

**PREPARATION, RADIOLABELING, AND CONJUGATION OF AN AURORA
KINASE B INHIBITOR TO ^{198}Au -ACTIVATED NANOPARTICLES FOR
CANCER IMAGING AND THERAPY**

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partial fulfillment of the requirement for the degree of Master of Science in
Chemistry

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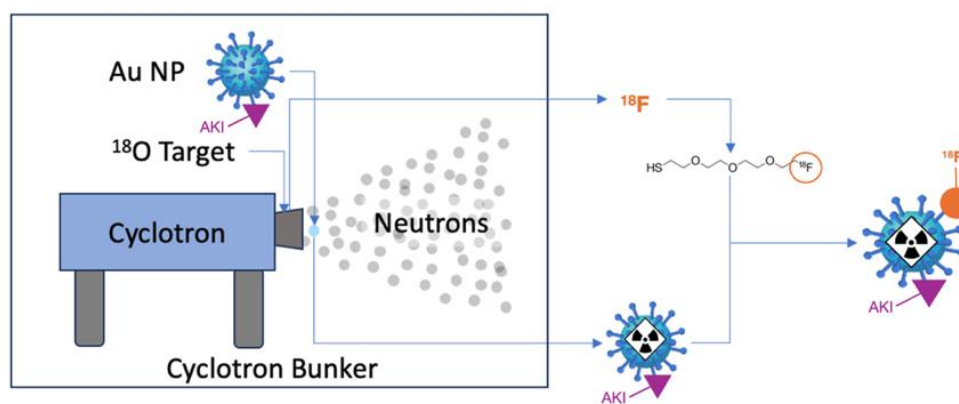
ABSTRACT

When targeting cancer, it is often necessary to attack the disease on multiple fronts. This work proposes the development of multifunctional nanoparticles (NPs) to deliver both a therapeutic radiation dose and a cancer-targeting (Aurora Kinase Inhibitor) chemotherapeutic directly to the tumour. Additionally, the incorporation of a diagnostic agent to the NP allows for a theranostic approach. The progression of the therapeutic agent can be subsequently monitored via the NPs as delivery vehicles, with the diagnostic agent allowing assessment of tumour dosage. The presence of leaky vasculature in many tumours facilitates the infiltration and accumulation of NPs in these tissues.

The preparation of polyethylene-glycol (PEG)-stabilized gold NPs (AuNPs) has been achieved and enables the targeted delivery of both therapeutic and diagnostic agents directly to the tumour. In a CCK-8 cytotoxicity assay, the AuNPs were found to have an IC_{50} of 0.06 ± 0.04 (n=10) mg/mL on the SiHa cell line, 0.08 ± 0.07 (n=4) mg/mL on the CaSki cell line, and 0.041 ± 0.002 (n=2) mg/mL on the C33A cell line. The subsequent use of cyclotron-based neutron activation to convert the AuNP into gold-198 NP ($^{198}\text{AuNP}$) can be used to provide a therapeutic radiation dose. This conversion of gold into ^{198}Au has been demonstrated and can facilitate the delivery of beta radiation directly to the tumour. The IC_{50} of the $^{198}\text{AuNPs}$ decreased with increasing radioactivity, indicating an increase in cytotoxicity. At lower activity levels (111 and 278 Bq/mL), only a minor increase in toxicity was observed; however, further increases in activity (1,145 and 2,290 Bq/mL) resulted in a continued decrease in IC_{50} , indicating enhanced cytotoxicity. The AuNP has been labelled with an aurora kinase inhibitor (AKI) as a chemotherapeutic and a cancer-targeting moiety.

The Aurora Kinase B inhibitor of interest was found to have an IC_{50} of $(1.0 \pm 1.2) \times 10^{-3}$ (n=3) mg/mL on the SiHa cell line, $(6.3 \pm 1.8) \times 10^{-5}$ (n=3) mg/mL on the CaSki cell line, and $(3.5 \pm 2.4) \times 10^{-3}$ (n=3) mg/mL on the C33A cell line. Conjugating this inhibitor enhances both specific cancer cell targeting in addition to the enhanced permeability and retention (EPR) effect that allows for the AuNPs to enter the tumour tissue, and the killing capacity of the ^{198}Au NP. The PEG chain used for the peptide linkage that connects the inhibitor to the AuNP has been synthesized, as well as the AuNP-AKI conjugate. The IC_{50} for the AuNP-AKI conjugate was determined to be 0.0298 ± 0.0008 (n=2) mg/mL with a 30:1 ratio of PEG:PEG-AKI on SiHa.

This work uniquely employs the use of a medical cyclotron for the simultaneous preparation of [^{18}F]-fluoride for PET labelling and the neutron activation of the prepared AuNPs via the $^{197}\text{Au}(n,\gamma)^{198}\text{Au}$ reaction. A model system for the PET-radiolabelling of the AuNPs has been investigated and a method has been proposed to achieve ^{18}F -labelling.



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LIST OF SYMBOLS AND ABBREVIATIONS

NPs	Nanoparticles
AuNPs	Gold Nanoparticles
¹⁹⁸ AuNPs	Gold-198 Nanoparticles
AURK	Aurora Kinase
AURK B	Aurora Kinase B
AKI	Aurora Kinase Inhibitor
PEG	Polyethylene Glycol
β^-	Beta Minus Particle (Electron)
TRT	Targeted Radionuclide Therapy
EPR	Enhanced Permeability and Retention
EGFR	Epidermal Growth Factor Receptor
PTT	Photothermal Therapy
ROS	Reactive Oxygen Species
DNA	Deoxyribonucleic Acid
β^+	Positron
PET	Positron Emission Tomography
ROI	Region of Interest
TEM	Transmission Electron Microscopy
TBAF	Tetrabutylammonium Fluoride
K2.2.2	Kryptofix 2.2.2
KF	Potassium Fluoride
NMR	Nuclear Magnetic Resonance
IR	Infrared
TLC	Thin Layer Chromatography
MeOH	Methanol
EtOH	Ethanol
MeCN	Acetonitrile
EtOAc	Ethyl Acetate
Hex	Hexanes
DCM	Dichloromethane

THF	Tetrahydrofuran
DMAP	4-dimethylaminopyridine
DMF	Dimethylformamide
NEt ₃	Triethylamine
PPh ₃	Triphenylphosphine
DIPEA	Di-isopropyl ethylamine
TIPS	Tri-isopropyl silane
EDCI	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
HOBt	1-hydroxybenzotriazole
CPC	Chromosomal Passenger Complex
Thr	Threonine
Ala	Alanine
Leu	Leucine
Val	Valine
Arg	Arginine
Glu	Glutamic Acid
Lys	Lysine
HPV	Human Papilloma Virus
CTAB	Cetyltrimethylammonium bromide
CCK-8	Cell Counting Kit-8
WST-8	Water-Soluble Tetrazolium 8
IC ₅₀	Half-maximal inhibitory concentration

1 NANOMEDICINE AND THE SYNTHESIS OF GOLD NANOPARTICLES

1.1 INTRODUCTION

Cancer continues to be a major global health concern and remains a leading cause of mortality worldwide, accounting for approximately 10 million deaths in 2020¹. Predictions suggest that annual cancer-related fatalities could reach 30 million by 2040, emphasizing the critical need for continual advancement in treatment strategies¹. Standard therapeutic techniques, such as chemotherapy, radiation, and surgical procedures, have significantly advanced cancer treatment; and typically a combination of these therapies is used to address the cancer from multiple fronts². Nonetheless, these approaches frequently encounter limitations including treatment resistance, off-target toxicity, tumor heterogeneity, and the length of time between therapy and evaluation^{1,2}. The diversity of cancer and its ability to rapidly change makes the development of universally effective treatments particularly challenging¹. The numbers suggest that traditional treatments are not independently sustainable, necessitating new approaches or their combinations to further improve outcomes.

Nanotechnology has emerged as a promising platform for personalized medicine and theranostic applications. One application of theranostics involves the incorporation of diagnostic and therapeutic functionalities on a single nanocarrier, enabling simultaneous disease detection, treatment, and monitoring³. The utilization of a single delivery vehicle for both the imaging agents and therapeutics allows for targeted delivery and enhanced tumour binding affinity to be achieved, while subsequently being able to monitor the treatment response *in vivo*^{3,4}. This combination has the potential to improve treatment efficacy with the ability to detect if it has reached

the tumour site and change courses, ultimately improving patient outcomes and reducing adverse effects⁵.

Nanoparticles (NPs) are among the most widely investigated nanocarriers in the field of theranostics³. Inorganic metal-based NPs are particularly attractive because their surface properties can be readily tailored for the desired applications³. Their dimensions are comparable to those of many biological structures at the cellular level, enabling efficient circulation and interaction with tissues throughout the body⁶. NPs typically range from 1-100 nm in size and can be synthesized in a variety of morphologies, both of which strongly influence their biological behavior and therapeutic performance^{5,7}. Their nanoscale dimensions are especially advantageous for tumour targeting and accumulation. Furthermore, NPs can carry multiple radionuclides and other therapeutic and diagnostic agents, making them highly relevant for nuclear medicine applications, as shown in **Figure 1.1**⁵.

Another important characteristic of NPs is their exceptionally high surface-area-to-volume ratio relative to mass. A 10 mg solution of 20 nm NPs will have much larger surface area than 10 mg of 100 nm NPs. As particle size decreases, the proportion of surface atoms increases, providing abundant sites for functionalization⁵. This enables the attachment of multiple therapeutic agents, radionuclides, imaging probes, and targeting ligands to a single NP platform⁵. The resulting multifunctionality can enhance tumour targeting, increase therapeutic payload delivery, and facilitate non-invasive monitoring of treatment response. When compared to conventional radiopharmaceuticals (**Figure 1.1**), the ability to carry several radionuclides as well as various therapeutic agents, targeting ligands, and diagnostic agents allows for better

treatment efficacy and the application of a theranostic approach⁵. As a result, NP-based systems are considered highly promising tools for the advancement of personalized cancer therapy.

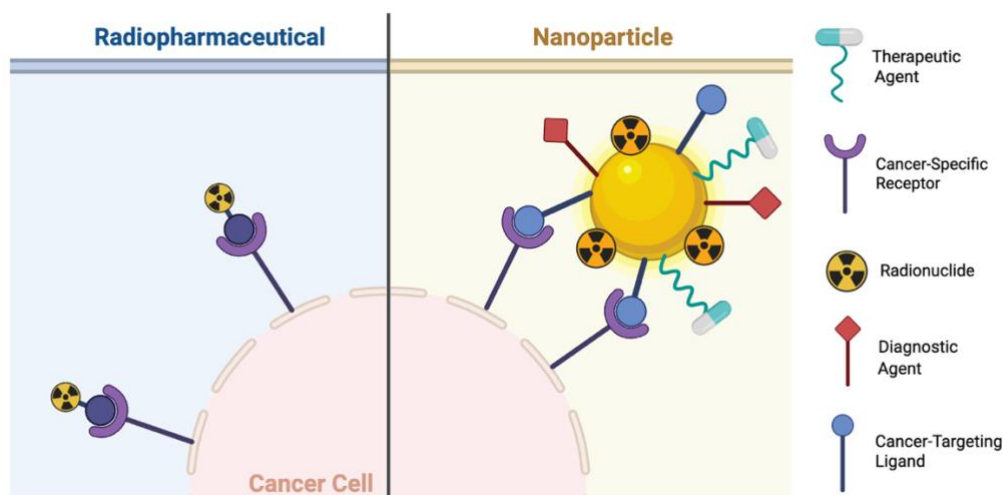


Figure 1.1 Conventional Radiopharmaceuticals vs. Nanoparticles in Nuclear Medicine (Biorender™). Schematic adapted from [5] and created using Biorender™.

The accumulation of NPs within tumours has traditionally been attributed to the enhanced permeability and retention (EPR) effect, shown in **Figure 1.2**⁵. Rapid tumour growth often results in abnormal and poorly organized vasculature characterized by gaps between endothelial cells that typically range from approximately 100-800 nm⁵. These vascular defects permit NPs to extravasate into tumour tissue, where they are likely retained due to inefficient lymphatic drainage⁵. Further, these NPs can accumulate within the tumour microenvironment and potentially increase the localized radiation dose delivered to malignant cells. Although the EPR effect has been well demonstrated in animal models, its contribution to NP delivery in human tumours remains under investigation⁵. Recent studies suggest that active trans-endothelial transport mechanisms may also play a significant role in NP uptake⁸. It has been shown

that inhibition of trans-endothelial transport pathways have reduced NP delivery such that only 3–25% of particles entered tumours through passive diffusion via endothelial gaps, indicating that additional transport mechanisms contribute to tumour accumulation⁸.

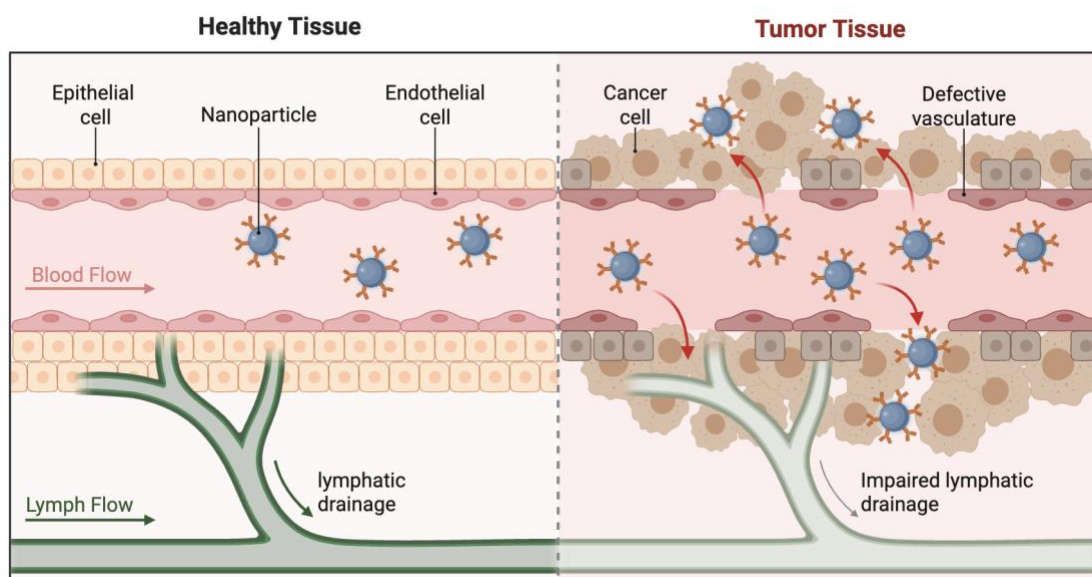


Figure 1.2 The Enhanced Permeability and Retention (EPR) Effect (Biorender™)⁵.

There are two delivery mechanisms that can be employed to bring the AuNPs to the tumour tissue, and then further inside the cell if required. The EPR effect, shown in **Figure 1.2**, is the main route of passive delivery to specific sites in the body⁹. In addition to improving the targeting of cancer drugs, this effect has also been shown to passively delivery to sites of infection and injury⁹. The EPR effect has been found to increase drug delivery and accumulation in tumour tissue by 20-30%⁹. Other parameters have to be considered to solely rely on the EPR effect as the size and shape of the AuNPs can impact the uptake, accumulation, and retention of the AuNPs⁹.

Studies have shown that smaller AuNPs have been found to have longer blood plasma circulation times, as the bigger AuNPs are cleared by the immune system faster⁹. The other mechanism that can be used is active targeting. It differs from passive delivery as it uses specific targets or disease markers in the body that are located on the surface of the cancer cells, compared to relying on physiological changes⁹. When specific markers are overexpressed on the cell surface, small molecules, peptides, or proteins, can be attached to the surface of the AuNPs in order to bring them directly to the desired site⁹.

Once inside the tumour tissue, entry into the cell can sometimes be required if the receptor is present on the inside of the cell. Passive cell entry can be achieved (**Figure 1.3A**), where the accumulation of the AuNPs in the tumour tissue via the EPR effect results in some diffusing into the cell to establish equilibrium¹⁰. It has been observed that AuNPs < 10 nm in size can readily diffuse through the cell membrane¹⁰. Once inside the cell, they can be used for controlled drug release, inducing apoptosis, diagnostic imaging, inducing cellular stress, producing ionizing radiation, and being employed for external radiation or photothermal therapy (PTT)¹⁰. Within the cell the NP has potential to diffuse through the nuclear membrane, if appropriate size¹⁰. Active cell entry can result in receptor-mediated endocytosis (**Figure 1.3B**) via binding to cancer specific receptors on the cell surface, such as the epidermal growth factor receptor (EGFR) using an antibody that is EGFR specific¹¹. This interaction triggers receptor-mediated endocytosis, internalizing the AuNP into an endosome¹¹. Within the endosome, the AuNPs can be programmed for controlled drug release, endosomal degradation, or endosomal escape¹¹.

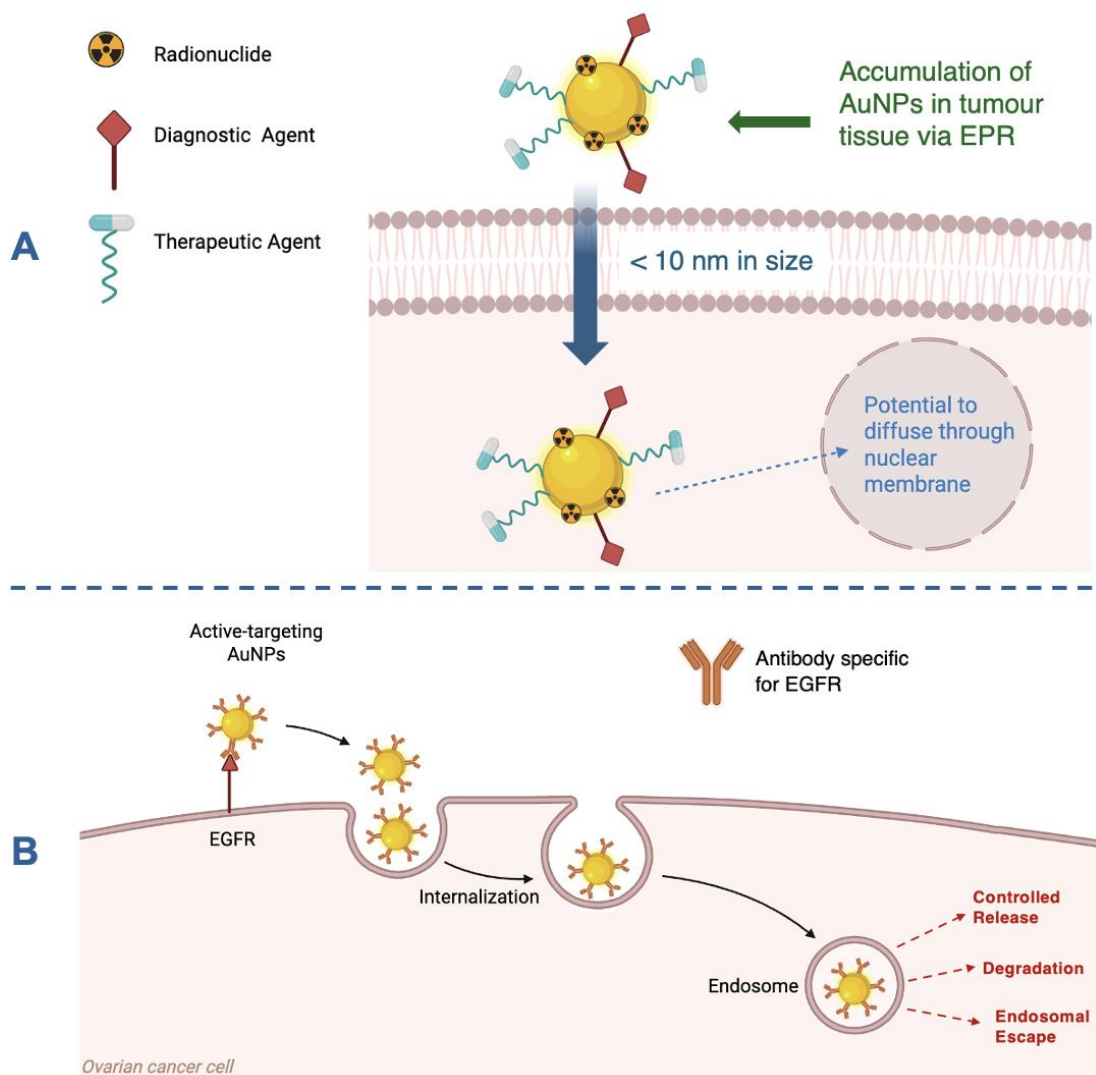


Figure 1.3 Passive (A) and active (B) cell entry mechanisms for AuNPs once inside the tumour tissue (Biorender™).

Gold nanoparticles (AuNPs) have emerged as particularly attractive nanocarriers for theranostic applications due to their ability to transport radionuclides and their diverse physicochemical properties⁵. The medicinal use of gold, including colloidal gold formulations, has been investigated for many decades¹². The optical properties of gold, specifically its light scattering and absorption characteristics, were first reported in 1857,

laying the foundation for the exploration of AuNPs in biomedical research¹².

Subsequent studies focused on understanding and optimizing NP synthesis methods as well as tailoring surface properties for specific applications¹³.

One of the earliest and most widely adopted synthesis methods involved the reduction of chloroauric acid with trisodium citrate, where citrate also functioned as a stabilizing ligand¹⁴. This approach produced AuNPs ranging from approximately 15-150 nm in diameter, with particle size dependent on the concentration of trisodium citrate employed¹⁴. The citrate reduction method subsequently served as the basis for numerous alternative synthetic strategies, enabling precise control over NP formation through adjustments in pH, temperature, and reducing agents¹⁴. More recently, environmentally friendly and non-toxic synthetic methods have attracted significant interest to further reduce environmental impact while maintaining the favorable biocompatibility associated with gold-based materials¹⁴.

Advances in synthetic techniques have enabled precise control over the size, morphology, and surface characteristics of AuNPs. These parameters directly influence NP stability, biodistribution, cellular uptake, and overall biological compatibility, allowing AuNPs to be engineered for specific biomedical purposes¹⁴. For drug delivery applications, NPs must possess sufficient stability to circulate within the body while also being capable of crossing physiological barriers, entering target cells, and accommodating therapeutic cargo on their surface¹⁴. To ensure NP stability and functionality, various surface-modifying agents are used including organic reducing compounds, surfactants, thiol-containing ligands, and biological targeting molecules¹⁵. Among these, polyethylene glycol (PEG)-thiol coatings of NPs have been extensively studied because of their excellent biocompatibility and favorable pharmacokinetic

properties¹⁵. PEGylation helps prevent NP aggregation, enhances cellular uptake, reduces immune recognition, and facilitates transport across biological barriers¹⁶. PEGylation contributes to the low toxicity of the AuNPs as gold itself is relatively inert compared to other chemotherapy agents⁵.

AuNPs have been shown to be effective drug and radionuclide carriers due to their large attachment surface and desirable surface chemistry⁵, shown in **Figure 1.4**. There are some examples of drugs being adsorbed onto the surface of the AuNPs, but typically they are attached to the AuNP surface through a thiol-containing ligand⁹. They can also be employed for controlled drug release via enzymatic, pH, photothermal or various other mechanisms⁹. Beyond their role as drug and radionuclide carriers, AuNPs can directly influence tumour biology through radiosensitization mechanisms⁵. Exposure to AuNPs has been associated with increased production of reactive oxygen species (ROS) and disruption of cellular antioxidant defense systems, leading to elevated oxidative stress⁵. These effects can promote DNA damage and impair cellular repair pathways, ultimately increasing tumour cell vulnerability⁵. Because cancer cells often exist under substantial intrinsic stress, the additional burden imposed by AuNP-mediated oxidative damage may reduce their ability to withstand subsequent treatments such as ionizing radiation, thereby enhancing therapeutic response⁵. Collectively, these properties make AuNPs a highly versatile platform for cancer therapy⁵.

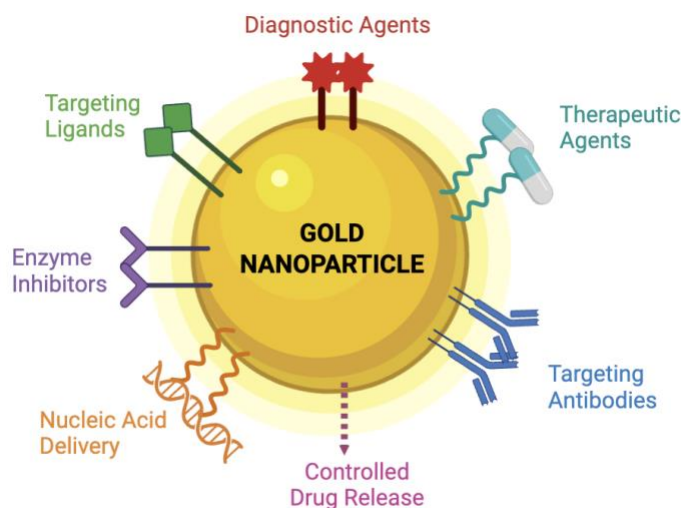


Figure 1.4 Applications of Gold Nanoparticles (AuNPs) (Biorender™).

AuNPs have also been employed in photothermal therapy (PTT)⁵, where upon exposure to light of an appropriate wavelength, collective oscillation of free electrons at the NP surface results in efficient conversion of light energy into heat¹⁷. This localized heat generation can induce hyperthermia within tumour tissue, causing irreversible cellular damage in the surrounding region⁵. In addition to directly inducing tumour cell death, photothermal treatment can enhance the release of therapeutics carried by the NPs, thereby improving treatment efficacy¹⁸. Recent developments in NP engineering and light-delivery technologies have expanded the potential of PTT to access deeper and anatomically challenging tumour sites, highlighting its promise as a complementary strategy alongside existing cancer therapies¹⁸.

AuNPs also possess advantageous radiological properties owing to the high atomic number of gold ($Z = 79$), which results in significantly greater X-ray attenuation over soft tissue⁵. This characteristic enhances image contrast while potentially reducing radiation exposure to surrounding healthy tissue⁵. A recent study showed that despite this, iodine

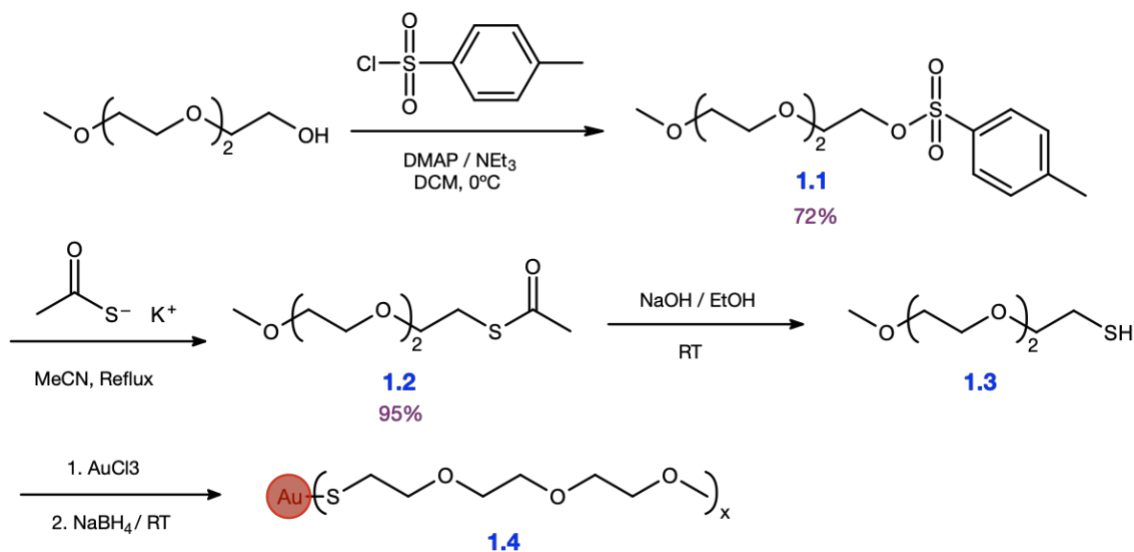
exhibits higher x-ray attenuation compared to gold, making iodine desirable under standard conditions, resulting in more efficient attenuation of diagnostic X-rays^{5,19}. AuNPs are also being extensively investigated as next-generation contrast agents capable of overcoming several limitations associated with conventional iodine-based imaging agents. Compared with iodine, gold exhibits prolonged blood circulation and increased stability, enabling longer imaging windows due to slower clearance from the body⁵. The imaging performance of AuNPs is also enhanced by their high gold atom density⁵. A 1.9 nm AuNP contains approximately 250 gold atoms, whereas conventional iodine-based contrast molecules typically contain only three to six iodine atoms⁵. As a result, radiolabeled AuNPs have the potential to improve both imaging quality and the therapeutic effectiveness of radionuclide-based cancer treatments⁵.

Overall, gold NPs (AuNPs) present a highly versatile and promising platform for advancing cancer theranostics due to their unique physicochemical properties, biocompatibility, and multifaceted functionality. Precise control over their synthesis and surface modification is critical to optimizing their stability, targeting capability, and therapeutic efficacy. As such, thorough synthesis and characterization of AuNPs form the essential foundation for harnessing their full potential in personalized cancer treatment and imaging. This chapter will focus on the detailed methods and analytical techniques employed to synthesize and characterize AuNPs, aiming to establish robust platforms for future biomedical applications. In addition, a medical cyclotron is used for the simultaneous preparation of [¹⁸F]-fluoride for PET labelling and the neutron activation of the prepared AuNPs via the $^{197}\text{Au}(n,\gamma)^{198}\text{Au}$ reaction (Chapter 2). A model system for the PET-radiolabelling of the AuNPs has been proposed to achieve ¹⁸F-

labelling (Chapter 3), in addition to the inhibitor conjugation (Chapter 4) and subsequent toxicity evaluation (Chapter 5).

1.2 RESULTS

The synthesis of the AuNPs was performed as shown in **Scheme 1.1**. Triethylene glycol monomethyl ether, 4-dimethylaminopyridine (DMAP), triethylamine (NEt_3), and p-toluenesulfonyl chloride were combined in dichloromethane (DCM) at 0°C to form **1.1**. This reaction was performed at a reduced temperature to minimize the elimination side reaction. **1.1** was obtained with a 72% yield and then subsequently reacted with potassium thioacetate to synthesize the ester-protected thiol chain (**1.2**) with a 95% yield. **1.2** was deprotected under basic conditions to produce the free thiol chain (**1.3**), that was immediately reacted with gold chloride (AuCl_3) under anaerobic conditions in the presence of a reducing agent to produce the PEG-stabilized AuNPs (**1.4**).



Scheme 1.1 Synthesis of PEGylated ligand and AuNPs.

The AuNPs were then dissolved in a small amount of water and added to a dialysis kit to remove any impurities (**Figure 1.5**)²⁰.



Figure 1.5. Purification of AuNPs via Dialysis.

Previous work was done to determine the optimal ratio of gold: thiol by examining the appearance, stability, and relative size distribution via transmission electron microscopy (TEM) images²⁰. The ratios of gold: thiol that were examined included 1:3, 1:2.5, 1:1.5, and 1:0.75. The optimal ratio was found to be 1:3, with a

characteristic deep red colour¹⁴, and consistent size of ~5 nm (**Figure 1.6**)²⁰. This ratio was used in all subsequent syntheses of the AuNPs.

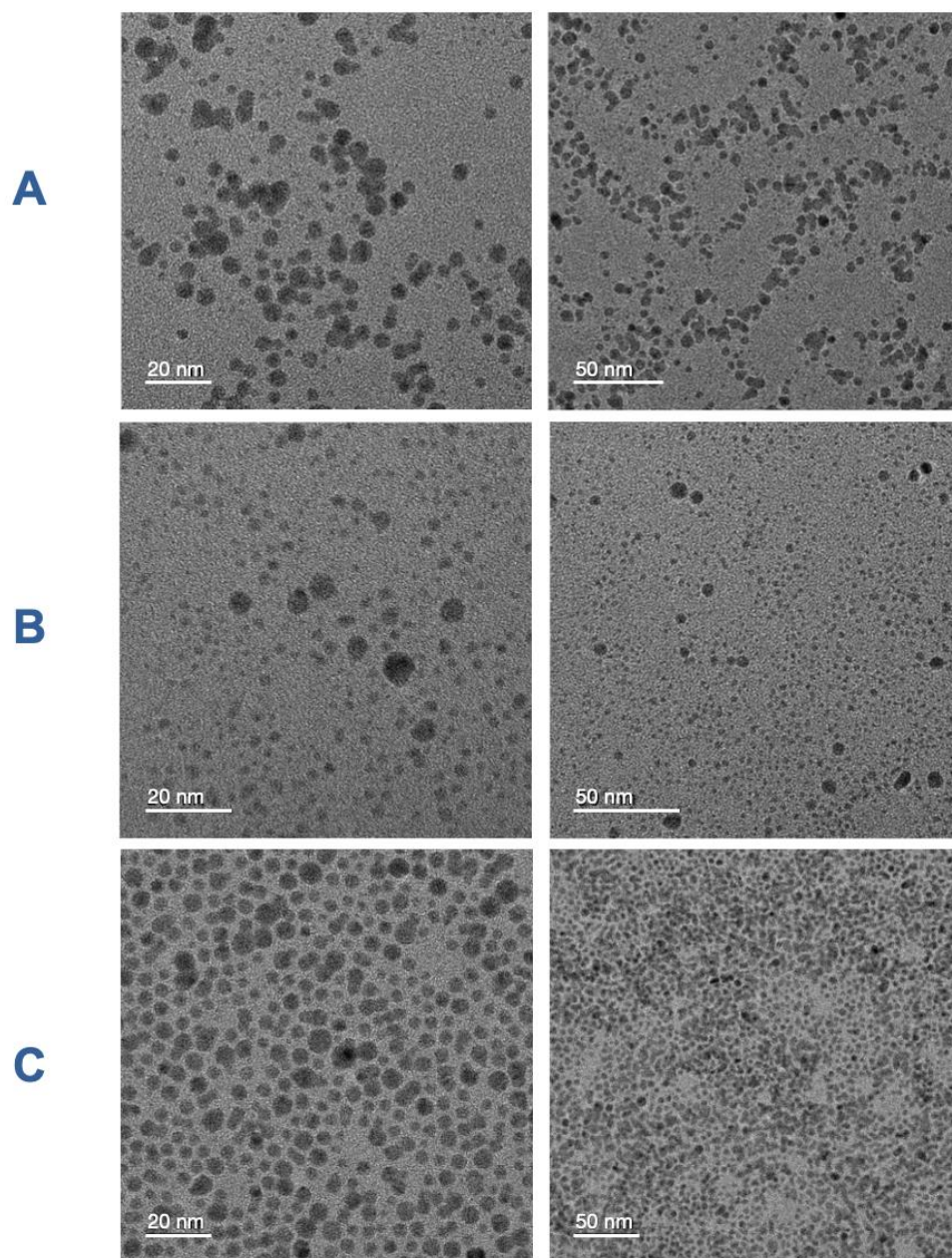


Figure 1.6 TEM Images of Thiol-Varied AuNPs. A) 1:1.5, B) 1:2.5, C) 1:3. The 20 nm scale is shown on the left and the 50 nm scale is shown on the right.

