Self-assembly and ion transport by stimuli-responsive ruthenium(II) complexes

Introduction

Cytoplasmic membrane transporters play a crucial role in metabolic pathways, cell communication, proper functioning, and maintenance. Malfunctions of the cellular transport can lead to disruptions of the ion gradients, lysosomal pH, the buildup of metabolites, and trigger programmed cell death. Consequently, the field of synthetic transporters development has seen a tremendous rise in recent years. Most current methods rely on the coordination compounds as they provide an easy way to design transporters' properties such as geometries, solubility, lipophilicity and polarisability.

Particularly, ruthenium complexes attracted considerable research attention for their unique chemical and physical properties. For example, their luminescence and affinity toward nucleic acids were successfully used to detect RNA and DNA mismatch mutations, as demonstrated in numerous research papers (1,2). The other useful property is their general stability in biological environments and photodissociation that can be induced via irradiation. It allows for the switchable behaviour of such complexes and controlled cytotoxicity and drug delivery applications since it can be manipulated to dissociate and release drugs at the desired microenvironments (3). Lastly, they've been demonstrated to undergo self-assembly, form larger aggregates, exhibit antimicrobial activity and increase the efficacy of antibiotics if applied together (4). Therefore, my research project aims to synthesise novel ruthenium complexes (Fig 1) and perform full characterisation with a series of experiments to assess their antimicrobial potential.

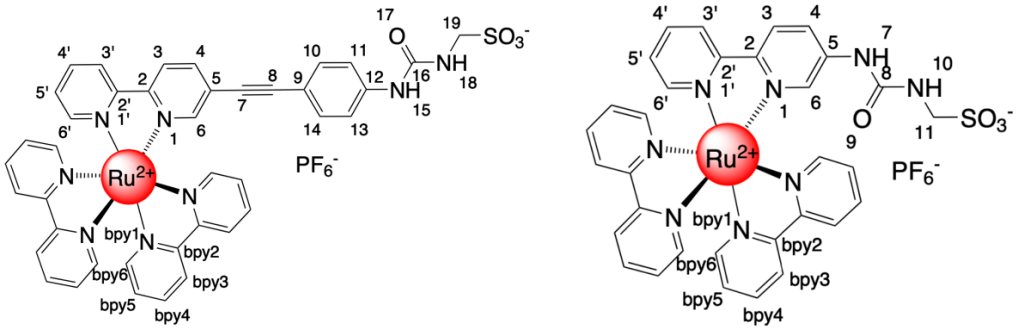

Fig 1 SSA 1 (on the left) and SSA 2 (on the right)

Ruthenium was selected as a central metal atom for the above-mentioned properties such as luminescence, photodissociation and antimicrobial activity. The anionic component of the complexes, on the other hand, was selected because its thio/urea component exhibits specificity and have a higher affinity towards phospholipids found in the cytoplasmic membrane of prokaryotes than that of eukaryotes (5,6). At the project's beginning, we also had two proposed action mechanisms for our compounds. The first one suggests that ruthenium compounds might produce reactive oxygen species (ROC) upon irradiation which will exhibit cytotoxicity by damaging bacterial DNA and cytoplasm since it was shown to be the case for similar ruthenium polypirydyl compounds (7). The alternative mechanism is that our compound will undergo self-association and insertion in the cytoplasmic membrane, which will either act as an ion

transporter and alter the ion gradients, eventually triggering the apoptosis or disrupt the phospholipid bilayer and compromise the cell's integrity. Therefore, our plan was to do a series of experiments to confirm their activity and differentiate between these two mechanisms.

