Synthesis of Metal Nanoparticles in a Deep Eutectic Solvent for Use in an Antimicrobial Wound Dressing

Joseph Eyre

A thesis submitted in partial fulfilment of the requirements of Nottingham Trent University for the degree of Doctor of Philosophy

March 2025

Table of Contents

Acknowledgements

I would like to thank my supervisors, Emma Smith, Samantha McLean and John Hunt, for their input and guidance on the project throughout my PhD.

I would also like to thank Hannah Beska, not only for assisting with some research but also for the procrastination coffee trips and lunches.

A thank you goes out to the staff of the Derby Royal A&E department for restarting my heart when half of it decided to stop working.

Abbreviations and Acronyms

Analysis of Variance (ANOVA)

Arbitrary unit – AU

Atom economy – AE

Betaine – Bet

Bovine serum albumin – BSA

Capric acid – Cap

Choline bitartrate – ChBit

Choline chloride – ChCl

Citric acid – CA

Didodecyldimethylammonium bromide - DDAB

Cobalt sulphide – CoS

Trisodium citrate – Na_3Cit

Colony forming units (CFU)

Deep Eutectic Solvents – DES's

Dimethyl sulphoxide – DMSO

Disodium ethylenediaminetetraacetate – Na_2EDTA

Dopamine hydrochloride – DA

Dynamic Light Scattering – DLS

Energy dispersive X-ray spectroscopy – EDS

Environmental factor – e-factor

Escherichia coli – E. coli

Glutaric acid – GA

Glycerol – Gly

Gold nanoparticle – AuNP

Gram - g

Green tea extract – GTE

Hexadecyltrimethylammonium bromide – CTAB

Hours – Hrs

Hydrogen bond – H-bond

Hydrogen bond acceptor – HBA

Hydrogen bond donor – HBD

Inductively coupled plasma mass spectrometry – ICP-MS

Inductively coupled plasma optical emission spectroscopy - ICP-OES

Iron oxide – Fe_2O_3

Lauric acid – Lau

Levulinic acid – Lev

Localized surface plasmonic resonance – LSPR

L-proline – Pro

MacConkey agar – MCA

Maleic acid – Mal

Malonic acid – MA

Mannitol salt agar – MSA

Medical Technology Innovation Facility – MTIF

Methanol – MeOH

Microgram - µg

Milligrams – mg

Millilitres – mL

Millimetre - mm

Millimolar -mM

Minimum bactericide concentration – MBC

Minimum inhibitory concentration – MIC

Minutes - min

Molecular liquid - ML

Mueller-Hinton agar – MHA

Mueller-Hinton broth – MHB

Nanometres – nm

Nanoparticle - NP

Nanoparticles – NPs

National Health Service - NHS

Natural Deep Eutectic Solvent - NADES

Nickel – Ni

Nitric acid – HNO_3

No significance – ns

Nottingham Trent University – NTU

Oleic acid – OA

Oleylamine – OAm

Optical density – OD

Oxalic acid – OxA

Parts per million – ppm

Phosphate buffered saline – PBS

Polytetrafluoroethylene – PTFE

Polyvinylpyrrolidone – PVP

Powder X-ray Diffractometer – PXRD

Process mass intensity - PMI

Real atom economy - RAE

Revolutions per minute – RPM

Scanning electron microscopy – SEM

Seconds – sec

Silver – Ag

Silver nanoparticles – AgNPs

Silver nanoparticles in DES – AgNP DES

Silver nitrate – $AgNO_4$

Silver sulphate – Ag_2SO_4

Sodium borohydride – $NaBH_4$

Sodium hyaluronate – SH

Sodium Hydroxide – NaOH

Staphylococcus aureus – S. aureus

Tetrabutylammonium borohydride – TBABH

Transmission Electron Microscopy – TEM

Ultraviolet-visible – UV-vis

Urea – Ure

X-ray diffraction – XRD

Abstract

Antimicrobial resistance has been predicted to cause more deaths than cancer by the year 2050.[1] This project proposes to use Deep Eutectic Solvents (DES's) to synthesise silver nanoparticles (AgNPs) for use in an antimicrobial wound dressing. DES's have previously been studied to synthesise AgNPs as a greener alternative to conventional aqueous methods.[2]

The synthesis of AgNPs using the DES (Bet:Gly 1:3),[3] and oleylamine (OAm) as a capping agent was initially studied producing spherical AgNPs that were 10±2.4 nm in size, characterised by UV-vis, DLS and TEM. Additional synthesis and extraction methods were then developed to remove the hazardous OAm to synthesise AgNPs in Bet:Gly 1:3 without any additional reagents which were 13±1.94 nm in size with a spherical morphology suitable for an antimicrobial wound dressing.

Novel three-component DES's, were developed with inherent antimicrobial activity that could also act as a reducing and capping agent for synthesis of AgNPs. A successful synthesis method with Cap:Lau:Bet 4:2:1 DES was developed which resulted in spherical AgNPs with size ranges of 6.36±2.89 nm. The antimicrobial properties of AgNPs in both a Bet:Gly 1:3 and Cap:Lau:Bet 4:2:1 were studied using colony biofilm assays, as well as minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC), for a *Gram*-positive bacteria (*S. aureus*) and a *Gram*-negative bacteria (*E. coli*). The best candidate for an antimicrobial wound dressing was AgNPs in Cap:Lau:Bet 4:2:1 which had a more potent antimicrobial effect against the biofilms with MIC and MBC concentrations for both bacterium found to be 24.1 μg/mL and 0.75 μg/mL respectively. This work advanced the synthesis of AgNPs in a DES; allowing for a greater control and stability of the AgNPs, as seen by the narrow size ranges produced, as well as demonstrating a more accurate protocol for the determination of the antimicrobial properties of DESs.

Chapter 1: Introduction

It is predicted that by 2050 more deaths will occur from antimicrobial resistance than from cancer.[1] Finding new drugs alongside antimicrobial materials to combat this potential epidemic is vital to save lives in the future. Currently used solutions include using AgNPs, which are a known antimicrobial agent, within wound dressings to help combat and prevent infections without the use of drugs. However, some currently used methods used to synthesise AgNPs use hazardous reagents, such as $NaBH_4$, or produce a lot of waste material, see **Chapter 1.2** for further discussion, which impacts the green and sustainability of the manufacturing process. Development into new methodologies for synthesising AgNPs using greener and more sustainable materials. The work presented in this thesis aims to improve on these AgNP wound dressings by using a Deep Eutectic Solvent (DES) to synthesise silver nanoparticles (AgNPs) to improve upon the methodology but also to develop a DES which is also antimicrobial to further enhance the antimicrobial properties of a wound dressing.

Nanoparticles (NPs) have been known in the scientific community since Faraday first synthesised gold nanoparticles in 1857 using chloroauric acid and phosphorus.[4] Since then many papers have been published on nanoparticle synthesis using new and updated methods. Synthesising nanoparticles can fall under two different categories, "top down" and "bottom up". A top-down synthesis involves taking bulk metal and processing this to smaller particles and then to nanomaterials. This could involve ball milling, laser ablation or sputtering. Generally, these materials form small nanoparticles with irregular shapes which means this method is not generally suitable for synthesising consistent nanoparticles.[5] The chemical reduction of metal salts to form nanoparticles come under the "bottom up" method for synthesising nanoparticles, also known as the co-precipitation method. Chemically reducing nanoparticles requires, a solvent, a reducing agent, and a capping agent. The reducing agent is required to reduce the anionic salt, specifically the metal cation in solution to a neutral metal atom, which results in the metal atoms bonding with each other to form the NPs which is also called nucleation. This process will continue unless the surface of the NP is blocked from further growth to larger sizes and different shapes. The capping agent is required to prevent the NPs from undergoing continuous growth during the nucleation step; choosing a capping agent that caps the NPs at a certain size is how the reaction can be tailored to the specific use. Capping agents also prevent aggregation of the nanoparticles. Another advantage of a chemical reduction method is having a tailorable synthesis by changing reaction conditions as well as the reducing and capping agents.[5] Generally the reducing agent will tend to

allow for the kinetic control of the formation of the NPs to proceed at a faster rate depending on the reducing agent chosen as well as the concentration which is used in the reaction.

The first recorded synthesis of AgNPs in 1889 by M.C. Lea used citrate to stabilise silver colloids in the size range of 7-9nm.[6] Further research in 1902 also showed that silver colloids can be stabilised with proteins which were called "collargol". These silver "collargol" nanoparticles have been commercialised since 1897 for use in medical applications. Using proteins as a capping agent for the AgNPs can be considered to be a greener alternative to harmful capping agents, such as CTAB and OAm discussed in **Section 1.2.1**, some other synthetic methods do include harmful capping agents which is not ideal if used in a medical application. This also applies for the reducing agents used in the synthesis as well, proteins as capping agents are fine but generally, they also require a reducing agent to assist the synthesis since the protein itself is not a good reducing agent. Using more hazardous reducing agent, such as $NaBH_4$, is not ideal if the AgNPs are going to be used in a medical application as this will require the AgNPs to be purified from any excess hazardous reagents before they can be used. This also includes any capping agents used, such as CTAB,[7] which has been labelled as a skin irritant and harmful to humans through prolonged exposure. Going forward the emphasis is on creating a greener and more sustainable way to synthesise NPs that stays away from harmful chemicals. The aim is to develop a synthetic method that can be scaled up for industrial production, is low cost and repeatable; with good stability of the AgNPs when stored in solution after synthesis.

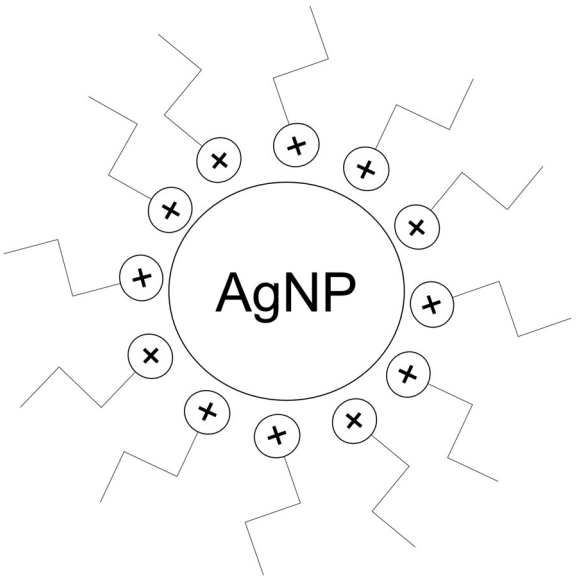


Figure 1: A diagram of a AgNP capped with a hydrophobic capping agent *e.g.* CTAB or OAm, which have a hydrophobic "tail" and polar "head".

There are a few ways to measure the green credentials of a synthesis, with one of the most common calculations being atom economy (AE), which determines the efficiency of the reaction to synthesise as much useful product from the reagents as possible, minimising waste.[8,9] If the weight of the products is known at the end of the synthesis then real atom economy (RAE) can be calculated, which will take into consideration the efficiency of the reaction and reaction yield. However, both the atom economy calculations only consider the conservation of reagents into products and not necessarily the solvents in which the synthesises are performed. Another more comprehensive calculation can be performed called environmental factor (E-factor), which takes into account all wastes produced from the reaction, including any solvent that is evaporated or is disposed of afterwards.[9] Similarly to E-factor, process mass intensity (PMI) is a more commonly used metric for green chemistry in which all reagents and solvents are compared to the mass of the product at the end of the synthesis, whilst E-factor only considers the waste compared to the product.[9] Both E-factor and PMI will be calculated and compared in the analysis of the different synthesises.

It is worth noting that there is a terminological difference between a green synthesis and a sustainable synthesis, a green synthesis may include non-hazardous reagents that have a low E-factor and high atom economy, however, if this green reaction requires a high energy input for long periods of time, with reagents that are difficult to synthesise, then the reaction may not be sustainable compared to other alternatives which may not be as green.[8] Finding a balance between green and sustainable synthesises is important to consider when scaling-up of reactions to an industrial level. Research into greener more sustainable methods of AgNP synthesis have involved using alternatives for hazardous reducing and capping agents, such as natural products or antimicrobial drugs, *e.g.* use of tyrosine and the antibiotic cefaclor.[10–12] More recently, Deep Eutectic Solvents (DES) have been used to synthesise nanoparticles using a variety of different methods.