The TEM images shown in **Figure 1.6** illustrate that to obtain an even size distribution with an average size of ~5 nm based on visual inspection, a 1:3 ratio of gold: thiol is required (**C**). When the ratio of thiol is decreased, the range of NP sizes increases, and the coverage decreases, as seen in **A**), with an average size range of 5-10 nm and about 50% surface coverage on the sample, and **B**), with an average size range of 2-10 nm and about 75% surface coverage.

Overall, the synthesis of the PEG-stabilized AuNPs was successfully completed (replicated more than 10 times) using the synthetic route shown in **Scheme 1.1**, producing reproducible NPs following purification by dialysis. Optimization of the gold-to-thiol ratio identified a 1:3 ratio as the most suitable condition for NP synthesis, consistently yielding AuNPs with a characteristic deep red colour in solution, high surface coverage, and an average diameter of approximately 5 nm, as confirmed by TEM analysis (**Figure 1.6**). These optimized synthesis conditions were employed for all subsequent NP preparations described throughout this thesis. The significance of these findings and their implications for the development of the multifunctional AuNP platform are discussed in the following section.

1.3 DISCUSSION

The synthesis of the AuNPs (**Scheme 1.1**) the PEG chain with the tosylate handle (**1.1**) was initially synthesized with a 72% yield. The ester-protected thiol chain was then synthesized (**1.2**) with a 95% yield. The ester was further deprotected to produce the free thiol (**1.3**), and then this compound was immediately reacted with the AuCl_3 under anaerobic conditions in the presence of NaBH_4 to form the AuNPs (**1.4**). The synthesis of each of these compounds was confirmed by $^1\text{H}/^{13}\text{C}$ NMR (Appendix 1). The AuNPs were extracted from the reaction mixture using toluene; the toluene was removed, and they were washed with cyclohexane to remove any excess thiol, then dissolved in water and purified via dialysis. The dialysis kit containing the AuNPs in DI H_2O is shown in **Figure 1.5**. This deep red colour is consistent with the literature for AuNPs that are <100 nm in size, as those >100 nm are typically more purple/blue in colour¹⁴.

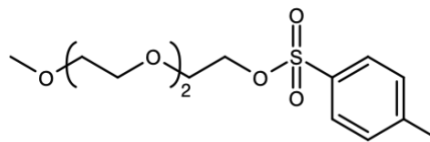
Figure 1.6 shows the TEM image results of the size variation experiment previously performed²⁰ to determine the optimal ratio of gold: thiol required to obtain the desired size of <10 nm. **Figure 1.6 A)** and **B)** show the 1:1.5 and 1:2.5 ratios, respectively. A 1:1.5 ratio yielded minimal coverage and an uneven shape distribution, and a 1:2.5 ratio had increased overall sample coverage with smaller NP sizes, but still an uneven size distribution. **Figure 1.6 C)** shows the 1:3 ratio, which yielded optimal sample coverage and a consistent average size of ~5 nm. This is likely due to the phenomenon in which AuNPs aggregate in solution to decrease their high surface energy, resulting in aggregation when there is less thiol present in solution as it acts as a stabilizing agent²¹. This occurs to ensure complete thiol coverage, whereas when there is

sufficient thiol present, a consistent size distribution is observed and they are not required to aggregate to obtain complete coverage.

Overall, the synthesis of the AuNPs was performed and replicated more than 10 times, confirming its reproducibility. Multiple TEM images were acquired on different batches, and all were comparable to **Figure 1.6 C)** with an average size of ~5 nm. Multiple batches were irradiated (Chapter 2), an Aurora Kinase B inhibitor was conjugated (Chapter 4), and the cytotoxicity was evaluated (Chapter 5).

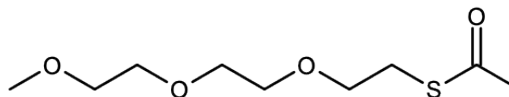
1.4 EXPERIMENTAL METHODS

1.4.1. 1-[2-(2-Methoxyethoxy)ethoxy]-2-(tosyloxy)ethane (**1.1**)⁵



Triethylene glycol monomethyl ether (9.7 mL, 61 mmol), triethylamine (21.2 mL, 152 mmol), and DMAP (1.74 g, 14.2 mmol) were added to a round bottom flask in DCM (400 mL). The reaction was cooled to 0 °C and 4-toluene sulfonyl chloride (17.4 g, 91.5 mmol) was added, and the reaction mixture was left to warm to RT overnight. The reaction progress was monitored via TLC using 3:7 EtOAc:DCM and stained with KMnO_4 . Once the reaction reached completion, the organic layer was washed with DI H_2O , dried over MgSO_4 , filtered, and the solvent was removed under reduced pressure. The crude product was purified via LC using 3:7 EtOAc:DCM. **1.1** was obtained as a colourless oil (14.5 g, 72% yield). ^1H NMR (CDCl_3 , 500 MHz): δ 7.78 (2H, d, J = 8.5 Hz), 7.35 (2H, d, J = 8.5 Hz), 4.15 (2H, t, J = 5 Hz), 3.50–3.68 (10H, m), 3.35 (3H, s), 2.43 (3H, s). ^{13}C NMR (CDCl_3 , 126 MHz): δ 144.3, 132.4, 129.3, 127.3, 71.28, 70.05, 69.88, 68.85, 68.02, 58.31, 21.00.

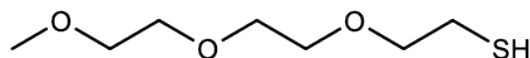
1.4.2. S-(2-(2-(2-methoxyethoxy)ethoxy)ethyl) ethanethioate (**1.2**)⁵



Compound **1.1** (14.0 g, 44 mmol) was dissolved in acetone (500 mL) in a 1 L round-bottom flask. Potassium thioacetate (6.30 g, 55 mmol) was added to the flask, fitted with a condenser, and heated to 50 °C. Formation of a precipitate was observed after 15 minutes, and the reaction progress was monitored via TLC with 1:1 Hex:EtOAc. Once the reaction was complete, it was cooled to RT and the solid was filtered out. The acetone was removed under reduced pressure from the filtrate, and the resulting crude

product was dissolved in DCM and washed with DI H₂O and brine. The organic layer was dried over MgSO₄, filtered, and the solvent was evaporated, resulting in a yellow oil. Purification on silica gel (1:1 Hex:EtOAc) yielded the desired product (9.97 g, 95% yield). ¹H NMR (CDCl₃, 500 MHz): δ 3.52–3.64 (10H, m), 3.35 (3H, s), 3.06 (2H, s), 2.30 (3H, s). ¹³C NMR (CDCl₃, 126 MHz): δ 195.2, 71.71, 70.32, 70.09, 69.52, 60.13, 58.77, 30.31, 28.59.

1.4.3. 3,6,9-Trioxa-1-decanethiol (1.3)⁵



Compound **1.2** (2.04 g, 9.2 mmol) was dissolved in MeOH (30 mL) in a round-bottom flask that was sealed with a septum and purged with N₂. 1 M NaOH (0.4 g) was dissolved in EtOH (10 mL) and was purged with N₂ for 15 minutes. The above ethanolic NaOH solution (9.2 mL) was added to the solution of **1.2** prepared above and stirred for 45 minutes. A degassed solution of HCl (1 M, 24 mL) was added to the flask. The solution was left to stir for 5 minutes, and the progress was monitored via TLC (1:1 Hex:EtOAc). The thiol was extracted with DCM, the organic phase was dried over MgSO₄, filtered, and the solvent was removed under reduced pressure. The desired product **1.3** was obtained and was immediately used in the next step to minimize disulfide formation. ¹H NMR (CDCl₃, 500 MHz): δ 3.53–4.11 (10H, m), 3.36 (3H, s), 2.67–2.70 (2H, m) 1.63 (1H, t, *J* = 5 Hz). ¹³C NMR (CDCl₃, 126 MHz): δ 72.15, 71.23, 69.83, 69.81, 69.49, 58.20, 23.53.

$$\text{Au}-(\text{S}-\text{CH}_2\text{CH}_2-\text{O}-\text{CH}_2\text{CH}_2-\text{O}-\text{CH}_2\text{CH}_2-\text{O}-)_x$$

20

2 CYCLOTRON-BASED NEUTRON ACTIVATION TO PRODUCE ^{198}Au

2.1 INTRODUCTION

The integration of multiple therapeutic modalities onto a single AuNP platform offers the potential to enhance treatment efficacy while overcoming mechanisms associated with therapeutic resistance⁵. Among the radionuclides investigated for this purpose, ^{198}Au has attracted considerable attention due to its β^- emission and relatively long half-life of 2.69 days, making it well-suited for therapeutic applications⁵. The presence of the accompanying 411 keV photons however can lead to exposure beyond the site of treatment. ^{198}Au decays into the stable isotope ^{198}Hg , which is excreted via the renal system. Since the ^{198}Au is contained in the core of the AuNPs, the PEG chains on the outside of the AuNP will prevent the ^{198}Hg from interacting with any biological structures. ^{198}Au has also been used alongside ^{125}I and ^{103}Pd as a low-dose brachytherapy agent for prostate cancer²², showing its promise as a cancer therapy agent.

The potential of PEG-functionalized ^{198}Au nanoparticles ($^{198}\text{AuNPs}$) has been demonstrated in both diagnostic and therapeutic applications. In studies investigating their use for small bowel cancer, the intrinsic optical properties of the NPs enabled tumour visualization when combined with a wireless fluorescence imaging system²³. This approach allowed the detection of early-stage tumours in the small intestine through external light stimulation of the NPs, providing a non-invasive method for tumour identification²³. In addition to their diagnostic capabilities, the $^{198}\text{AuNPs}$ delivered therapeutically relevant radiation doses directly to tumour tissue while

exhibiting minimal toxicity toward healthy cells, highlighting their promise as a theranostic platform²³.

Therapeutic radionuclides that emit β^- particles are commonly employed because they can deliver cytotoxic radiation doses directly to tumour cells⁵. Beta particles are a form of ionizing radiation capable of removing electrons from atoms, resulting in ion formation and subsequent cellular damage (**Figure 2.1**)^{24,25}. These radionuclides are also frequently utilized in Targeted Radionuclide Therapy (TRT), where radioactive isotopes are linked to tumour-targeting molecules to improve tumour localization and therapeutic efficacy while reducing non-specific exposure⁵.

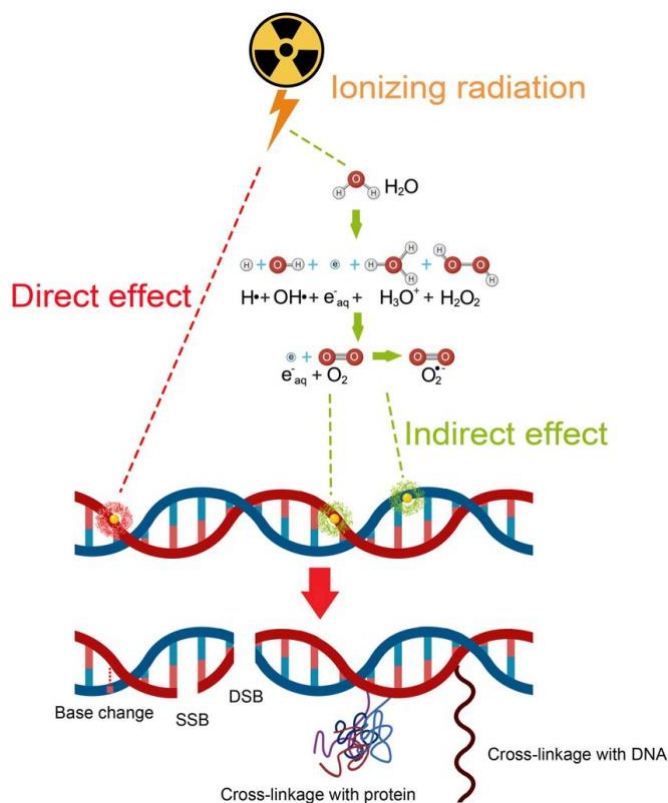


Figure 2.1 Mechanisms of Ionizing Radiation Damage to DNA²⁵.

Traditionally, the preparation of ^{198}Au NPs involves the initial production of radioactive ^{198}Au through neutron irradiation in a nuclear reactor, followed by NP

synthesis using the activated gold precursor¹⁵. This method is limited as access to nuclear reactors is expensive and produce a significant amount of waste. Also, the synthesis and purification become more challenging as the gold is already activated to ¹⁹⁸Au and cannot be handled using traditional methods. More recently, alternative production methods have been explored including the use of medical cyclotrons. Although cyclotrons are primarily utilized for the generation of positron-emitting radionuclides for diagnostic imaging, they have also been shown to facilitate the production of several β^- -emitting radionuclides through neutron activation processes²⁶.

A cyclotron accelerates charged particles using alternating electromagnetic fields, causing them to travel along an expanding spiral trajectory until they achieve sufficient energy to initiate nuclear reactions²⁷. During radionuclide production, secondary neutrons are generated as byproducts and can be harnessed for neutron activation of suitable target materials²⁶. These excess neutrons may be directed toward samples positioned within the cyclotron vault, enabling the production of neutron-activated isotopes without requiring access to a nuclear reactor²⁶.

This chapter outlines production of ¹⁹⁸Au using the waste neutrons produced in a medical cyclotron through the ¹⁸O(p,n)¹⁸F reaction, during routine ¹⁸F production²⁶. In this approach, a sample containing AuNPs in solution is placed within the cyclotron bunker in front of the ¹⁸O-water target to maximize neutron exposure and activation efficiency²⁶. Specialized handling procedures have been developed to safely transfer samples into and out of the cyclotron vault while minimizing radiation exposure to personnel²⁶. This methodology provides a potentially accessible alternative for the production of ¹⁹⁸Au and many other neutron-rich isotopes via the cyclotron and neutrons

that would otherwise be considered waste²⁶. This process allows for the AuNPs to be activated after synthesis and subsequent conjugation of other therapeutic agents.

2.2 RESULTS

In order to minimize the radiation exposure and allow for quick sample collection, the sample transport system shown in **Figure 2.2** was developed²⁶. The red tube is the sample transfer tube where the samples are placed, and the air compressor is used to move them into the vault via the trench under the floor. This places the sample in the correct position to be irradiated where it will receive the maximum neutron exposure, in front of the ^{18}O -water target. The blue compressed air return line is used to retrieve the samples once they have been irradiated. This is done using the air compressor to bring the samples back to the outside of the vault through the sample transfer tube in the floor, where they can be collected. This allows for the AuNPs to be irradiated simultaneously as the Fluorine-18 isotope is being produced for diagnostic use.

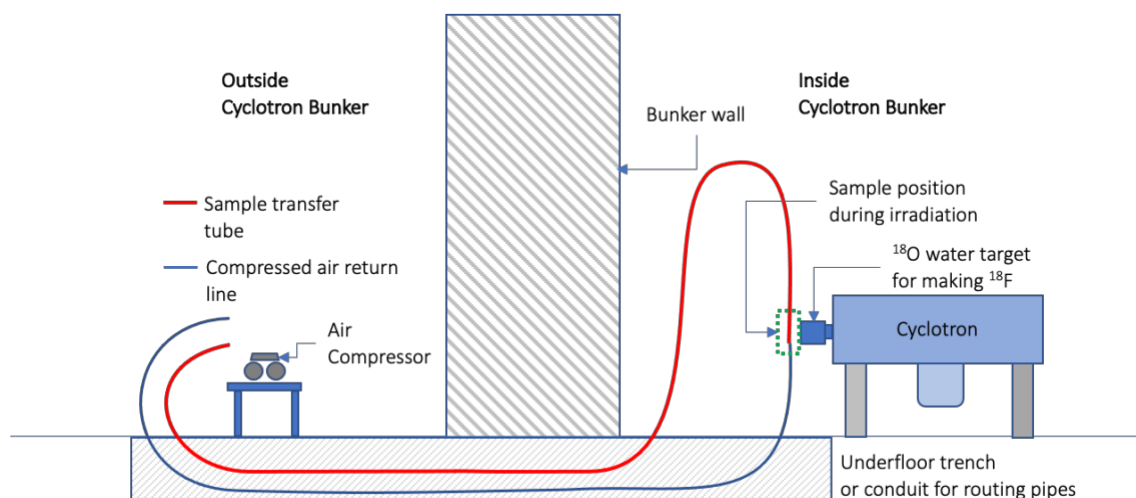
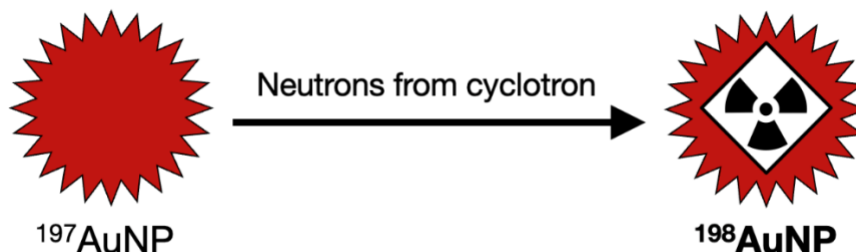


Figure 2.2 Cyclotron Sample Transport System²⁶. The red tube is the sample transfer tube and the air compressor is used to transport them into the vault, placing the sample in front of the ^{18}O target. The blue compressed air return line is used to retrieve the samples once they have been irradiated.

1 mL of a 37.5 mg/mL AuNP stock solution in DI H_2O was transferred into the 2 mL polyethylene sample tubes with screw-sealed caps. The sample was loaded into the vault using the sample transport system in **Figure 2.2**. The sample was irradiated at

9216 $\mu\text{A}\cdot\text{minutes}$ total (5574 $\mu\text{A}\cdot\text{mins}$ + 3642 $\mu\text{A}\cdot\text{mins}$) with a proton beam energy of 18 MeV, shown in **Scheme 2.1**.



Scheme 2.1 Neutron Activation of Gold-197 NPs to Gold-198 NPs via the Cyclotron.

The resulting gamma spectrum obtained on the sample is shown in **Figure 2.3**, with the 411 keV peak highlighted in red, corresponding to ^{198}Au . The lack of any other peaks in the spectrum indicates that no other isotopes were produced and no contamination was observed.

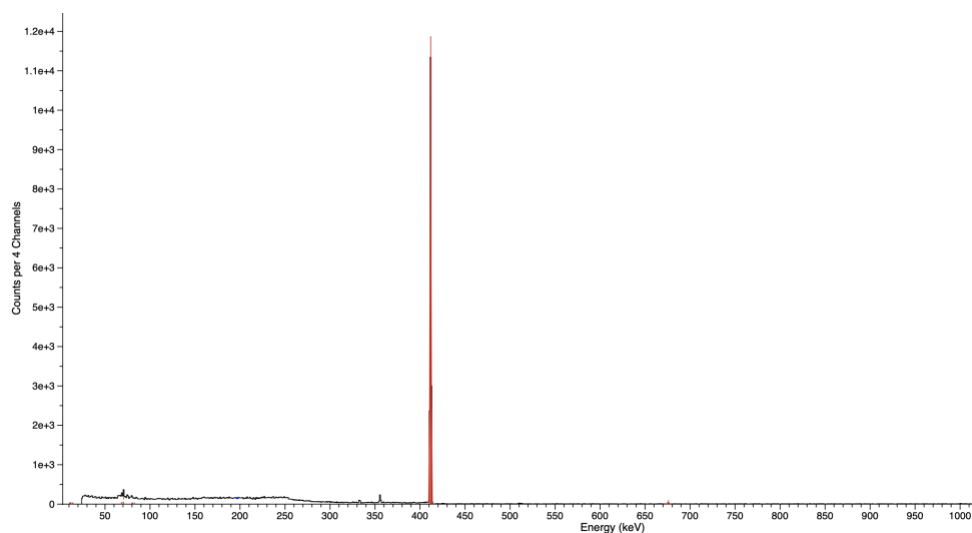


Figure 2.3 Gamma Spectrum of $^{198}\text{AuNPs}$. This spectrum was obtained from a 10-minute detection cycle of the $^{198}\text{AuNPs}$ irradiated over 9216 $\mu\text{A}\cdot\text{minutes}$ with a proton beam energy of 18 MeV on the [^{18}O] water target. The 411 keV peak corresponds to ^{198}Au .

A well counter was used to determine the activity in Becquerels (Bq) of the same sample from above, shown in **Figure 2.4**.

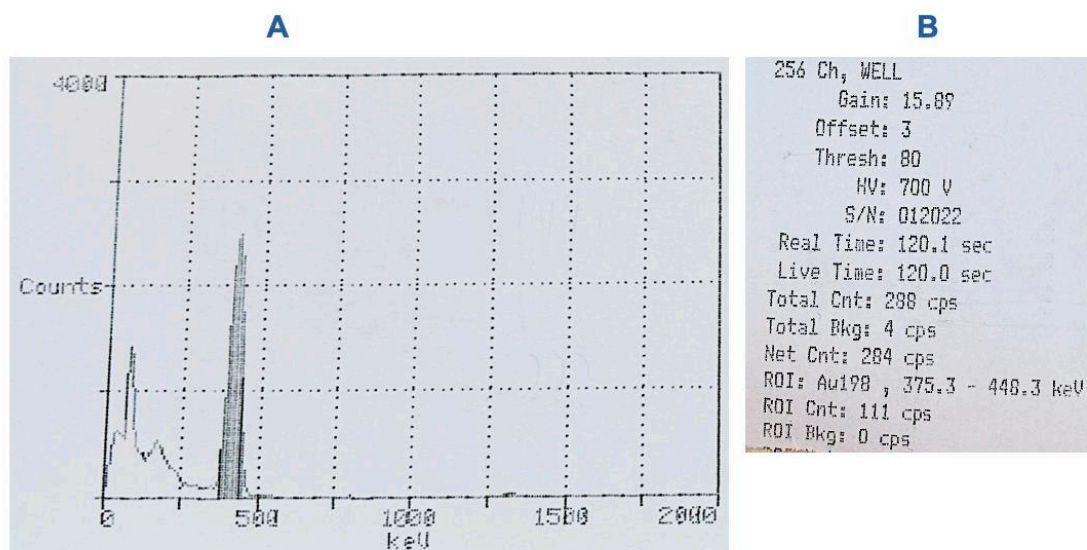


Figure 2.4 Well Count of Gold-198 NPs. This spectrum was obtained from a 2-minute detection cycle of a 0.4 mL solution of ^{198}Au NPs irradiated over 9216 $\mu\text{A}\cdot\text{minutes}$ with a proton beam energy of 18 MeV. A) shows the plot with the darkened ROI corresponding to ^{198}Au (375.3-448.3 keV). B) shows the parameters of the scan as well as the total counts and the counts in the ROI.

The 2-minute scan detected 288 cps total, which is equivalent to 288 Bq, and 111 cps or 111 Bq in the ROI for ^{198}Au (375.3-448.3 keV). This results in a radioactive concentration of 278 Bq/mL for the 0.4 mL solution. The other counts detected are likely from the activation of the vial itself or other Au isotopes that decay at lower energies, which also can result in X-ray peaks from the β -particles interacting with the lead walls of the detector or the Au atoms.

2.3 DISCUSSION

The AuNPs produced in **Scheme 1.1** underwent neutron activation to produce ^{198}Au NPs (**Scheme 2.1**), using the sample transport system shown in **Figure 2.2**. This system was developed to allow for the sample to be retrieved immediately after neutron bombardment without having to enter the vault, minimizing radiation exposure and ensuring optimal activity. This system works by inserting the sample into the red sample transfer tube and turning on the air compressor to transport the sample under the floor into the vault and in front of the ^{18}O -water target. A green light will turn on outside the vault once the sample is in position to ensure it has travelled all the way through the tube and is placed in front of the desired target. Once the neutron bombardment is complete, the sample can be retrieved immediately by blowing air into the compressed air return line and the sample can be collected from the red sample transfer tube and characterized.

Sample characterization includes a 10-minute gamma spectrum cycle to visualize the 411 keV peak representing ^{198}Au followed by a 2-minute well count to determine the sample activity in Becquerels. **Figure 2.3** displays a 10-minute gamma spectrum of a sample that was irradiated for 9216 $\mu\text{A}\cdot\text{minutes}$ total (5574 $\mu\text{A}\cdot\text{mins}$ + 3642 $\mu\text{A}\cdot\text{mins}$) with a proton beam energy of 18 MeV. This spectrum was analyzed using the Interspec software. The large peak highlighted in red corresponds to the 411 keV peak of ^{198}Au . The absence of any other peaks indicate that no other isotopes were produced and the sample was pure. **Figure 2.4** shows the 2-minute well count that was obtained on the same sample. **Figure 2.4 A)** shows the plot is produced with the ROI highlighted corresponding to ^{198}Au , and **B)** shows the parameters of the scan, including the total

counts and the counts in the ROI. The total counts were 288 cps which is equivalent to 288 Bq, and the counts in the ROI for ^{198}Au (375.3-448.3 keV) were 111 cps or 111 Bq. The other counts likely correspond to the activity from the vial itself or resulting X-ray peaks from the interaction of the sample with the lead walls of the chamber. All other neutron activations followed the same procedure and characterization methods.

2.4 EXPERIMENTAL METHODS

2.4.1 Neutron Activation of AuNPs

The AuNPs were dissolved in ~1 mL of DI H₂O and some of the solution was transferred into the 2 mL polyethylene irradiation vial. 2 mg of the AuNPs were in the vial with a serial number of 12108741. Once at the cyclotron facility, the sample was loaded into the vault using the schematic shown in **Figure 2.2**, to minimize radiation exposure. The vial was placed in the sample transfer tube, and the air compressor was turned on to transfer the sample through, using the trench under the floor to enter the vault. The sample was then kept in the transfer tube, where it sits in front of the ¹⁸O water target that is used to produce the ¹⁸F. Once the sample was irradiated at 5000 $\mu\text{A}\cdot\text{minutes}$ over approximately 50 minutes with a proton beam energy of 18 MeV, the air compressor was placed in the return line and turned on to bring the sample out of the vault where it was collected in a lead box. The sample was then placed in the gamma spectrometer where its activity was measured during a 10-minute detection cycle.

3 MODEL SYSTEM FOR PET-RADIOLABELING OF GOLD NANOPARTICLES

3.1 INTRODUCTION

To leverage the diagnostic aspect of the AuNPs, it is necessary to include a diagnostic tag such as a positron-emitting radionuclide. Positron emission tomography (PET) imaging is the most common imaging modality used in cancer detection and treatment²⁸. This imaging technique uses positron (β^+)-emitting isotopes in order to detect a signal in tumour tissue and produce 3D images of the functional processes in the body²⁸. These images are produced via the detection of the pair of gamma rays that are emitted after the annihilation events occur from the β^+ -emitting radionuclide²⁸.

Since PET imaging is quantitative, the concentration of imaging agent in the tumour tissue can be determined²⁸. The theranostic technique enables the quantification of the therapeutic agent through the diagnostic agent, facilitating the monitoring of therapeutic agent delivery, and allows for *in vivo* therapy evaluation²⁹. **Table 3.1** shows the comparison of various treatment characteristics between traditional and theranostic approaches to cancer treatment. It can be seen that the theranostic approach allows for enhanced targeting with the damage to healthy cells being limited, the treatment can be tailored to each patient, there are generally less side effects, the treatment can be evaluated and adjusted in real-time, and lastly the theranostic approach typically has increased efficacy³⁰.

Table 3.1 Comparison of Treatment Characteristics Between Traditional and Theranostic Approaches³⁰.

Treatment Characteristic	Traditional Approach	Theranostic Approach
Precision of Targeting	Not specific; also affects healthy cells	Very specific and targeted; damage to healthy cells limited
Patient Personalization	Limited; treatment the same for all patients	Tailored to each patient
Adverse / Side Effects	Often severe	Generally fewer
Treatment Monitoring	Evaluation after several treatment courses	Real-time in vivo monitoring via diagnostic imaging
Ability to Adjust Treatment	Slow; based on slow or indirect results	Quicker; adjusted with real-time data
Overall Efficacy	Variable; depends on cancer type and patient	Higher efficacy; for certain cancer types

The most widely used PET radionuclide is Fluorine-18 (^{18}F), which possesses favourable nuclear and chemical properties for PET applications, including an optimal half-life of 109.8 minutes and positron emission at 635 keV²¹. The emitted positron is thermalized and collides with an electron, causing an annihilation event, where the two coincident photons are diverging at approximately 180° relative to each other with an energy of 511 keV²⁸. The detection of these coincident photons is used to construct the PET image²⁸. These characteristics enable both off-site synthesis and subsequent transport of the radionuclide, as well as extended imaging protocols²¹. Other PET isotopes include ^{15}O , ^{13}N , and ^{11}C , which all have significantly shorter half-lives compared to ^{18}F of 2.04, 9.97, and 20.4 minutes³¹. In addition, fluorine is a favourable molecule for drug development due to its ability to be incorporated into various molecules via the formation of a strong and thermally stable bond with carbon, and its low positron energy that is able to increase the resolution of PET images³¹. Many

selective fluorination agents have been developed for both nucleophilic (F^-) and electrophilic (F^+) substitutions, making fluorine more useful³¹.

^{18}F can be synthesized in large quantities with a cyclotron by the irradiation of ^{18}O with an accelerated proton (1H)³¹. The nuclear reaction used to produce the nucleophilic ^{18}F -fluoride is $^{18}O(p,n)^{18}F$, which is very efficient and produces a high amount of radioactivity³¹. Nucleophilic fluorination methods are used in most radiolabelling cases due to the ease of synthesis and the high specific activity³¹. This process includes the production of the aqueous $^{18}F^-$ solution via the cyclotron, followed by the extraction of the isotope from the solution. This is crucial to the process, as the fluoride ion hydrogen bonds to the water molecules in the aqueous solutions, making it unreactive. In order to mitigate this, its solubility in nonaqueous solvents and reactivity is enhanced using either a phase transfer catalyst (i.e., Kryptofix 2.2.2 and potassium) or a bulky tetrabutylammonium cation³¹. These reactions require a base that is also a poor nucleophile (i.e., carbonate or bicarbonate ions)³¹. The water then must be thoroughly removed so the reaction can be carried out in a polar aprotic solvent. This is typically done by azeotropic drying in acetonitrile³¹. Once dry, the fluorine can then be added to the reaction mixture and subsequently to the desired molecule to be radiolabelled.

Radiolabelling of AuNPs with ^{18}F can be achieved through either direct or indirect approaches. In direct radiolabelling, the ^{18}F -labelled moiety is incorporated into the organic surface coating of the AuNP, typically via surface-bound ligands or functional chains⁵. In contrast, indirect radiolabelling employs a linker, such as an aromatic phenol derivative, which carries the ^{18}F label and facilitates its conjugation to the AuNP

surface⁵. The inclusion of ^{18}F as a diagnostic component enables the imaging functionality within a theranostic platform.

This chapter developed the methodology necessary for the indirect radiolabelling of the AuNPs via nucleophilic fluorination using ^{19}F . The methods developed can later be translated using the ^{18}F radionuclide and the automated synthesis equipment in a hot cell. Fluorination reactions were explored using tetrabutylammonium fluoride (TBAF) and the Kryptofix 2.2.2/potassium fluoride (K2.2.2/KF) system to evaluate their suitability for incorporation into the proposed radiolabelling strategy (**Figure 3.1**).