Synthesis

During the first week of the project, I went to Otto-Diels Institue of Organic Chemistry in Kiel, Germany and had a general introduction to the work that Dr Anna McConnel's research group has done. For the first few days, I was primarily reading and doing a literature search to familiarise myself with the methodology they use and get a conceptual understanding of the field. Then I had supervised training with Dr Tjorge Neumann in the synthesis of ruthenium compounds, 2D NMR characterisation using TopSpin software, and UV-vis spectroscopy so that I could perform it myself on my return to UCL. Then during the second week in London, while I was waiting for the reagents from Kiel to arrive, I had an introduction to the lab, safety training, revision of 1D NMR characterisation and studied the standard protocol for vesicle screening experiments. The next week after we received the reagents, I did all preliminary work for the synthesis, which included: writing risk assessment, doing stoichiometrical calculations to figure out the masses of compounds required, calculating the theoretical yield and number of moles of the product we expect to get.

Once it was all done, I performed a small-scale synthesis of 2 ruthenium complexes under the supervision of Dr Kylie Yung. The first step of the procedure for both compounds included: suspending [Ru(bipy)2]Cl2 and corresponding tetrabutylammonium salt in ethanol and heating the mixture under reflux at 80 °C for 20 hours. However, due to the high lipophilicity and viscosity of the SSA 2, liquid nitrogen was required to freeze it and transfer it to the flask. I then sonicated the mixture and applied a heat gun because the compound had lower solubility and stuck to the sides of the glassware. After that, I left the flasks with our reaction mixture to cool down to room temperature, added 10 ml of deionised water and an excess amount (1 full spatula) of ammonium hexafluorophosphate powder to each flask and left them in the fridge at -4 °C for 24 hours so that that precipitation could take place. Finally, the precipitated product was filtered off, washed with 5 ml amount of deionised water to remove unreacted ammonium salts and dry them to obtain red crystals. However, when I did NMR to check our product's purity, it had many TBA impurities entrapped within the crystalline structure. Therefore, after consultation with Dr Kylie Yang, we decided to perform another round of vacuum filtration with methanol instead of water. We hypothesised that due to differences in solubility, TBA would completely dissolve and pass through, leaving our products behind as a residue.

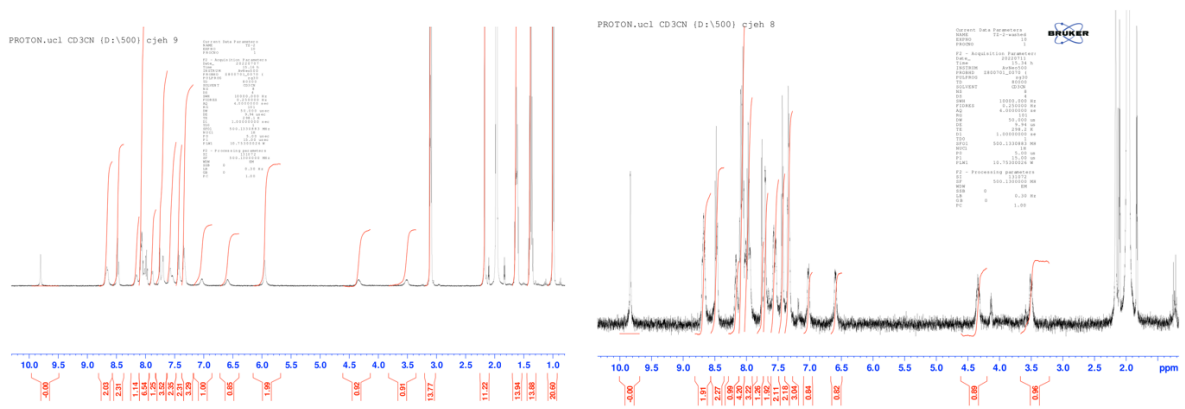

Fig 2 crude SSA 2 (on the left) and after methanol wash (on the right)

It worked really well, and I managed to purify complexes. As can be seen from Fig 2, three peaks in the 1-2 ppm region are absent in the washed sample, indicating that TBA has been removed and confirming our initial hypothesis. Lastly, I weighted out a small amount of SSAs into separate vials to perform X-ray crystallography later, which will be useful to elucidate the hydrogen-bonding mode adopted by the compounds.