DES's have been studied since 2004 and are synthesised by mixing a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) in different molar ratios which hydrogen bond (H-bond) together to form a large bonding network that's melting point is lower than that of the individual components.[13] DES's have been used for many different applications due to their unique ability to have tailorable properties to suit different methods such as electrochemical, organic synthesis and more recently metal nanoparticle synthesis .[13–15]

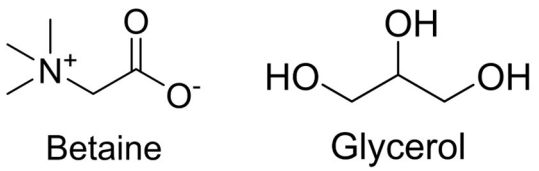
DES's have been used in a number of different bottom-up methods to synthesise nanomaterials. These include the main method which is heating and stirring which

has been used to synthesise a variety of different metal nanoparticles such as platinum, gold, tin oxide, iron oxide and silver, the latter of which is the focus of this project, due to its inherent antimicrobial properties but also its lower cost than other materials such as platinum and gold.[2,16–19] Further metal nanoparticles have been synthesised using other methods for example electrochemically synthesised platinum, gold, silver and copper.[20–23] As well as sonication or calcination by furnace which have been used to synthesise magnetic iron nanoparticles as well as zinc oxide nanoparticles.[24,25] The majority of these methods in the literature use the DES and add to it an aqueous solution or organic solvent to synthesise the nanoparticles.[26,27] Using water in DES's can disrupt the hydrogen bond network and change the physical properties of the solution, at large quantities the DES breaks down resulting in a solution of the DES components; meaning that the synthesis is not using a DES as stated and instead it is a conventional aqueous synthesis with a mixture of DES components. Finding a novel synthesis where additional solvents do not need to be added and instead have the DES act simultaneously as the solvent, reducing agent and capping agent would allow for the nanoparticles in DES to be used for a chronic wound dressing without the need for additional reaction steps to remove hazardous chemicals.

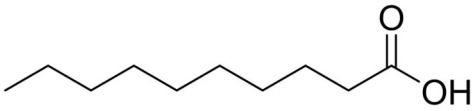
Another important benefit of using DES's to synthesise and suspend AgNPs for wound dressings is the potential antimicrobial activity of the DES itself. DES's have undergone antimicrobial testing in the literature using different methods such as disk diffusion assays and minimum inhibitory concentration (MIC) assays.[28,29] The problem with some of these methods is that they require the DES to be dissolved in a solvent before use, whether that is water or DMSO,[29] this is not ideal since the hydrogen bond network will be disrupted and the DES will break down into a mixture of the primary components, therefore cannot be classed as a DES. Hence, the antimicrobial studies would be on the components of the DES rather than that of the pure DES. This is an issue since the DES may have different antimicrobial properties to that of the components of the DES in solution. If the components of the DES are antimicrobial, it should not be assumed that the DES is also antimicrobial, especially if the intended use is for medical applications. Here we will perform method development so the antimicrobial testing can be performed without breaking down the DES.

Colloidal AgNPs have already been established as having antimicrobial properties at sizes <10nm which alongside an antimicrobial DES would make a promising material for use in a wound dressing.[30] Whilst DES are useful for NP synthesis as well as having antimicrobial properties, one major drawback is the usability of the DES in analytical

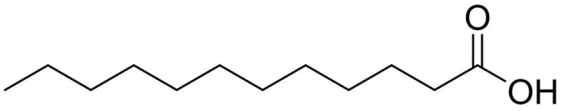
techniques. For instance, to analyse the NPs synthesised in a DES, they first must be extracted from the DES itself. This may cause issues since it will be unknown whether the extraction method will alter the size or shape of the NPs which may affect their usefulness for their intended use. This extraction of the NPs is not ideal if the AgNP DES is to be used as a wound dressing since the intended use of the NPs require an accurate size and shape analysis. Developing new analytical techniques to characterise NPs in a DES will allow for an accurate size and shape analysis without requiring extraction.



Betaine Glycerol



Capric acid



Lauric acid

331

In this work AgNPs will be synthesised using a DES to create a nanomaterial with antimicrobial properties. A Betaine:Glycerol 1:3 DES will be used that has already been reported in the literature,[3] as well as synthesizing brand-new molecular liquids that haven't been reported in the literature performing as the reducing agent, capping agent and as the solvent in a AgNP synthesis. These AgNPs in a DES will be used to create a prototype antimicrobial wound dressing for use on chronic wounds with the aim to inhibit the growth of bacteria as well as preventing antimicrobial resistant microbes from developing.[31]

1.1 Aims and Objectives

The aim of this project is to develop new methods for synthesising AgNPs in a DES as well as determining new protocols for analysing these AgNPs DES's for antimicrobial properties that do not disrupt the hydrogen bond network in the DES, to allow for an accurate determination of the antimicrobial properties of the DES. A new DES will be designed that has inherent antimicrobial properties for use in a wound dressing which is suitable to act as the solvent, capping agent and reducing agent for synthesising AgNPs with the intention of using this as a prototype antimicrobial wound dressing. This newly designed DES will incorporate fatty acids which can act as a capping agent so that additional hazardous reagents are not needed. The objectives of this project are to:

- develop new DES's, that are tailored for use in an antimicrobial wound dressing with the purpose of also acting as a solvent, capping agent and reducing agent to synthesise AgNPs without the addition of harmful reagents for a more green and sustainable synthesis.
- determine a new extraction protocol, which allows for the AgNPs in the DES to be extracted after the synthesis by changing the capping agent from the DES itself to OAm whilst maintaining a sufficient yield of AgNPs to allow for characterization by UV-vis, DLS, XRD and TEM.
- determine the stability of the AgNPs within the DES to establish if the AgNPs will have a long shelf life when used as a wound dressing, and to assess if any aggregation occurs which might impede antimicrobial activity.
- determine the concentration of the AgNPs formed within a DES using ICP-MS.
- design new antimicrobial testing methods to determine antimicrobial activity against *E. coli* and *S. aureus* bacteria, as well as MIC and MBC assays, for use with a DES that minimises contact with water.
- perform a simulated wound test, using 2 antimicrobial resistance strains of *Gram*-positive and *Gram*-negative bacteria to test the AgNP in a DES.
- develop a DES wound dressing prototype to be used on a wound simulation which then can be further studied for human toxicity.

1.2 Literature Review

The use of silver as an antimicrobial material has increased in more recent years due to AgNP's exhibiting properties which makes them more desirable over other metal NP's, *e.g.* gold and platinum, due to the silver metal salts having a lower cost, whilst maintaining a high antimicrobial potency of the AgNPs. AgNPs exhibit a high surface-area-to-volume ratio which increases their antimicrobial activity whilst also showing minimal cytotoxicity and immunological response.[32] In order for AgNPs to be antimicrobial, the size of the AgNP does have to be <10 nm, which depends on the synthesis method as well as the reducing and capping agents used.[30] The size of the AgNP is not the only factor in determining the antimicrobial properties, the morphology of the AgNP also plays an important role. Spherical AgNPs do not show as much antimicrobial activity as prismatic shapes with shaper edges that can penetrate the bacteria more easily.[33,34]

One of the aims of this project is to improve the green synthesis of AgNPs. If we consider some of the 12 principles of green chemistry such as:[35]

- Atom economy, to maximise all materials used in a process to synthesise the final product.
- Less hazardous chemical syntheses, using and generating substances that possess little or no toxicity to human health and environment.
- Designing safer chemicals, designed to preserve efficacy of function while reducing toxicity.
- Safer solvents and auxiliaries, hazardous solvents and auxiliary substances should be made unnecessary wherever possible.
- Design for energy efficiency, wherever possible synthesises should be performed close to ambient temperature and pressure.
- Use of renewable feedstocks, raw material or feedstock should be renewable and not deplete natural resources.
- Inherently safer chemistry for accident prevention, chosen chemicals should minimize the potential for chemical accidents, including releases, explosions and fires.

This is important for industrial scale up since any hazardous chemical at the lab synthesis stage becomes much more hazardous in such large quantities required for industry synthesis. Industrial scale up whilst not being an objective in this project, was still considered since the overall aim is to produce an antimicrobial wound dressing that would need to be synthesised at an industrial scale for

commercialization. Taking this into consideration, other factors that would also affect the industrial scale up are the synthesis method, in terms of the cost of the materials, the cost of performing the synthesis, as well as the turnover rate of the products and does the reaction take too long to synthesise, which also relates back to the cost. So, an ideal reaction synthesis would use cheap starting materials, have a low cost for the production and generate the AgNPs in a reasonable timeframe, *e.g.* less than a day.

When comparing different methods, either in the literature or between methods developed in this project, calculations for atom economy (RAE), environmental factor (E-factor) and process mass intensity (PMI) (see **Equation 1-3**) will be performed to determine how impactful it is to the environment.[9] An atom economy calculation can provide information about how efficient the AgNP synthesis is from converting the starting reagents to products and E-factor will determine how much waste is generated from each synthesis.

$$Real\ Atom\ Economy\ (\%) = \frac{Actual\ wei \quad of desired\ product\ (kg)}{Total\ weight\ of\ all\ raw\ materials\ in\ process\ (kg)} x100 \quad (\mathbf{1})$$

$$Environmental\ factor\ (AU) = \frac{mass\ of\ wastes\ (g)}{mass\ of\ product\ of\ interest\ (g)} \quad (\mathbf{2})$$

$$Process\ mass\ intensity\ (AU) = \frac{Total\ mass\ used\ in\ the\ process\ (g)}{Mass\ of\ product\ (g)} \quad (\mathbf{3})$$

When considering literature methods for synthesising AgNP's, this will be difficult to quantify accurately since it will be unknown how much capping agent and reducing agent is left unreacted in the solvent, for these calculations, it will be assumed that these reagents are in excess to ensure that all the silver salt is fully reacted. Another limitation with calculating atom economy, is that depending on the size range of the AgNPs, some of them will be beyond a useable size range for antimicrobial purposes, quantifying the mass of usable AgNPs will not be possible, so it will be assumed that all AgNPs will be in a desired size range, but the true atom economy value may be lower. These issues also apply to the E-factor and any calculations will be stated as potentially being higher than calculated.

This project looks at the use of a Bet:Gly 1:3 and a Cap:Lau:Bet 4:2:1 molecular liquid as an alternative solvent, reducing agent and capping agent for AgNP synthesis, which would fulfil the greener synthesis requirements of the industrial scale up by using non-hazardous, low-cost materials. This project will also study improving the AgNP synthesis itself making DES's a more desirable solvent than the traditional

aqueous synthesises by reducing the quantity of additional reagents, such as capping agents, required. The molecular liquids were designed with primary metabolites so in theory they possess little to no toxicity to humans, however this would need to be confirmed with human toxicology testing.

1.2.1 Traditional methods for synthesis of AgNPs

1.2.1.1 Aqueous methods for synthesis of AgNPs

A bottom up co-precipitation synthesis was the method used when the first silver colloid was discovered in 1889 by M.C.Lea using citrate to stabilise the AgNPs in solution.[6] Whilst these methods are more commonly used in the literature, there are some concerns that are raised if the AgNPs are to be used for a medical application such as the toxicity of the capping and reducing agents used, such as sodium borohydride ($NaBH_4$) or CTAB, and whether they are suitable to introduce to a wound as well as if the AgNP synthesised fits the antimicrobial criteria of being >10 nm.[30] When performing comparisons between different literature publications it is worth nothing that finding direct comparisons is difficult due to the tailorability of the co-precipitation reactions resulting in many different reaction conditions that can be changed as well as the number of different reducing agents and capping agents that can be chosen.

The stronger reducing agents used in these traditional methods as seen in **Table 1,** tend to be the more harmful chemicals, for example $NaBH_4$ which releases flammable gases when in contact with water and a reproductive toxin.[32] When compared with the other reducing agents used for the aqueous methods, $NaBH_4$ does speed up the nucleation of AgNPs resulting in a quicker reaction time which would reduce the amount of energy required for the reaction, however, if the intended use for the AgNPs is for a medical field a purification step would be needed to remove all the $NaBH_4$ before the AgNPs could be considered for medical use.