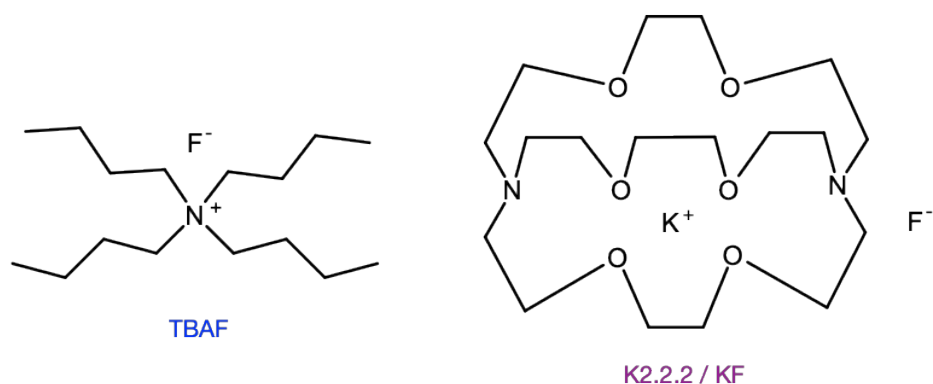
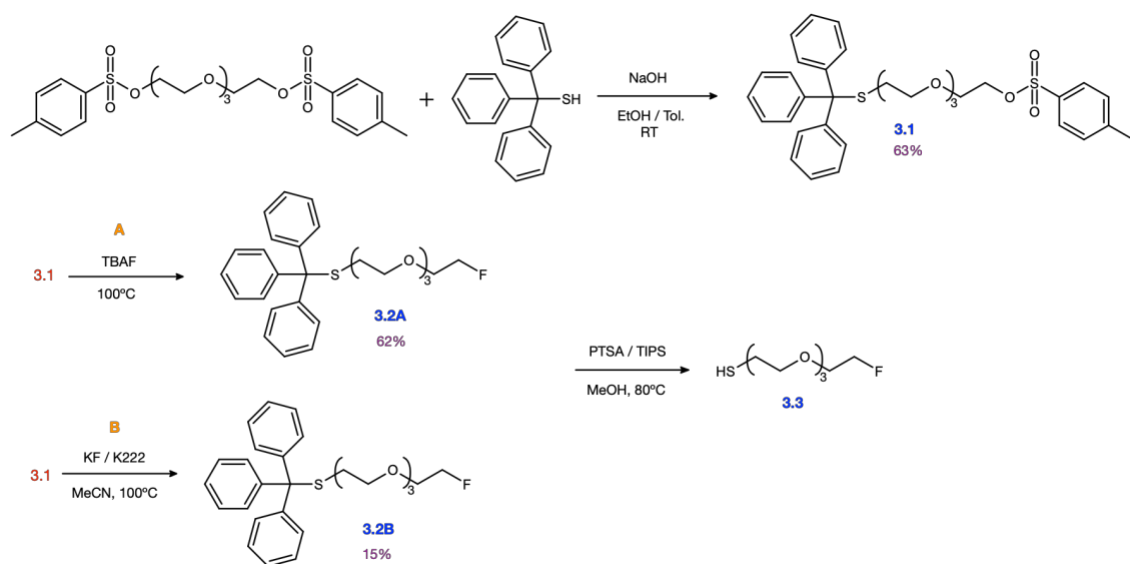


Figure 3.1 Tetrabutylammonium fluoride (TBAF) on the left and Kryptofix 2.2.2/Potassium Fluoride (K2.2.2/KF) on the right.

The results presented in this chapter establish the feasibility of these fluorination methods and assess their potential for the future synthesis of ^{18}F -labelled AuNPs as a multifunctional theranostic platform.

3.2 RESULTS

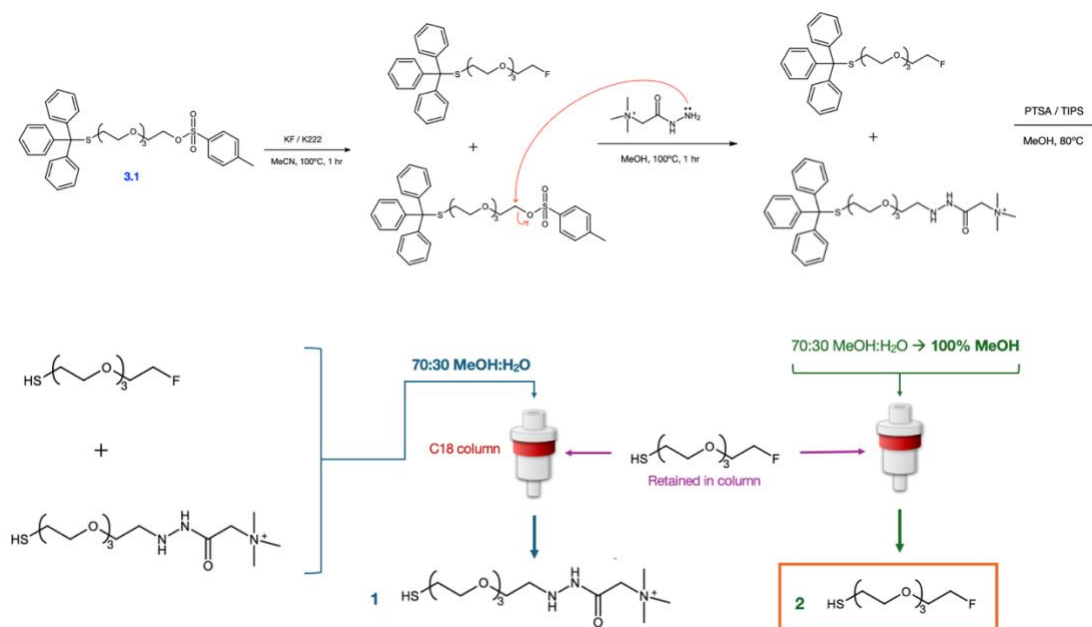
The synthesis of the radiolabelling chain with ^{19}F is shown in **Scheme 3.1**. The bifunctional PEG chain was reacted with triphenyl methanethiol to add the thiol handle to attach to the AuNPs. **3.1** was produced with a 63% yield. The addition of the fluorine atom was then investigated with both A) TBAF and B) K2.2.2/KF to produce **3.2**. The reaction with TBAF resulted in a 62% yield, and the reaction with K2.2.2/KF had a 15% yield. Lastly, the triphenyl group was deprotected under mild conditions to produce the fluorinated free thiol (**3.3**).



Scheme 3.1 Synthesis of Radiolabelling Chain with ^{19}F .

A “one-pot” synthesis of the radiolabelling chain that can be utilized in the synthetic hot cell is shown in **Scheme 3.2** with ^{19}F . **3.1** was synthesized as in **Scheme 3.1**, and then the fluorine was added using K2.2.2/KF. Due to the stoichiometry of a radio-fluorination reaction, there will be a large amount of unreacted starting material left over. To remove the unreacted tosylate precursor, the acetonitrile was removed and

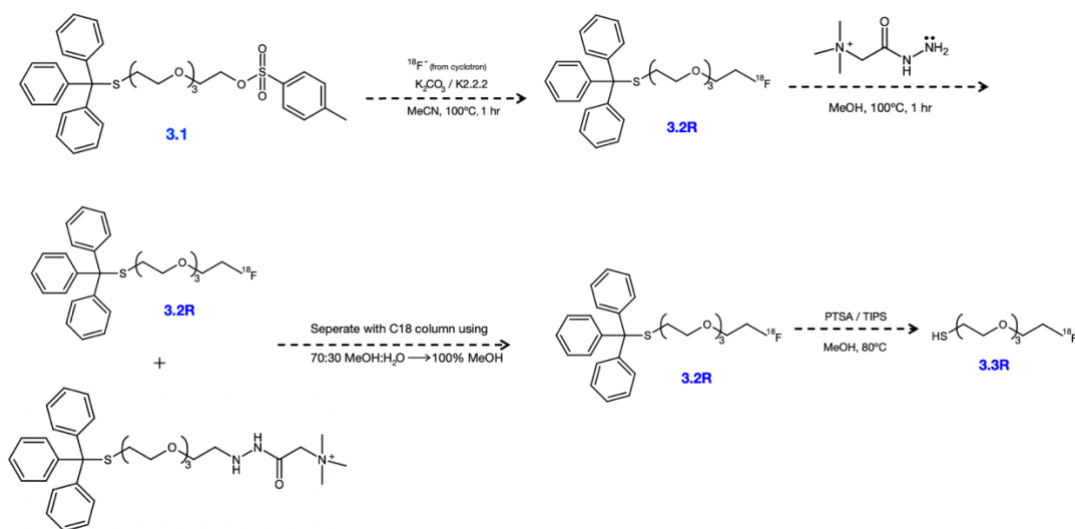
Girard's reagent was added in MeOH to react with the tosylate and increased its polarity. This difference in polarity allows for easier separation. The removal of the triphenyl group was performed using PTSA and TIPS, through the direct addition to the reaction mixture. Once the reaction is complete, it was loaded onto a C18 column in 70:30 MeOH:H₂O to elute the precursor, and then the solvent ratio was increased to 100% MeOH to elute the product. This product can then be immediately reacted with the AuNPs to add the radiolabelling chain (**Scheme 3.4**).



Scheme 3.2 Model System for Fluorination with ¹⁹F.

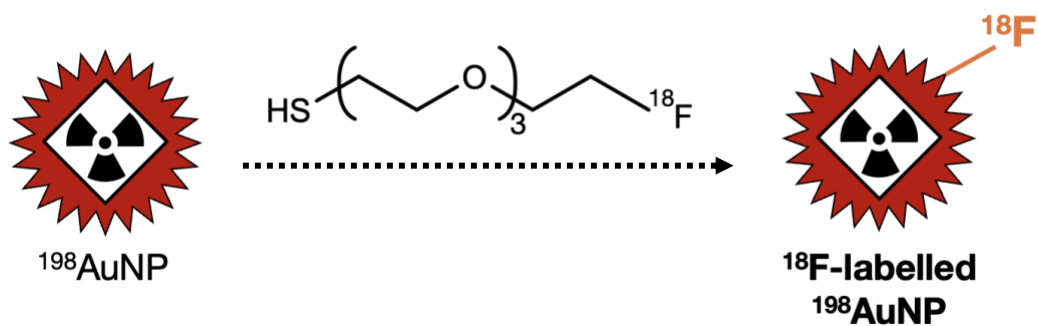
Scheme 3.3 shows the proposed synthesis of the radiolabelling chain with ¹⁸F, that can be performed in the hot cell. **3.1** was synthesized as shown in **Scheme 3.1**, and then ¹⁸F-fluoride can be added directly from the cyclotron with K₂.2.2 and K₂CO₃ to produce **3.2R**. The acetonitrile would then be removed, followed by Girard's reagent added in MeOH to react with any excess tosylate precursor. Once this reaction is

complete, the PTSA and TIPS can be added directly into the reaction mixture to free the thiol group via removal of the triphenyl group. The product can be purified on a C18 cartridge. With a starting mixture of 70:30 MeOH:H₂O, the precursor will elute almost immediately from the column, and the desired fluorinated product (**3.3R**) can be eluted by increasing the percentage of MeOH to 100%. The resulting solution can be directly reacted with the AuNPs according to **Scheme 3.4**, to achieve radiolabeling.



Scheme 3.3 Proposed Synthesis of Radiolabeling Chain with ^{18}F in the Hot Cell.

Since the surface of the AuNPs is covered in the normal PEG chain with the methyl group, there will be in an equilibrium shift once the fluorinated chain is added, resulting in exchange. Only one attached ^{18}F -ligand is required to visualize the AuNP.



Scheme 3.4 Radiolabeling of the $^{198}\text{AuNPs}$.

Overall, the synthesis of the chain for radiolabelling the AuNPs was synthesized with ^{19}F , and the potential radiolabelling route was investigated to be performed in the synthetic hot cell.

3.3 DISCUSSION

The synthesis of the fluorinated chains used to radiolabel the $^{198}\text{AuNPs}$ with ^{18}F was optimized with ^{19}F . As ^{18}F has a short half-life and is radioactive, any subsequent reactions must be carried out with suitable radiation protection and automation³¹. This was required as the chain must be synthesized, deprotected, and then reacted with the $^{198}\text{AuNPs}$ before the half-life is reached. **Scheme 3.1** shows the step-by-step synthesis of the radiolabeling chain with ^{19}F . The synthesis of the precursor for radiolabeling (**3.1**) was performed starting with the bifunctional PEG chain to add the thiol handle via triphenyl methanethiol with a 63% yield. This adds a chromophore, allowing for subsequent reactions to be monitored via TLC. This starting material also allows for the functionalization of the PEG chain with different moieties. Two methods were investigated to add the fluorine to the chain (**3.2**), first using TBAF (A) and second using K₂2.2.2/KF (B). The reaction with TBAF resulted in a significantly higher yield compared to the K₂2.2.2/KF; nonetheless, the K₂2.2.2/KF reaction is preferred due to the availability of all necessary reagents and our proficiency with this method. Lastly, Compound 3.3 was produced using PTSA and TIPS as mild conditions are required for the synthetic hot cell.

Scheme 3.2 shows the “one-pot” synthesis of the radiolabelling chain that can be utilized in the synthetic hot cell, with ^{19}F . The tosylate precursor for radiolabelling was synthesized as shown in **Scheme 3.1**, and then the addition of the fluorine was performed with K₂2.2.2/KF in acetonitrile. After ~1 hr, the acetonitrile was removed and then Girard’s reagent was added in MeOH to react with any excess tosylate precursor. This helps mitigate the challenges of separating the tosylate precursor from the fluorinated product as they are similar in polarity. They can be separated using liquid

chromatography and a silica column (**Scheme 3.1**), but when using ^{18}F , a C18 cartridge is required, which has a nonpolar stationary phase. When reacting the tosylate precursor with Girard's reagent, the polarity significantly increases and the compound is now positively charged, resulting in it readily flowing through the C18 cartridge and the fluorinated product being retained. The best solvent ratio was found to be 70:30 MeOH:H₂O, allowing for the precursor to be readily eluted from the column, followed by the fluorinated product once the ratio is increased to 100% MeOH. Some challenges observed with this method included finding a solvent system that would be able to dissolve the very polar Girard's reagent, the tosylate starting material, and the fluorinated product. Different ratios of MeOH/MeCN, MeCN/H₂O, and MeOH/H₂O were investigated, with a ratio of 70:30 MeOH:H₂O being found as optimal for the initial loading of the column and separation. In both methods, the triphenyl group was removed prior to the purification, but this made detection of elution difficult as the chromophore was removed. In the case of synthesis in the hot cell with ^{18}F , radio-TLC can be used to detect product elution. Alternatively, high-performance LC was found to be able to separate out the products, allowing for radio-HPLC to also be used in the case with ^{18}F .

Scheme 3.3 shows the proposed radiolabelling with ^{18}F , with the same synthetic route as **Scheme 3.2**. The tosylate precursor can be radiolabeled with $^{18}\text{F}^-$ (from the cyclotron) and K2.2.2/K₂CO₃. K2.2.2 is a phase transfer agent that specifically complexes the potassium ion³², and K₂CO₃ is the base required. This reaction can be performed in MeCN and is hypothesized to take less than one hour when heated to 100°C, as there is a significant excess of the tosylate precursor, based upon the stoichiometric ratios required during radio-fluorination. Once this reaction is complete,

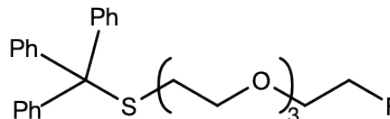
the reaction mixture will be dried to remove any excess water and to remove the MeCN prior to the addition of Girard's reagent in MeOH. This reacts with the excess tosylate precursor to make separation easier via the C18 cartridge, by making a larger difference in its polarity compared to the desired fluorinated product. Before the mixture is loaded onto the C18 column, the triphenyl group will be removed via PTSA and TIPS which will act as a carbocation scavenger to stabilize the trityl cation and prevent it from reacting with the free thiol again. The product can then be collected from the C18 column and detected via radio-TLC. As mentioned above, radio-high-performance LC could also be used to purify the reaction mixture and collect the final radiolabeled product. Once the radiolabeled product with the free thiol is collected, it must be immediately reacted with the $^{198}\text{AuNPs}$ to minimize disulfide formation (**Scheme 3.4**). Since the mixture will be in equilibrium, the attachment of at least one radiolabelling chain to every few NPs should be observed via ligand exchange. This is sufficient, as only one radiolabeling chain must be present on every few AuNPs to employ the theranostic approach and be able to detect them via PET imaging.

Overall, the synthesis of the radiolabelling chain with ^{19}F was optimized and performed in both a step-by-step and a one-pot approach to simulate the conditions in the synthetic hot cell. The starting material used allows for the chain to be functionalized with different moieties, making it applicable to more than just radiolabelling applications. The ongoing challenge of being able to separate out the tosylate precursor from the fluorinated product observed with most radiolabelling reactions was solved using Girard's reagent. This reagent can react with the tosylate on the starting material, making the PEG chain significantly more polar compared to the fluorinated product, allowing for much easier separation. The solvent ratio optimal for

this separation was also investigated, and it was found that starting with 70:30 MeOH:H₂O allowed for the mixture to be dissolved and loaded onto the column, and continuing with this system eluted the starting material. Increasing the ratio to 100% MeOH allowed for the fluorinated product to be released from the cartridge. The synthesis of the radiolabelling chain with ¹⁸F could be attempted with the conditions provided, creating a model system for the PET radiolabeling of the AuNPs.

c1ccccc1C(c2ccccc2)(c3ccccc3)SCCOCCOCCOCCOC(=O)c4ccccc4c1ccccc1C(c2ccccc2)(c3ccccc3)SCCOCCOCCOCCOC(=O)c4ccccc4c1ccccc1C(c2ccccc2)(c3ccccc3)S(=O)(=O)CCOC(=O)c4ccccc4

3.4.2. 1-{2-[2-(2-Fluoroethoxy)ethoxy]ethoxy}-2-(tritylthio)ethane (3.2)



A) Tetrabutylammonium fluoride (TBAF) (3.2A)

Compound **3.1** (250 mg, 0.411 mmol) was placed in a 20 mL vial and TBAF (1 M in THF, 2.5 mL) was added. The reaction was heated to 80 °C, and after 1 hr, completion was determined via TLC in 2:1 Hex:EtOAc. The reaction was allowed to cool to RT, the solvent was removed under reduced pressure, and the residue was dissolved in EtOAc. The solution was washed with sat. NaHCO₃, dried over MgSO₄, filtered, and the solvent was removed from the organic phase under reduced pressure. The product was purified by LC using 2:1 Hex:EtOAc and **3.2A** was obtained as a colourless oil (0.107 g, 62% yield). ¹H NMR (CDCl₃, 500 MHz): δ 7.41 (6H, d, *J* = 7.5 Hz), 7.18–7.28 (9H, m), 4.57 (1H, t, *J* = 4.5 Hz)*, 4.48 (1H, t, *J* = 4 Hz)*, 3.56–3.75 (8H, m), 3.45 (2H, t, *J* = 4 Hz), 3.20 (2H, t, *J* = 7.5 Hz), 2.43 (2H, t, *J* = 7 Hz). ¹³C NMR (CDCl₃, 126 MHz): δ 144.8, 129.6, 127.8, 126.6, 83.74* (*J* = 170 Hz), 82.40*, 70.73, 70.59, 70.45, 70.41, 70.26, 70.11, 69.55, 66.54, 31.60.

*Corresponds to fluorine coupling.

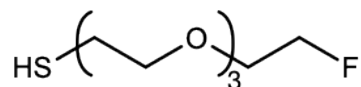
B) Potassium Fluoride (KF) and Kryptofix 2.2.2. (K222) (3.2B)

Compound **3.1** (100 mg, 0.197 mmol) was placed in a 20 mL vial with MeCN (5 mL). KF (13 mg, 0.217 mmol) and K222 (82 mg, 0.217 mmol) were added to the vial. The reaction was heated to 100 °C, and after 1 hr, completion was determined via TLC in 2:1 Hex:EtOAc. The solvent was removed under reduced pressure, the residue was dissolved in EtOAc, and the solution was washed with brine, dried over MgSO₄, filtered, and the solvent was removed

under reduced pressure. The product was purified by LC using 2:1 Hex:EtOAc and **3.2B** was obtained as a colourless oil (10 mg, 15% yield). ¹H NMR (CDCl₃, 500 MHz): δ 7.41 (6H, d, *J* = 7.5 Hz), 7.18–7.29 (9H, m), 4.58 (1H, t, *J* = 4 Hz)*, 4.48 (1H, t, *J* = 4.5 Hz)*, 3.56–3.70 (8H, m), 3.45 (2H, t, *J* = 4.5 Hz), 3.30 (2H, t, *J* = 7 Hz), 2.42 (2H, t, *J* = 7 Hz).

*Corresponds to fluorine coupling protons.

3.4.3. 2-{2-[2-(2-Fluoroethoxy)ethoxy]ethoxy}ethanethiol (**3.3**)



Compound **3.2** (100 mg, 0.245 mmol) was added to a 20 mL vial with *p*-toluenesulfonic acid (140 mg, 0.735 mmol), tri-isopropyl silane (0.1 mL), and MeOH (5 mL). The reaction was heated to 100 °C, and after 1 hr, completion was determined via TLC in 2:1 Hex:EtOAc. The reaction mixture was cooled to RT, the solvent was removed under reduced pressure, and the mixture was dissolved in DCM and washed with sat. NaHCO₃. The organic layer was dried over NaSO₄, filtered, and the solvent was removed under reduced pressure. The product was purified by LC using 2:1 Hex:EtOAc → 100% MeOH and **3.3** was obtained as a colourless oil. ¹H NMR (CDCl₃, 500 MHz): δ 4.61 (1H, t, *J* = 4 Hz), 4.52 (1H, t, *J* = 4 Hz), 3.59–3.72 (12H, m), 2.89 (2H, t, *J* = 7 Hz).

3.4.4. Model System for Fluorination with ¹⁹F.

Compound **3.1** (100 mg, 0.197 mmol) was placed in a 25 mL round-bottom flask with MeCN (5 mL). KF (13 mg, 0.217 mmol) and K₂S₂O₈ (82 mg, 0.217 mmol) were added to the flask. The reaction flask was heated to 100 °C and equipped with a reflux condenser for ~1 hr. The reaction progress was checked after ~1 hr via TLC in 2:1

Hex:EtOAc, the solvent was removed under reduced pressure, and Girard's Reagent (0.099 g, 0.591 mmol) was added in MeOH (5 mL). The reaction was heated to 100°C, equipped with a condenser, and stirred for ~1 hr. The reaction progress was checked after ~1 hr via TLC in 2:1 Hex:EtOAc to confirm that the tosylate precursor reacted via the polarity difference. P-toluenesulfonic acid (0.109 g, 0.573 mmol) and tri-isopropyl silane (0.05 mL) dissolved in MeOH (2 mL) were added to the reaction mixture and stirred for ~1 hr. The reaction progress was monitored via TLC in 2:1 Hex:EtOAc. Once complete, the MeOH was removed under reduced pressure, and the reaction mixture was dissolved in 70:30 MeOH:H₂O. The crude mixture was loaded onto a C18 cartridge, the starting material was eluted using this ratio, and the product was eluted by increasing the solvent ratio to 100% MeOH.

4 CONJUGATION OF AN AURORA KINASE B INHIBITOR AND SYNTHESIS OF OTHER CONJUGATION HANDLES

4.1 INTRODUCTION

The Aurora Kinases are a family of serine/threonine kinases that are subclassified into Aurora Kinase A, B, and C³⁴. They are activated via phosphorylation at different threonine residues, and their main function is to maintain chromosomal integrity during both the mitotic and meiotic cell division processes³⁴. Aurora Kinases A and B regulate mitosis, which occurs in somatic cells, whereas Aurora Kinase C regulates meiosis, which is restricted to germ cells³⁵. Both Aurora A and B are frequently overexpressed in various cancers and are associated with oncogenic properties, whereas the role of C is less understood³⁵. The overexpression of the Aurora kinase enzymes and their correlation with both genetic instability and aneuploidy implies that there is a potential for numerous types of cancers to respond to inhibition of these enzymes³⁵. Aurora kinase inhibitors are mostly ATP-competitive, so the binding is depicted by the ATP-binding region of the enzyme. Since the kinase domains only differ by a few amino acid residues in the hinge region between the subtypes, achieving selectivity is challenging³⁶.

Aurora Kinase A plays a role in centrosome maturation, spindle assembly/orientation, and meiotic maturation, making it critical for maintaining genomic stability³⁵. Its kinase activity is dependent on the phosphorylation of the threonine 288 (Thr288) residue at the C-terminal lobe in the activation loop³⁵. Selective inhibition of Aurora A results in the halt of the autophosphorylation at Thr288, monopolar spindles, and early cell cycle arrest³⁵. Aurora Kinase B is activated via autophosphorylation of

Thr232³⁷, and forms a part of the Chromosomal Passenger Complex (CPC), along with three non-enzymatic units³⁵. The CPC is crucial for chromosome condensation, orientation, the spindle assembly checkpoint, correcting errors during mitosis, and cytokinesis³⁵. Since Aurora A has been found to have dual-functionality in early embryonic development and as a tumour suppressor, its complete inhibition is likely fatal³⁴, making the selective inhibition of Aurora B is compelling for a lower-toxicity cancer target³⁶.

Selective inhibitors of Aurora B have been highly desired and sought after in cancer drug development. It has been found that ~90% of human malignancies contain aneuploid cells, which defines cells that contain an abnormal number of chromosomes³⁴. This can result from multiple processes during mitosis, which are all modulated by Aurora B³⁴. Overexpression of Aurora B has been observed in numerous malignancies and has been found to induce aneuploidy, leading to genetic instability and subsequent tumorigenesis, eliciting their classification as oncogenes³⁴. This has been confirmed via multiple *in vivo* and *in vitro* studies over the last few decades³⁴.

An ideal characteristic of any small molecular inhibitor is the ability for the null organism to function normally once the target is inhibited, either by the function being dispensable or replaced³⁴. In the interest of Aurora B, gene-knockout studies in mice have shown that it is expendable, as Aurora C can compensate for the function of B in early phases of cell development³⁴. This is because the kinase domain is highly conserved between all subtypes of Aurora kinases, with sequence homology of 71% for AurA/B, 60% for AurA/C, and the highest between AurB/C being 75%³⁸.

This ATP-binding kinase domain is composed of a β -stranded N-terminal lobe and an α -helical C-terminal lobe that are linked together by a hinge region that is

responsible for the active conformation of the protein³⁸. **Figure 4.1** shows the binding pocket of Aurora kinase A, which is based on the crystal structure. The only difference in Aurora Kinase B is the position of the two amino acids in the sequence that are displayed in the hinge region. It displays the five regions of the ATP kinase binding domain, including the hinge region, the buried region, the phosphate binding region, the sugar region, and the solvent-accessible region^{39,40}. The hinge region is a significant factor contributing to the binding affinity of the inhibitor, as it creates a hydrogen bonding network between the amino acid residues in the region^{39,40}. The ATP binding site residues are highly conserved between all the Aurora kinases, with the residues around the hinge residues in Aurora A and B only differing by 4 residues. In Aurora A there are Leu215, Thr217, Val218, and Arg220 compared to Arg159, Glu161, Leu162, and Lys164 in Aurora B³⁶. When looking at the amino acid differences, Aurora A contains more hydrophobic amino acids (Leu215 and Val218) compared to Aurora B, which only contains one (Leu162). Aurora B also contains a positively charged Arg159 residue in place of the hydrophobic Leu215 residue in Aurora A, and a negatively charged Glu161 residue in place of the polar Thr217 residue in Aurora A. Both contain a different positively charged amino acid in the final spot, Arg220 in Aurora A and Lys164 in Aurora B³⁶. These residue differences can be used to confer selectivity for Aurora kinase B.

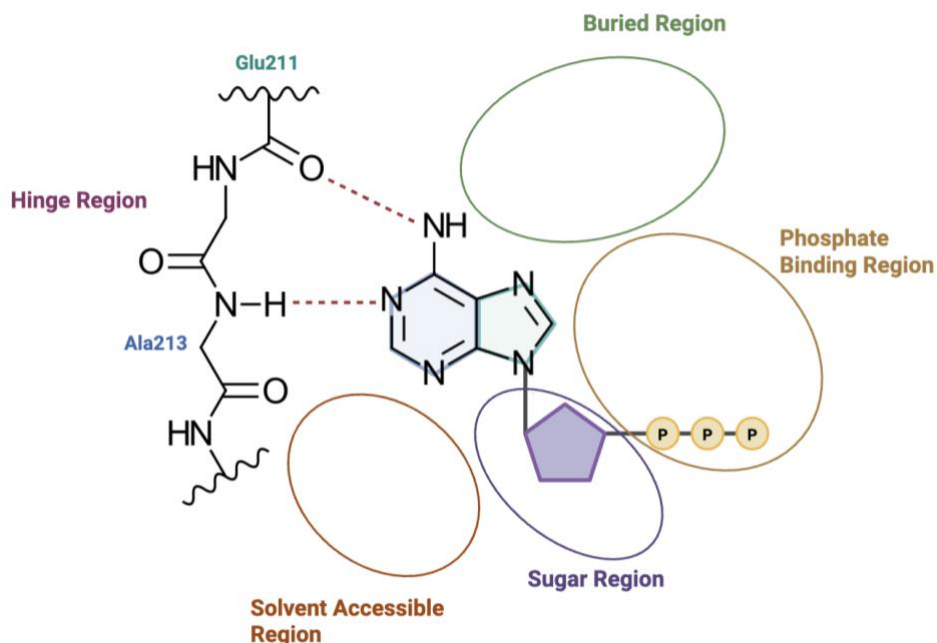


Figure 4.1 ATP-Binding Pocket of Aurora Kinase A (Biorender™). Schematic adapted from [39,40] and created using Biorender™.

Overall, Aurora Kinase B plays many crucial roles in the regulation of genetic information and cell division, making it a desirable target for inhibition³⁶. Its overexpression has been observed in a variety of malignancies including prostate, liver, leukemia, and breast cancers³⁴. Its inhibition has manifested both anti-proliferative and pro-apoptotic effects in some cancers⁴¹, making it a promising target moving forward. These effects were also found to be stronger for Aurora Kinase B compared to Aurora A, specifically on acute leukemia cells, making the Aurora B inhibition a new treatment strategy for both acute lymphoblastic and acute myeloid leukemia⁴².

The Aurora Kinase B inhibitor developed based on a known inhibitor, VX-680 (**Figure 4.2**) offers multiple advantages once conjugated to the AuNPs. The only change in the inhibitor is the removal of the methyl group on the piperazine, allowing for further modification at this site. First, it can act as a chemotherapeutic and inhibit the

Aurora Kinase B enzyme overexpressed in cancer once in the cancer tissue, but it can also act as a targeting agent to ensure the AuNP-conjugate makes it to the desired site. The same PEG chains with the free thiol group that were used to synthesize the AuNPs can be modified to add the Aurora Kinase B inhibitor.

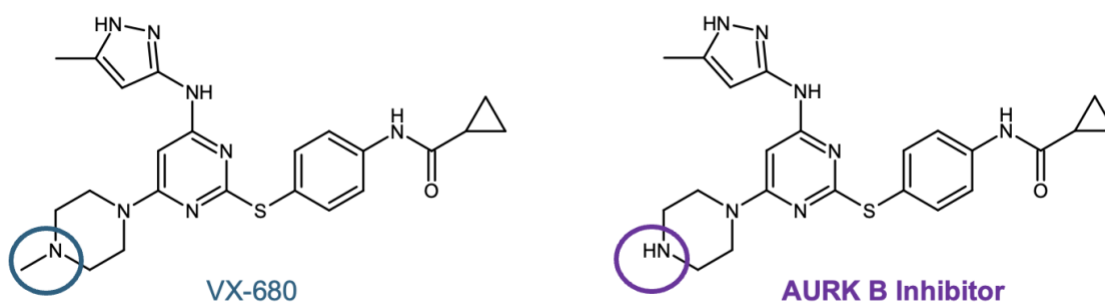


Figure 4.2 Direct Comparison of VX-680 and the Aurora Kinase B Inhibitor Developed Based on VX-680³⁹.

The crystal structure of Aurora kinase B in complex with INCENP and VX-680 has been investigated via molecular docking studies (**Figure 4.3**)^{43,45}. The crystal structure (A) shows that the region that is being modified containing the piperazine does not participate in any major interactions and is solvent exposed⁴⁵. (B) shows the main amino acid interactions based on docking studies, with notable interactions being the hydrogen bonding via Ala157 with the pyrazolidine and the linker nitrogen, hydrophobic interactions with Leu83 and Val91, and pi-pi stacking with Phe88 and the benzene ring in VX-680^{43,44}.

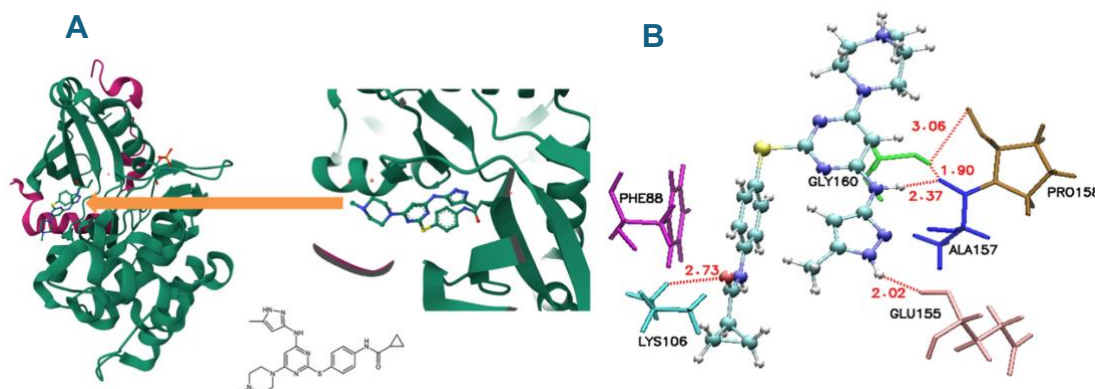
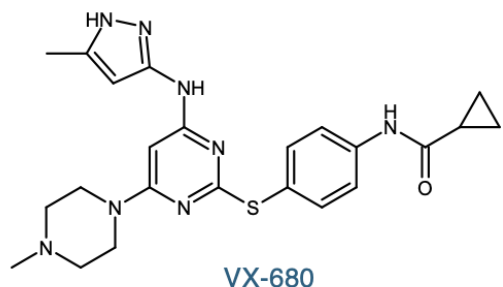


Figure 4.3 (A) Crystal Structure of Aurora Kinase B in complex with VX-680⁴⁵. (B) Molecular Docking Results for VX-680 with Human Aurora Kinase B⁴³. Important active site residues and their corresponding interactions are represented by the line and stick models in red⁴³.