Fluorescent spectroscopy

The first task upon my arrival in Kent was to carry out full NMR characterisation and IR spectroscopy to confirm the identity and purity of the samples before doing any experiments on them. Then I did a melting point test and carried out fluorescence spectroscopy using the microplate reader. Its purpose was to figure out the absorption, excitation and emission wavelengths of the compounds since it would be required to adjust the filter excitation range for the subsequent fluorescent microscopy experiments with living cells.

Name	Concentration	Absorption	Excitation	Emission
SSA 1	3.1 µg/mL	1st peak at ~360nm, 2nd peak at ~456nm	~459.2 nm	~672 nm
SSA 2	3.1 µg/mL	1 peak at ~448nm	~457.2 nm	~636 nm

Fig 3 Fluorescent spectroscopy summary

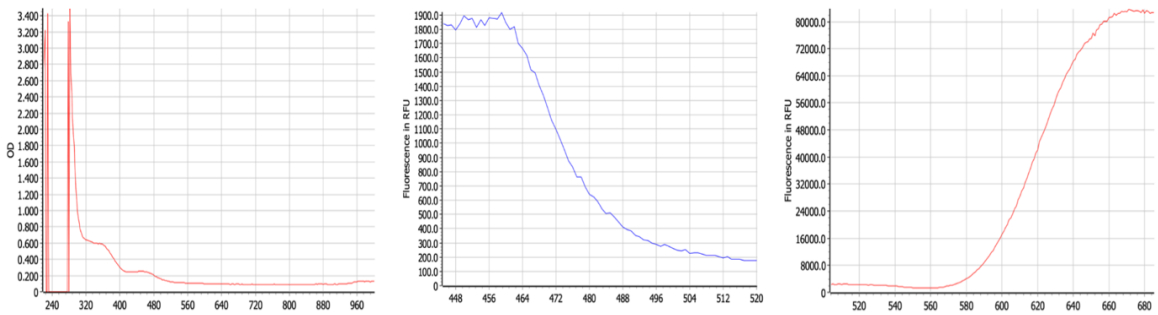

Fig 4 SSA 1 absorption (left), excitation (middle) and emission (right) spectra

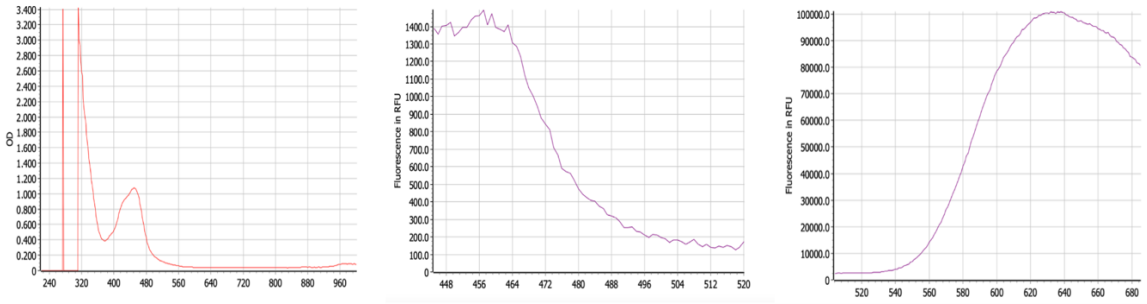

Fig 5 SSA 2 absorption (left), excitation (middle) and emission (right) spectra

During the experiments, I got a few artefacts in my spectra, such as solvent and Raman peaks, which arose due to the inelastic scattering of the photons of light in the spectrometer. Therefore, additional processing and formatting were necessary to get concordant results and draw conclusions. Figures 4 and 5 show that both compounds have similar absorption and excitation wavelengths in the blue region of the visible light spectrum and more divergent emission wavelengths in the red region. In turn, fluorescent spectroscopy demonstrated that compounds crystallise instead of adhering to the bacterial membranes.

Then, I weighted a small amount of compounds for Dr Hiscock's research group, so they can continue the project once I return to UCL and do the MIC (minimum concentration inhibition) studies on e. Coli and MRSA to confirm and quantify their antimicrobial activity. It is also important to conduct control experiments with reagents only to ensure that the efficacy of the complexes is significantly higher than that of the reagents.