Looking at **Table 1** where $NaBH_4$ was the reducing agent the timescale for formation of AgNPs ranged from almost immediately,[36] to up to 30 minutes.[37] In these cases the other reaction conditions will be influencing the time of reaction as seen by the capping agent being PVP or trisodium citrate and the synthesis of the AgNPs requiring two different reaction temperatures. The instant reaction was performed at room

temperature,[36] whilst the 30-minute reaction was performed at a much colder temperature of ~0°C (in an ice bath).[37] This was not the only difference in the reaction, the capping agents used for the synthesis were different with the short reaction time using trisodium citrate,[36] and the longer reaction time using PVP.[37] Due to the different conditions of each reaction, it is unknown whether it is the temperature controlling the speed of reaction, or the capping agents, or whether there is a combined effect. AgNP synthesis could be made more sustainable by changing to less hazardous reducing agents, whilst keeping the short reaction times as close to room temperature as possible.

Naturally occurring reducing agents have been used in the literature that would not need to be removed prior to medical use, such as β-D-Glucose or tyrosine.[11,12,38] Whilst these reducing agents could remove the requirement for an extra purification step, this comes at a cost of potentially having longer reaction times since these are weaker reducing agents. As seen in **Table 1** the β-D-Glucose requires a much higher temperature compared to $NaBH_4$ to obtain the same reaction time of 30 min, this trend is also the same for the L-tyrosine.[11,37,38]

Table 1: Comparison of AgNP synthesised in aqueous solution looking at particle size and morphology with the changing reagents and reaction conditions. *determined with UV-vis ** No data provided

Reducing Agent	Capping Agent	Time	Temperature /°C	Size /nm	Morphology	Yield /%	Reference
pH controlled Trisodium citrate ($NaOH$, HNO_3)	Trisodium citrate	10-55 min	100	58±6.38	Spherical	**	39
Sodium Borohydride	Polyvinyl pyrrolidone (PVP)	30 min	0 (in ice bath)	24.74±13.13	Spherical (aggregated)	**	37
Sodium Borohydride	Trisodium citrate	immediate	Room	2.3-3.7*	-	**	36
Sodium Borohydride	Trisodium citrate	15+ min	Room	5-8	Spherical	**	40
Tyrosine	Cetyltrimethylammonium bromide (CTAB)	180 min	25	5-25	Spherical (aggregated)	**	12
L-Tyrosine (pH controlled with NaOH)	L-Tyrosine	30 min	90	13-33	Spherical + Hexagonal	**	11
B-D-Glucose	B-D-Glucose	30 min	100	20-40	Spherical+Rods	**	38

Unlike the reducing agents which can be removed from the reaction afterwards using purification, the capping agent cannot be removed due to its important role protecting the AgNP from aggregating in solution. The other essential reagents used in these synthesises are the capping agents which are important to control the size of the AgNPs. Without the capping agent, the NPs are less stable due to the availability of the NP surface to undergo further nucleation.

Capping agents can be naturally occurring molecules such as glucose and tyrosine as discussed above, or they can be much larger molecules such as PVP or CTAB (see **Figure 2**).[11,12,37,38] Just like with the reducing agents, if these capped AgNPs are to be used in a medical capacity, it is preferable to have the more naturally occurring molecules, however as previously mentioned the size of the AgNPs will also determine their antimicrobial properties, which is controlled by the capping agent.[30] Depending on the structure of the capping agent will depend on if the NPs can be suspended in an aqueous or organic solvent. For example, as seen in **Figure 2**, CTAB has a polar head and a non- polar long chain hydrocarbon tail, the polar head will adsorb and interact with the NP surface whilst the non-polar tail will interact with the solvent allowing the NP to be suspended in solution.[41] The capping agent does not just control the size of the AgNP synthesised but also the morphology. For example, research by Dong *et. al.* researched how the pH of a citrate mediated synthesis would affect the shape of AgNPs.[39] The change in pH would affect the structure of the citrate depending on whether the solution was more acidic or more basic. This change in the citrate structure during the synthesis allowed for a range of morphologies to be seen. At a high pH, the AgNPs were shown to have a mix of spherical NPs as well as observing rod shaped NPs as well. On the other side of the scale, at a low pH the shape was seen to be more prismatic and triangular.[39]

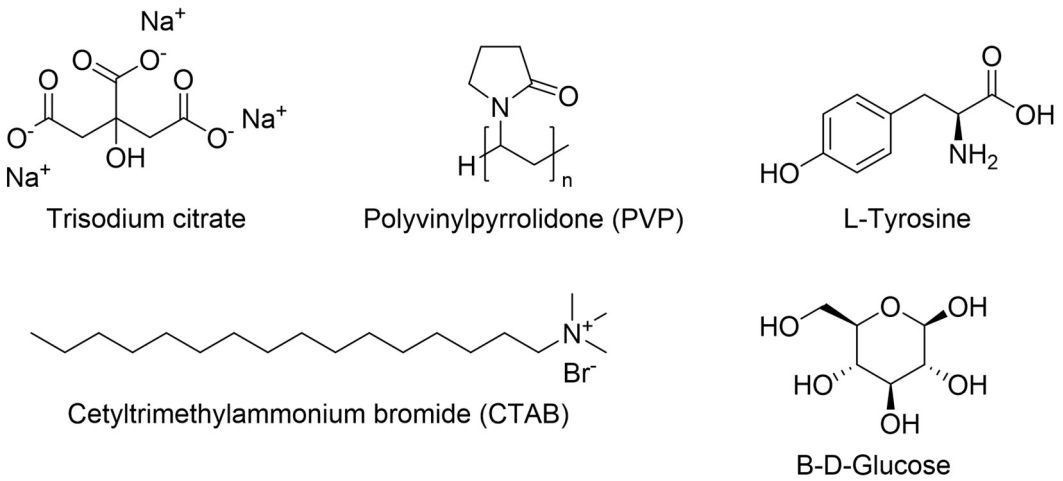


Figure 2: Structures of the capping agents used in the aqueous synthesise of AgNPs in **Table 1**.

In **Figure 3**, the TEM images from some of the publications have been collated in order to examine how the capping agent chosen effects the AgNP diameter as well as morphology. **Figure 3a** shows AgNPs synthesised with the trisodium citrate acting as the capping and reducing agent. The reaction was pH controlled at 7.7 with nitric acid and sodium hydroxide (NaOH). NaOH itself is also a strong reducing agent and if present in the reaction solution will also act as the reducing agent alongside the stated trisodium citrate. As seen in **Table 1**, these nanoparticles have a small size range, which makes them ideal for use in industrial applications due to having a more consistent size. However, for the use as an antimicrobial material, these are not ideal due to their relatively large size of 58 nm.[39] Desai *et.al.* also uses trisodium citrate as a capping agent but uses $NaBH_4$ as the reducing agent. The size of the NPs is stated as being 5-8 nm, however the scale of the TEM does not highlight this very well in the publication.[40] The change of AgNP size is to be expected since the $NaBH_4$ is a strong reducing agent and the reaction would occur much quicker instead of having a slow growth over time which can lead to larger particles forming.[42]

PVP is a much larger capping agent and we can see in **Figure 3b** that the size of the AgNPs is smaller than that of the trisodium citrate capped AgNPs, but does not have the same size control with the size range being over 50% of the average AgNP size, compared to the trisodium citrate being just over 10% of the average.[37,39] So whilst these particles are smaller and closer to the size range that would be suitable for antimicrobial applications, the control of the nucleation is not as good as the citrate capped particles. Comparing **Figure 3a** and **3b**, the PVP capped AgNPs also show more aggregation than citrate, this could be due to the fact that the PVP is not fully capping the AgNPs due to their small size not accommodating such larger capping agents. This can also be seen in **Figure 2c** where CTAB is used which is also a large molecule and the size of the AgNPs were 5-25 nm as seen in **Table 1**.

The last two TEM images show the AgNPs which have been capped with glucose and tyrosine, shown in **Figure 3d** and **Figure 3e**. These two molecules both produced similar sizes of AgNPs as seen in **Table 1**.[11,38] These AgNPs have shown some deviations in morphology to also include spherical AgNPs with some rod-shaped particles (glucose) as well as hexagonal shaped (tyrosine). These AgNPs could exhibit a higher antimicrobial activity due to the sharp edges of the NPs being able to penetrate into the bacteria more effectively,[33,34] however as seen in **Table 1**, the size ranges of these AgNPs are too large for effective antimicrobial activity, since larger particles result in a less efficient cellular uptake into the bacteria for antimicrobial effects.[33] If these synthesis methods can be improved to have smaller sized AgNPs (<15 nm) then they would be ideal for use in an antimicrobial wound dressing.

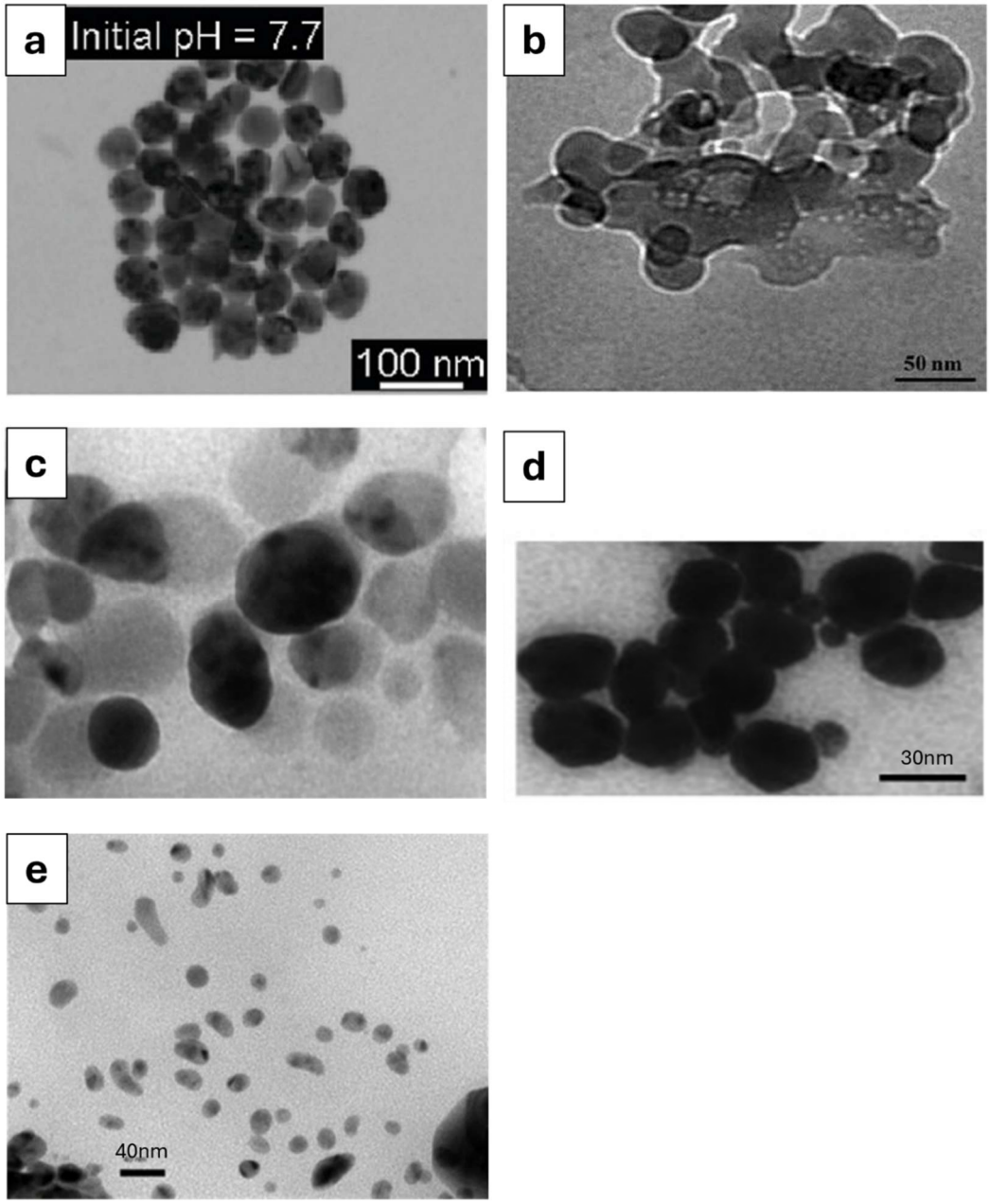


Figure 3: TEM comparison of aqueous synthetic methods from the literature to synthesise AgNPs to compare size and morphology. Reaction conditions a) trisodium citrate 100 °C for 10-55 min.[39] (Reprinted (adapted) with permission from X. Dong, X. Ji, H. Wu, L. Zhao, J. Li and W. Yang, *The Journal of Physical Chemistry C*, 2009, **113**, 6573–6576. Copyright 2009 American Chemical Society.) b) sodium borohydride, PVP, 0 °C for 30 min.[37] c) tyrosine, CTAB, 25 °C for 180 min, no scale bar provided.[12] d) B-D-Glucose, 100 °C for 30 min.[38] ** e) L-tyrosine, 90 °C for 30 min.[11] ***

* Reprinted from Colloids and Surfaces B: Biointerfaces, 89, Zoya Zaheer, Rafiuddin, Silver nanoparticles formation using tyrosine in presence cetyltrimethylammonium bromide, 211–215, Copyright (2012), with permission from Elsevier.
** Reprinted from Toxicology in Vitro, 41, Elisa Panzarini,Stefania Mariano,Cristian Vergallo,Elisabetta Carata,Gian Maria Fimia,Francesco Mura,Marco Rossi,Viviana Vergaro,Giuseppe Ciccarella,Marco Corazzari,Luciana Dini, Glucose capped silver nanoparticles induce cell cycle arrest in HeLa cells, 64-74, Copyright (2017), with permission from Elsevier.
*** Reprinted from Environmental Toxicology and Pharmacology, 51, Sireesh Babu Maddinedi,Badal Kumar Mandal,Kiran Kumar Anna, Tyrosine assisted size controlled synthesis of silver nanoparticles and their catalytic, in-vitro cytotoxicity evaluation, 23-29, Copyright (2017), with permission from Elsevier.