The kinase selectivity of a known compound, VX-680, was determined using the ADP-Glo™ Kinase Assay³⁹, as it is very similar to that of the Aurora Kinase B inhibitor displayed in **Figure 4.2**. The structure of this compound and the results of this assay are shown in **Table 4.1**. This shows that VX-680 is moderately selective for Aurora Kinase A, but it is considered a pan-inhibitor⁴⁴. This can also be expected for the Aurora B Inhibitor.

Table 4.1 Selectivity Results for VX-680, performed in house and obtained from the literature using ADP-Glo™ Kinase Assay^{39,46}.

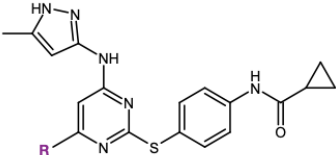
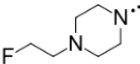
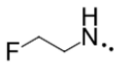


	Aurora Kinase IC ₅₀ (nM)		
	A	B	C
In House	14.3	36.4	67.2
Literature	0.6	18	4.6

Analogues were synthesized and tested in Dr. Hou's lab using the same conditions while modifying the piperazine ring shown in **Table 4.2**. The results of this

assay show that as the length of the chain at this position is increased, the selectivity for Aurora Kinase B is also increased. This is likely due to this part of the molecule being in the solvent-exposed region, changing the way that the inhibitor can interact with the hinge residues^{43,44}, making it more selective for Aurora B over A. This data was then used when determining where to attach the Aurora B inhibitor to the AuNPs, with this being the optimal site.

Table 4.2 Selectivity Results while Modifying the R Group on the Piperazine Ring of VX-680, using ADP-Glo™ Kinase Assay³⁹.

	Compound	R Group	Aurora Kinase IC ₅₀ (nM)		
			A	B	C
	1		32.9 ± 11.2	17.7 ± 2.9	35.7 ± 9.7
	2		25.3 ± 13.6	0.44 ± 0.24	25.0 ± 27.2

AuNPs are non-specific and as discussed in Chapter 1, they can enter the tumour tissue and further the cell via either a passive or active entry mechanisms⁹. AuNPs enter the tumour tissue though passive accumulation via the EPR effect due to the leaky vasculature in tumours⁹. Once inside the tumour tissue, they may be able to diffuse through the cell membrane since they are less than 10 nm in size⁹. Since the AuNPs will also contain some ¹⁹⁸Au atoms, they may also have a radiosensitization effect on the cells, making passive entry more likely. Radiosensitization can alter the tumour microenvironment and cell membrane permeability, making it easier for the AuNPs to penetrate the membrane and enter the cells⁴⁷.

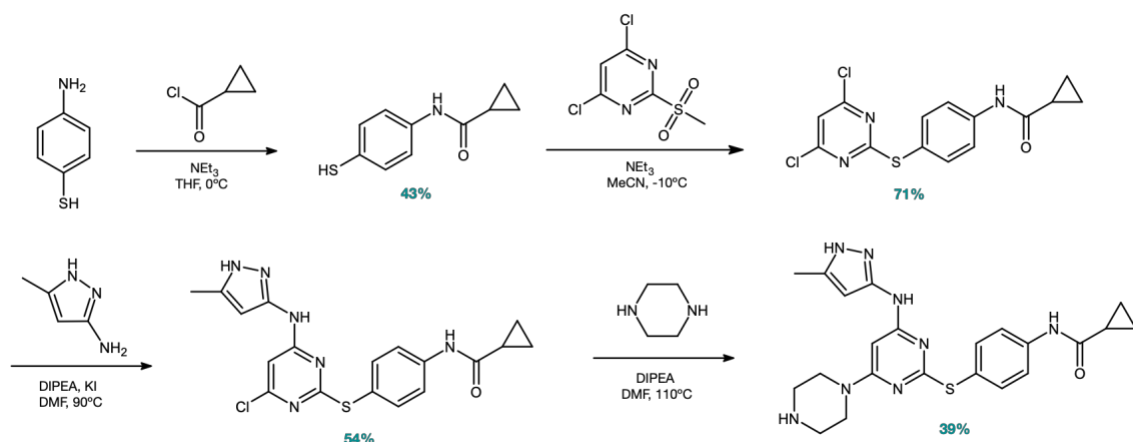
Once inside the tumour tissue, if passive diffusion cannot occur, an antibody can be conjugated to the AuNPs as well to trigger receptor-mediated endocytosis. The

Epidermal Growth Factor Receptor (EGFR) has been found to be co-overexpressed with Aurora Kinase B on the cell surface, allowing for the use of the Cetuximab antibody, which specifically binds the EGFR⁴⁸. In addition to this, AuNPs have also been found to enhance the tumour-growth-suppressing effects of the Cetuximab antibody and radiotherapy in vitro and in vivo⁴⁸. Other PEG chains like the one above to attach the Aurora B inhibitor have been developed with an azide functional group to allow for click chemistry applications as well as an amine functional group for antibody or other drug attachments.

This chapter focuses on the synthesis and characterization of PEG-functionalized AuNP conjugates incorporating the Aurora Kinase B inhibitor synthesized in our laboratory, as well as the preparation of additional PEG linkers containing amine and azide functional groups. The conjugation strategy was designed to attach the inhibitor through the piperazine moiety to preserve its kinase inhibitory activity while providing a stable linkage to the NP surface. Together, these conjugation strategies establish a versatile and multifunctional AuNP platform capable of supporting targeted chemotherapy while providing opportunities for future incorporation of antibodies and other biomolecules to further enhance tumour specificity.

4.2 RESULTS

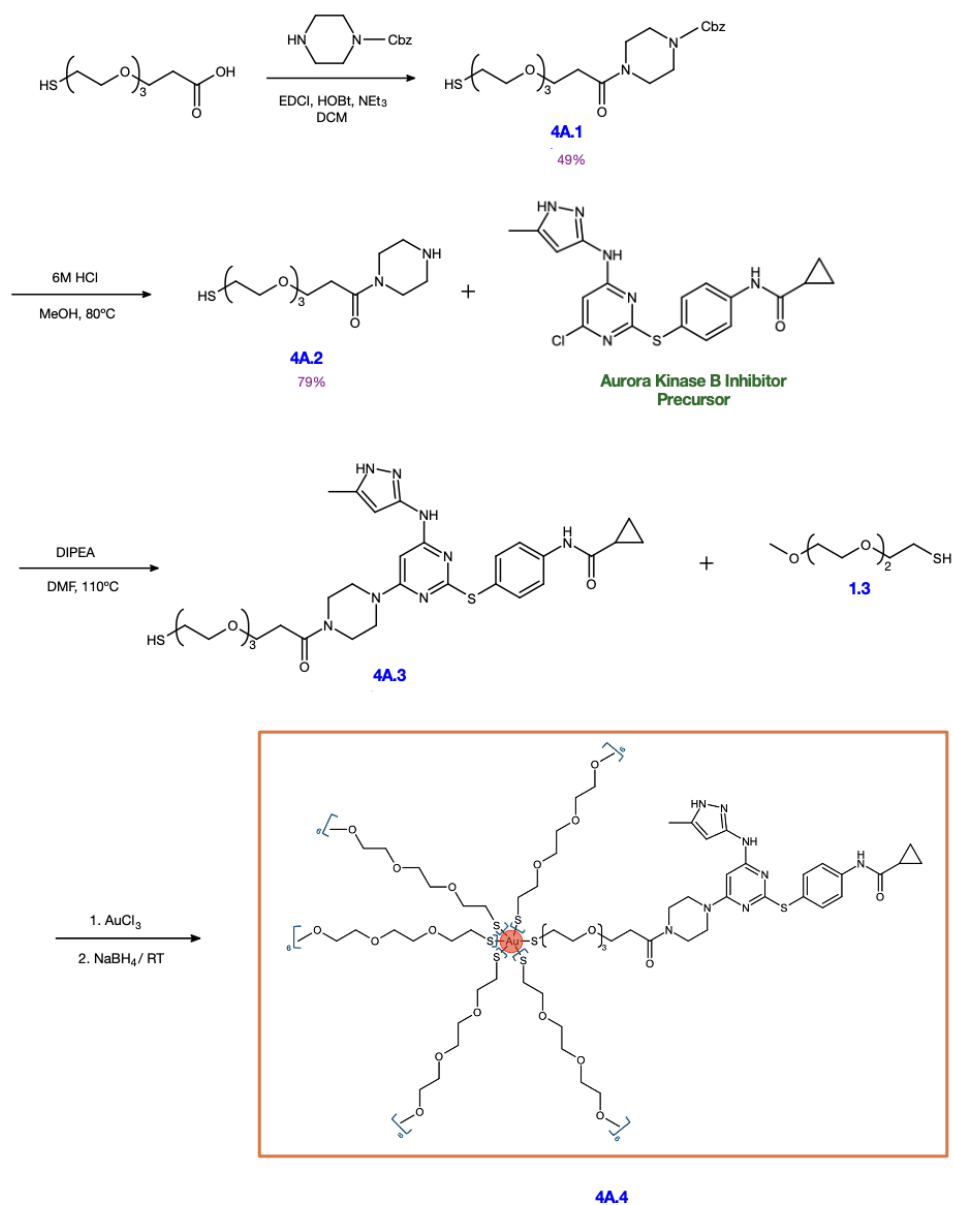
The Aurora Kinase B inhibitor was synthesized following the synthetic route developed in Dr. Hou's laboratory, as outlined in **Scheme 4.1**³⁹. The synthesis involved sequential functional group transformations to produce the inhibitor containing a free secondary amine on the piperazine ring, providing a site for subsequent conjugation while preserving the pharmacophore responsible for kinase inhibition. The final product was previously synthesized³⁹, and the last intermediate was used for subsequent conjugation reactions.



Scheme 4.1 Synthesis of Aurora Kinase B Inhibitor³⁹.

To enable attachment of the inhibitor to the AuNP surface, the free amine on the piperazine ring was reacted with a bifunctional PEG linker containing a carboxylic acid group and terminal thiol functionality (**Scheme 4.2**). The carboxylic acid selectively reacted with the piperazine amine in the presence of a base and the coupling agent EDCI to form **4A.1** with a 49% yield. The protecting group was then removed under acidic conditions (**4A.2**), with a 79% yield. **4A.2** was then reacted with the Aurora Kinase B inhibitor to form **4A.3**. This was immediately reacted in a 1:30 ratio with the

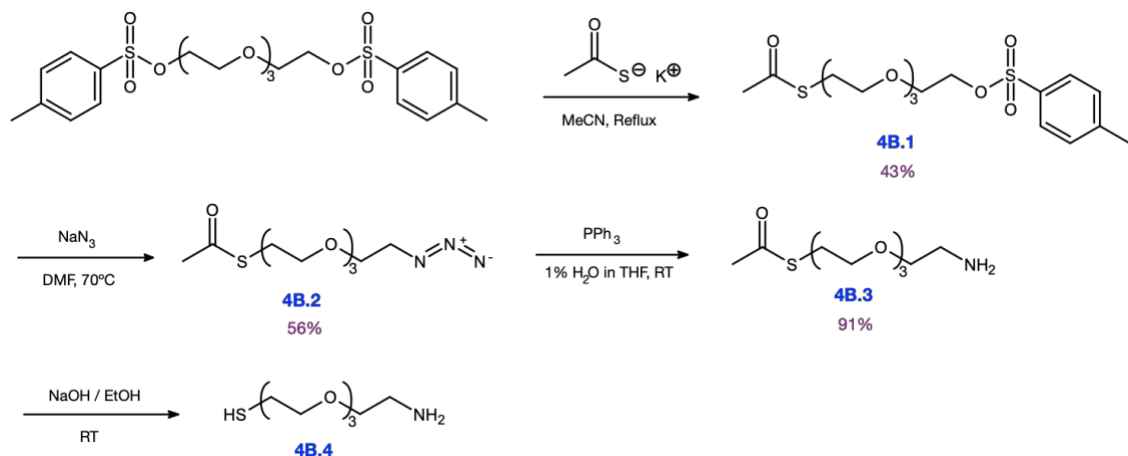
original PEG chain with AuCl_3 and NaBH_4 to form the AuNP-AKI conjugate. This conjugation strategy was selected to preserve the inhibitor binding region while providing a method for NP functionalization.



Scheme 4.2 Conjugation of the Aurora Kinase B Inhibitor to the AuNPs.

In addition to the Aurora Kinase B inhibitor conjugate, two additional PEG-functionalized linkers were synthesized to expand the versatility of the NP platform

(**Scheme 4.3**). These included PEG derivatives terminating in either a primary amine or an azide functional group, allowing for future conjugation of antibodies, imaging agents, or other therapeutic molecules through conventional amide coupling or click chemistry approaches. The synthesis of both derivatives begins with a bifunctional tosylate molecule, with the protected ester being added to one side as the thiol handle, forming **4B.1** with a 43% yield. The azide handle is added via sodium azide (NaN_3) to form **4B.2** with a 75% yield. Lastly, the azide can be reduced to an amine via a Staudinger reduction with resin-bound triphenylphosphine (PPh_3). This produced **4B.3** with a 91% yield. Either the azide chain or the amide chain can be deprotected to form **4B.4** to enable attachment to the AuNPs via the free thiol. These linkers provide a modular platform capable of supporting a wide range of multifunctional NP formulations.



Scheme 4.3 Synthesis of Other Handles for Molecule Attachment and Functionalization.

Lastly, an overview of all the synthesized PEG-functionalized chains is presented in **Figure 4.4**. **A)** denotes the regular PEG chain used to synthesize the AuNPs, **B)** the PEG chain with the Aurora Kinase B inhibitor conjugated, **C)** the PEG

chain with the azide moiety that can be employed for click chemistry applications, **D**) the radiolabelling PEG chain with ^{18}F for diagnostic applications, and lastly **E**) the PEG chain with the amine functionality that can be used for other antibody or drug attachment. Each construct contains a terminal thiol group for attachment to the AuNP surface while incorporating a distinct functional handle to enable subsequent bioconjugation. The successful preparation of these molecules establishes a flexible platform for the development of targeted AuNP-based therapeutics capable of incorporating multiple functional components.

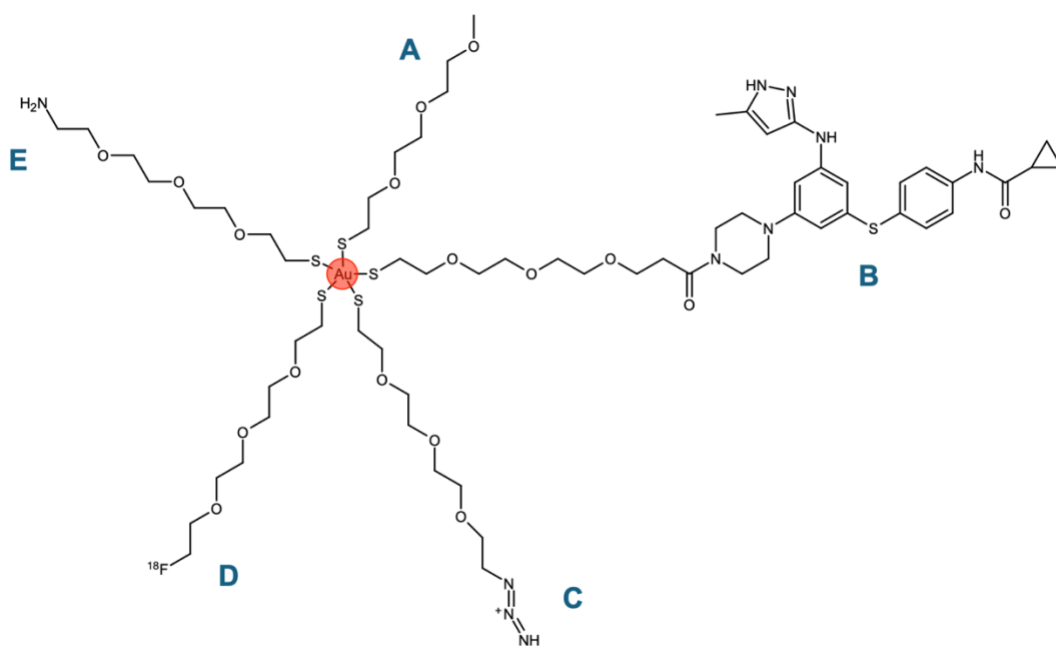


Figure 4.4 Overview of Various Chains Synthesized that can be used for Attachment to the AuNPs.

Overall, the synthetic strategies described in this chapter successfully produced the Aurora Kinase B inhibitor conjugate and a series of PEG-functionalized linkers for AuNP surface modification. The incorporation of multiple functional handles, including the Aurora Kinase B inhibitor, azide, amine, and radiolabelling functionalities (Chapter 3),

establishes a versatile NP platform capable of supporting targeted drug delivery and future multifunctionalization. The significance of these synthetic approaches and their implications for the development of AuNP-based therapeutics are discussed in the following section.

4.3 DISCUSSION

The objective of this chapter was to develop a method to conjugate the Aurora Kinase B inhibitor (**Figure 4.2**) to the AuNPs. The synthesis of this inhibitor was previously done and is shown in **Scheme 4.1**³⁹. The conjugation of this inhibitor to the PEG-chain with the free thiol used for attachment to the AuNPs is shown in **Scheme 4.2**. A 1:30 ratio of inhibitor to regular thiol chain was found to be optimal for AuNP attachment based upon the IC₅₀ of the inhibitor. This is further discussed in Chapter 5.

A major consideration in the design of the Aurora Kinase B inhibitor conjugate shown in **Scheme 4.2** was the preservation of the inhibitor's biological activity following conjugation. The kinase assay results presented in **Table 4.1** and **Table 4.2** provided the rationale for selecting the piperazine ring as the site of modification. Although the parent compound VX-680 exhibits only moderate selectivity between Aurora Kinase A and B, systematic modification of the substituent attached to the piperazine ring demonstrated that increasing the length of this side chain slightly improved selectivity toward Aurora Kinase B while maintaining kinase inhibition. This also showed that modifying this region of the compound did not have a large impact on the selectivity. **Figure 4.3** shows the crystal structure of Aurora Kinase B (A) and the main amino acid interactions based on docking studies (B). These studies confirmed that the site for modification containing the piperazine likely extends into the solvent-accessible portion of the ATP-binding pocket rather than directly participating in the critical hinge-region interactions responsible for target binding^{38,43,45}. Rather than modifying regions of the inhibitor that contribute to ATP-binding interactions³⁸, the PEG linker was introduced at a position shown to tolerate structural modification while simultaneously maintaining

Aurora Kinase selectivity. In addition to maintaining the biological activity of the inhibitor, the PEG linker offers several advantages for NP delivery.

Beyond the Aurora Kinase B inhibitor conjugate, the additional PEG-functionalized ligands synthesized in **Scheme 4.4** expand the versatility of the AuNP platform. The azide-functionalized PEG chain provides a handle for click chemistry, enabling efficient attachment of a wide range of biomolecules under mild reaction conditions. Similarly, the amine-functionalized derivative provides a convenient site for conventional amide coupling reactions, allowing antibodies, peptides, fluorophores, or additional therapeutic agents to be incorporated onto the NP surface. Together with the previously developed ^{18}F -labelled PEG chain for PET imaging (Chapter 3) and the Aurora Kinase B inhibitor conjugate, these molecules establish a multifunctional library of PEG ligands that can be incorporated onto the same AuNP surface in controlled ratios.

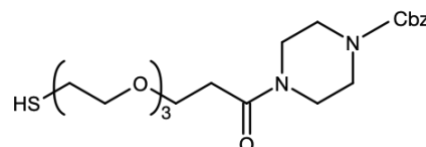
The significance of this approach is illustrated in **Figure 4.4**, which summarizes the five PEG-functionalized ligands developed throughout this work. The original PEG ligand provides NP stabilization, the Aurora Kinase B inhibitor ligand enables targeted chemotherapy, the azide and amine derivatives provide versatile conjugation handles for future biomolecule attachment, and the ^{18}F -labelled PEG chain introduces diagnostic imaging capability. Because each ligand contains the same terminal thiol functionality, they can all be incorporated onto the AuNP surface using the same gold-thiol chemistry employed during NP synthesis. This design allows the final NP formulation to be tailored by varying the ratio of each PEG derivative, providing considerable flexibility for future therapeutic and diagnostic applications.

Overall, the synthetic strategies described in this chapter establish a versatile and multifunctional AuNP platform capable of supporting therapeutic, diagnostic, and

targeting applications within a single nanoconstruct. Importantly, the conjugation strategy was guided by the kinase selectivity studies, ensuring that attachment of the PEG linker occurred at a position predicted to preserve Aurora Kinase B inhibition. Likewise, the complementary PEG derivatives substantially broaden the functionality of the platform by providing opportunities for future incorporation of imaging agents, targeting ligands, and additional therapeutics. Together, these PEG-functionalized molecules establish a strong chemical foundation for the biological evaluation presented in the following chapter and for the continued development of multifunctional AuNP-based theranostic systems.

4.4 EXPERIMENTAL METHODS

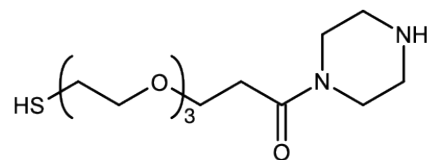
A) PEG-Linker for Inhibitor Attachment



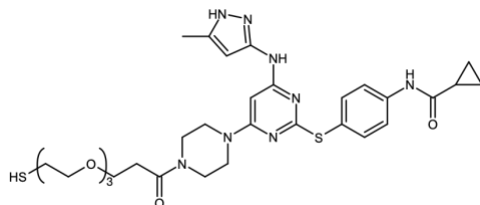
4.4.1 Benzyl 4-(3-{2-[2-(2-mercaptoethoxy)ethoxy]ethoxy}propionyl)-1-piperazinecarboxylate (**4A.1**)

3-{2-[2-(2-Mercaptoethoxy)ethoxy]ethoxy}propionic acid (0.403 g, 1.68 mmol) was added into a 100 mL round-bottom flask with DCM (20 mL). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) (0.593 g, 3.02 mmol) and hydroxybenzotriazole (HOBt) (0.479 g, 3.02 mmol) were added into the flask. The flask was equipped with a septum and placed under N₂ atmosphere while stirring for 15 minutes. In a separate flask, 1-Z-piperazine (1.14 g, 5.03 mmol) and triethylamine (NEt₃) (0.635 g, 3.77 mmol) were dissolved in DCM (20 mL). After 15 minutes of stirring, the 1-Z-piperazine solution was added into the 100 mL round-bottom flask and stirred overnight at room temperature. The reaction progress was checked via TLC in 9:1 DCM:MeOH with 1% NH₄OH and stained with ninhydrin. Once the reaction was complete, it was quenched with 10% HCl and washed with H₂O/brine. The organic layer was dried over MgSO₄, filtered, and the DCM was removed under reduced pressure. The product was purified via LC in 9:1 DCM:MeOH → 100% MeOH with 1% NH₄OH and **4A.1** was obtained (0.627 g, 49% yield). ¹H NMR (CDCl₃, 500 MHz): δ 7.36 (5H, s), 5.15 (2H, s), 3.49–3.80 (20H, m), 2.87 (2H, t, *J* = 6.5 Hz), 2.63 (2H, t, *J* = 6.5 Hz). ¹³C NMR (CDCl₃, 126 MHz): δ 169.7, 154.9, 136.3, 128.4, 128.1, 127.9, 71.32, 70.73, 70.41, 70.36, 70.33, 70.24, 69.49, 67.32, 67.30, 45.36, 41.20, 38.27, 33.57.

4.4.2 3-{2-[2-(2-Mercaptoethoxy)ethoxy]ethoxy}-1-(1-piperazinyl)-1-propanone (4A.2)



Compound **4A.1** (500 mg, 1.136 mmol) was added into a 100 mL round-bottom flask with 25 mL of 6 M HCl in MeOH. The reaction was heated to 80°C, equipped with a reflux condenser and stirred overnight under an N₂ atmosphere. The reaction progress was checked via TLC using 1:1:0.2 Hex:EtOAc:MeOH with 1% NH₄OH, cooled to RT and some MeOH was removed under reduced pressure. The mixture was then dissolved in EtOAc and the organic layer was washed with NaHCO₃ and DI H₂O. The organic layer was dried over MgSO₄, filtered, and the EtOAc was removed under reduced pressure to obtain **4A.2** (70 mg, 79% yield). ¹H NMR (CDCl₃, 500 MHz): δ 3.63–3.78 (20H, m), 2.89 (2H, m), 2.60 (2H, t, *J* = 6.5 Hz). ¹³C NMR (CDCl₃, 126 MHz): δ 172.1, 70.56, 70.50, 70.43, 70.41, 70.34, 69.65, 69.60, 66.61, 51.69, 38.41, 34.88, 29.70.



4.4.3 N-(4-((4-(4-(3-(2-(2-(2-mercaptoethoxy)ethoxy)ethoxy)propanoyl)piperazin-1-yl)-6-((5-methyl-1H-pyrazol-3-yl)amino)pyrimidin-2-yl)thio)phenyl)cyclopropanecarboxamide (4A.3)

Compound **4A.2** (50 mg, 0.173 mmol) was added to a 50 mL round-bottom flask with 3 mL of DMF. Aurora Kinase B Inhibitor Precursor (*p*-[4-Chloro-6-(5-methyl-1H-pyrazol-3-ylamino)-2-pyrimidinylthio]phenylamino)cyclopropylformaldehyde) (20 mg, 0.0865 mmol) was added to the flask with DIPEA (13.4 mg, 0.104 mmol). The reaction was heated to 80°C, equipped with a reflux condenser, and stirred overnight under an N₂ atmosphere. The reaction progress was checked via TLC using 9:1 DCM:MeOH with

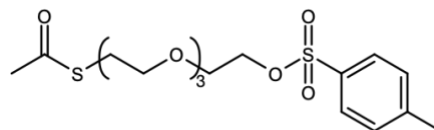
1% NH₄OH, and the mixture was diluted with EtOAc and washed with sat. NaHCO₃, followed by brine. The organic layer was dried over MgSO₄, filtered, and the solvent was removed under reduced pressure. The product was purified via LC in 9:1 DCM:MeOH → 100% MeOH with 1% NH₄OH to produce **4A.3**. ¹H NMR (CDCl₃, 500 MHz): δ 7.54 (2H, m), 7.13 (4H, m), 6.81 (2H, d, *J* = 8 Hz), 3.29–3.79 (20H, m), 2.88 (2H, t, *J* = 5 Hz), 2.60 (2H, t, *J* = 6.5 Hz), 2.15 (3H, s), 1.58 (1H, m), 0.74–0.92 (4H, m).

4.4.4 Synthesis of Aurora Kinase B Inhibitor-AuNP Conjugate (4A.4).

AuCl₃ (14.80 mg, 49 μmol) was purged with N₂ in a vial. MeOH (7.5 mL) and glacial acetic acid (1.25 mL) were added to the vial using a syringe. Compound **1.3** (25.2 mg, 140 μmol) and Compound **4A.3** (4.02 mg, 6 μmol) were added to the vial, and the solution was stirred vigorously for 1 hour. NaBH₄ (0.0185 g, 490 μmol) dissolved in DI H₂O (1.25 mL) was added to the vial dropwise, and the solution turned a dark red/brown colour, indicating the formation of AuNPs, and was left to stir overnight. The solution was concentrated under reduced pressure, and the product was dissolved in DI H₂O (20 mL). The NPs were extracted with toluene, and a small amount of brine was added to the aqueous phase after each extraction. The toluene was removed from the collected organic fractions, and the NPs were dissolved in ~1 mL of toluene and transferred to a clean round-bottom flask, leaving behind the precipitate. Once the toluene was removed under reduced pressure, the AuNPs were washed with ~1 mL of cyclohexane to remove any unreacted thiol. The cyclohexane was then removed under reduced pressure and the AuNPs were dissolved in DI H₂O and added to a dialysis kit (Slide-A-Lyzer™ G3 Dialysis Cassettes, 2K MWCO, 3 mL capacity). The kit was placed in 1 L of DI H₂O, stirring slowly. The DI H₂O was changed after 2 hours and then was

left overnight. The solution was removed from the dialysis kit, and the DI H₂O was evaporated.

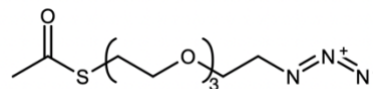
B) PEG-Linkers for Other Applications



4.4.5 *S*-(2-(2-(2-(2-(tosyloxy)ethoxy)ethoxy)ethoxy)ethyl)ethanethioate (4B.1)

p-[2-(2-{2-[2-(p-Tolylsulfonyloxy)ethoxy]ethoxy}ethoxy)ethoxysulfonyl]toluene (2.00 g, 3.98 mmol) was added into a 250 mL round-bottom flask with acetonitrile (60 mL) and the solution was stirred. Potassium thioacetate (0.454 g, 3.98 mmol) was added to the flask and the reaction was stirred overnight at RT. The reaction progress was checked via TLC in 1:1 Hex:EtOAc. The acetonitrile was removed under reduced pressure, and the solution was extracted with DCM and washed with DI H₂O. The organic layer was dried over MgSO₄, filtered, and the solvent was removed under reduced pressure. The crude product was purified by LC using 1:1 Hex:EtOAc and **4B.1** was obtained as a yellow oil (0.691 g, 43% yield). ¹H NMR (CDCl₃, 500 MHz): δ 7.79 (2H, d, *J* = 8 Hz), 7.35 (2H, d, *J* = 8 Hz), 4.16 (2H, t, *J* = 4.5 Hz), 3.69 (2H, t, *J* = 5 Hz), 3.58 (10H, m), 3.07 (2H, t, *J* = 6.5 Hz), 2.45 (3H, s), 2.33 (3H, s). ¹³C NMR (CDCl₃, 126 MHz): δ 195.6, 144.8, 133.0, 129.8, 127.9, 72.87, 70.73, 70.56, 70.28, 69.25, 68.70, 63.53, 30.56, 28.80, 24.24, 21.63.

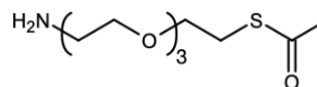
4.4.6 1-{2-[2-[2-(2-



azidoethoxy)ethoxy]ethoxy]ethylsulfanyl}ethan-1-one (4B.2)

Compound **4B.1** (3.00 g, 7.38 mmol) was added into a 200 mL round-bottom flask with DMF (75 mL) and the solution was stirred. NaN₃ (2.40 g, 36.9 mmol) was added to the flask equipped with a reflux condenser, and the mixture was heated to 90°C and stirred overnight. The reaction progress was checked via TLC in 1:1

Hex:EtOAc and stained with 10% PPh₃ in DCM solution first, and then ninhydrin to detect the presence of the azide. The crude product was purified via liquid chromatography with 1:1 Hex:EtOAc and **4B.2** was obtained (1.597 g, 75% yield). ¹H NMR (CDCl₃, 500 MHz): δ 3.43–3.56 (12H, m), 3.25 (2H, t, *J* = 5 Hz), 2.93 (2H, t, *J* = 6.5 Hz), 2.20 (3H, s). ¹³C NMR (CDCl₃, 500 MHz): δ 193.7, 69.57, 69.51, 69.47, 69.21, 68.98, 68.62, 49.57, 29.32, 27.71. IR Spectrum: azide peak (2097.8 cm⁻¹).



4.4.7 1-({2-[2-[2-(2-aminoethoxy)ethoxy]ethoxy]ethylsulfanyl}ethan-1-one (**4B.3**)

Compound **4B.2** (50 mg, 0.180 mmol) was added to a 20 mL scintillation vial equipped with a stir bar. 1% H₂O in THF (8 mL) was added into the vial and the mixture was stirred. Polymer-bound triphenylphosphine (PPh₃, 100-200 mesh, extent of labeling: ~3 mmol/g triphenylphosphine loading) (0.182 g, 0.270 mmol) was added to the vial, and a septum was added and the contents were placed under an N₂ atmosphere. The vial was heated to 40°C and stirred overnight. The reaction progress was checked via TLC in 1:1 Hex:EtOAc and stained with ninhydrin. The resin was filtered out, and the aqueous layer was washed with ethyl acetate. The organic layer was dried over MgSO₄, filtered, and the solvent was removed under reduced pressure. The crude product was purified via LC with 9:1 DCM:MeOH and **4B.3** was obtained (0.041 g, 91% yield). The procedure used to deprotect the thiol to produce compound **1.3** can be used to deprotect the thiols in compounds **4B.2** and **4B.3**. ¹H NMR (CDCl₃, 500 MHz): δ 3.59–3.66 (14H, m), 3.09 (2H, t, *J* = 6.5 Hz), 2.34 (3H, s). ¹³C NMR (CDCl₃, 126 MHz): δ 195.3, 72.51, 70.20, 70.08, 69.93, 69.83, 69.42, 39.01, 30.27, 28.40. IR Spectrum: lack of azide peak (2097.8 cm⁻¹), N-H stretch x2 for primary amines (3200-3500 cm⁻¹).