Quantitative NMR

The next step was quantifying the fraction of molecules undergoing self-association in the aqueous solution. Hence, I conducted quantitative NMR of the samples and, knowing the height of the standard peaks, calculated the percentage loss of compound, which corresponds to the percentage that self-associated and formed larger aggregates. Figs 6 and 7 demonstrate that 33% and 25% loss was observed for SSA 1 and 2, respectively.

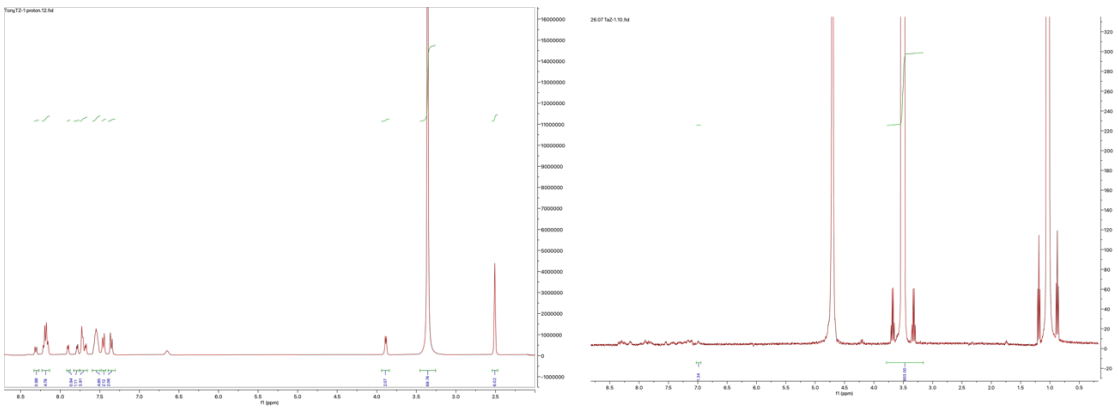

Fig 6 SSA 1 in deuterated acetonitrile (on the left) and in 1:19 EtOH:H20 solution (on the right)

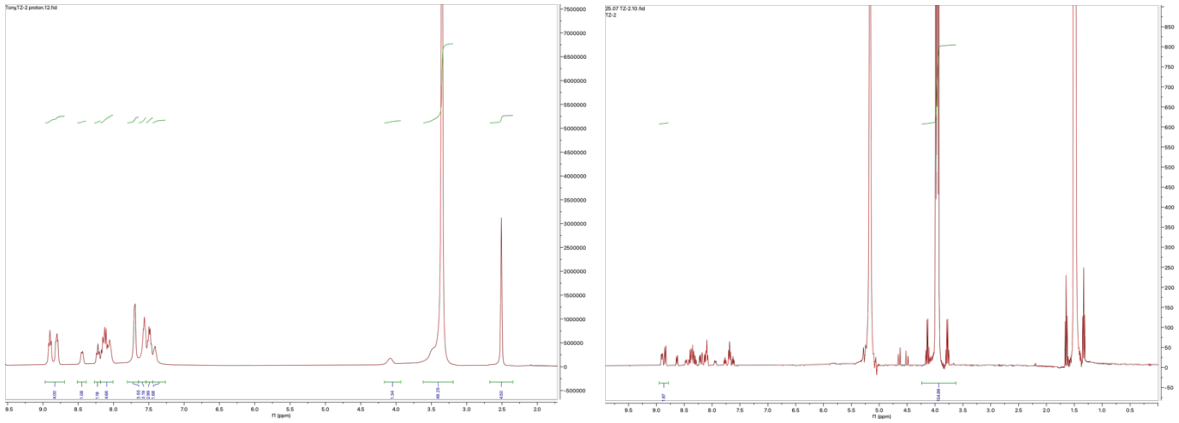

Fig 7 SSA 2 in deuterated acetonitrile (on the left) and in 1:19 EtOH:H20 solution (on the right)

Similarly to the synthesis step, increased lipophilicity of SSA 2 meant that a higher dilution factor was necessary to run the experiment, leading to a lower signal-to-noise ratio and reduced quality of the spectra in general. Additional measures, such as mixture sonication and heat-gun application, were required to dissolve crystals completely.