With the goal of producing an antimicrobial wound dressing using AgNPs, the only reaction seen in **Table 1** that produces AgNPs that are all within the antimicrobial size range was the work by Wojtysiak *et.al.*[40] Since the mass of the AgNPs at the end of the synthesis was not reported by the authors, RAE will be calculated using **Equation 1** and **Table 2**, assuming all silver salt is reacted to AgNPs, in this case at a molar ratio of 1:1 from $AgNO_3$ to Ag^0 as well as assuming all of the trisodium citrate is used in capping the AgNPs.

Table 2: Mass of reactants and products for green metric calculations of AgNPs synthesised by Wojtysiak *et.al.*[40]

Reactants	Molar mass /g mol^{-1}	Moles /mmol	Mass /g	Products	Molar mass /g mol^{-1}	Moles /mmol	Mass /g
$AgNO_3$	169.87	0.2	0.0340	**AgNPs**	107.87	0.2	0.0216
Na_3Cit	294.1	0.62	0.1823	**Na_3Cit**	294.1	0.62	0.1823
$NaBH_4$	37.83	0.004	0.0002				

$$Real\ Atom\ Economy\ (\%) = \frac{0.0216 + 0.1823\ (g)}{0.0340\ +\ 0.1823\ +\ 0.0002\ (g)} x100 = 94.2\%$$

The RAE calculated for this reaction is ≤94.2%, which is a good value for a green synthesis however, 6% of the silver precursor is wasted in the reaction, the true number will be lower than this considering that this calculation assumed a complete synthesis. Using the data in **Table 2** a calculation was also performed for E-factor and PMI since the total volume of water was known (220.02 mL which was converted to mass (219.62 g).

$$Environmental\ factor\ (AU) = \frac{219.62 + 0.0002\ (g)}{0.0216 + 0.1823\ (g)} = 1077$$

$$Process\ mass\ intensity\ (AU) = \frac{219.62 + 0.0340 + 0.1823 + 0.0002\ (g)}{0.0216 + 0.1823\ (g)} = 1078$$

The E-factor for this reaction was calculated to be ≥1077 and a PMI of ≥1078, which is a considerable high number compared to other chemical industries.[43] An ideal E-factor would be close to 0, which means that the synthesis produces no waste products from the reaction and an ideal PMI value close to 1, which means the total mass of raw materials produces a similar mass of products. A value of over 1000 for

both E-factor and PMI shows how much water was required to synthesise a small quantity of AgNPs compared to how little product was synthesised. Comparing this value to the pharmaceutical industry, E-factors have been stated to be in the range of 20-100,[43] the synthesis of these AgNPs in an aqueous solvent is not very sustainable and scaled up to industrial levels would produce a significant quantity of contaminated wastewater which would need treatment to remove the sodium borohydride. Aqueous co-precipitation methods are not the only way to synthesise AgNPs since they can also be synthesised in organic solvents. Whilst these are not ideal for use as a wound dressing due to the toxic nature of the organic solvent, it is still worth a look at these methods as a comparison.

1.2.1.2 Organic Methods for synthesis of AgNPs

Whilst aqueous methods are more commonly found in the literature, there are some AgNPs that have been synthesised using organic solvents. Whilst these are not suitable for use as a wound dressing due to the toxicity of the solvents, looking at these methods will give a complete picture of AgNP synthesis in the literature.

As seen in **Table 3**, a popular choice for the reducing agent/capping agent when synthesising AgNPs in an organic solvent, is to use oleylamine (OAm) and oleic acid (OA), these two reagents are very similar to each other (see **Figure 4**), both consisting of a unsaturated long carbon chain tail with a polar head of either an amine group, for OAm, or a carboxylic acid group, for OA.[44–46] These long carbon chains allow for the AgNPs when capped, to easily suspend in organic solvents such as toluene. However, when compared to the aqueous synthesise the time and temperatures required to synthesise AgNPs in an organic solvent is generally much higher than that required for an aqueous solvent.

Table 3: Comparison of AgNP synthesised in organic solvents looking at particle size and morphology with the changing reagents and reaction conditions. * No data provided

Reducing Agent	Capping Agent	Time	Temperature /°C	Size /nm	Morphology	Yield /%	Reference
Dodecylamine (in toluene)	Dodecylamine	12 h	110	10-20	Spherical (aggregated)	*	47
Tetrabutylammonium borohydride ($TBABH_4$) (in toluene and ethylene glycol)	Oleic acid	11-20 min	Room	8.5	Spherical	*	46
Oleylamine	Oleylamine	1 h	180	8.44±1.73	Spherical	*	44
Oleylamine (in toluene)	Oleylamine	Overnight (8-24 hr)	110	~9	Spherical	*	45

When OAm is used with toluene as the solvent the reaction times varied depending on the temperature of the reaction. These ranged from being short with 1-hour reactions at 180 °C, to requiring overnight reactions at 110 °C. This is likely due to the fact that at a higher temperature of 180 °C the synthesis of AgNPs can be achieved in a shorter time than at the lower temperature of 110 °C. Both these temperatures however are still higher than the higher temperature methods for aqueous synthesis which was 100 °C as seen in **Table 1**. These high temperatures and times mean that the sustainability of these methods compared to that of the aqueous methods are poor. The exception for this is when a strong organic reducing agent, tetrabutylammonium borohydride ($TBABH_4$), is used which lowered the temperature and time of reaction.[46]

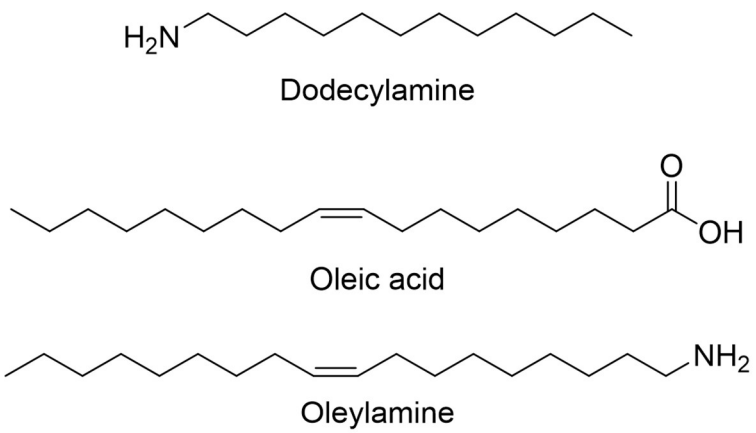


Figure 4: Structures of the capping agents in the organic synthesises in **Table 3**.

As seen in **Figure 5**, the AgNPs synthesised in organic solvents are spherical of sizes <10 nm. The OAm and OA synthesises, seen in **Figure 5b-5d**, show similar size and shaped AgNPs which is to be expected due to the similarity of the capping agent chosen, the difference between the sizes could be attributed to the reaction conditions which may indicate that the reactions are less sensitive to changes in reaction temperatures and time in organic solvents.[44–46] **Figure 5a** which is an SEM image of the AgNPs synthesised also shows spherical nanoparticles. The size of these AgNPs were not directly stated in the publication but from **Figure 5a**, it can be determined that the size can be estimated at ~10-20 nm.

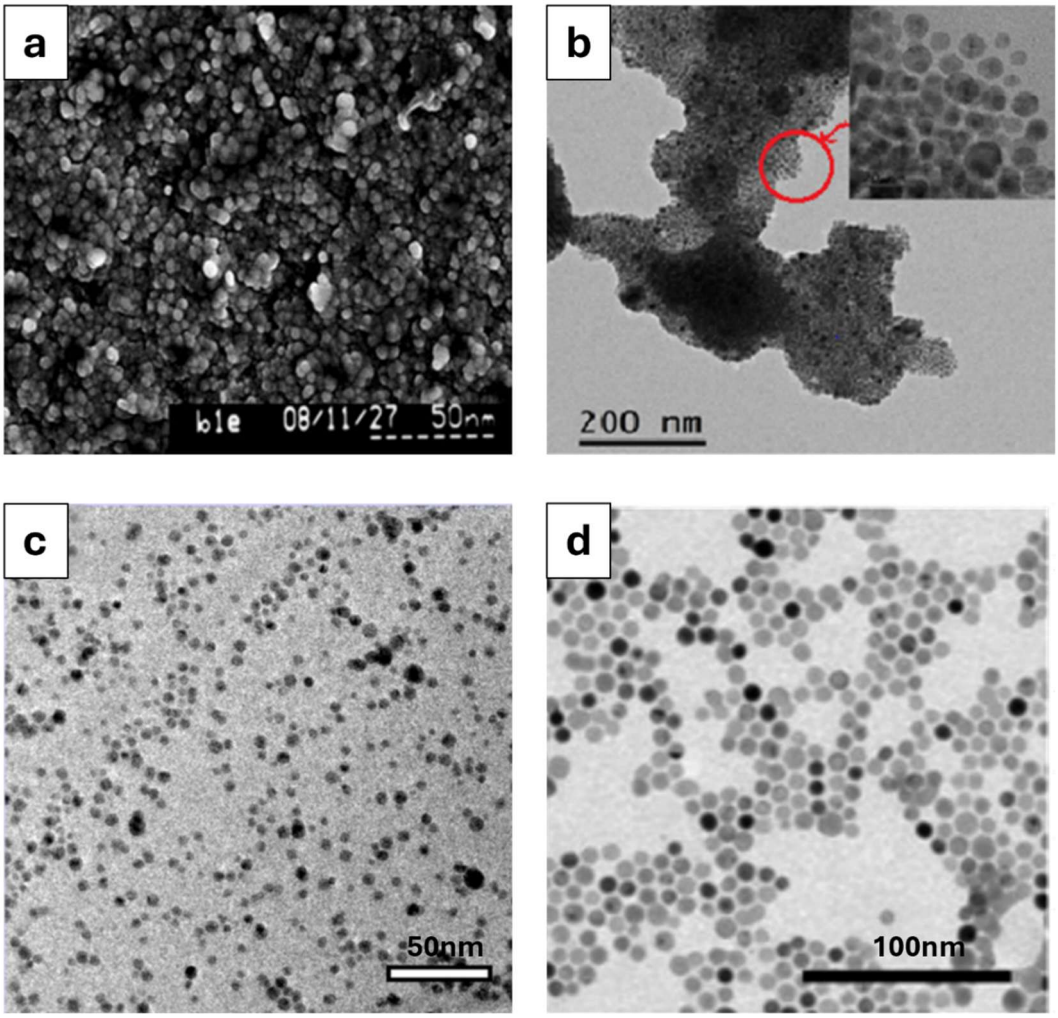


Figure 5: Comparison of organic synthetic methods from the literature using SEM for a) and TEM for b-d) to compare size and morphology. Reaction conditions a) dodecylamine in toluene, 110 °C for 12 hr.[47] * b) OAm, 180 °C for 1 hr.[44] c) tetrabutylammonium borohydride, oleic acid in toluene and ethylene glycol, RT for 11-20 min.[46] ** d) OAm in toluene, 110 °C overnight (8-24 hr).[45] ***

* Reprinted from Colloids and Surfaces B: Biointerfaces, 101, Thomas V. Mathew,Sunny Kuriakose, Studies on the antimicrobial properties of colloidal silver nanoparticles stabilized by bovine serum albumin, 14-18, Copyright (2013), with permission from Elsevier.
** Used with permission of Royal Society of Chemistry, from A biphasic batch and continuous flow synthesis of hydrophobic gold and silver nanoparticles, M. Distaso, W. Peukert, 8, 8,2016; permission conveyed through Copyright Clearance Center, Inc
*** Reprinted (adapted) with permission from H. Hiramatsu and F. E. Osterloh, *Chemistry of Materials*, 2004, **16**, 2509–2511. Copyright 2004, American Chemical Society.