5 EVALUATION OF CYTOTOXICITY AND EFFICACY

5.1 INTRODUCTION

The successful development of targeted NP-based therapeutics requires not only the synthesis and physicochemical characterization of the complex but also the evaluation of its biological activity. While the preceding chapters described the preparation of AuNPs, their neutron activation and radiolabelling, and the conjugation strategies for the attachment of an Aurora kinase B inhibitor, it is essential to determine whether these formulations exhibit anticancer activity *in vitro*. Biological evaluation provides critical insight into the cytotoxic effects of the NPs and establishes their potential as therapeutic agents for future preclinical investigation.

AuNPs can have varying impacts on biological activity based upon their size, morphology, and surface functionalization⁴⁹. Size has been shown to effect both cellular uptake and cytotoxicity, with higher and more efficient uptake being observed for smaller particles via active transport mechanisms. This is likely due to less energy being required to uptake particles of smaller size⁴⁹. In a cytotoxicity study done on mice, it was found that AuNPs with sizes of 10-60 nm commonly exhibited some adverse effects including cell shape alterations, inhibition of cell proliferation, DNA mutations, and liver and kidney damage⁴⁹. Other studies have shown that smaller particles have a high surface area to volume ratio, resulting in higher absorption capacity, increased interactions with other biomolecules, and easier travel to other sites in the body⁴⁹. Smaller particles are desirable due to the increased uptake and toxicity, but additional precautions need to be used to ensure that they are reaching the desired site to minimize adverse effects.

AuNPs exert varying impacts on biological activity depending on their size, morphology, and surface functionalization. Particle size significantly affects both cellular uptake and cytotoxicity⁴⁹. Smaller AuNPs exhibit higher and more efficient cellular internalization via endocytosis because they require less energy to be internalized and interact with fewer cell-surface receptors, whereas larger particles can be limited by insufficient available surface receptors⁴⁹. In an *in vivo* cytotoxicity study, PEG-coated AuNPs sized 10–60 nm caused adverse effects in mice; including cell shape alterations, inhibition of cell proliferation, and DNA mutations, whereas PEG-coated particles sized 5–30 nm demonstrated no toxicity⁴⁹. Mechanistically, the increased toxicity observed for smaller particles is attributed to their high surface-area-to-volume ratio, which expands surface absorption capacity and increases interactions with biomolecules⁴⁹. However, because findings across the broader literature regarding size-dependent cytotoxicity remain contradictory, particle size alone cannot dictate biocompatibility and must be evaluated alongside surface coating and charge properties.

The morphology of the AuNPs also influences their biological activity. The “edge effect” is a phenomenon surrounding nanomaterials, describing how different shapes can result in the ability for different interactions to occur, ultimately contributing to their toxicity⁴⁹. The distribution of surface atoms varies depending on the shape, so nanomaterials with more angles and edges will have more atoms at these sites, causing stronger interactions with other biomolecules⁴⁹. This results in nanorods and nanostars exhibiting more toxicity and increased surface interactions than NPs⁴⁹. This is

favourable for the application of the AuNPs, as there should be decreased adverse effects due to this phenomenon.

The surface ligands present have a large impact on the shape exhibited by the nanomaterials, their characteristics, and their cell uptake and recognition⁴⁹. It has been reported that the surface coating on the AuNPs has a greater impact on NP toxicity than the gold core itself, with CTAB-coated AuNPs exhibiting substantially higher cytotoxicity toward human dermal fibroblasts than PEG- or serum-coated NPs⁵⁰. Similarly, it has been demonstrated that surface ligands play a critical role in determining the physicochemical properties of AuNPs, thereby influencing their interactions with cells⁵¹. This is described as "cell vision," which proposes that the unique composition of proteins, carbohydrates, and phospholipids present on the cell membrane governs how cells recognize and interact with NPs⁵¹. Because these membrane components differ among cell types, NP uptake and the resulting cellular responses vary accordingly⁵¹. Surface charge is another key determinant of NP behaviour in biological systems, influencing stability in physiological media as well as interactions with cell membranes and proteins⁴⁹. Studies have shown that cationic AuNPs tend to be more cytotoxic, promoting stronger interactions with cells and plasma proteins, whereas anionic and neutral NPs generally exhibit reduced toxicity⁵². This difference is partly attributed to the formation of a "protein corona," which is more pronounced for positively charged particles and can enhance uptake by immune cells such as macrophages, leading to stronger immune responses⁵². However, NP-cell interactions are not governed solely by electrostatics, as additional forces including hydrophobic interactions, hydrogen

bonding, and ligand-receptor binding also contributes significantly to cellular uptake and biological response.

Lastly, AuNP toxicity has also been shown to be dose-dependent, with higher NP concentrations leading to increased cytotoxic effects in cell models such as mouse fibroblasts⁵³. Following uptake, AuNPs can undergo changes in aggregation state and surface properties due to interactions with biological components, including adsorption of serum proteins that form a protein corona⁵³. This corona formation can in turn trigger various cellular responses, including apoptosis, lysosomal membrane disruption, and inflammatory signalling⁵³. Overall, these findings highlight that the biological effects of AuNPs are governed by an interplay of size, shape, surface chemistry, charge, and dose, all of which collectively influence NP-cell interactions, uptake mechanisms, and downstream cytotoxic responses.

The biological performance of NP formulations is commonly assessed using *in vitro* cell viability assays, which provide a rapid and quantitative measure of cellular response following treatment⁵⁴. Among these methods, the Cell Counting Kit-8 (CCK-8) assay is widely used to assess cell viability due to its high sensitivity, simplicity, and reproducibility. The assay is based on the reduction of the water-soluble tetrazolium salt WST-8 by metabolically active cells to produce a coloured formazan product, the quantity of which is directly proportional to the number of viable cells (**Figure 5.1**)⁵⁴. The decreases in cell viability following treatment can be used to evaluate the cytotoxic

effects of candidate therapeutic agents.

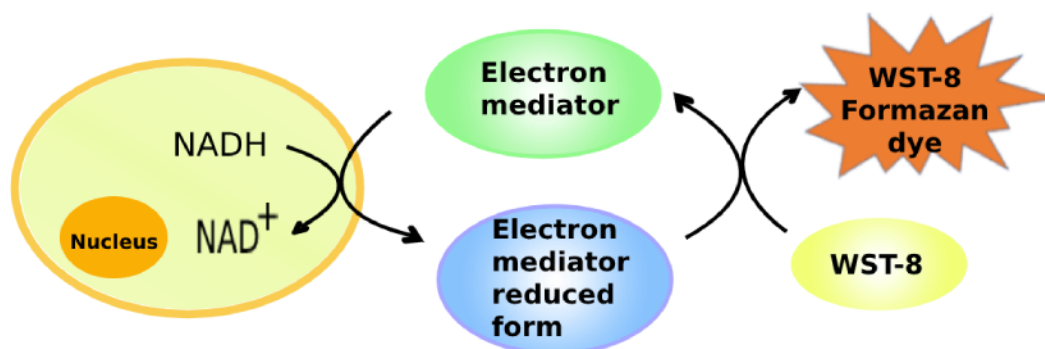


Figure 5.1 Principle of the CCK-8 Cell Viability Assay⁵⁴. Metabolically active cells reduce the water-soluble tetrazolium salt WST-8 to a water-soluble orange formazan product via intracellular dehydrogenase enzymes. The amount of formazan produced is directly proportional to the number of viable cells and is quantified by measuring absorbance at 450 nm.

In this study, the CCK-8 assay was employed to investigate the cytotoxicity of several NP formulations and treatment conditions. Cell viability following exposure to AuNPs alone, ¹⁹⁸AuNPs, and the free Aurora kinase B inhibitor was evaluated to characterize the individual contributions of each component. In addition, cells were treated with combinations of AuNPs and the Aurora kinase B inhibitor at their respective half-maximal inhibitory concentration (IC₅₀) values to investigate whether the AuNPs and the Aurora B inhibitor produce a synergistic effect prior to the conjugate being tested. This was also performed to determine the molar equivalents of Aurora B inhibitor needed to be conjugated to the AuNPs to reach the desired IC₅₀ value. These studies establish a biological baseline against which the activity of the AuNP-AKI conjugates can be compared.

The objective of this chapter is to evaluate the *in vitro* cytotoxicity of the AuNPs, ¹⁹⁸AuNPs, the AKI, and AuNP-AKI conjugate using the CCK-8 assay and to assess their potential as targeted anticancer therapeutics. These findings provide an initial assessment of the biological performance of the developed nanoplatform and guide the continued optimization of multifunctional radiolabelled AuNP-based drug delivery systems.

5.2 RESULTS

The cytotoxic effects of the Aurora Kinase B inhibitor (AKI), gold nanoparticles (AuNPs), and their combination were evaluated using the CCK-8 cell viability assay in three human cervical cancer cell lines: SiHa, CaSki, and C33A. SiHa cells are HPV-16-positive squamous cell carcinoma cells⁵⁵, CaSki cells are HPV-16-positive cervical epidermoid carcinoma cells containing a high copy number of integrated HPV DNA⁵⁶, and C33A cells are HPV-negative cervical carcinoma cells⁵¹. Cells were cultured under standard conditions and seeded at a density of 2,000 cells per well in 96-well plates. The optimal cell density was determined by testing 2,000, 4,000, and 6,000 cells/well. The optimal amount of CCK-8 reagent added was also tested in this experiment, with 10 μ L providing the best results. Following overnight attachment, cells were treated with the indicated drug concentrations and incubated for 72 hours before cell viability was assessed. Each experiment was performed independently at least twice, with multiple replicates included for each treatment condition. The resulting dose-response curves were used to calculate IC_{50} values via Graphpad Prism (Appendix 5).

The cytotoxicity of the Aurora Kinase B inhibitor (AKI) was evaluated in the SiHa, CaSki, and C33A cervical cancer cell lines by determining IC_{50} values (**Table 5.1**). The inhibitor demonstrated varying levels of potency across the three cell lines, with the lowest IC_{50} observed in CaSki cells ($0.14 \pm 0.04 \mu\text{M}$ or $(6.3 \pm 1.8) \times 10^{-5} \text{ mg/mL}$), followed by SiHa cells ($2.2 \pm 2.7 \mu\text{M}$ or $(1.0 \pm 1.2) \times 10^{-3} \text{ mg/mL}$). C33A cells exhibited the highest IC_{50} value ($7.8 \pm 5.3 \mu\text{M}$ or $(3.5 \pm 2.4) \times 10^{-3} \text{ mg/mL}$), indicating the lowest sensitivity to AKI treatment.

Table 5.1 IC₅₀ Values for the Aurora Kinase B Inhibitor tested on three cell lines: SiHa, CaSki, and C33A using the CCK-8 assay. These values were obtained by averaging the IC₅₀ values from three separate assays (n=3).

Cell Line	Aurora Kinase B Inhibitor IC ₅₀ (n=3)	
	μM	mg/mL
SiHa	2.2 ± 2.7	(1.0 ± 1.2) x 10 ⁻³
CaSki	0.14 ± 0.04	(6.3 ± 1.8) x 10 ⁻⁵
C33A	7.8 ± 5.3	(3.5 ± 2.4) x 10 ⁻³

The cytotoxicity of the original AuNPs was also assessed in the three cervical cancer cell lines (**Table 5.2**). IC₅₀ values were comparable among the cell lines, ranging from 0.08 ± 0.07 mg/mL in CaSki cells to 0.06 ± 0.04 in SiHa cells, to finally 0.041 ± 0.02 mg/mL in C33A cells. The effect of neutron activation on AuNP cytotoxicity was subsequently evaluated by comparing non-radioactive ¹⁹⁷AuNPs with activated ¹⁹⁸AuNPs (**Table 5.3**). Similar IC₅₀ values were obtained for the AuNPs and those that were activated to ¹⁹⁸AuNPs with the lower activity of 278 Bq/mL, with values of 0.111 ± 0.002 mg/mL and 0.11 ± 0.01 mg/mL, respectively. When they ¹⁹⁸AuNPs were left in the vault for an extended period of time, reaching an activity level of 2290 Bq/mL, the IC₅₀ decreased to a value of 0.0386 ± 0.0005 mg/mL.

Table 5.2 IC₅₀ Values for the AuNPs tested on three cell lines: SiHa, CaSki, and C33A, using the CCK-8 assay. The number of replicates completed to determine this value is denoted by n in the table.

Compound	IC ₅₀ (mg/mL)		
	SiHa	CaSki	C33A
AuNPs	0.06 ± 0.04	0.08 ± 0.07	0.041 ± 0.002
	(n=10)	(n=4)	(n=2)

Table 5.3 IC₅₀ Values for the same batch of AuNPs, one non-radioactive and the other after undergoing a neutron activation, for two different activities. These values were determined using the CCK-8 Assay with the SiHa cell line using a 36 mg/mL stock solution for both the AuNPs and the ¹⁹⁸AuNPs. The activity in becquerels (Bq) was determined by performing a two-minute well count to measure the activity in counts per second, and then this value was converted to Bq. The radioactive concentration was determined by dividing the activity in Bq by the sample volume. These values were obtained by averaging the IC₅₀ values from two separate assays (n=2).

Compound	Radioactive Concentration (Bq/mL)	IC ₅₀ (mg/mL) (n=2)
AuNPs	0	0.112 ± 0.002
¹⁹⁸ AuNPs	278	0.11 ± 0.01
	2290	0.0386 ± 0.0005

The combined effects of AKI and AuNP treatment were investigated by measuring cell viability following exposure to varying concentrations of one agent while maintaining the other at a constant concentration around their IC₅₀ value (**Table 5.4**). Increasing the concentration of AuNPs while maintaining a constant AKI concentration resulted in reduced cell viability across all three cell lines. Similarly, increasing the AKI concentration at a constant AuNP concentration produced a concentration-dependent decrease in cell viability. The greatest reduction in viability was observed when high

concentrations of both treatments were used, with cell viability approaching 1% in all cell lines. **Table 5.5** shows the IC₅₀ values for the AuNP-AKI conjugate that was tested on SiHa in duplicate. The average IC₅₀ value of 0.0298 ± 0.0008 mg/mL was obtained using a 30:1 ratio of PEG:AKI-PEG.

Table 5.4 Percent cell viability following treatment with varying AuNP concentrations at a constant AKI concentration, and varying AKI concentrations at a constant AuNP concentration. This was performed on SiHa, CaSki, and C33A cell lines.

[AKI] (mg/mL)	[AuNP] (mg/mL)	Cell Viability (%)		
		SiHa	CaSki	C33A
3.07 x 10 ⁻³	0	50.5%	70.1%	55.9%
3.07 x 10 ⁻³	0.032	46.4%	62.3%	38.2%
3.07 x 10 ⁻³	0.284	1.15%	4.18%	3.12%
0	0.136	25.9%	34.1%	7.12%
1.98 x 10 ⁻⁴	0.136	15.7%	19.3%	1.02%
0.0128	0.136	1.05%	0.25%	0.85%

Table 5.5 IC₅₀ Values for the AuNP-AKI conjugate tested on the SiHa cell line, using the CCK-8 assay. A 25 mg/mL stock solution was used to perform the serial dilution. These values were obtained by averaging the IC₅₀ values from two separate assays (n=2).

Compound	IC ₅₀ (mg/mL) (n=2)
AuNP-AKI	0.0298 ± 0.0008

Figure 5.2 shows the UV absorption spectra for the **A)** AuNPs and **B)** the AuNP-AKI conjugate, measured at a scan rate of 600 nm/min over a 1.0-minute cycle. **Figure 5.3** shows these two spectra overlayed to emphasise the peak shift observed once the AKI is added to the AuNP.

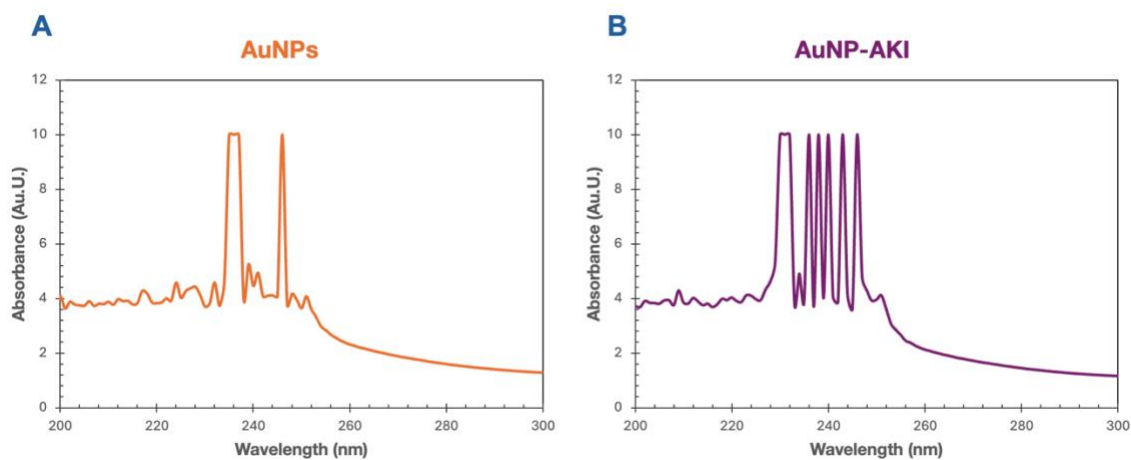


Figure 5.2 Ultraviolet (UV) Absorption Spectra for AuNPs (A) and the AuNP-AKI Conjugate (B). These spectra were measured using a scan rate of 600 nm/min, a data interval of 1.0 nm, a cycle time of 1.0 min, and a cycle count of 1.

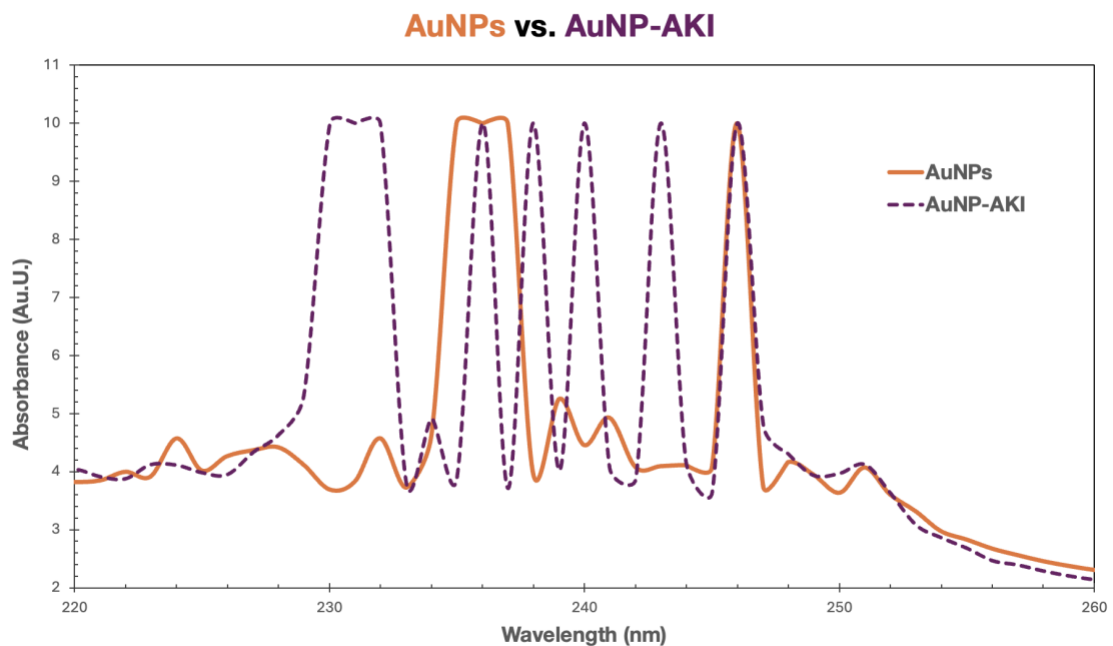


Figure 5.3 Overlaid Ultraviolet (UV) Absorption Spectrum of the AuNPs and AuNP-AKI Conjugate.

Overall, both the Aurora Kinase B inhibitor and AuNPs exhibited cytotoxic activity in the cervical cancer cell lines examined, with differences in sensitivity observed between cell lines. The activation to $^{198}\text{AuNPs}$ showed a small decrease in the toxicity values, but more replicates would need to be obtained to confirm this decrease, as there are many variable factors between assays. Combination treatment further reduced cell viability compared to either treatment alone, particularly at higher concentrations. The significance of these findings and their implications for the therapeutic potential of this treatment strategy are discussed in the following section.

5.3 DISCUSSION

The biological evaluation performed in this study demonstrated that both the Aurora kinase B inhibitor (AKI) and the AuNP formulations exhibited cytotoxic effects against cervical cancer cells, with the greatest reduction in cell viability observed when the two treatments were used in combination. These findings support the continued development of AuNP-based drug delivery systems for Aurora kinase inhibition and provide an important biological baseline for the future evaluation of covalently conjugated AuNP-AKI constructs.

The free Aurora kinase B inhibitor displayed cytotoxic activity in all three cervical cancer cell lines tested, with IC_{50} values of $(1.0 \pm 1.2) \times 10^{-3}$ mg/mL (2.2 ± 2.7 μ M), $(6.3 \pm 1.8) \times 10^{-5}$ mg/mL (0.14 ± 0.04 μ M), and $(3.5 \pm 2.4) \times 10^{-3}$ mg/mL (7.8 ± 5.3 μ M) for SiHa, CaSki, and C33A cells, respectively (**Table 5.1**). The inhibitor used in this study was derived from the well-characterized Aurora kinase inhibitor VX-680, which has been reported to exhibit IC_{50} values in the range of approximately 15-130 nM in various cancer cell lines⁴⁶. This suggests that functionalization of the inhibitor did not substantially compromise its biological activity and supports its suitability for NP conjugation. The free Aurora kinase B inhibitor displayed cytotoxic activity in all three cervical cancer cell lines tested. Although the lowest IC_{50} value was observed in the CaSki cell line, indicating greater apparent sensitivity to the inhibitor, this result was associated with relatively large experimental variability, as reflected by the corresponding error.

The AuNPs alone also demonstrated cytotoxicity toward all three cell lines. (**Table 5.2**). However, neutron activation of the NPs produced little additional effect, as

evidenced by the similar IC₅₀ values obtained for the non-radioactive (AuNPs) and radioactive (¹⁹⁸AuNPs) formulations (**Table 5.3**). This observation is likely explained by the relatively low activity of the activated NPs, which measured only 278 Bq/mL (111 Bq) for ¹⁹⁸Au at the time of treatment. At this activity, the radiation dose delivered to the cells over the 72-hour incubation period was expected to be low, limiting the contribution of the radioactive emission to the overall cytotoxic response. This is also around only one half-life of the ¹⁹⁸Au, limiting the radiation exposure to the cells. Increasing the specific activity of the NPs in future studies may result in greater radiotherapeutic efficacy and allow the therapeutic benefits of ¹⁹⁸Au to be more clearly distinguished. There was a slight decrease in IC₅₀ observed when the activity was increased to 2290 Bq/mL (1145 Bq) (**Table 5.3**), but subsequent assays would need to be performed to ensure that this decrease is statistically significant. There are multiple factors that can affect the ability to measure the cytotoxicity of the AuNPs including the optical overlap with the dye used in the CCK-8 assay and the interactions between the AuNPs and other assay components^{49, 57}. To mitigate this, an absorbance measurement was obtained prior to the addition of the reagent. This value was subtracted from the absorbance measured after the reagent was added. It is also challenging to control the exact amount of AuNPs present in each aliquot of the stock solution, causing variation in the toxicity values for both the AuNPs and the ¹⁹⁸AuNPs.

The three cervical cancer cell lines included two HPV-positive models (SiHa and CaSki)^{55,56} and one HPV-negative model (C33A)⁵⁸, allowing the influence of HPV status on treatment response to be assessed. Despite differences in the sensitivity of each cell line to the free Aurora kinase inhibitor, no consistent trend was observed between HPV-

positive and HPV-negative cells for either the AuNPs or the activated AuNPs. This suggests that the cytotoxic effects of these formulations are not strongly dependent on HPV status and may instead reflect intrinsic differences in cellular uptake, proliferation rate, or Aurora kinase expression among individual cell lines.

The combination studies further demonstrated that simultaneous treatment with AuNPs and the AKI resulted in greater reductions in cell viability than either treatment alone, shown in **Table 5.4**. Increasing the concentration of AuNPs while maintaining a constant concentration of AKI around its IC_{50} value, as well as increasing the AKI concentration at a constant AuNP concentration around its IC_{50} value, consistently decreased cell viability in all three cell lines. Treatment with higher concentrations of both agents reduced cell viability to approximately 1% across all cell lines. These findings suggest that the combined treatment produces an enhanced cytotoxic effect that is greater than would be expected from either component individually, consistent with a synergistic interaction. This also allowed for the determination of how much inhibitor is required to be conjugated onto the surface of the AuNPs to produce a significant therapeutic effect. This was determined to be a 30:1 ratio of regular PEG-chain to inhibitor. By delivering both therapeutic modalities within a single nanopatform, it may be possible to further enhance tumour cell killing while simultaneously improving drug localization and reducing systemic exposure.

The complete AuNP-AKI conjugate was tested in duplicate on SiHa, and the resulting IC_{50} values are shown in **Table 5.5**. Since the IC_{50} value of the AuNPs alone were not found to be significantly low, it would likely not have a large effect on the IC_{50} of the conjugate. Also, a 30:1 ratio of PEG:AKI-PEG was used to synthesize the

particles, accounting for the small increase in toxicity. Additional assays would be required to determine the statistical significance in these IC_{50} values.

UV absorption spectra were obtained for both the AuNPs alone, and the AuNP-AKI conjugate (**Figure 5.2**). **Figure 5.3** shows the two spectra-overlaid to emphasize the large peak shift from 236 nm (AuNPs) to 230 nm (AuNP-AKI). These samples were diluted almost 200X and still reached near-maximum absorbance, making it difficult to conclude exactly what was occurring. Since there was a peak shift between the samples, it can be hypothesized that there was a chemical change after the inhibitor was added, as well as additional peaks observed accounting for the absorbing species in the inhibitor. These samples could be further analyzed if this experiment was re-run with a larger dilution factor.

Overall, the results of this study demonstrate that the modified Aurora kinase B inhibitor retains potent anticancer activity following functionalization, while the AuNP platform provides a suitable carrier for future therapeutic development. Although the low activity of the neutron-activated NPs limited the radiotherapeutic contribution in the present work, the enhanced cytotoxicity observed during combination treatment supports continued investigation of AuNP-AKI conjugates. Future studies should evaluate covalently conjugated formulations with higher specific activities, investigate NP uptake and intracellular localization, and assess treatment efficacy in more complex in vitro and in vivo models to fully characterize the therapeutic potential of this multifunctional nanoplatform.

5.4 EXPERIMENTAL METHODS

5.4.1 Cell Culture

Cervical cancer cell lines CaSki and SiHa (HPV-positive) and C33A (HPV-negative) were kindly provided by Dr. Guillem Dayer (Lakehead University and Thunder Bay Regional Health Research Institute). All cells were cultured in DMEM (Dulbecco's modified Eagle medium) with 10% FBS (fetal bovine serum) and 100 units/mL penicillin and 100 µg/mL streptomycin in 37°C in a water-saturated atmosphere with 5% CO₂.

5.4.2 Cytotoxicity (CCK-8) Assay

Cytotoxicity of the aurora kinase inhibitors and AuNPs against cancer cell lines was determined using CCK-8 kit (Sigma-Aldrich). Briefly, cells were seeded in a 96-well plate (2000 cells/well) with triplicates and grown overnight and then treated with designated dosages of inhibitor or AuNPs with fresh culture medium for 72 h. For wells treated with AuNPs, absorbance at 450 nm was measured prior to the addition of CCK-8 reagent to account for background absorbance from the nanoparticles. This background absorbance was subtracted from subsequent measurements. After that, CCK-8 reagents (10 µL/Well) were added and then incubated for 3 h. The absorbance at 450 nm was recorded by a microplate reader (Bio TEK) every hour. Obtained results were plotted using GraphPad (GraphPad Prism version 6.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com) to determine the IC₅₀. The positive control without inhibitor was set as 100% viability, and the sample without cell and inhibitor was set as background. At least two independent experiments were performed. Data was presented as mean ± SD (Standard deviation).

6 GENERAL EXPERIMENTAL

6.1 Reagents

*All reagents were used directly as received from supplier or obtained from the laboratory without any further purification or modifications.

Chemical	Abbreviation	CAS #	Chapter(s) Used
Triethylene glycol monomethyl ether	-	112-35-6	1
Triethylamine	NEt ₃	121-44-8	1, 4
4-Toluenesulfonyl chloride	TsCl	98-59-9	1
4-Dimethylaminopyridine	DMAP	1122-58-3	1
Potassium thioacetate	-	10387-40-3	1
Potassium permanganate	KMnO ₄	7722-64-7	1
Sodium hydroxide	NaOH	1310-73-2	1, 3
Magnesium sulfate	MgSO ₄	7487-88-9	1, 3, 4
Sodium chloride	NaCl	7647-14-5	1, 3, 4
Sodium bicarbonate	NaHCO ₃	144-55-8	1, 3, 4
Sodium sulfate	Na ₂ SO ₄	7757-82-6	3
Ninhydrin	-	485-47-2	1, 4
Hydrochloric acid	HCl	7647-01-0	1, 4
Gold(III) chloride	AuCl ₃	13453-07-1	1, 4
Sodium borohydride	NaBH ₄	16940-66-2	1, 4
Ammonium hydroxide	NH ₄ OH	1336-21-6	4
Sodium azide	NaN ₃	26628-22-8	4
Triphenylphosphine	PPh ₃	603-35-0	4

Chemical	Abbreviation	CAS #	Chapter(s) Used
Triphenylphosphine, polymer-bound	PPh ₃ resin	39319-11-4	4
1-Ethyl-3-(3- dimethylaminopropyl)c arbodiimide hydrochloride	EDCI HCl	25952-53-8	4
Hydroxybenzotriazole	HOBt	123333-53-9	4
1-Z-piperazine	-	31166-44-6	4
Di-isopropyl ethylamine	DIPEA	7087-68-5	4
Aurora Kinase B Inhibitor Precursor	-	N/A	4
Tetrabutylammonium fluoride	TBAF	429-41-4	3
Potassium fluoride	KF	7789-23-3	3
Kryptofix 2.2.2	K2.2.2	23978-09-8	3
p-Toluenesulfonic acid	PTSA	104-15-4	3
Triisopropylsilane	TIPS	6485-79-6	3
Girard's reagent	-	123-46-6	3
Triphenyl methanethiol	-	3695-77-0	3

6.2 Solvents

*All solvents were obtained from the supplier and used without further purification.

Solvent	Abbreviation	Chapter(s) Used
Dichloromethane (DCM)	DCM	1, 3, 4
Methanol (MeOH)	MeOH	1, 3, 4, 5
Ethanol (EtOH)	EtOH	1, 3
Acetone	-	1
Ethyl acetate (EtOAc)	EtOAc	1, 3, 4
Hexanes (Hex)	Hex	1, 3, 4
Toluene (Tol.)	Tol.	1, 3
Cyclohexane	-	1, 4
Acetonitrile (MeCN)	MeCN	3, 4
Dimethylformamide (DMF)	DMF	3, 4
Tetrahydrofuran (THF)	THF	4
Deionized water (DI H ₂ O)	DI H ₂ O	1–5

6.3 Biological Materials

*All biological materials were used directly as received from supplier or obtained from the Thunder Bay Regional Health Research Institute (TBRHRI) without any further purification or modifications.

Chemical	Abbreviation	Chapter Used
SiHa Cell Line	-	5
CaSKi Cell Line	-	5
C33A Cell Line	-	5
Dulbecco's Modified Eagle Medium	DMEM	5
Fetal Bovine Serum	FBS	5
Penicillin	Pen.	5
Streptomycin	Strep.	5
Cell Counting Kit-8	CCK-8	5

6.4 Instruments

Instrument / Equipment	Location	Manufacturer / Model	Application
NMR spectrometer	Lakehead University	Bruker / 500 MHz Bruker Avance NEO Liquid and Solid-State NMR	^1H and ^{13}C NMR
FTIR spectrometer	Lakehead University	Thermo Fischer Scientific / Everest™ Diamond ATR Accessory for the Nicolet™ Summit Spectrometer	Characterization of compounds, e.g. azide
UV-Vis spectrophotometer	Lakehead University	Agilent / Cary 60	AuNP characterization
Transmission electron microscope (TEM)	Lakehead University	JEOL / JEM-2100Plus	AuNP morphology and size
Cyclotron	TBRHRI – Cyclotron Facility	Advanced Cyclotron Systems Inc. / TR-24 PET Cyclotron	Neutron activation of AuNPs
Gamma spectrometer	TBRHRI – Cyclotron Facility	Variable (Multiple at Facility)	Measuring ^{198}Au activity after neutron activation
Well Counter	TBRHRI – Cyclotron Facility	Capintec CAPRAC®-t	Shielded Multi-Channel Analyzer (MCA) for isotope identification
Microplate reader	TBRHRI – Cell Culture Lab	BioTek / PowerWave HT 340	CCK-8 absorbance at 450 nm

7 CONCLUSIONS AND FUTURE DIRECTIONS

This work describes the development of the components necessary to prepare a multi-functionalized AuNP. Combined, it will allow for a theranostic platform designed to integrate targeted chemotherapy, radiotherapy, and molecular imaging within a single moiety. Through the synthesis of PEG-functionalized AuNPs, neutron activation to produce therapeutic radionuclides, development of radiolabelling strategies, conjugation of a targeted Aurora Kinase B inhibitor, and preliminary biological evaluation, the work establishes a strong chemical and biological foundation for the continued development of multifunctional NP therapeutics.