Vesicles screening experiments

Lastly, to test compounds' fusion and activity in the phospholipid bilayer, we aimed to perform vesicle screening experiments. Conceptually, it can be split into four steps: preparation of the synthetic LUVs (large unilamellar vesicles) from the lipids via the standard procedure, hydration with a predetermined solution, experimental compound's fusion with the bilayer by using appropriate solvent and voltage measurement across the membrane to quantify ionic transport. To accomplish this, I first prepared intravesicular and extravesicular buffered solutions to maintain pH and ionic strength constant. When adjusting the pH of the buffers, it was crucial to add carefully selected acids or bases to avoid introducing extra ions into the system. Then I removed the solvent from the chloroform solution of the lipid using a rotary evaporator and dried it under a high vacuum for 2 hours. Later, I hydrated dried lipids in the round bottom flask with the intravesicular buffer and used a vortexer to agitate the mixture thoroughly and ensure that it was not stuck to the walls of the flask.

It was also essential to do nine freeze-thaw cycles using liquid nitrogen to break down large lipid particles, so they could pass through the 200 nm polycarbonate membrane of the mini-extruder and form uniformly sized vesicles. Once it was done, I assembled the extruder as shown in Fig 8, passed the lipid suspension through 25 times and collected it from the opposite syringe to where they were added to avoid contamination of the newly produced vesicles. Finally, I transferred the mixture to the dialysis tubing and stirred it gently in the 1.5 litres of the extravesicular buffer for 2 hours to purify the vesicles.

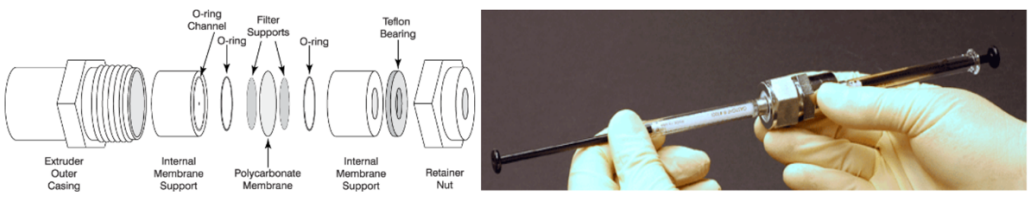

Fig 8 Structure of the extruder (left), assembled extruder (right)

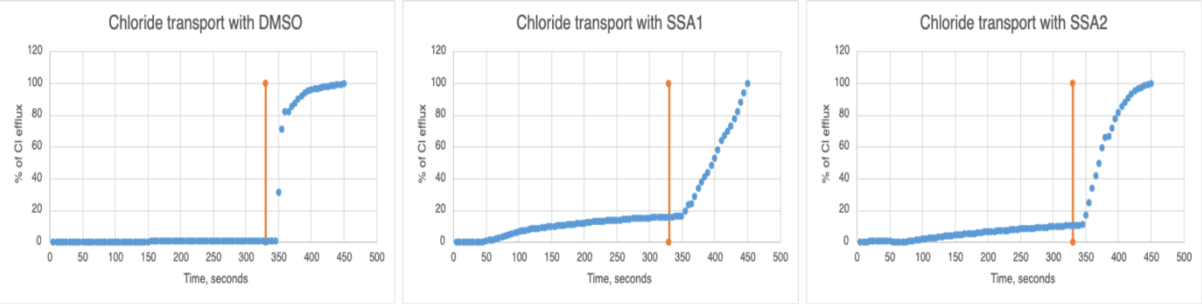

Fig 9 results of vesicle screening experiments with DMSO (left), SSA1 (middle) and SSA 2 (right)

After all necessary preparation and purification, I first did a negative control experiment with DMSO solvent to confirm that the vesicles have been produced correctly and behave as expected with zero transport observed (Fig 9). Then, I repeated the experiments with the compounds and measured chloride efflux after adding SSA 1 and 2. After 5 min 30 sec, I added a few drops of the detergent to break open the vesicles and calculate a 100% chloride efflux that will serve as a baseline for future calculations and plots. As can be seen from the graphs in Fig 9, SSA 1 and SSA 2 produced up to 20% and 10% chloride efflux, respectively.