From the comparison between the organic methods in **Table 3**, the work by Distaso *et.al*. using oleic acid as a capping agent would be the ideal reaction for synthesis of antimicrobial AgNPs, due to the oleic acid being non-hazardous. Using **Equation 1** and **Table 4** the RAE can be calculated.

Table 4: Mass of reactants and products for green metric calculations of AgNPs synthesised by Distaso *et. al.*[46] Calculations performed in Excel without rounding values.

Reactants	Molar mass /g mol^{-1}	Moles /mmol	Mass /g	Products	Molar mass /g mol^{-1}	Moles /mmol	Mass /g
$AgNO_3$	169.87	5.0	0.8493	**AgNPs**	107.87	5.0	0.5394
OA	282.46	0.79	0.2231	**OA**	282.46	0.79	0.2231
$TBABH_4$	257.31	1.0	0.2573				
DDAB	462.63	0.5	0.2313				

$$Real\ Atom\ Economy\ (\%) = \frac{0.5393 + 0.2231\ g}{0.8493 + 0.2231 + 0.2573 + 0.2313\ g} x100 = 48.8\%$$

Following the same set of assumptions from **Section 1.2.1.1** (assuming the reaction has gone to completing and no excess regents are used) the RAE was calculated to be ≤48.8%, which compared to the aqueous method shows that the reaction is less efficient and has more waste products. Further calculations were performed for E-factor and PMI using **Table 4** as well as the known volume of solvents used in the publication converted to mass (6.551 g).

$$Environmental\ factor\ (AU) = \frac{6.551 + 0.2573 + 0.2313\ (g)}{0.5394 + 0.2231\ (g)} = 9.23$$

$$Process\ mass\ intensity\ (AU) = \frac{6.551 + 0.2573 + 0.2313 + 0.5393 + 0.2231\ (g)}{0.0216 + 0.1823\ (g)} = 10.6$$

Calculating the E-factor showed that the organic synthesis produces less overall waste, compared with the aqueous method, with a value of ≥9.23, which compared again to the pharmaceutical industry (E-factor 25-100)[43] was more sustainable. The PMI value for the organic synthesis was calculated to be ≥10.6, which is much lower than the aqueous synthesis meaning that more of the raw material is being converted to the products, most likely due to the lower volume of additional solvents used. However, it can be argued that the greener and more sustainable method would be the aqueous method due to the contaminated wastewater being less hazardous than the contaminated organic waste.

Whilst the organic synthesis route is not ideal due to the toxicity of the solvents used, as well as the capping and reducing agents, some of these methods produced AgNPs that are <10nm in diameter that would be suitable for use as an antimicrobial wound dressing.[30] However, the AgNPs would need to be purified to remove toxic reagents, which was seen in the work from Mathew *et. al.* in which the capping agent was exchanged from dodecylamine to BSA [47] On a larger scale synthesis this could prove to be costly and time consuming to ensure all traces are removed. Looking at greener alternatives to hazardous chemicals is a popular research field and more greener solutions involving biomolecules have been discovered for the synthesis of AgNPs.

1.2.2 Current state-of-the-art aqueous green synthesis of AgNPs

Considering the 12 principles of green chemistry,[35] discussed previously at the start of **Chapter 1.2**, in particular; less hazardous chemical synthesis, using and generating substances that possess little or no toxicity to human health and environment, the use of natural products would satisfy this criterion. However, there is not one definitive way to achieve a green synthesis of AgNPs due to the wide variety of natural products that can be used (see **Table 5**). Some of the more popular choices in the literature include chemicals that can be extracted from plant material such as tea leave or fruit extracts. These are ideal candidates for green synthesis since they can be grown naturally without the need for chemical processing which would be required for synthesising some of the reagents discussed previously, *e.g.* sodium borohydride or oleylamine. However, when using a natural product extracted from plant material you may end up with a solution that is heterogeneous as all the natural products in the plant will be extracted, which make cause issues with the synthesis by the AgNPs being capped with different molecules. This would result in a larger range of sizes due to different capping agents on the surface of the AgNPs. Plant matter is not the only natural products that can be used, some research has looked at using fungi as an alternative reducing and capping agent for AgNP synthesis, but also looking at chemicals naturally produced in nature such as chitosan found in shellfish. These are much more sustainable to source from a readily available and replenishing stock of plant and animal material. However, whilst the reagents are considered to be green and sustainable, the extraction method may not, it will depend on the solvent used, so both the synthesis and extraction protocol have to be considered. Due to a large variety of green synthesis components that can be used,

it is expected that the size and shape of the AgNPs will also be different depending on the different natural products in the synthesis.

The greener synthesises tend to use much lower reaction temperatures than that of the previous aqueous and organic methods, which result in a much more sustainable synthesis. As seen in **Table 5**, the majority of the reviewed articles reported that the synthesis of AgNPs can be done at room temperature, and this is shown to work with a variety of different biomolecules. One such example is the work from Taher Mohammed *et. al.* looking at the use of the fungus *Fusarium graminearum* as the reducing agent and capping agent.[48] This reaction was performed at room temperature however the time of reaction was not given, and the endpoint was judged based on a colour change which is not ideal since this cannot be accurately defined depending on each individual's perspective on colour. This method produced large nanoparticles at 94 nm which are beyond the size range for antimicrobial effects.

Vigneshwaran *et. al.* also researched using fungi to synthesise AgNPs but increases the temperature from room temperature to 37 °C, however, this reaction does use a different species of fungus so a direct comparison cannot be performed. As seen in **Table 5**, these AgNPs in **Figure 6a** are much smaller than the other fungus capped AgNPs, and within the size limits needed for antimicrobial activity. One other fungus was reviewed, *Trichoderma app.* however, the reported size of these AgNPs was 150 nm, which is not within the range of being classed as a NP, and more than 10x the size of what is required for antimicrobial activity. These AgNPs were synthesised at 32 °C which is between the two temperatures previously mentioned but has a very long reaction time of 216 Hrs (9 days), which would not be suitable for scaling up to industry production since other faster options are available.[49]

Table 5: Comparison of state-of-the-art green AgNP synthesised in aqueous solution looking at particle size and morphology with the changing reagents and reaction conditions. * No data provided

Reducing Agent	Capping Agent	Time	Temperature /°C	Size /nm	Morphology	Yield /%	Reference
Fungus – *Aspergillus flavus*	Fungus – *Aspergillus flavus*	72 h	37	8.92±1.61	Spherical	*	50
Fungus – *Trichoderma spp.*	Fungus – *Trichoderma spp.*	216 h	32	150	Spherical	*	49
Fungus -*Fusarium graminearum*	*Fusarium graminearum*		room	94	Spherical	*	48
Persea Americana extract	*Persea Americana* extract	24 h	room	16±5		*	51
Glucose	Bamboo hemicellulose	120 s	Microwave irradiation	8.39-14.72	Spherical	*	52
Thyme extract	Thyme extract		45	66±7.70	Spherical	*	53
Black elderberry extract	*Black elderberry* extract		45	20±4.80	Spherical	*	53
Carica papaya extract	*Carica papaya* extract		room	10-60	Spherical	*	54
NaOH	*Ammannia baccifera* extract	24 h	room	13-60	Spherical	*	55
Hagenia abyssinica extract	*Hagenia abyssinica* extract	24 h	50	8-42	Mostly Spherical Prismatic Cylindrical	*	56
Green tea leaf extract	Green tea leaf extract		45	Reported as 9±0.025	Spherical	*	57
Chitosan	Chitosan		Room/sunlight	4-20	Spherical	*	58

Fungi are not the only biomolecules that can be used for AgNPs synthesis, other research has been performed on using plant extracts as the capping agent as well as the reducing agent. Since there are many different genomes of plants there are a lot of potential extracts to be tested for synthesis of AgNPs. Some of the examples of plant extracts used in the literature are found in **Table 5**. Similarly with the fungi methods, these plant extracts methods can be performed at room temperature but also tends to require a long reaction time. **Figure 6b** shows the AgNPs synthesised using bamboo hemicellulose as a capping agent with glucose as a reducing agent. Glucose can be classed as a green sustainable reagent as it is still a product that can be produced by plants. This synthesis uses microwave irradiation which can be a greener alternative to heating the solutions on a hotplate, depending on the time of reaction, in this case the time of reaction is 120 seconds which is the shortest time of reaction reported for biomolecule synthesis of AgNPs.[32,52] These AgNPs were a similar size to the fungus capped AgNPs shown in **Figure 6a**, but are synthesised much faster and both have a spherical morphology and would be a suitable size for antimicrobial activity.[50]

Other plant extracts have also been tested but instead use a conventional heating method rather than a microwave synthesis. The work shown in **Figure 6c** and **Figure 6d**, were both reported in the same publication by Tavukcuoglu *et. al.* comparing the plant extracts of thyme (thymol) and black elderberries (cyanidin).[53] These methods used the same reaction conditions of 45 °C but did not specify a time of reaction instead relying on a colour change to determine the end point.[48] Having the same reaction conditions, to an extent, allows for a good comparison of how the plant extract chosen can affect the size of the AgNPs. As seen in **Table 5**, the AgNPs produced from thyme extract would not be able to be used as an antimicrobial agent due to their large size (66±7.70 nm), when using black elderberry extract the size of the AgNPs were 3x smaller (20±4.8 nm). Whilst this is not suitable for antimicrobial activities, these may be suitable for other applications and confirms that the capping agent in these aqueous synthesises are very important for size control.