Chapter 1 outlines the reproducible synthesis of PEG-stabilized AuNPs with an average diameter of approximately 5 nm, a size well suited for biomedical applications due to its favourable cellular uptake and tumour accumulation characteristics. Optimization of the 1:3 gold-to-thiol ratio produced uniform NPs with batch-to-batch reproducibility, providing a reliable platform for subsequent functionalization. These NPs were successfully neutron activated in Chapter 2, using the excess neutron flux generated during routine cyclotron production of ^{18}F , producing pure ^{198}Au NPs without detectable radioactive contaminants. While increased cellular toxicity was not observed for the irradiated NP, this likely can be overcome through irradiation at higher energy and for longer durations. Longer exposure of the cells to the NP may also increase cell mortality. The development of a dedicated sample transport system further enabled rapid retrieval of activated samples while minimizing radiation exposure, demonstrating the practicality of this activation approach for future radiopharmaceutical production.

A model system for ^{18}F -radiolabelling strategy was developed with ^{19}F in Chapter 3, to allow for a theranostic approach. Optimization of the fluorination chemistry using non-

radioactive ^{19}F established both stepwise and one-pot synthetic approaches that are compatible with the time constraints associated with ^{18}F radiochemistry. These studies provide a practical route for future radiolabelling within the synthetic hot cell and establish the methodology required to generate PET-visible AuNPs capable of tracking NP biodistribution in vivo.

To enable targeted chemotherapy, an Aurora Kinase B inhibitor previously developed in Dr. Hou's laboratory was successfully adapted for NP conjugation in Chapter 4. The conjugation strategy was guided by kinase selectivity studies demonstrating that modification of the piperazine substituent improved Aurora Kinase B selectivity while preserving inhibitory activity, allowing PEG attachment at a position predicted to minimally affect target binding. In addition to the Aurora Kinase B inhibitor conjugate, complementary PEG-functionalized ligands containing azide and amine functionalities were synthesized, expanding the versatility of the AuNP platform for future incorporation of targeting ligands, antibodies, imaging agents, or additional therapeutics. Together with the radiolabelling PEG chain, these molecules establish a small library of PEG ligands that can be incorporated onto the NP surface in controlled ratios to produce multifunctional agents.

Lastly, the biological evaluation in Chapter 5 demonstrated that both the Aurora Kinase B inhibitor and AuNP formulations exhibited cytotoxic activity against cervical cancer cell lines. Although neutron activation produced only a small increase in cytotoxicity because of the relatively low specific activity of the ^{198}Au NPs, combination treatment with the Aurora Kinase B inhibitor and AuNPs produced substantially greater reductions in cell viability than either treatment alone, consistent with a synergistic interaction. These studies also established the optimal ratio of Aurora Kinase B inhibitor

to PEG ligand for future NP synthesis, providing an important parameter for the preparation of conjugated AuNP-AKI formulations. This conjugate was also tested on the cervical cancer cell lines, yielding IC₅₀ values slightly lower than the individual components.

Collectively, the work presented in this thesis establishes each of the individual components required to produce a complete AuNP-based theranostic platform. The synthesis of reproducible PEG-stabilized AuNPs, successful neutron activation to generate therapeutic ¹⁹⁸Au, the development of a model system for ¹⁹F-labelling in order to further develop an ¹⁸F radiolabelling strategy, development of a small PEG conjugation library, and demonstration of promising biological activity together represent important advances toward the multifunctional AuNPs capable of simultaneous targeted chemotherapy, radiotherapy, and diagnostic imaging. **Figure 7.1** shows the overall view of the conjugate attacking a cancer cell.

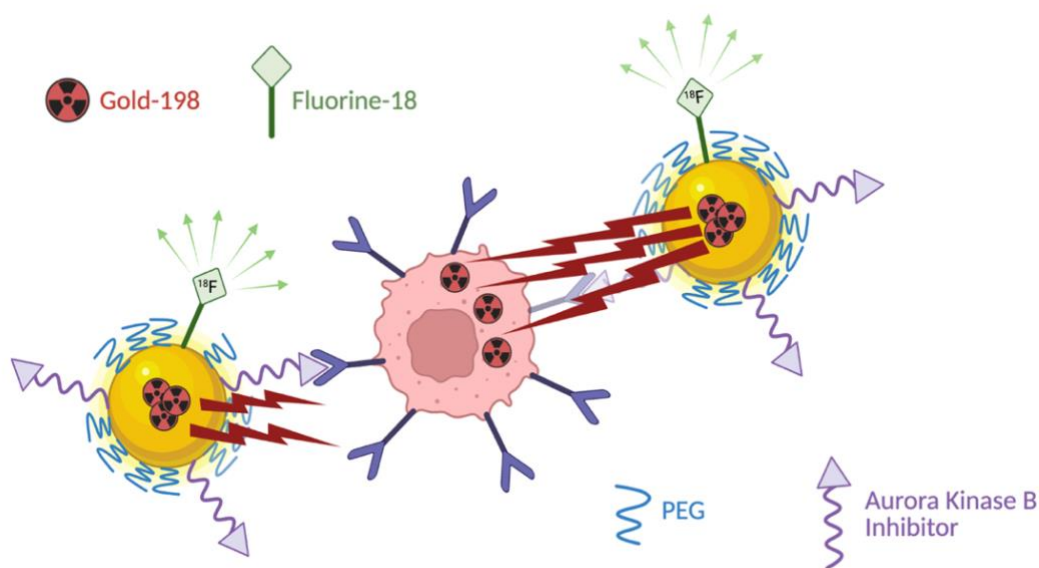


Figure 7.1 Complete Radiolabeled ¹⁹⁸AuNP-AKI Conjugate attacking a cancer cell (Biorender™).

The full conjugate contains; the AuNPs containing a few atoms activated to gold-198 are shown in gold and red, respectively, with the blue PEG chains surrounding to increase biocompatibility and solubility. The AuNP acts as a vehicle for delivery with various optical properties, as well as the few radioactive gold-198 atoms that release beta-electrons in order to damage the cancer cells and increase the therapeutic effect. The conjugated aurora kinase B inhibitor is shown in purple, which can bind to cancer specific receptors on the cell and as well as inhibit the kinase activity, decreasing the cancer cell proliferation. Lastly, the fluorine-18 diagnostic agent is shown in green, which allows for the diagnostic aspect of the theranostic approach, including dose determination and *in-vivo* monitoring of treatment. Overall, this highlights the multifaceted approach used in this work to target cancer and evaluate treatment efficacy using a single conjugated complex.

Future work should focus on completing the biological evaluation of the fully assembled nanoplatform. The priority will be to synthesize the $^{198}\text{AuNP}$ -Aurora Kinase B inhibitor conjugate and evaluate its cytotoxicity in cervical cancer cell lines using the CCK-8 assay to determine the corresponding IC_{50} values to quantify the combined therapeutic contributions of Aurora Kinase B inhibition and β^- emission. Other assays may also need to be used that can allow for longer incubation time and for the AuNPs to have higher specific activity. Based upon the model system developed for fluorination, the optimized ^{18}F radiolabelling strategy should then be implemented using cyclotron-produced ^{18}F and the method to use the synthetic hot cell to prepare the final ^{18}F -labelled $^{198}\text{AuNP}$ -AKI construct. Successful preparation of this fully functionalized nanoplatform will enable simultaneous PET imaging and targeted radionuclide therapy, allowing NP biodistribution and therapeutic efficacy to be evaluated *in vivo*. Additional

studies should also investigate NP uptake, intracellular localization, pharmacokinetics, and tumour targeting, ultimately progressing toward preclinical animal models to fully assess the therapeutic potential of this multifunctional theranostic system.

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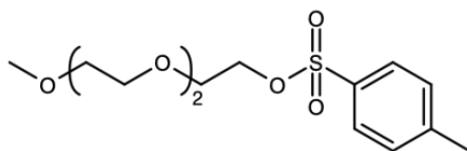
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APPENDICIES

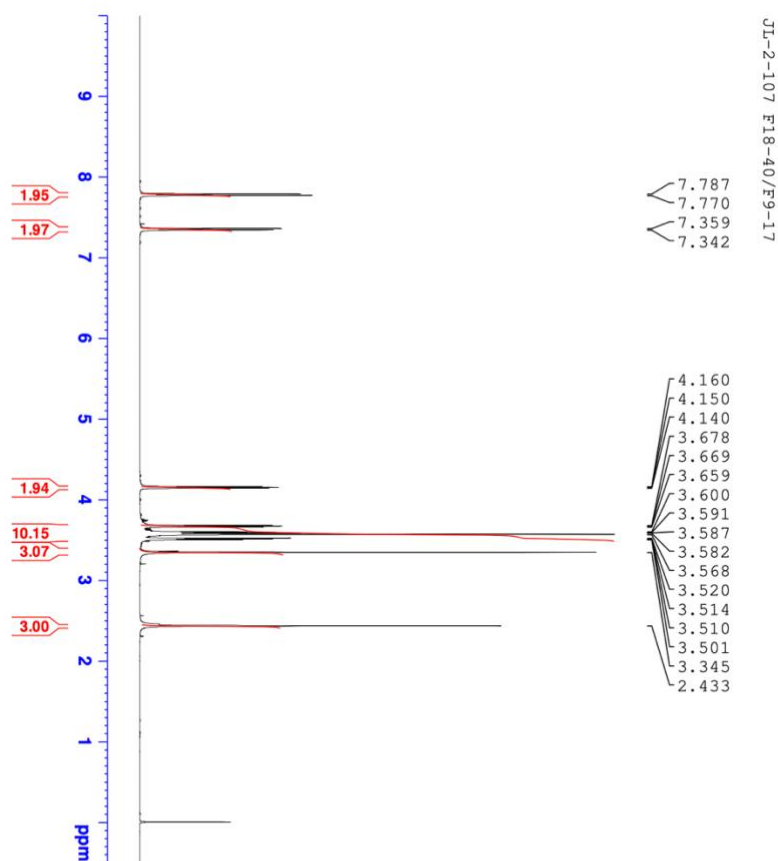
*All NMRs were performed in d-chloroform, acquired at 500 Hz, and integrated using Bruker TopSpin.

1. APPENDIX 1

1.1. Compound 1.1 (1-[2-(2-Methoxyethoxy)ethoxy]-2-(tosyloxy)ethane)



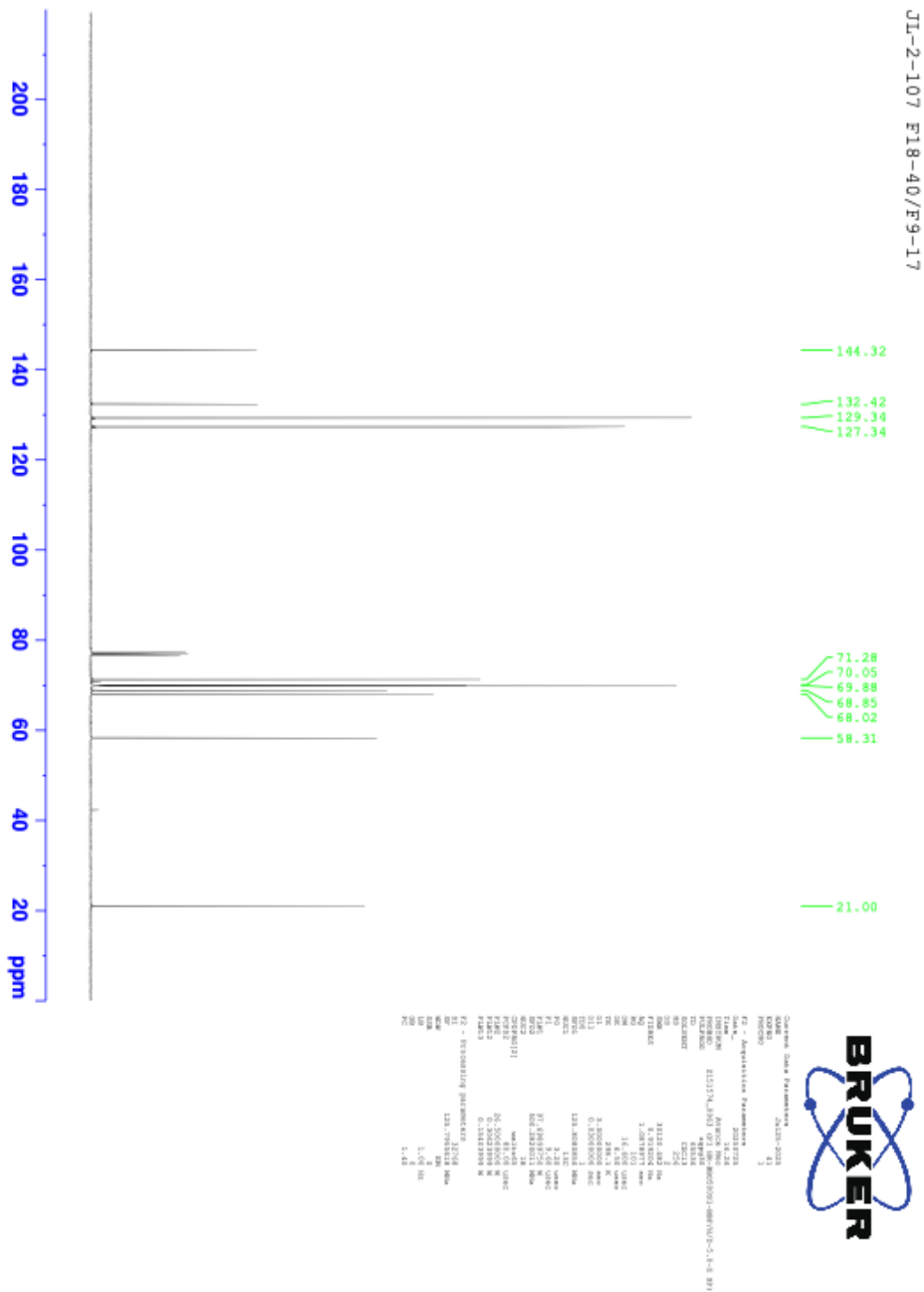
1.1.1. ¹H NMR



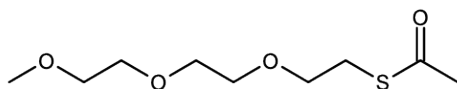
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EXPNO: 1
PROCNO: 1
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Date_Exp: 2015-02-16 11:11:40
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Time: 11:11:40



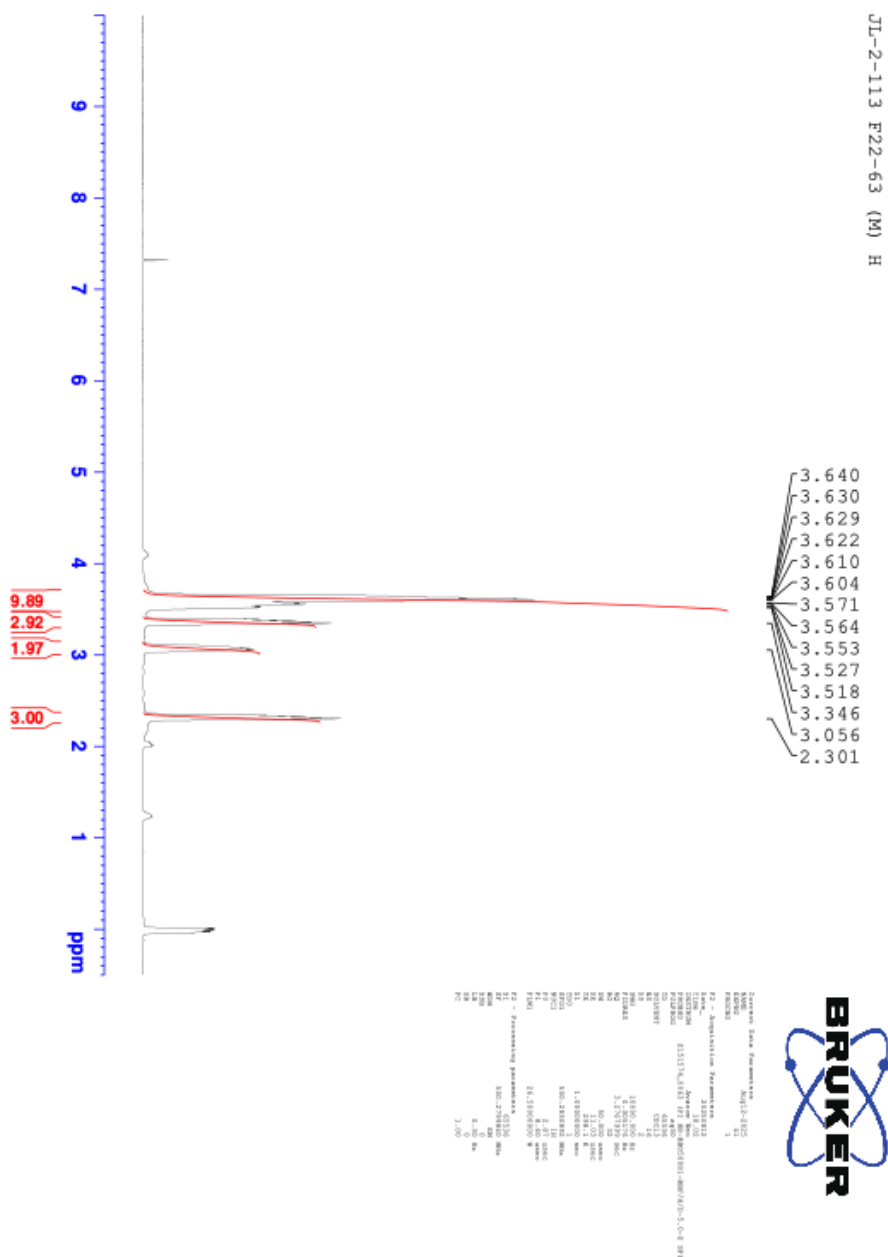
1.1.2. ^{13}C NMR



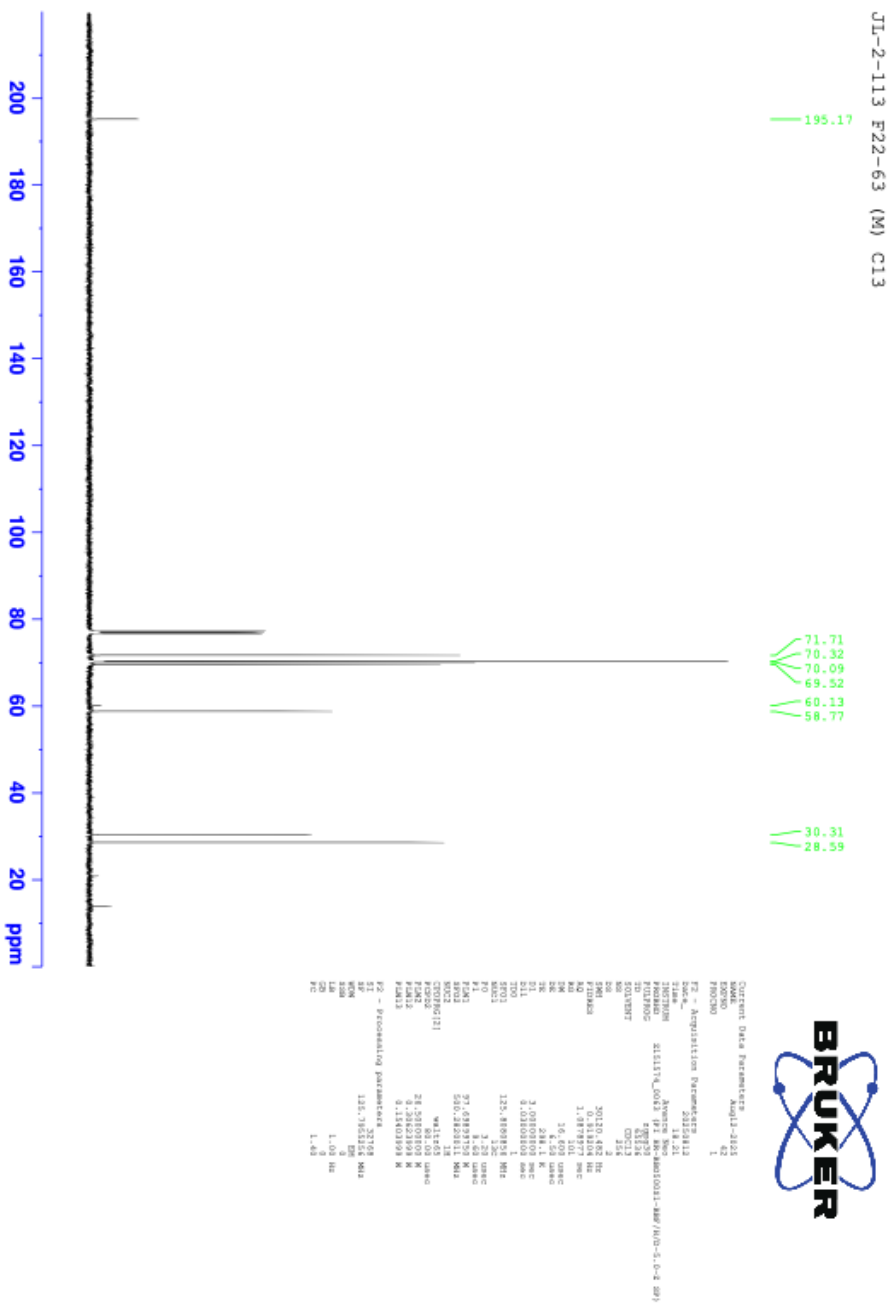
1.2. Compound 1.2 (S-(2-(2-(2-methoxyethoxy)ethoxy)ethyl)ethanethioate)



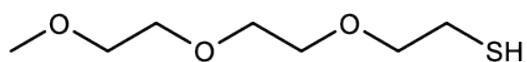
1.2.1. ^1H NMR



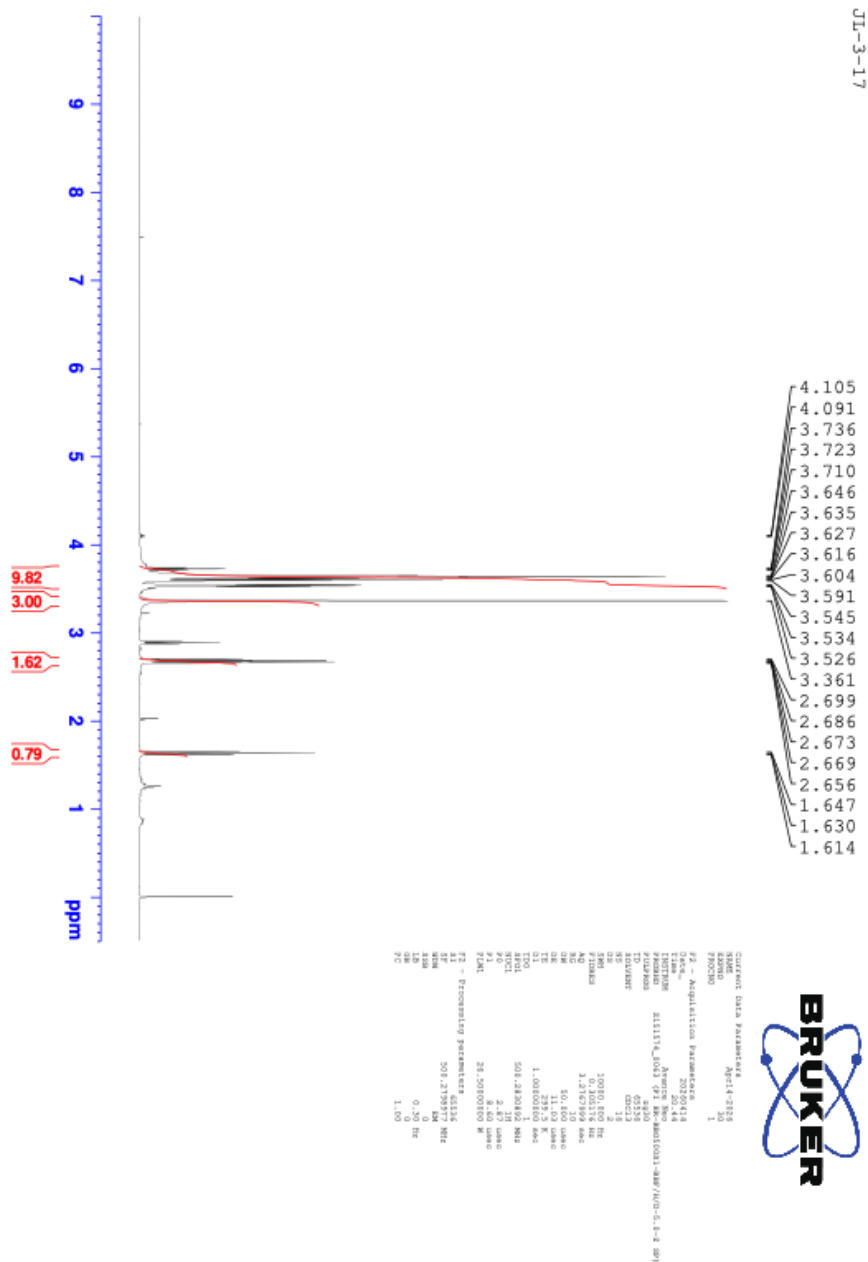
1.2.2. ¹³C NMR



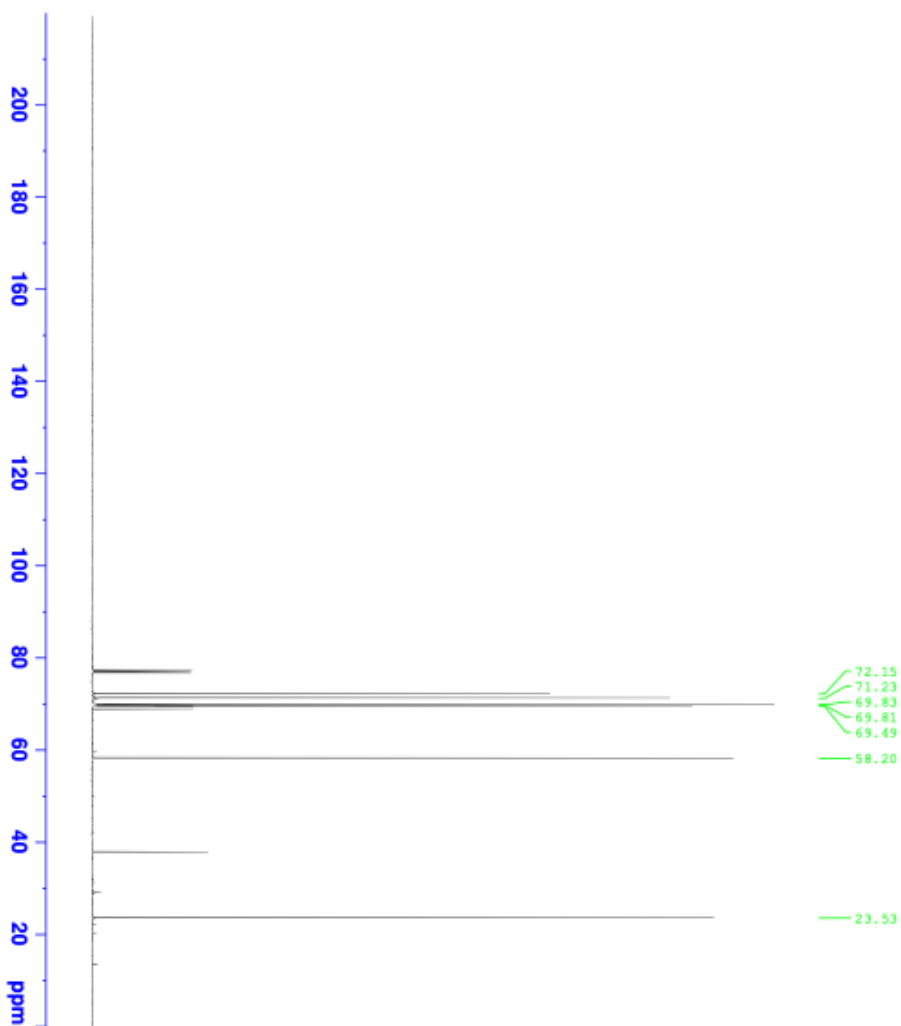
1.3. Compound 1.3 (3,6,9-Trioxa-1-decanethiol)



1.3.1. ¹H NMR

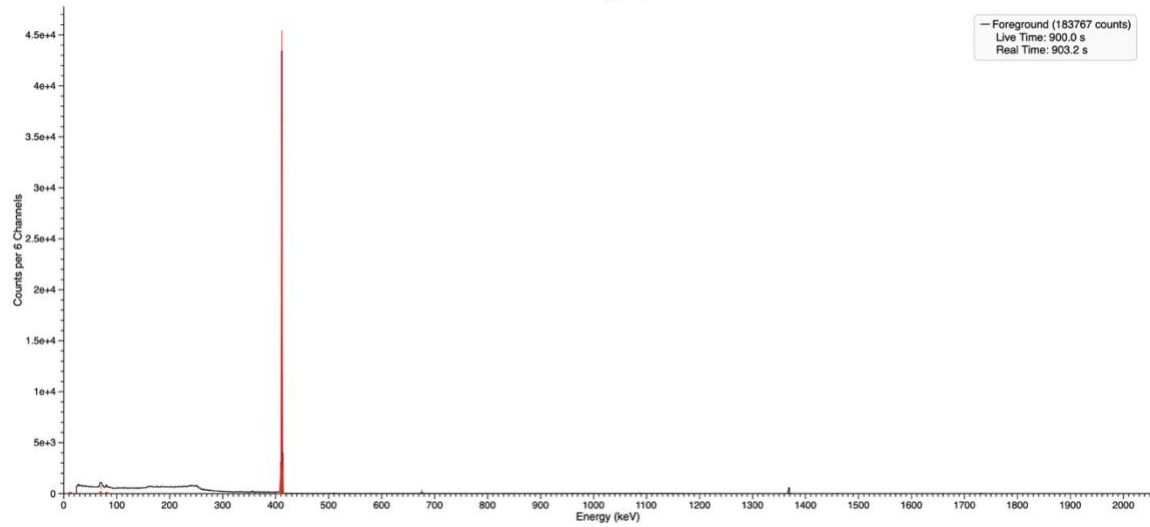
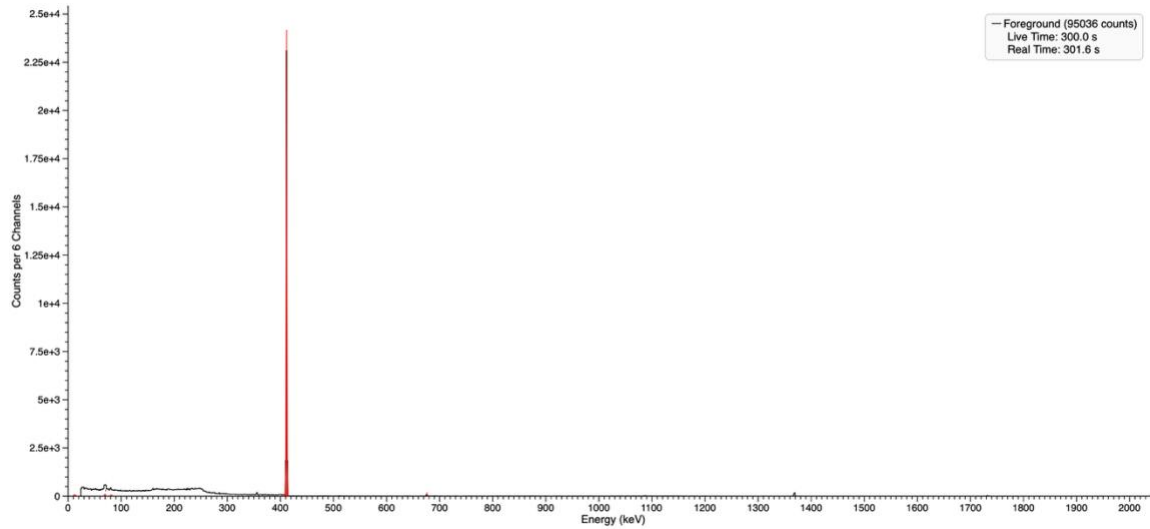