During the last week of my research project, I presented my results at the quarterly international consortia meeting to colleagues from partner institutions and representatives from East Kent NHS Foundation Trust. Additionally, I made a short presentation to Dr Haynes' research group to provide an update on the work that I've done, summarise my findings and make conclusions on the future of the project. In the end, I received valuable feedback from Dr Kylie Yang, which allowed me to greatly improve my communication and presentation skills, as well as boost my understanding of the workflow and project management within the department of Chemistry. Lastly, the literature reading, experiment planning, and conduction significantly enhanced my laboratory skills, leadership and managerial competencies whilst also making me a more independent researcher capable of critical thinking and systematic appraisal of complex natural phenomena.

Conclusions

Results from qNMR and vesicle studies show that only a small fraction of molecules undergo self-association. SSAs also produced small ionic movement across the membrane, which most likely won't generate any significant therapeutic effects. Despite this, the results indicate that the additional hydrophobic moiety in SSA 1 improved the compound's performance, perhaps by strengthening intramolecular interaction (favouring self-association) and facilitating fusion with the phospholipid bilayer, which increases the fraction of complexes embedded in the membrane. Overall, it can be used to develop more potent derivatives for future experiments. Currently, results favour the hypothesis of antimicrobial activity via the production of reactive oxygen species. However, a minimum of three replicates of the experiments will be needed to perform statistical analysis (calculate mean values, standard deviation and error bars) and get more representative results. It will also be useful to do an anion exchange column and substitute the SSA anion component from hexafluorophosphate to more biologically relevant chloride to see how it influences movement across the membrane and provide insight into their mode of action. Additionally, results from fluorescent microscopy showed that SSAs mostly crystallised instead of adhering to the bacterial membranes, which questions their ability to permeabilise and disrupt cell membranes. It also suggests that further modifications to the chemical structure will be needed to improve their cytotoxicity and efficacy.

Further advancements in the ruthenium complex research and characterisation will require synthesising more SSAs to produce a Hill plot and gain an insight into the pharmacokinetic and pharmacodynamical properties of the compounds. A higher amount of SSAs will also be required to conduct CMC (critical micelle concentration) studies due to the zwitterion nature of compounds. Additionally, after the weekly meeting devoted to experiment planning, I concluded that it would be essential to modify the synthesis procedure and do methanol wash directly instead of rinsing SSAs with water. It will make the synthesis less time-consuming and should significantly improve the yield. The alternative option would be to select a ligand with a different counterion than TBA to make the purification step more straightforward. Lastly, although this research project revealed some unexpected technical challenges with our compounds, it also showed a huge potential for improvements and the discovery of novel antimicrobial agents.

Glossary

- Anion – a negatively charged ion.
- Apoptosis – a programmed cell death that occurs in multicellular organisms.
- Eukaryotes – organisms with cells that have a membrane-bound nucleus and complex organelles (e.g. animals, plants, fungi, protists)
- Lipophilicity – the affinity for lipids and hydrophobic molecules.
- Membrane transporter – membrane protein involved in the movement of ions and small molecules across the biological membranes.
- MRSA – methicillin-resistant Staphylococcus aureus.
- Photodissociation – dissociation of a compound by the action of light.
- Polarisability – a tendency of matter to acquire electric dipole moment when subjected to the electric field.
- Prokaryotes – single-celled organisms without membrane-bound nuclei (e.g. bacteria, cyanobacteria)
- SSA – supramolecular self-associating amphiphilic salts (Chemical classification of the ruthenium complexes synthesised during the research).
- Stoichiometry – the relationships between the relative quantities of the substances taking part in the chemical reaction

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