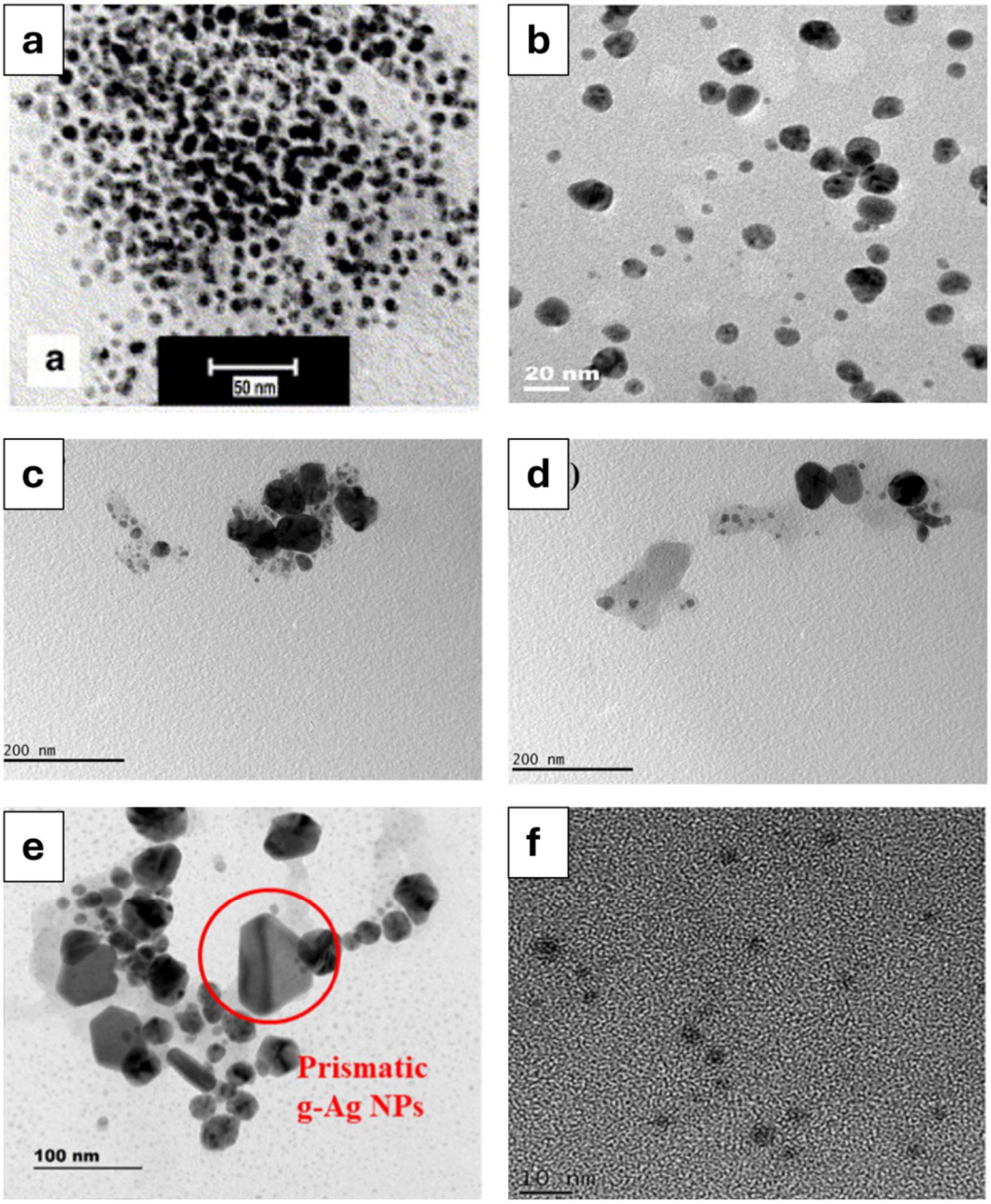
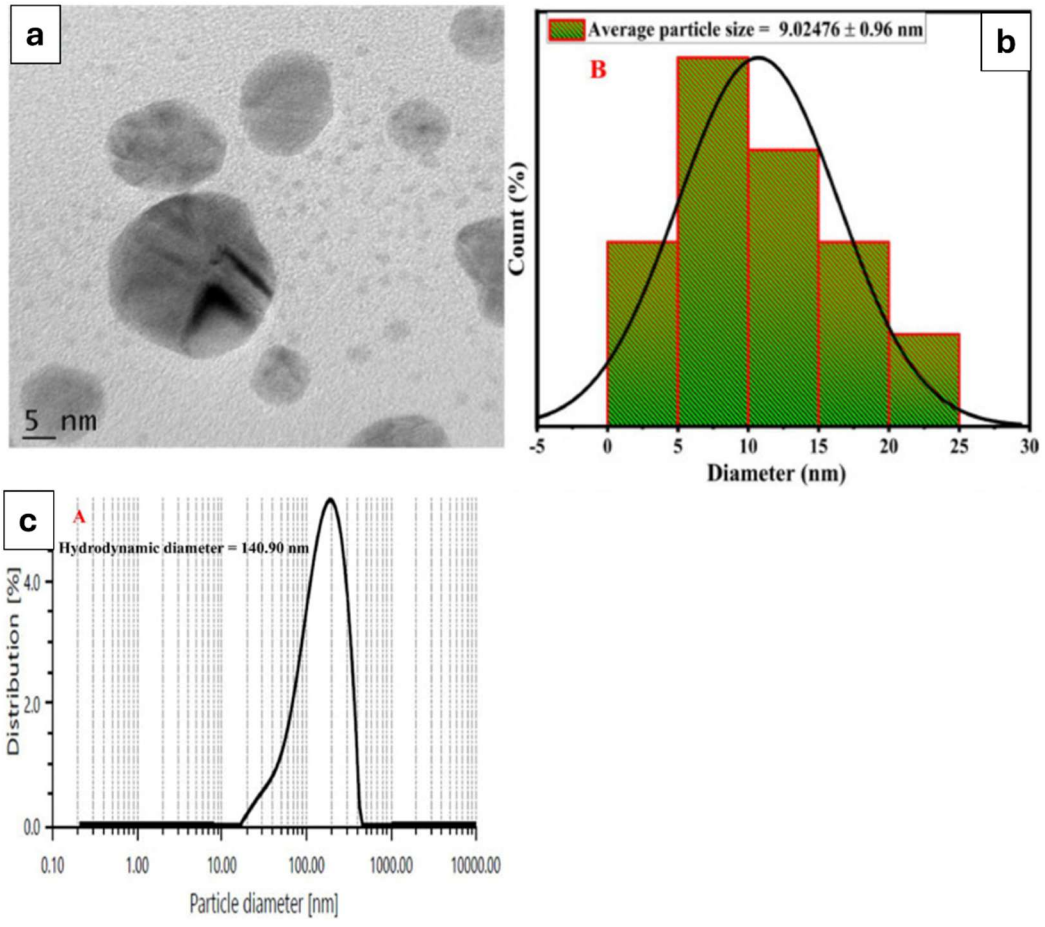


Figure 6:Comparison of state-of-the-art green synthetic methods from the literature to synthesise AgNPs using TEM analysis to compare size and morphology. Reaction conditions a) *Aspergillus flavus*, 37 °C for 72 Hr.[50] * b) glucose, bamboo hemicellulose, microwave irradiated for 120 s.[52] ** c) thyme extract, 45 °C, time not stated.[53] *** d) black elderberry extract, 45 °C, time not stated.[53] *** e) *Hagenia abyssinica* extract, 50 °C for 24 hr.[56] f) chitosan, RT in direct sunlight.[58] ****

* Reprinted from Materials Letters, 61, N. Vigneshwaran,N.M. Ashtaputre,P.V. Varadarajan,R.P. Nachane,K.M. Paralikar,R.H. Balasubramanya, Biological synthesis of silver nanoparticles using the fungus Aspergillus flavus, 1413-1418, Copyright (2007), with permission from Elsevier.
** Reprinted from Carbohydrate Polymers, 91, Hong Peng,Anshu Yang,Jianghua Xiong, Green, microwave-assisted synthesis of silver nanoparticles using bamboo hemicelluloses and glucose in an aqueous medium, 348-355, Copyright (2013), with permission from Elsevier.
*** Reprinted from Industrial Crops and Products, 222, Ozlem Tavukcuoglu,Nilüfer Evcimen Duygulu,Aylin Altinbay,Fatih Ciftci, Green synthesis of silver nanoparticles from Thymus vulgaris and Sambucus nigra extracts in poly (vinyl alcohol) nanofiber matrix: In vitro evaluation, 119825, Copyright (2024), with permission from Elsevier.
**** Reprinted from International Journal of Biological Macromolecules, 252, Gautham Devendrapandi,Deepakrajasekar Padmanaban,Rabi Thanikasalam,Anthoniammal Panneerselvam,Ragasudha Palraj,Jothi Ramalingam Rajabathar,Nagappan Rajendiran,Ranjith Balu,Tae Hwan Oh,Subramaniyan Ramasundaram, Direct sunlight induced room temperature synthesis of anticancer and catalytic silver nanoparticles by shrimp shell waste derived chitosan, 126205, Copyright (2023), with permission from Elsevier

Some plant extracts seen in **Table 5** produce very large ranges of AgNP size, with the largest range from 10-60 nm when *Carica papaya* extract (papain, chymopapainis and vitamins A, B1, C and E) was used.[54] Other plant extracts that have a large size range of AgNPs are, *Ammannia baccifera* extract (polyphenol-type compounds, *e.g.* pyrogallol, guanosine), which also included a reducing agent NaOH, and *Hagenia abyssinica* extract (polyphenols of tannins).[55,56] **Figure 6e**, is representative of the type of AgNPs produced, synthesised from *Hagenia abyssinica,* as an example that the plant extracts do not have an acceptable control over the size range of the AgNPs for use in a wound dressing.[56] Interestingly, the AgNPs synthesised using this method show some variations in morphology, which links back to different morphologies having an increased antimicrobial activity.[33,34] Up to this point most of the AgNPs discussed have been spherical but as seen in **Figure 6e**, some of these AgNPs are prismatic and cylindrical which would be more penetrating in the bacteria.[33,34] It is also worth noting that from **Table 5** there was also a synthesis involving green tea extract which produced AgNPs which were reported as the average size being 9±0.025 nm.[57] However, this is misleading, as seen in **Figure 7** below, the actual sizes range from 0-25nm, which whilst the majority of the AgNPs are within the size range for antimicrobial properties it faces a similar issue to the method in which *Hagenia abyssinica* is used in which some of the particles are outside of this range.[56]

It is not just flora that can be used as inspiration for biomolecules to use for AgNP synthesis. Chitosan, which is extracted from shellfish, has also been used as a reducing agent and capping agent as seen in **Table 5**. This method from Devendrapandi *et. al.* also reports the reaction temperature to be room temperature, however this reaction is left in direct sunlight to assist the formation of the AgNPs.[58] This does mean that the actual temperature of the solution is unknown due to the effect of direct sunlight. The time of the reaction was also not stated due to waiting on a colour change like previous methods discussed. The AgNPs synthesised can be seen in **Figure 6f** and were stated to be between 4-20 nm. Whilst this method seems to have the most sustainable synthesis done at "room temperature" using the suns UV rays to synthesise the AgNPs, it does have the issue of the variability of the reaction conditions used.



3

Figure 7: AgNP size analysis synthesised using green tea extract at 45 °C.[57] * a) TEM analysis of AgNPs. b) AgNP size count from TEM analysis. c) DLS analysis of aqueous AgNPs

The issue with relying on the sun for a synthetic method is that not all countries in the world have the weather conditions or location for this to be a reliable synthesis method. For locations near the equator this method is fine, but for locations further away, such as in the United Kingdom, which does not have a lot of consistent sunny days then this reaction is not ideal compared to other reactions. The chitosan synthesis can be argued to be the most sustainable since it does not rely on microwave synthesises or hotplates and just the power of the sun, which is a renewable energy source, as well as using a naturally occurring chitosan. Unfortunately, it is not a method can be reliably used across the globe.

Looking at these green methods compared to aqueous and organic solvents discussed previously in **Section 1.2.1.1** and **Section 1.2.1.2**, the biomolecule synthesises require lower temperatures to form AgNPs compared to the average temperature of conventional aqueous synthesis and the higher temperatures of organic synthesis. However, the downside is that the time of reaction for these green methods is a lot longer, with the longest time being 216 hr (9 days). Yet the size control of the AgNPs can be achieved using biomolecules whilst maintaining the non-toxicity of the components used.

Two of the methods shown in **Table 5** can be used to synthesise AgNPs that would have antimicrobial properties, the work by Vigneshwaran *et.al.* using the fungus *Aspergillus flavus* by heating and stirring and the work by Peng *et.al.* using bamboo hemicelluloses and glucose in a microwave reactor. For the calculations for atom economy and E-factor, the consideration of the plant extraction process will not be included as the methods in the literature do not specifically say what quantity of solvent is used for each extraction process. The extraction of the plant material however would be an important factor to consider in a scaled-up industrial process. RAE, E-factor and PMI was calculated using **Table 6** as well as the known volume of water used in the reaction converted to mass (99.82 g).

Table 6: Mass of reactants and products for green metric calculations of AgNPs synthesised by Vigneshwaran *et.al.*[50]

Reactants	Mass /g	Products	Mass /g
$AgNO_3$	0.0170	**AgNPs**	0.0170
Aspergillus flavus	5.0	***Aspergillus flavus***	5.0

$$Real\ Atom\ Economy\ (\%) = \frac{0.0170 + 5.0\ g}{0.0170 + 5.0\ g} x100 = 100.0\%$$

$$Environmental\ factor\ (AU) = \frac{99.82\ (g)}{0.0170 + 5.0\ (g)} = 19.90$$

$$Process\ mass\ intensity\ (AU) = \frac{99.82 + 0.0170 + 5.0\ (g)}{0.0170 + 5.0\ (g)} = 20.90$$

The calculated RAE value for the *Aspergillus flavus* synthesis was calculated to be ≤100%, however, this assumes that all the fungus added is used to cap the AgNPs, and the actual value of fungus required for the capping of the AgNPs is unknown. The same assumption was also considered for the E-factor and PMI calculations using the fungus, E-factor was calculated to be ≥19.90 and PMI was calculated to be ≥20.90.

The same calculations were also performed on the AgNPs capped with bamboo hemicellulose using **Table 7** as well as the known mass of water used (19.964 g).

Table 7: Mass of reactants and products for green metric calculations of AgNPs synthesised by Peng *et.al.*[52] Calculations performed in Excel without rounding values.