JL-3-17

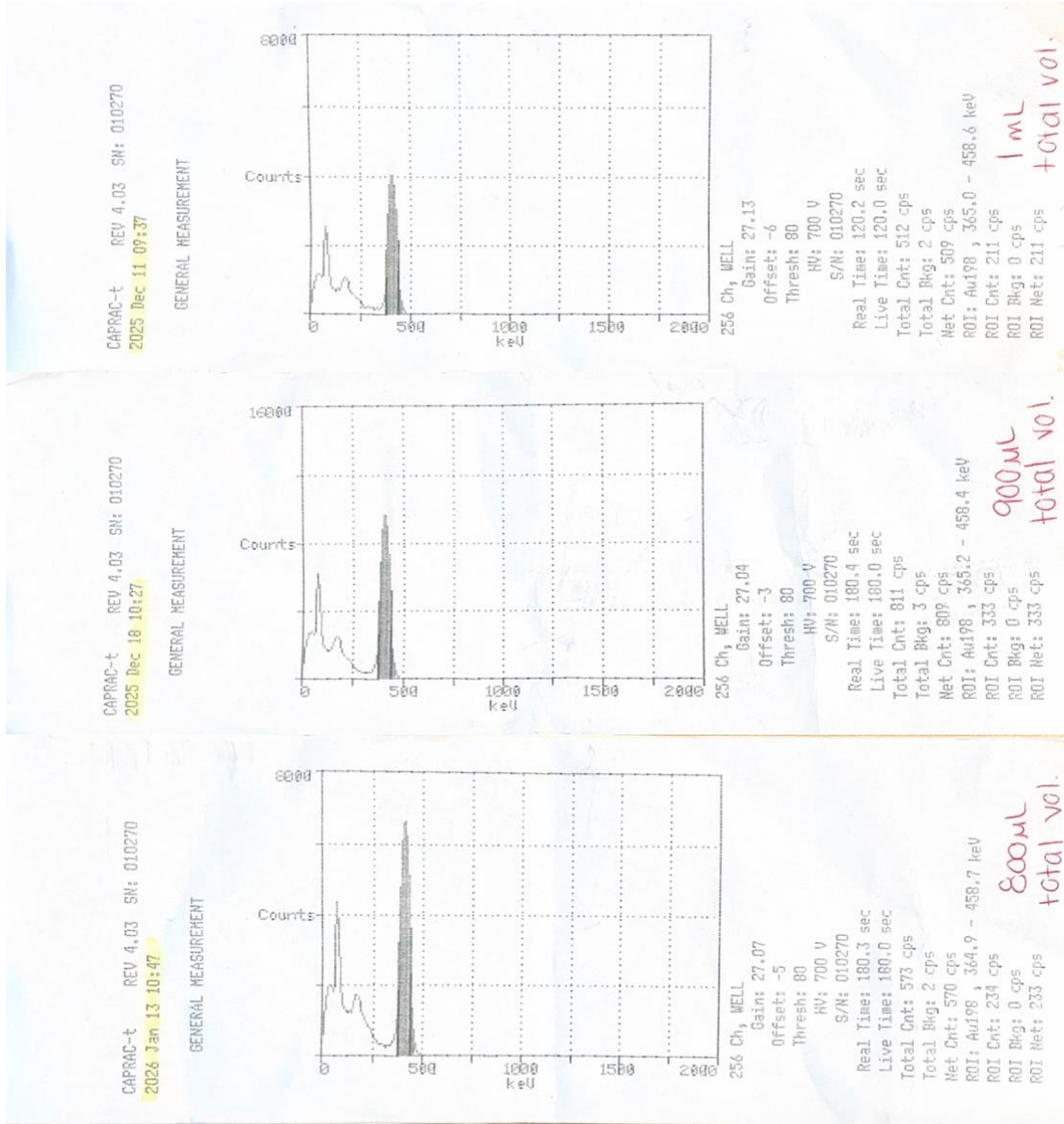
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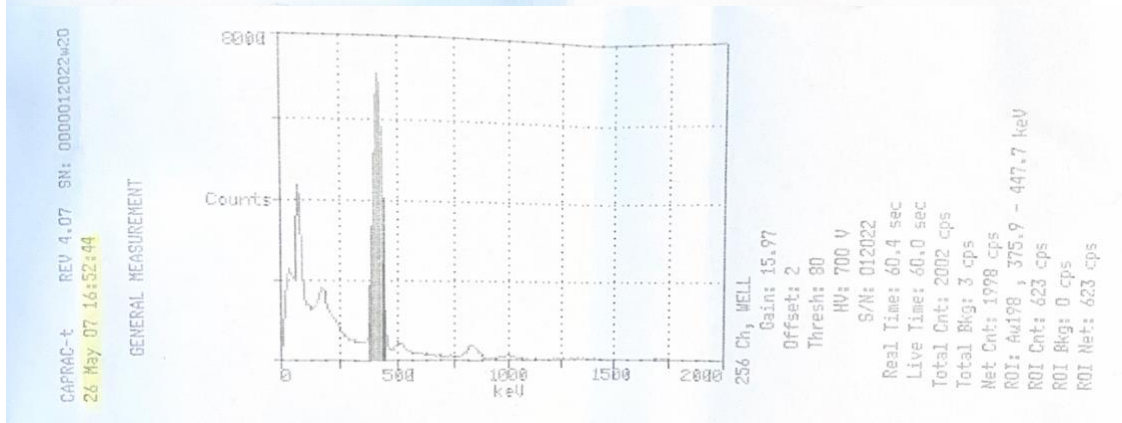
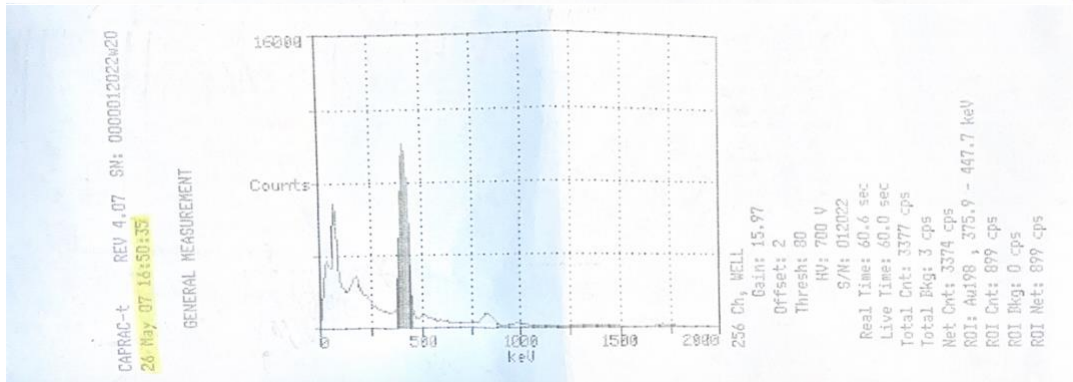
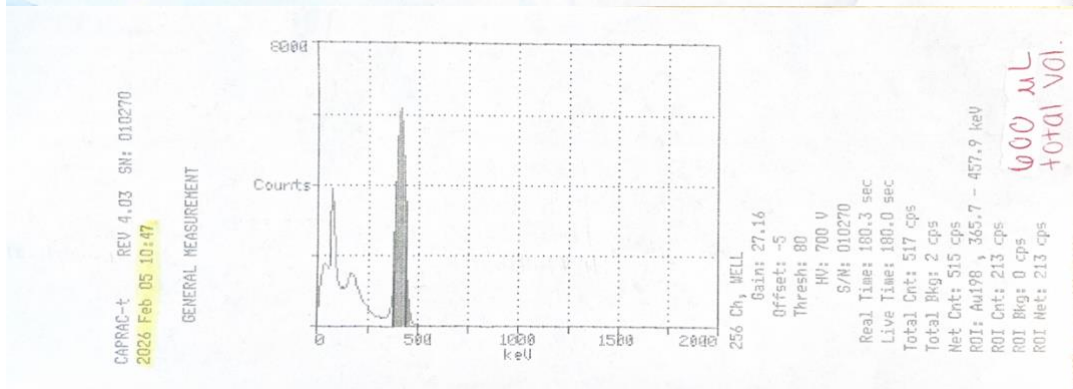
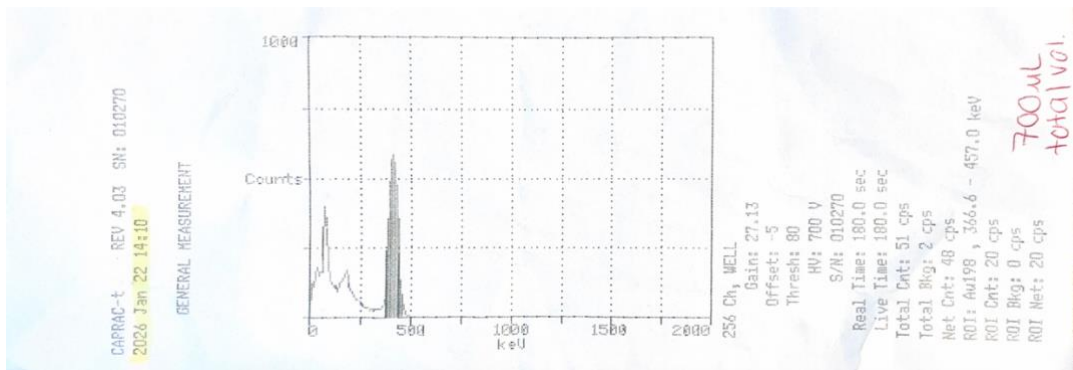
2. APPENDIX 2

2.1. Other Gamma Spectra Obtained



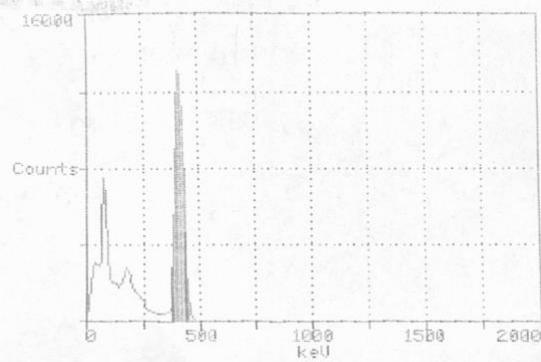
2.2. Other Well Counts Obtained





CAPRAD-t REV 4.07 SN: 0000012022#20
26 Jul 09 16:02:05

GENERAL MEASUREMENT



256 Ch, WELL

Gain: 15.97

Offset: 3

Thresh: 80

HW: 700 V

S/N: 012022

Real Time: 60.5 sec

Live Time: 60.0 sec

Total Cnt: 2721 cps

Total Bkg: 3 cps

Net Cnt: 2718 cps

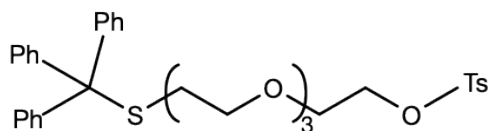
ROI: Au198 , 374.6 - 449.0 keV

ROI Cnt: 1145 cps

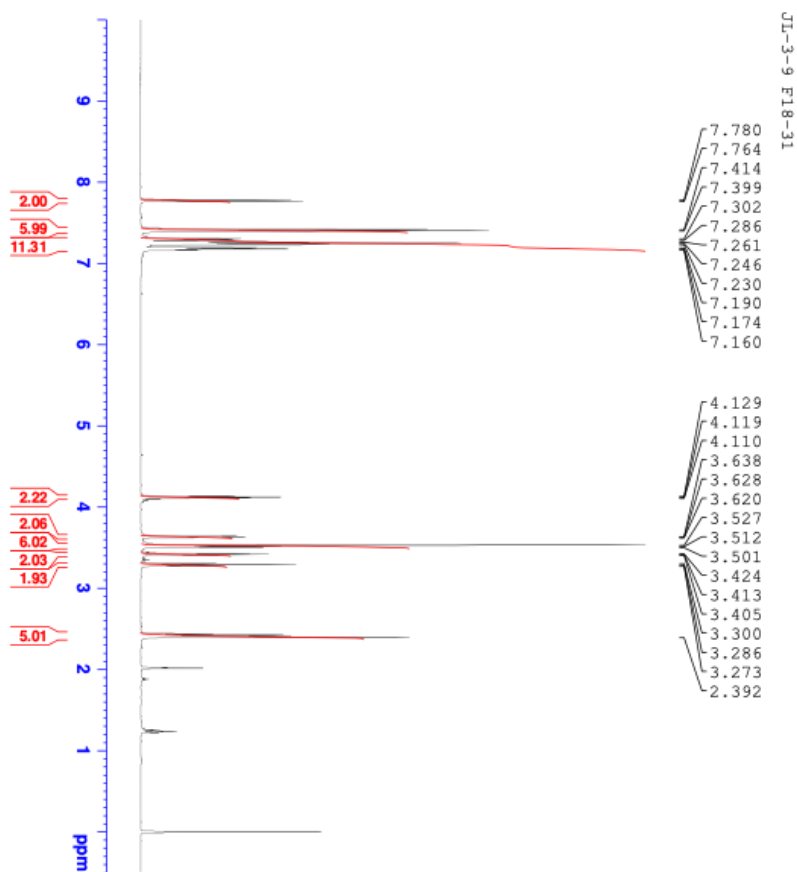
3. APPENDIX 3

3.1. Compound 3.1 (*p*-[2-(2-{2-[2

(Tritylthio)ethoxy}ethoxy)ethoxysulfonyl]toluene)



3.1.1. ^1H NMR

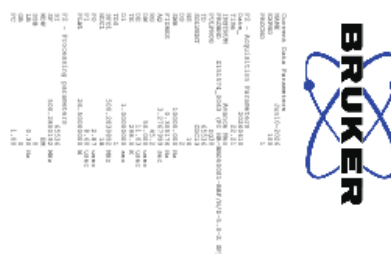
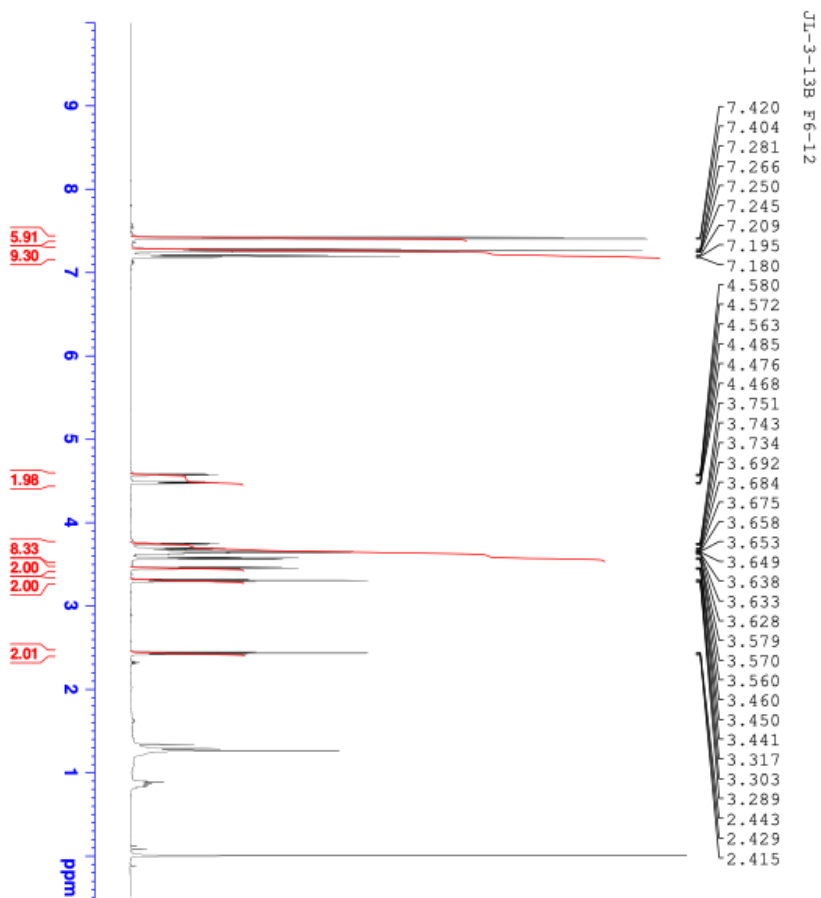
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JL-3-9 F18-31

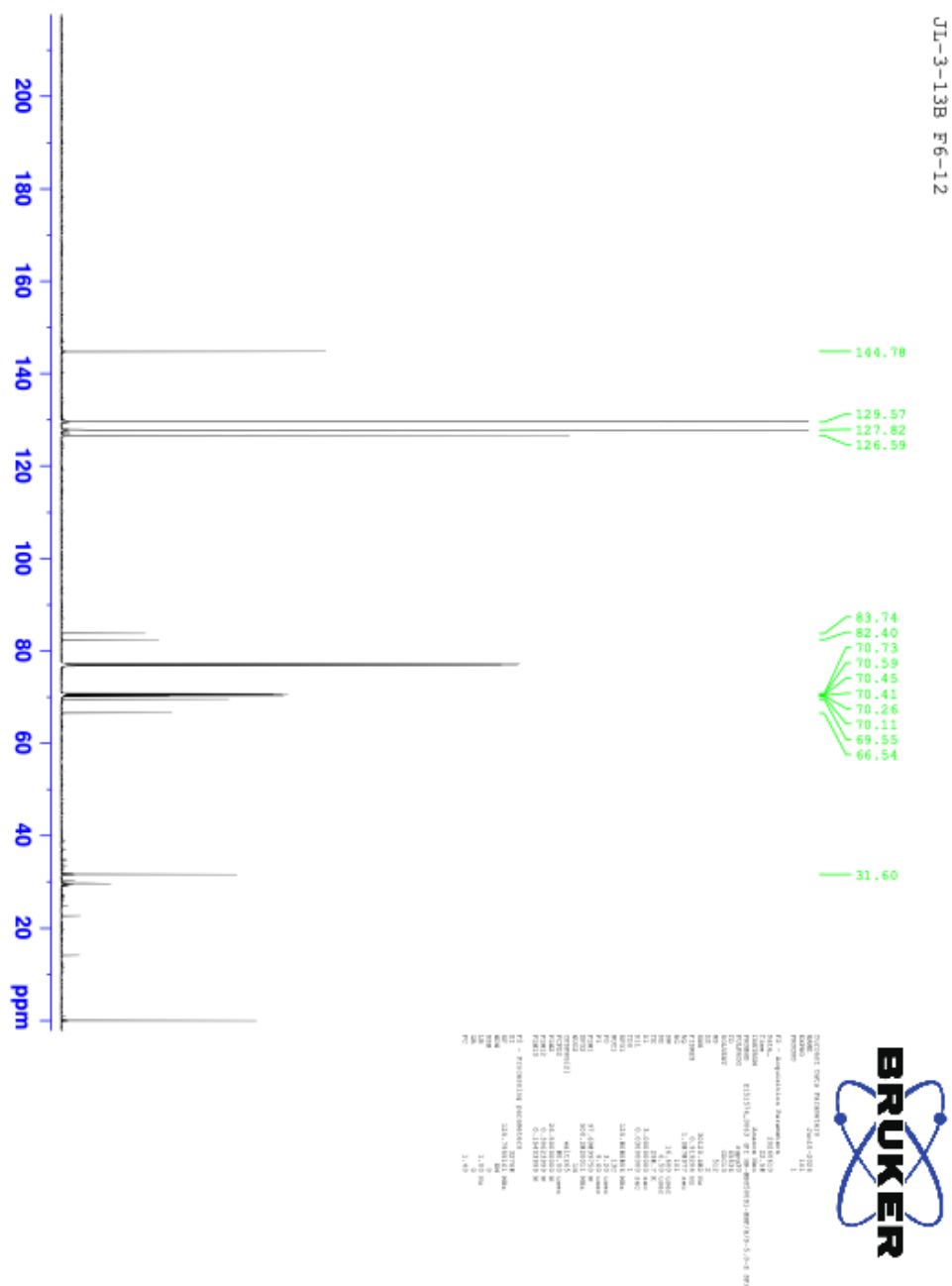


FCFSS(C1=CC=CC=C1)(C1=CC=CC=C1)C1=CC=CC=C1

3.2.1. ^1H NMR

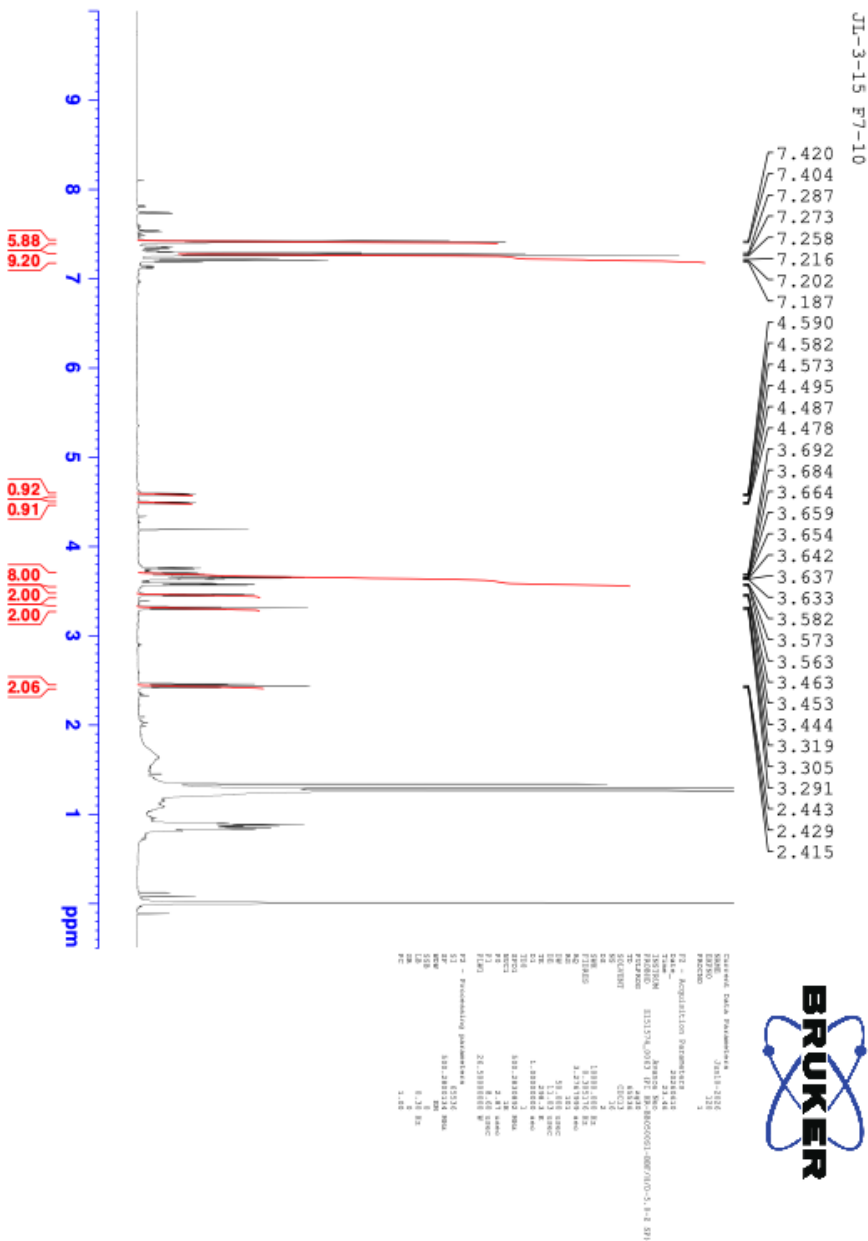


3.2.2. ^{13}C NMR



B) K.2.2.2/KF:

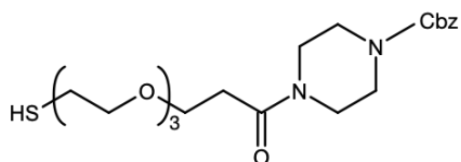
3.2.3. ^1H NMR



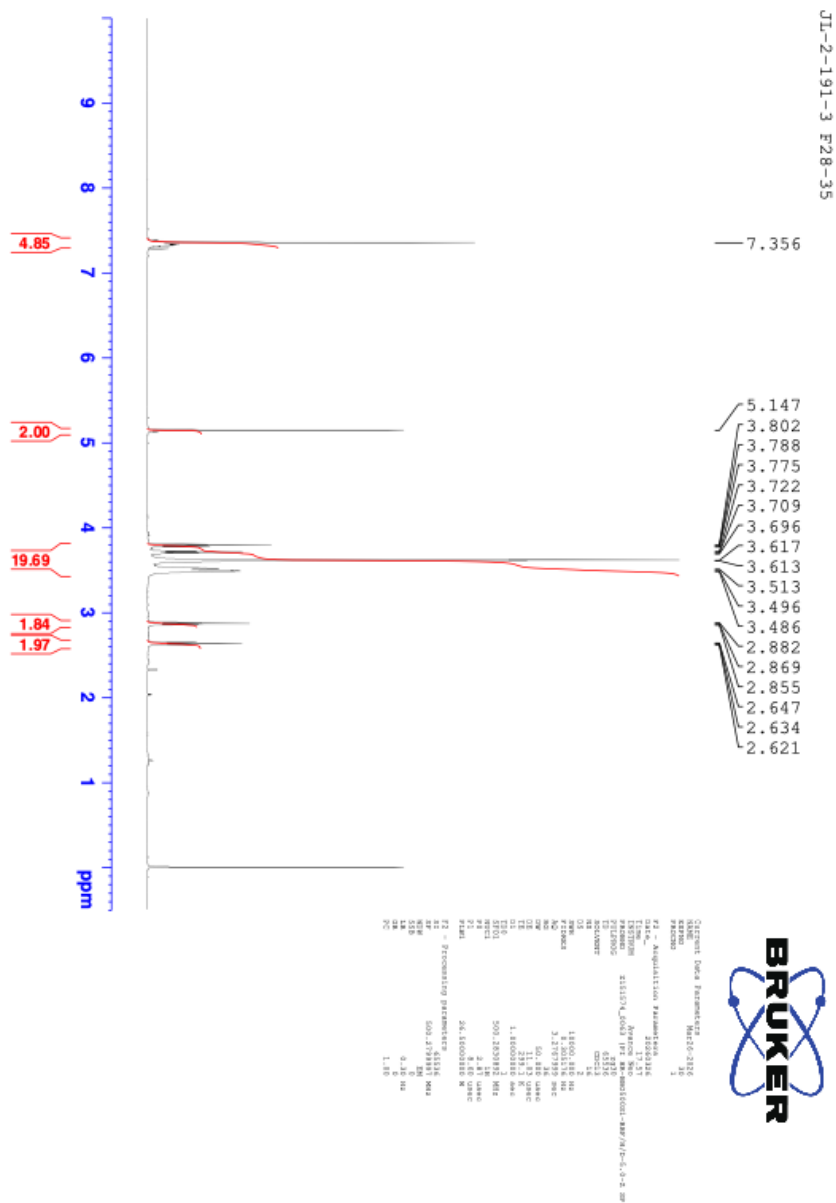
4. APPENDIX 4

A) Synthesis of Chains for Inhibitor Attachment:

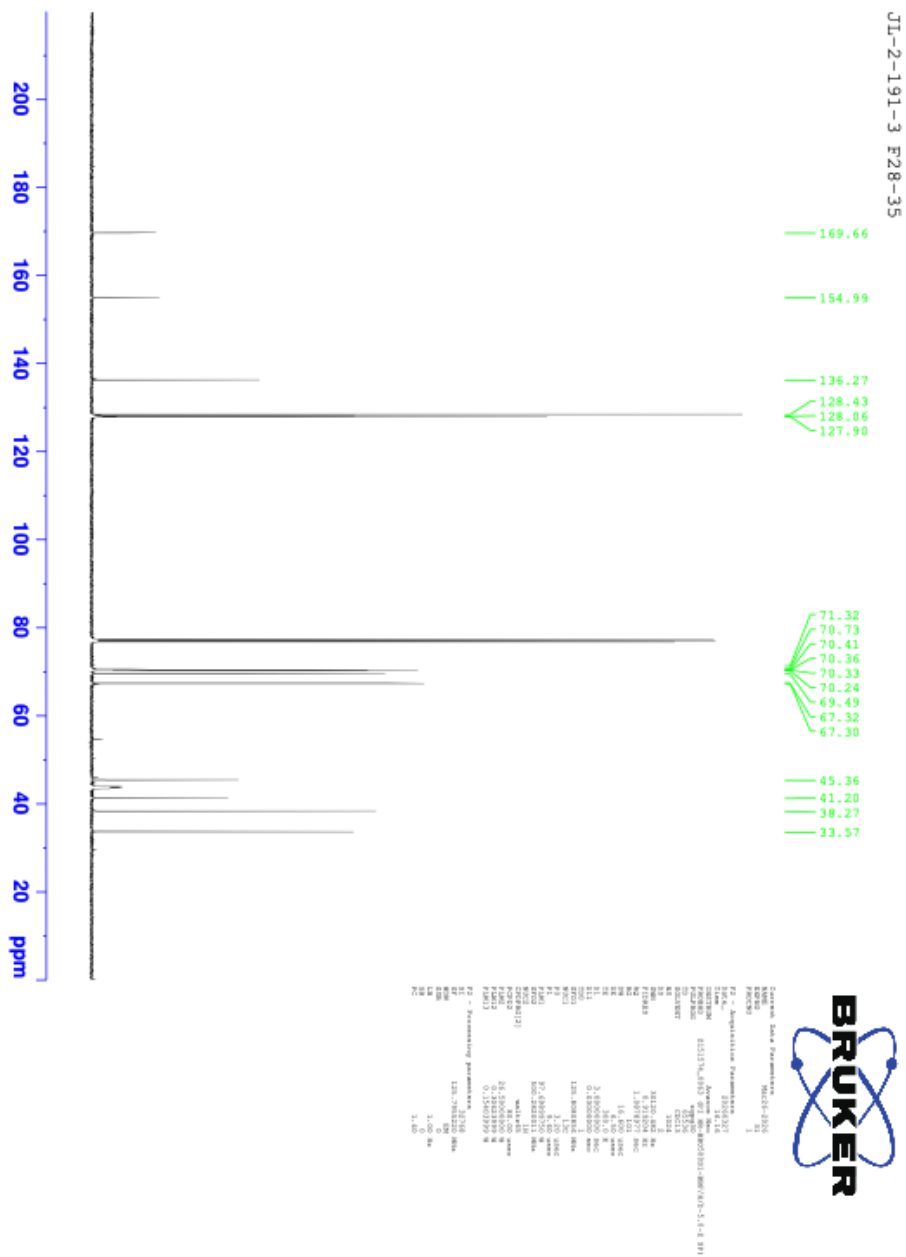
4.1. Compound 4.1 (Benzyl 4-(3-{2-[2-(2-mercaptoethoxy)ethoxy]ethoxy}propionyl)-1-piperazinecarboxylate)



4.1.1. ¹H NMR



4.1.2. ^{13}C NMR



OSCCOCCOCCOCC(=O)N1CCNCC1

1H NMR spectrum of compound 1l-3-11. The x-axis is chemical shift in ppm, ranging from 0 to 10. The spectrum shows a broad peak at ~3.6 ppm (20.10H), a multiplet at ~2.6-3.0 ppm (2.30H and 2.00H), and a small peak at ~1.2 ppm. The chemical structure of 1l-3-11 is shown in the top right corner.

Chemical structure of 1l-3-11: CC1(C)CC(C)(C)C(C)(C)C1

Peak list (ppm): 3.781, 3.774, 3.769, 3.761, 3.747, 3.733, 3.720, 3.690, 3.656, 3.649, 3.640, 3.634, 3.631, 2.913, 2.901, 2.888, 2.875, 2.621, 2.608, 2.595.

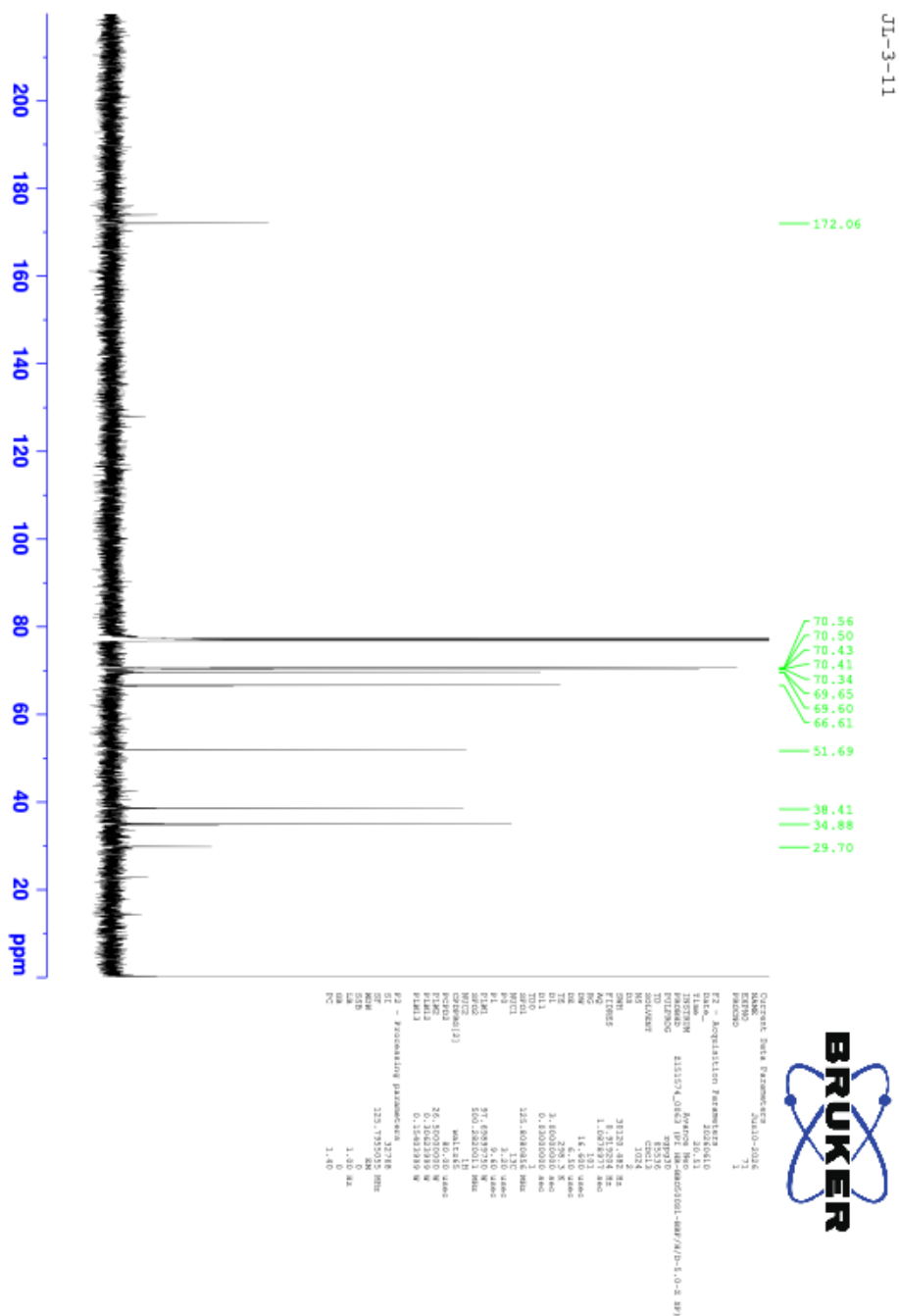
Integration values: 20.10, 2.30, 2.00.

