.16*	*0.31*	*0.63*	*1.25*	*2.5*	*5*
0	42.09%	54.32%	63.20%	65.67%	66.15%	69.32%
1	20.94%	29.76%	43.54%	53.39%	64.02%	63.20%
2	19.29%	36.56%	42.78%	60.74%	71.26%	72.84%
7	21.06%	32.51%	44.83%	57.33%	72.03%	87.24%
9	19.07%	37.36%	42.95%	64.41%	71.55%	88.18%
13	27.41%	42.02%	51.79%	54.39%	57.64%	57.36%
15	52.79%	68.96%	68.83%	75.21%	65.53%	68.35%
19	20.91%	37.64%	54.87%	72.65%	73.74%	81.76%
23	13.88%	24.73%	37.22%	52.05%	63.28%	73.31%
31	13.33%	25.18%	35.80%	45.07%	54.73%	58.34%

B.2 Immunoreactivity Fraction

Table 2: Experimental data for theiImmunoreactivity fraction the cell binding for different ages on the immunoconjugate.

Age of Immunoconjugate [Days]	*Immunoreactivity Fraction*
0	0.70
1	0.69
2	0.86
7	0.84
9	0.95
13	0.65
15	0.73
19	1.01
23	0.87
31	0.72