Reactants	Molar mass /g mol^{-1}	Moles /mmol	Mass /g	Products	Molar mass /g mol^{-1}	Moles /mmol	Mass /g
$AgNO_3$	169.87	0.08	0.0136	**AgNPs**	169.87	0.08	0.0136
Hemi-cellulose			0.04	**Hemi-cellulose**			0.04
Glucose	180.16		0.04				

$$Real\ Atom\ Economy\ (\%) = \frac{0.0136 + 0.04\ g}{0.0136 + 0.04 + 0.04\ g} x100 = 57.26\%$$

$$Environmental\ factor\ (AU) = \frac{19.964 + 0.04\ (g)}{0.0136 + 0.04\ \ (g)} = 373.28$$

$$Process\ mass\ intensity\ (AU) = \frac{19.964 + 0.0136 + 0.04 + 0.04\ (g)}{0.0136 + 0.04\ (g)} = 374.28$$

The same calculations were also performed on the AgNPs capped with bamboo hemicellulose with an RAE of ≤57.26%, an E-factor of ≥373.28 and a PMI of ≥374.28. The *Aspergillus flavus* synthesis is the greener and more sustainable synthesis due to the high RAE and low E-factor/PMI compared to the bamboo hemicellulose synthesis. When compared to the aqueous and organic methods previously discussed in **Section 1.2.1.1** and **Section 1.2.1.2**, this biomolecule synthesis is an improvement on the sustainability of the aqueous method with a much lower E-factor of ≥19.90 compared to ≥1077, however the RAE cannot be directly compared as it is unknown how much of the *Aspergillus flavus* was unreacted in the reaction.

However, when considering the green criteria of these biomolecule AgNP synthesises, it is important to remember that plant extraction first needs to occur, which will add to the environmental impact of the synthesis and depending on the extraction solvent and the conditions required for the extraction, may not be considered "green". Additionally, for an industrial-scale synthesis this would require lots of plant matter, which would not be an issue if the biomaterial were obtained from current waste sources, *e.g.* green tea leaves, which would otherwise be disposed of, however if they

require fresh biomatter, then this would also be costly and require lots of space to grow the crops or raise the animals and could have negative implications for the food production industry potentially leading to destroying habitats to accommodate the additional demand. Other synthetic methods such as electrochemical reduction, sonication and calcination by furnace,[20–25] which may also be a greener alternative to conventional synthesises, the aim of this project was to develop an antimicrobial wound dressing using AgNPs in a DES, since DES's can also have antimicrobial properties and combining the two would potentially have an increased antimicrobial effect. This thesis therefore focuses on using a DES, which could prove more desirable than the green methods discussed so far as on average less time is required to synthesise the AgNPs giving much more reasonable time frames if considering a large-scale synthesis for an antimicrobial wound dressing, as well as requiring lower temperatures to synthesise small AgNPs of spherical morphology. The DES's also do not require a purification/extraction step in the synthesis if the DES itself can be used as the reducing and capping agent, minimalizing hazardous waste resulting in a greener synthesis.

1.2.3 AgNP synthesis in Deep Eutectic Solvents

The most popular method used for nanoparticle synthesis in a DES is chemical reduction by heating and stirring following the same techniques as the aqueous methods. This usually involves a DES mixed with a metal salt with the addition of an additive and stirred for a set time at a specific temperature.[26] Heating and stirring using a hotplate is the preferred method but may instead involve the use of microwave reactors to introduce the heat instead.[2] Though, using a microwave reactor may not be as desirable as a hotplate depending on the time needed to synthesise the AgNPs due to a higher energy input required to produce the microwaves as well as the need for specialized technical equipment.

Table 8 shows some of the different DES's that have been used to synthesise AgNPs using a chemical reduction method. Due to the larger number of possible DES combinations, there are thousands of possibilities of solvents to synthesise AgNPs. This does make comparisons between methods difficult due to the different physicochemical properties of each DES possibly affecting the size of the AgNPs produced. This is supported by the size comparisons in **Table 8**, ranging from 7 nm to 100 nm. One common factor between most of the synthesis conditions is the use

of a halide-free DES, this is due to the fact that using a component which has a halide, such as chloride from choline chloride, you are more likely to synthesise NPs of the Ag halide salt rather than just pure AgNPs.[59]

DES synthesis of AgNPs publications tend to do a straightforward adaptation from aqueous synthesises by replacing aqueous solvent with the DES itself. However, one publication deviates from the rest and uses a DES hydrogel with dopamine hydrochloride (DA) and sodium hyaluronate (SH), named DES-DASH hydrogel.[60] This is the only method that does not use a liquid DES but instead opts to suspend the DES-DASH hydrogel into an aqueous solution of $AgNO_3$. It can be argued that this is not technically a synthesis of AgNPs in a DES and is more like the methods discussed in **Section 1.2.1.1** as the nanoparticles are synthesised in an aqueous solution and that adhere to the surface of the DES-DASH hydrogel. Whilst this is still using the DES as the reducing agent, it does seem that the growth control will be limited due to the lack of a capping agent to control the size of the particles on the surface, this is seen in the size range that was stated to be between 50-100nm.[60] This is a very large size range compared to the other papers using DES's as well as being above the recommended size limit for antimicrobial properties.[30] With the goal of making AgNPs for use as a wound dressing these large sizes with such a wide range of nanoparticle sizes makes it unsuitable. The other methods look to use the DES in liquid form to either facilitate the reaction with a capping agent, or to act as a capping agent themselves.[32]

One important consideration for the synthesis of AgNPs in a DES for use as a wound dressing is the purity of the final product. One impurity that would be present is the counter ion for the silver salt precursor, typically a nitrate anion, which in the aqueous synthesis can be removed during the extraction process. However, this can not easily be achieved with a DES as the solvent. Since the DES is also being used as part of the wound dressing, the counter ion in the silver salt will also be present in the DES, which may cause some unforeseen issues with toxicity during the wound healing process. As seen in **Table 8**, one solution to this issue is to have the silver incorporated in the DES. This removes the counter ions in the DES's however it does have its own problems with the synthesis. Since the DES is formed using a silver containing component, when that silver is removed as part of the AgNP synthesis the resulting DES may be a different structure to the original silver containing DES at the start of the reaction. This may also affect the toxicity during the wound healing process, as the resulting DES would have to be tested for human toxicity as well as the silver containing DES precursor. Adhikari *et. al.* has published three papers on AgNP synthesis in DES's, using a different DES in each publication, one of these opt

to use a DES which includes silver ions in the form of silver triflate: acetamide 1:4.[27] Out of all the AgNPs synthesised in DES's that was reviewed at the time, this is the only paper to incorporate the silver salt as part of the DES and not added afterwards, with no major changes in the AgNPs size or the synthesis conditions seen in **Table 8**.

In order to analyse the AgNPs in a TEM/SEM, they need to be extracted from the DES due to the DES exhibiting little to no vapour pressure, which means that they will not evaporate.[61] This is an issue since for TEM/SEM imaging, the AgNPs need to be stationary on a surface in order to image for size analysis. If the AgNPs were suspended in the DES, then they are free to move around the solvent resulting in blurry images. The addition of capping agents is needed so when the DES is broken down, the AgNPs won't aggregate and can be resuspended in a volatile solvent, that will evaporate leaving the AgNPs on a solid surface, for TEM/SEM analysis.

OAm was used as a capping agent in the Adhikari *et. al.* papers so the AgNPs can be extracted into toluene for analysis.[2,27,62] OAm has been used previously in organic solvent methods of AgNP synthesis but required different reaction conditions.[44] The Adhikari *et. al.* synthesis used a microwave reactor, which brought the reaction temperature down from 180 °C to 150 °C and the reaction time down from 1 hour to 30 seconds.[2] Using **Figure 8a** as an example of the sizes of AgNPs obtained from the work of Adhikari *et. al.* we can see that the size difference to organic synthesis is minimal and both reactions obtain AgNPs capped with OAm at ~10 nm. The use of a DES in this situation has shown to not impact the size of the AgNPs as well as making the reaction greener due to the lower reaction conditions.

Table 8: Comparison of literature for the synthesis of AgNPs looking at DES's used, the method of synthesis and the size of the AgNPs. * mg per gram of total reaction system. ** Data not available

DES	Additive	Time	Temperature /°C	Size /nm	Morphology	Yield	Reference
Choline nitrate:glycerol 1:2	Oleylamine	30 s	50-230	~10nm	Spherical	**	2
Silver Triflate:Acetamide 1:4	Oleylamine	2 min	200	15.7±2.1	Spherical	36.4 *	27
Dimethylammonium nitrate:triethylene glycol 1:1 Dimethylammonium nitrate:ethylene glycol 1:1 Dimethylammonium nitrate:glycerol 1:1	Oleylamine	19 min	180	7.0-15.1	Spherical	26 mg min^{-1}	62
ChCl:Glucose DASH hydrogel		20 min	Room Temperature	50-100	Spherical	*	60
Betaine:Glycerol 1:2 Glycerol:Urea 1:1 Betaine:Urea 1:2	Green tea extract, water	24 Hrs	Room Temperature	57.04±11.51 39.12±5.33	Spherical	58.2-94.8 %	26
D-glucose: urea: glycerol 1:1:2	Ascorbic Acid	30 min	Room Temperature	30.61	Elongated sphere	*	59
Lactic acid:Betaine 1:2	Grape Pomace, water	15 min	40	20±0.18	Spherical	*	63

However, using OAm is not ideal as this is a hazardous chemical and would only decrease the green credentials of a AgNP synthesis in DES's. Other papers have decided to combine the DES's with capping agents that are much less hazardous by using more natural products. Ascorbic acid was used alongside a DES as a reducing agent that would also aid with capping the AgNPs alongside the components of the DES.[59] This is an ideal solution to allow for the AgNPs to be characterised as well as keeping the reagents natural so there would be no issue in using the AgNPs-DES as a wound dressing. This was not the only paper to use more natural products, instead these papers look at using plant extracts which is a technique seen before with aqueous methods to cap the particles instead.[26,63] Whilst these more natural products are ideal for use as a wound dressing due to their low toxicity, the AgNPs produced were all >15 nm which is not ideal to use as antimicrobial materials.[30] An example of these AgNPs are seen in **Figure 8b**, where a solution of grape pomace was used as the capping agent for AgNP synthesis in a DES. The grape pomace was extracted using the DES itself before being added to an equivalent volume of silver nitrate aqueous solution and produced AgNPs of 20 nm.[63] Comparing this DES method with the state-of-the-art green methods seen in **Section 1.2.2** it can been seen that the reaction conditions are very similar. Both methods use low temperatures, however the addition of the DES can potentially lower the reaction time from up to a day to just 15 mins. For a large industrial scale synthesis of AgNPs this would make the DES method much more favourable due to a higher turnover per day of product.

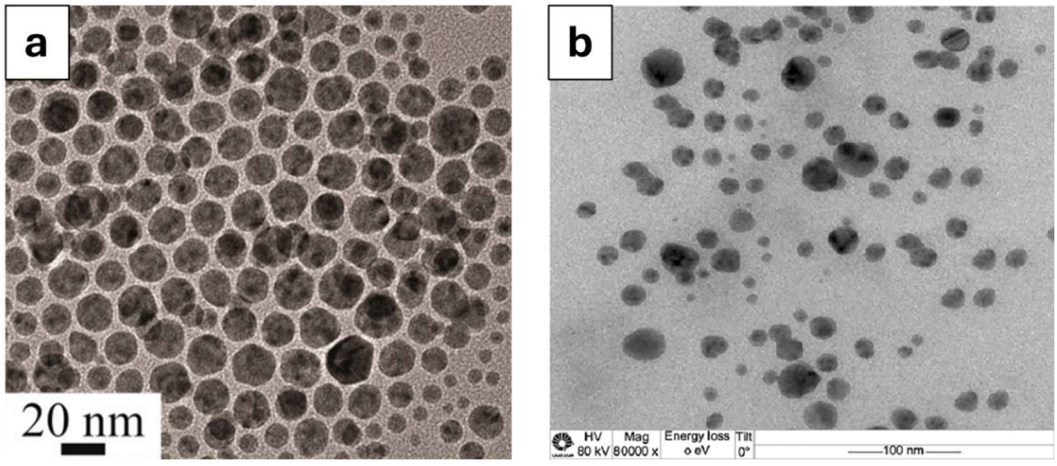


Figure 8: TEM analysis of AgNPs formed using a DES. a) DES was choline nitrate:glycerol 1:2, OAm used as the capping agent, heated in a microwave reaction at 150 °C for 30 s at 200 W.[2] b) DES was lactic acid:betaine 1:2, grape pomace/water was used as the capping agent, heated at 40 °C for 15 min.[63] (license obtained for reuse)

A more direct comparison to the biomolecule methods in **Section 1.2.2** and the DES methods for synthesising AgNPs would be to compare the methods using green tea extract (GTE). **Figure 7** shows the AgNPs synthesised for the biomolecule synthesis and has been discussed previously, whilst **Figure 9** shows the DES synthesis of AgNPs using GTE with 3 different DES's. As seen in **Figure 9**, 4 different DES's were used, and all show a difference in AgNP size. This could have a few different explanations to why this is, due to the different DES's used it could be that the physical properties of some of the DES's were less suited to extracting the necessary GTE required to fully encapsulate all the AgNPs in the solution, this resulted in larger AgNPs forming due to the lack of capping agent. There is also an argument that the DES's themselves are contributing to the capping of the AgNPs and thus the different DES's cap the AgNPs at different sizes.