JL-3-11

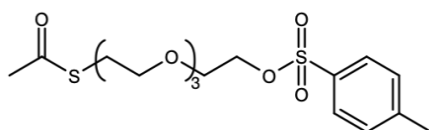


P1 - Training parameters		P2 - Augmentation Parameters	
Epochs	100	Epochs	200,000
Batch Size	128	Batch Size	128
Learning Rate	0.001	Learning Rate	0.001
Weight Decay	0.0001	Weight Decay	0.0001
Dropout Rate	0.5	Dropout Rate	0.5
Number of Layers	4	Number of Layers	4
Activation Function	ReLU	Activation Function	ReLU
Optimizer	Adam	Optimizer	Adam
Loss Function	CrossEntropy	Loss Function	CrossEntropy
Validation Split	0.2	Validation Split	0.2
Early Stopping	True	Early Stopping	True
Patience	10	Patience	10
Checkpointing	True	Checkpointing	True
Device	CPU	Device	CPU
Parallelism	False	Parallelism	False
Random Seed	42	Random Seed	42
Gradient Clipping	True	Gradient Clipping	True
Warmup Epochs	5	Warmup Epochs	5
Warmup LR Multiplier	0.1	Warmup LR Multiplier	0.1
Warmup LR Scheduler	Linear	Warmup LR Scheduler	Linear
Warmup LR End Epoch	5	Warmup LR End Epoch	5
Warmup LR Start Value	0.0001	Warmup LR Start Value	0.0001
Warmup LR End Value	0.001	Warmup LR End Value	0.001
Warmup LR Scheduler	Linear	Warmup LR Scheduler	Linear
Warmup LR End Epoch	5	Warmup LR End Epoch	5
Warmup LR Start Value	0.0001	Warmup LR Start Value	0.0001
Warmup LR End Value	0.001	Warmup LR End Value	0.001
Warmup LR Scheduler	Linear	Warmup LR Scheduler	Linear
Warmup LR End Epoch	5	Warmup LR End Epoch	5
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Warmup LR End Value	0.001	Warmup LR End Value	0.001
Warmup LR Scheduler	Linear	Warmup LR Scheduler	Linear
Warmup LR End Epoch	5	Warmup LR End Epoch	5
Warmup LR Start Value	0.0001	Warmup LR Start Value	0.0001
Warmup LR End Value	0.001	Warmup LR End Value	0.001
Warmup LR Scheduler	Linear	Warmup LR Scheduler	Linear
Warmup LR End Epoch	5	Warmup LR End Epoch	5
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Warmup LR End Epoch	5	Warmup LR End Epoch	5
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Warmup LR End Epoch	5	Warmup LR End Epoch	5
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Warmup LR End Value	0.001	Warmup LR End Value	0.001
Warmup LR Scheduler	Linear	Warmup LR Scheduler	Linear
Warmup LR End Epoch	5	Warmup LR End Epoch	5
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Warmup LR End Value	0.001	Warmup LR End Value	0.001
Warmup LR Scheduler	Linear	Warmup LR Scheduler	Linear
Warmup LR End Epoch	5	Warmup LR End Epoch	5
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Warmup LR End Value	0.001	Warmup LR End Value	0.001
Warmup LR Scheduler	Linear	Warmup LR Scheduler	Linear
Warmup LR End Epoch	5	Warmup LR End Epoch	5
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Warmup LR End Value	0.001	Warmup LR End Value	0.001
Warmup LR Scheduler	Linear	Warmup LR Scheduler	Linear
Warmup LR End Epoch	5	Warmup LR End Epoch	5
Warmup LR Start Value	0.0001	Warmup LR Start Value	0.0001
Warmup LR End Value	0.001	Warmup LR End Value	0.001
Warmup LR Scheduler	Linear	Warmup LR Scheduler	Linear
Warmup LR End Epoch	5	Warmup LR End Epoch	5
Warmup LR Start Value	0.0001	Warmup LR Start Value	0.0001
Warmup LR End Value	0.001	Warmup LR End Value	0.001
Warmup LR Scheduler	Linear	Warmup LR Scheduler	Linear
Warmup LR End Epoch	5	Warmup LR End Epoch	5
Warmup LR Start Value	0.0001	Warmup LR Start Value	0.0001
Warmup LR End Value	0.001	Warmup LR End Value	0.001
Warmup LR Scheduler	Linear	Warmup LR Scheduler	Linear
Warmup LR End Epoch	5	Warmup LR End Epoch	5
Warmup LR Start Value	0.0001	Warmup LR Start Value	0.0001
Warmup LR End Value	0.001	Warmup LR End Value	0.001
Warmup LR Scheduler	Linear	Warmup LR Scheduler	Linear
Warmup LR End Epoch	5	Warmup LR End Epoch	5
Warmup LR Start Value	0.0001	Warmup LR Start Value	0.0001
Warmup LR End Value	0.001	Warmup LR End Value	0.001
Warmup LR Scheduler	Linear	Warmup LR Scheduler	Linear
Warmup LR End Epoch	5	Warmup LR End Epoch	5
Warmup LR Start Value	0.0001	Warmup LR Start Value	0.0001
Warmup LR End Value	0.001	Warmup LR End Value	0.001
Warmup LR Scheduler	Linear	Warmup LR Scheduler	Linear
Warmup LR End Epoch	5	Warmup LR End Epoch	5
Warmup LR Start Value	0.0001	Warmup LR Start Value	0.0001
Warmup LR End Value	0.001	Warmup LR End Value	0.001
Warmup LR Scheduler	Linear	Warmup LR Scheduler	Linear
Warmup LR End Epoch	5	Warmup LR End Epoch	5

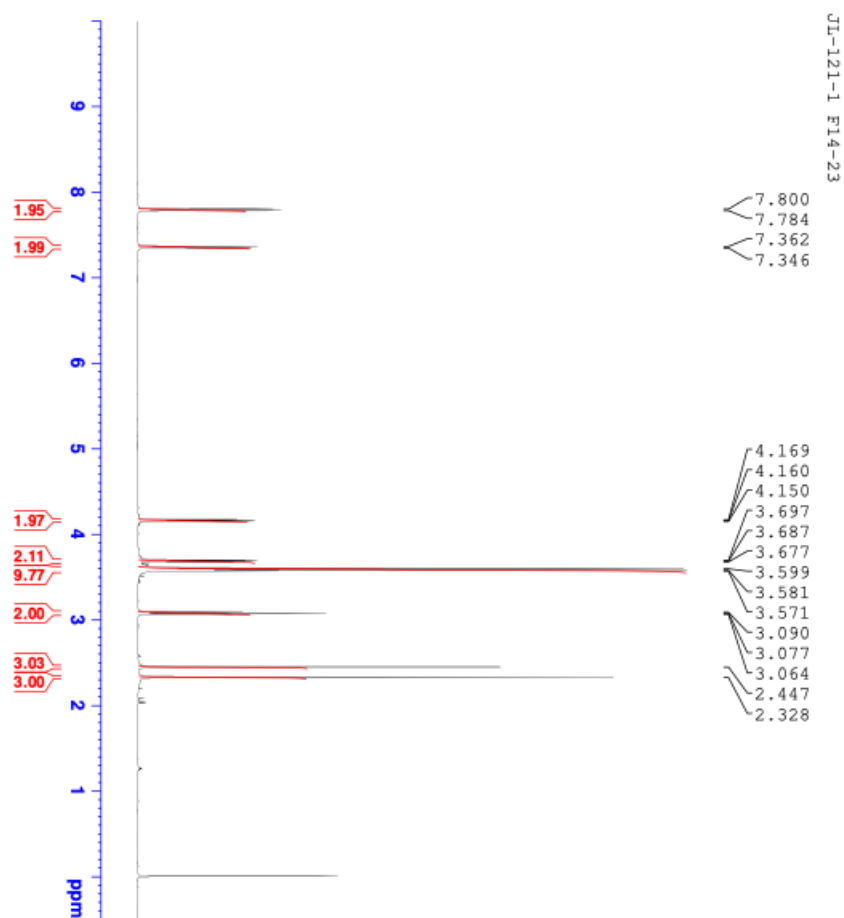
122



4.3. Compound 4B.1 (S-(2-(2-(2-(2-(tosyloxy)ethoxy)ethoxy)ethoxy)ethyl)ethanethioate)



4.3.1. ¹H NMR

[illegible]

13C NMR spectrum (CDCl₃) of compound 14. The spectrum shows several sharp peaks. Key peaks are labeled with their chemical shift values: 195.57, 144.81, 133.01, 129.83, 127.98, 72.87, 70.73, 70.56, 70.28, 69.25, 68.70, 63.53, 30.56, 28.80, 24.24, and 21.63.

Training Data Parameters		Validation Data Parameters	
Epochs	100	Epochs	100
Batch Size	32	Batch Size	32
Learning Rate	0.001	Learning Rate	0.001
Optimizer	Adam	Optimizer	Adam
Loss Function	CrossEntropy	Loss Function	CrossEntropy
Model Architecture	ResNet-18	Model Architecture	ResNet-18
Pre-training	Yes	Pre-training	Yes
Transfer Learning	True	Transfer Learning	True
Feature Extraction	True	Feature Extraction	True
Freeze Layers	18	Freeze Layers	18
Unfreeze Layers	2	Unfreeze Layers	2
Warmup Epochs	5	Warmup Epochs	5
Warmup LR	0.0001	Warmup LR	0.0001
Warmup Strategy	Linear	Warmup Strategy	Linear
Dropout Rate	0.1	Dropout Rate	0.1
Batch Normalization	True	Batch Normalization	True
Gradient Clipping	True	Gradient Clipping	True
Gradient Norm	5.0	Gradient Norm	5.0
Early Stopping	True	Early Stopping	True
Patience	10	Patience	10
Checkpointing	True	Checkpointing	True
Checkpoint Path	/path/to/checkpoints	Checkpoint Path	/path/to/checkpoints
Device	CPU	Device	CPU
Parallelism	None	Parallelism	None
Random Seed	42	Random Seed	42
Verbose	True	Verbose	True



CC(=O)S(CCO)CC[N+]#N

1H NMR spectrum of compound 10b in CDCl₃.

Chemical Shifts (ppm): 7.2, 6.8, 6.4, 5.8, 4.8, 4.2, 0.0 (TMS).

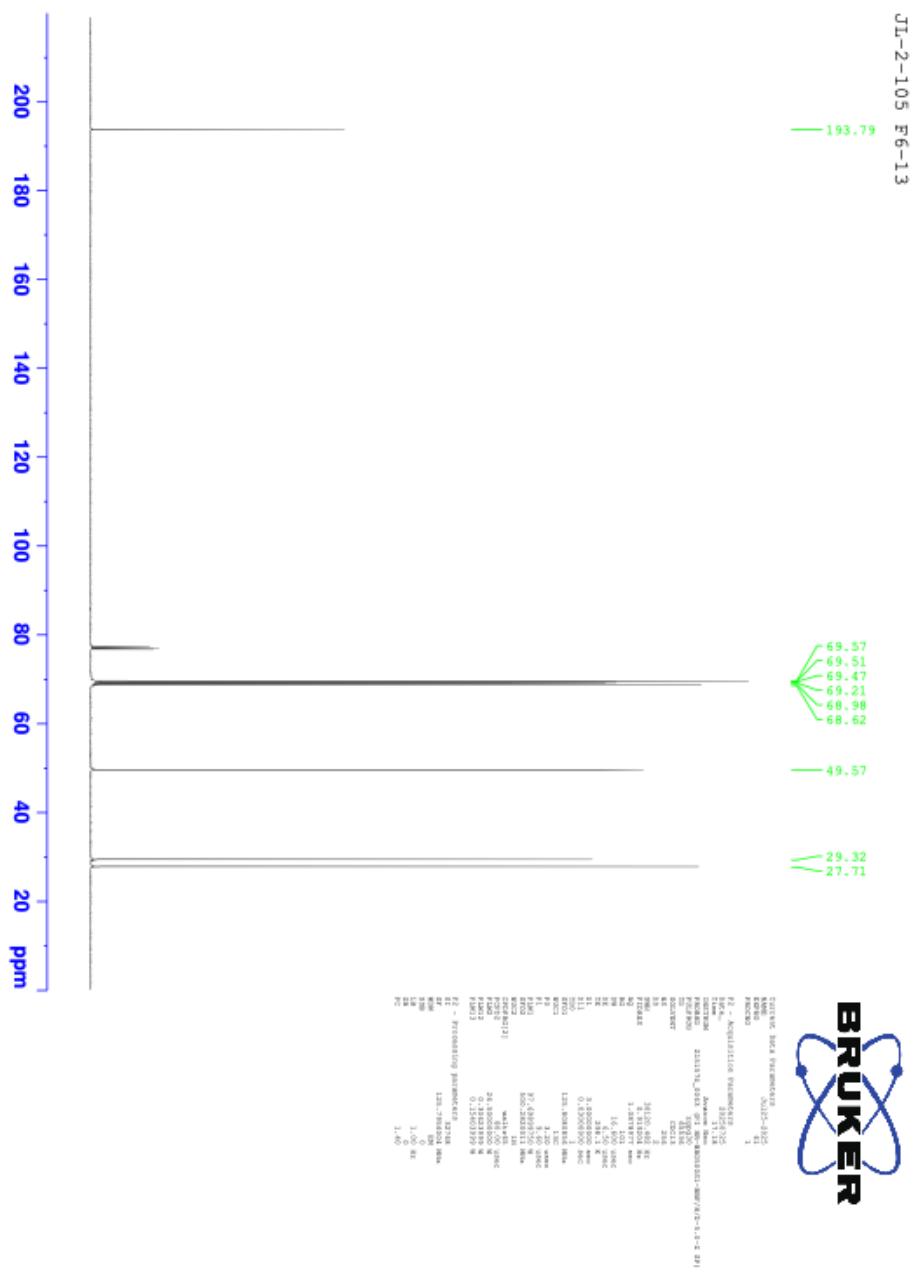
Integrations: 1.99, 1.99, 1.99, 3.00, 11.71, 1.99.

Peak Labels: 7.2, 6.8, 6.4, 5.8, 4.8, 4.2, 0.0 (TMS).

Integration Curve: A red line showing the cumulative integration of the peaks.

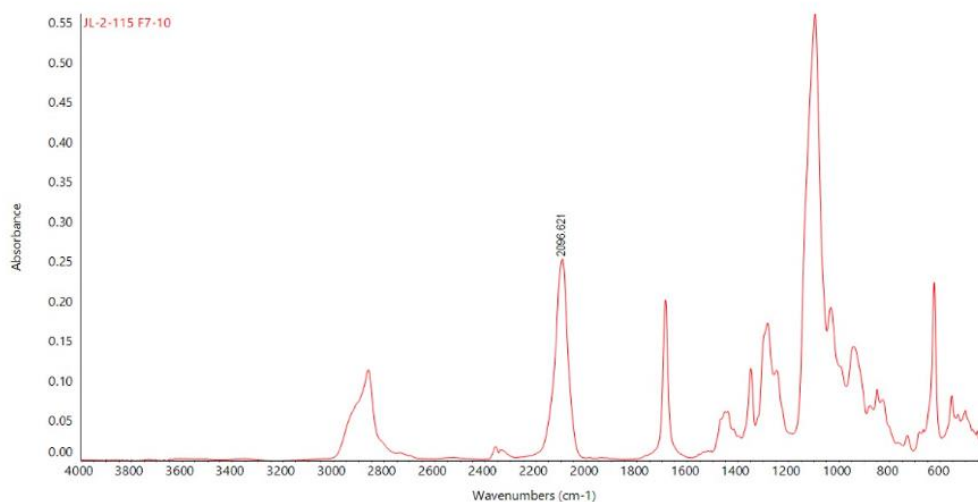
Reference: TMS (0 ppm).

4.4.2. ^{13}C NMR



4.4.3. IR Spectrum

JL-2-115 F7-10



Title: JL-2-115 F7-10

Measurement date: 2025-09-04 4:51:28 PM

Number of sample scans: 16

Number of background scans: 16

Instrument Serial: BHT2510099

Smart accy: B122116

Model: Nicolet Summit X

Source: IR

Detector: DTGS KBr

Smart Accessory Title: Everest ATR

Smart Accessory ID: B122116

Crystal type: : Diamond

Beamsplitter: KBr

Sample spacing: 1.0

Digitizer bits: 24

Optical velocity: 0.4747

Aperture: 100.0

Sample gain: 1.0

High pass filter: 20.0

Low pass filter: 11000.0

Comments: None

Regions:

Region 1: 2380.14-2020.14

Threshold: 0.188

Sensitivity: 50.000

Position	Intensity
2096.621	0.253

NC(CCO)CCSC(=O)C

1H NMR spectrum of compound 14a in CDCl₃.

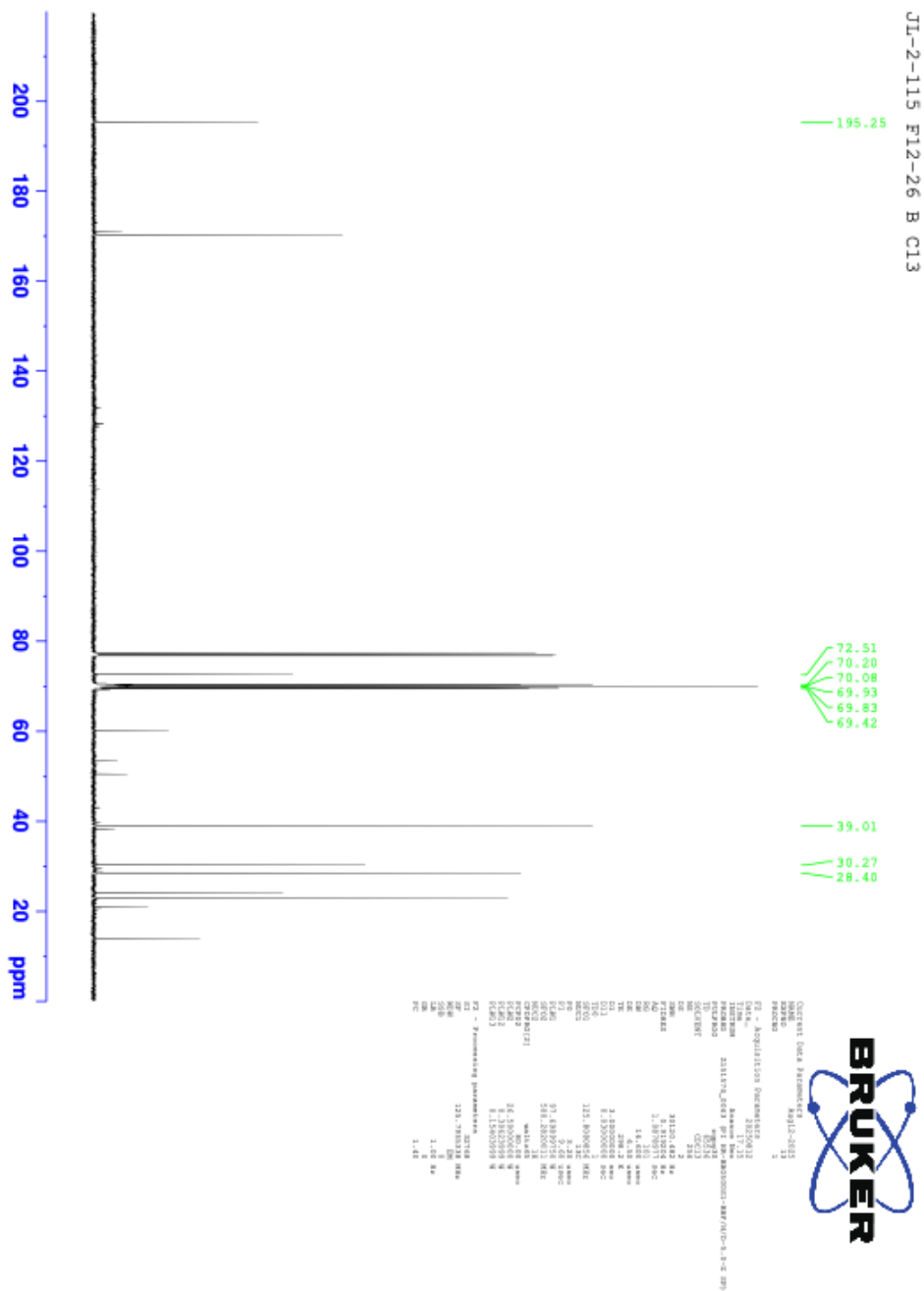
Chemical Shifts (ppm): 7.2, 6.8, 6.4, 5.8, 5.2, 4.8, 4.2, 3.8, 3.2, 2.8, 2.2, 1.8, 1.2, 0.8, 0.2, -0.2, -0.8, -1.2, -1.8, -2.2, -2.8, -3.2, -3.8, -4.2, -4.8, -5.2, -5.8, -6.4, -6.8, -7.2.

Integration values: 14.40, 2.29, 3.00.

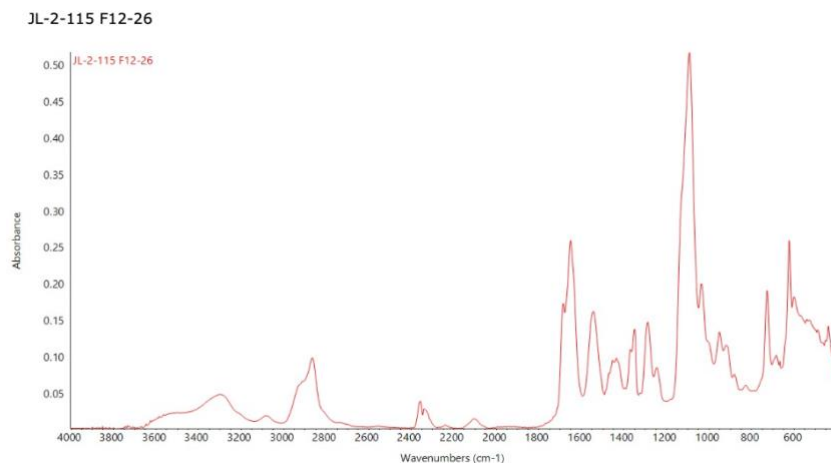
Peak assignments:

- 7.2 ppm: 3H, multiplet
- 6.8 ppm: 2H, multiplet
- 6.4 ppm: 2H, multiplet
- 5.8 ppm: 2H, multiplet
- 5.2 ppm: 2H, multiplet
- 4.8 ppm: 2H, multiplet
- 4.2 ppm: 2H, multiplet
- 3.8 ppm: 2H, multiplet
- 3.2 ppm: 2H, multiplet
- 2.8 ppm: 2H, multiplet
- 2.2 ppm: 2H, multiplet
- 1.8 ppm: 2H, multiplet
- 1.2 ppm: 2H, multiplet
- 0.8 ppm: 2H, multiplet
- 0.2 ppm: 2H, multiplet
- 0.2 ppm: 2H, multiplet
- 0.8 ppm: 2H, multiplet
- 1.2 ppm: 2H, multiplet
- 1.8 ppm: 2H, multiplet
- 2.2 ppm: 2H, multiplet
- 2.8 ppm: 2H, multiplet
- 3.2 ppm: 2H, multiplet
- 3.8 ppm: 2H, multiplet
- 4.2 ppm: 2H, multiplet
- 4.8 ppm: 2H, multiplet
- 5.2 ppm: 2H, multiplet
- 5.8 ppm: 2H, multiplet
- 6.4 ppm: 2H, multiplet
- 6.8 ppm: 2H, multiplet
- 7.2 ppm: 3H, multiplet

4.5.2. ^{13}C NMR



4.5.3. IR Spectrum



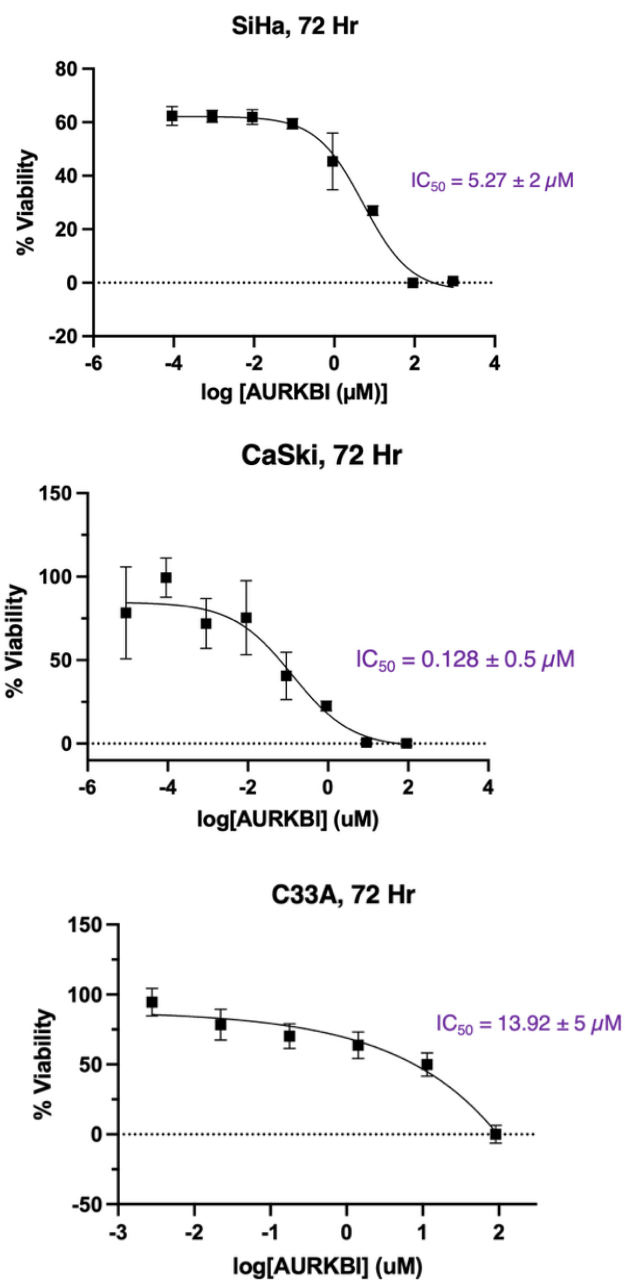
Title: JL-2-115 F12-26
Measurement date: 2025-09-04 4:53:07 PM
Number of sample scans: 16
Number of background scans: 16
Instrument Serial: BHT2510099
Smart accy: B122116
Model: Nicolet Summit X
Source: IR
Detector: DTGS KBr
Smart Accessory Title: Everest ATR
Smart Accessory ID: B122116
Crystal type: : Diamond
Beamsplitter: KBr
Sample spacing: 1.0
Digitizer bits: 24
Optical velocity: 0.4747
Aperture: 100.0
Sample gain: 1.0
High pass filter: 20.0
Low pass filter: 11000.0
Comments: None

5. APPENDIX 5

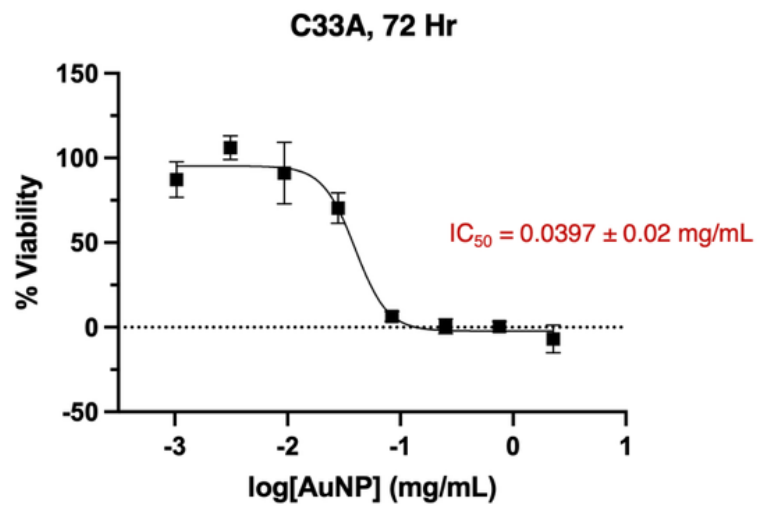
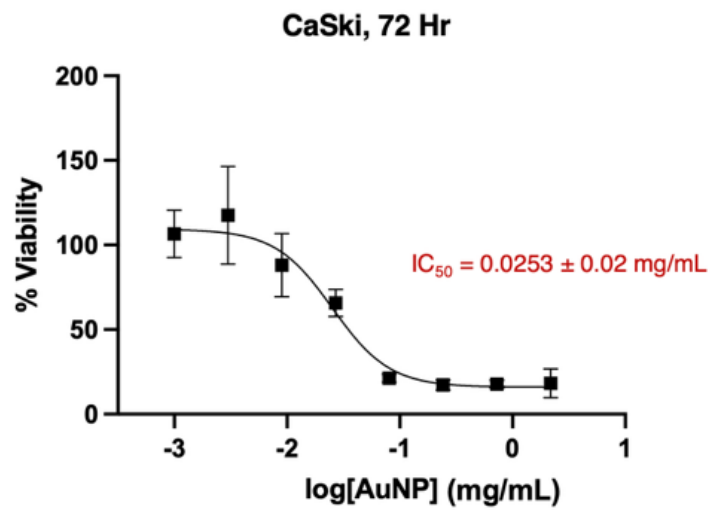
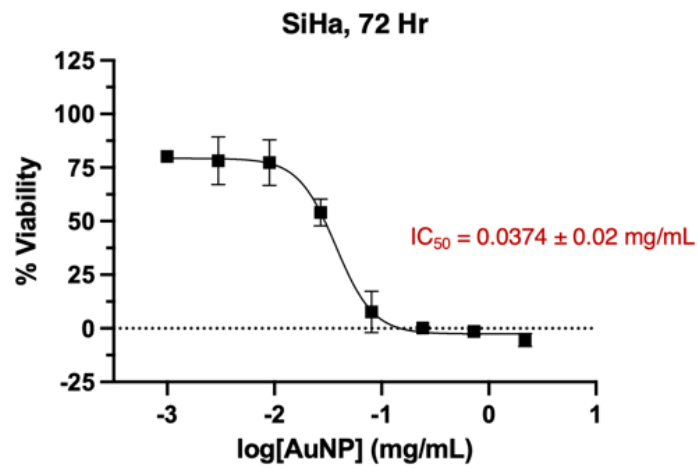
**All data was interpreted and all IC₅₀ values were calculated using GraphPad Prism™.

5.1. Cytotoxicity Data

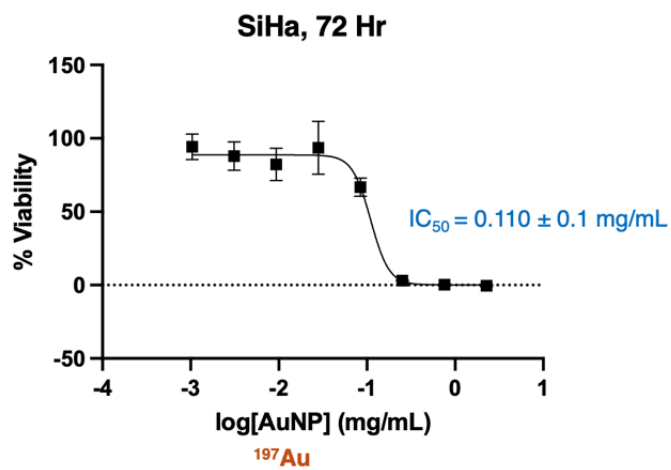
Aurora Kinase B Inhibitor



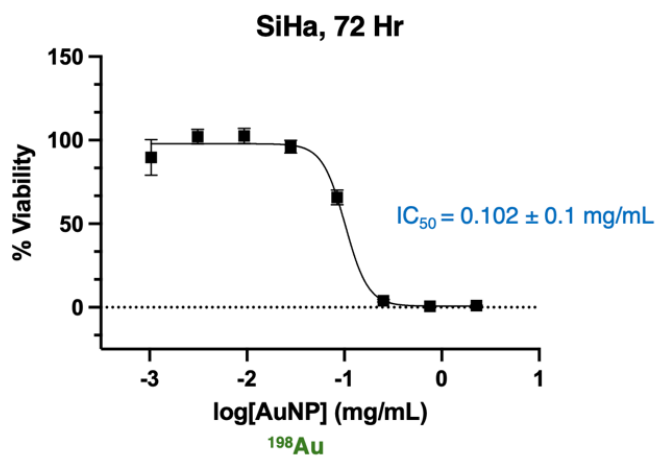
AuNPs



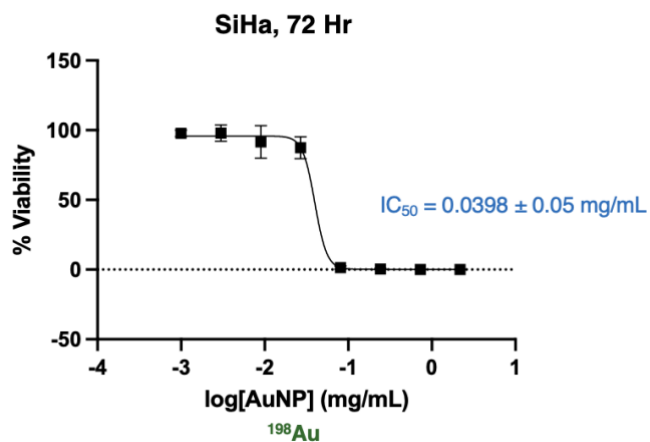
¹⁹⁸AuNPs



111 Bq:



1145 Bq:



AuNP-AKI Conjugate