Another possibility is that the aqueous solvent added during the GTE extraction step could have disrupted the H-bond network in the DES resulting in different physiochemical properties which have also affected the size of the AgNPs.[15] For the purpose of antimicrobial activity, the DES that shows the smallest size is the $GU_{(h)}$ DES which was Glycerol:Urea 1:1 synthesised at a higher temperature of 80 °C, which was stated in **Figure 9** to be 14.31±2.30 nm. This range of AgNPs are on the larger size of the range needed for antimicrobial activity, however when compared to the state-of-the-art synthesis the AgNPs have greater size control. With the correct reaction conditions and DES chosen this size could be decreased further putting the size range within the acceptable limit. With regards to the reaction conditions, it is difficult to compare the reaction time since the time taken was not stated in the state-of-the-art method, however the DES method did require 24 Hrs to synthesise. This could be a longer synthesis and is not ideal for industrial scale up if there are quicker methods available. The temperature of the DES reaction was lower (at room temperature) compared to the state-of-the-art method which would require less energy to synthesise. Out of these comparisons it is difficult to decide which method is most suitable for antimicrobial AgNP synthesis due to each having their own advantages and disadvantages depending on the situation.

There was another publication reviewed that uses peach pomace as a capping agent, however in the literature itself, it does not directly specify how the AgNPs were synthesised so was omitted from the comparison due to require more speculative analysis on the method used, however the AgNPs produced were 10-20 nm in size so would make them ideal for antimicrobial uses.[64]

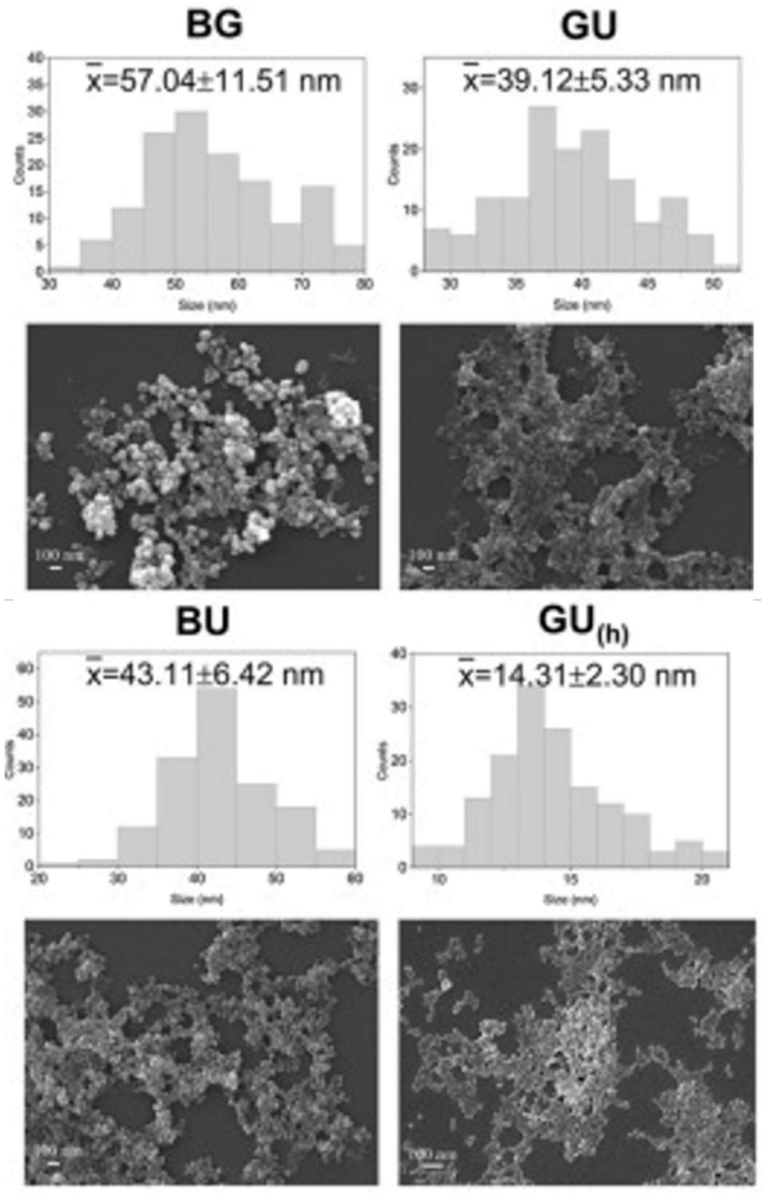


Figure 9: SEM images from the work of Liu *et. al.* synthesising AgNPs in a DES with green tea extracts (GTE). These reactions were performed at room temperature, stirring overnight in the dark. BG = Betaine:Glycerol 1:2, GU = Glycerol:Urea 1:1, BU = Betaine:Urea 1:2 and $GU_{(h)}$ = Glycerol:Urea 1:1 synthesised at a higher temperature ~80 °C.[26] *

It should also be mentioned that for most of the DES methods discussed in **Table 8**, the DES was added to another solvent during the synthesis. When a volume of additional solvent is added to the DES it can start to break down the H-bonds and no longer be classified as a DES and instead is just a solution of its components. This volume has been stated to be around 50% v/v for a variety of DES's that have been tested, but depending on the DES the water content could be greater or lower before it breaks down.[15,65]

* Reprinted from Journal of Cleaner Production, 379, Yuli Liu, Seulgi Kang,Ke Li,Jingyan Chen,Boyeon Bae,Inseon Hwang,Eun-Young Ahn,Youmie Park,Kwang-Hoon Chun,Jeongmi Lee, Ecofriendly and enhanced biogenic synthesis of silver nanoparticles using deep eutectic solvent-based green tea extracts, 134655, Copyright (2022), with permission from Elsevier.

In these methods, water, as part of an aqueous solution of silver salt, or OAm was added to the DES's which would most likely result in the DES H-bonds being disrupted and potentially breaking down the DES. This is especially the case with the addition of water at ~ 50% v/v, whilst these methods have been reviewed as DES methods, due to the amount of water added some of these may be better classified as aqueous methods instead. The only methods that could be considered to be true DES methods, was the work by Adhikari *et. al.* since the addition of hydrophobic OAm would not be miscible with the more hydrophilic DES's used and would have had minimal interactions and disruptions with the DES. The downside to using OAm is introducing a reagent that is harmful to humans and could not be used as a wound dressing.

All the methods of synthesis used in **Table 8** use a hotplate to stir and heat the sample. Publications from Adhikari *et. al.* are the only researchers that have currently synthesised AgNPs using a microwave reactor.[2,27] This is due in part to the requirement of high temperatures needed for both these synthesises (between 50-230 °C) which is much easier to achieve in a microwave reactor than on a hotplate due to the high energy inputs. However, as discussed previously, using a microwave reactor for long periods of time is not as sustainable and green as a hotplate. When looking at the best method for synthesizing AgNPs with the aim of having an antimicrobial wound dressing, sustainability is also an important factor due to the increased push for more sustainable practices in industry, during this project the mindset of developing a sustainable synthesis was just as important as developing the antimicrobial wound dressing to ensure that the methods would be suitable for scale up in industry.

Out of the Adhikari *et. al.* papers, the method using Choline Nitrate:Glycerol 1:2 was chosen to analyse RAE and E-factor,[2] due to the AgNPs having the correct size range for antimicrobial properties, as well as the synthesis using a DES that is formed from components that are non-hazardous and biocompatible with human tissues. The aim of this project is to use the AgNPs alongside the DES for the antimicrobial wound dressing, so the calculations of RAE, E-factor and PMI will be performed, one where the AgNPs are extracted, and another where they are not. Taking into account that OAm is not miscible with the Choline Nitrate:Glycerol 1:2 it was decided to include the mass of the OAm as a waste product for the non-extracted AgNPs calculation, it is to be noted however that the inclusion of the OAm is to extract the AgNPs from the DES so would not be required. The RAE, E-Factor and PMI was calculated using **Table 9** and the known volumes of the solvents used, which were converted to mass (20.17 g).

Table 9: Mass of reactants and products for green metric calculations of AgNPs synthesised by Adhikari *et.al.*[2] Calculations performed in Excel without rounding values.

Reactants	Mass /g	Products	Mass /g
$AgNO_3$	0.005	**AgNPs**	0.005
DES	1.0	**DES**	1.0
OAm	0.3		

$$Real\ Atom\ Economy\ \ (\%) = \frac{0.005 + 1.0\ g}{0.005 + 1.0 + 0.3\ g} x100 = 77.0\%$$

$$Environmental\ factor\ (AU) = \frac{20.17 + 0.3\ (g)}{0.005 + 1.0\ \ (g)} = 20.4$$

$$Process\ mass\ intensity\ (AU) = \frac{20.17 + 0.3 + 0.005 + 1.0\ (g)}{0.005 + 1.0\ (g)} = 21.4$$

The RAE calculated for the non-extracted AgNPs was ≤77.0% (assuming a completed reaction, similar with previous sections), which would be ≤100% if the OAm was not included. The formation of a DES is 100% atom economy since all components in the mixture are used to form the DES, which means in the AgNP synthesis without the OAm the only waste would be the nitrate ions in solution after the reduction of the silver ions. Since these nitrate ions will be <5 mg in a 1 g solution of DES, the mass differences are negligible. The E-Factor for using AgNPs in a DES was calculated to be ≥8.16 and the PMI calculated to be ≥9.16, including the waste products, the OAm and the ethanol used to dry the $AgNO_3$ before the synthesis. This DES synthesis has the lowest E-factor and PMI out of the previously calculated values for the aqueous synthesis, organic synthesis and the state-of-the-art synthesis in previous sections, however, the previous calculations were performed when the AgNPs were suspended in either an aqueous or organic solvent and whilst this project intends to use these AgNPs in a DES, Adhikari *et.al.* performed an extraction step after synthesis to suspend the AgNPs in toluene which needs to be accounted for.

Whilst the RAE would be affected by the extraction into toluene due to loss of product during this step, the known mass of obtained AgNPs is unknown and cannot be included in the calculation, however the E-Factor including this extraction step was calculated to be ≥20.4 and PMI was calculated to be ≥21.4. The extraction step to remove the AgNPs from the DES to suspend in the toluene, increased the E-factor

and PMI so that this method was more environmentally impactful that the organic synthesis and the state-of-the-art green synthesis. This highlights the importance of using the AgNPs within the DES itself to eliminate the need for extracting the AgNPs and reducing the environmental impact.

With an objective of making an antimicrobial wound dressing using AgNPs DES's, some of these literature methods are more suitable than others due to the size of AgNPs synthesised. The work of Adhikari *et. al.* produce AgNPs that are the correct size for antimicrobial properties, however the capping agent chosen is not ideal due to the hazardous nature of OAm. However, this is a good starting point for further development of a greener, less toxic synthesis and can be used as inspiration going forward.

1.2.4 Antimicrobial Testing of Deep Eutectic Solvents

Antimicrobial testing of NPs has previously been performed in the literature and has shown that they have numerous disruptive pathways to cause toxicity in prokaryotic cells.[30] AgNPs in this case have been chosen as the antimicrobial NP for the wound dressings alongside a DES. AgNPs that are synthesised through a variety of different methods and thus have different capping agents on the surface, have been tested for their antimicrobial activity. In most cases a disk diffusion assay as well as minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assay has been performed. AgNPs are a well-known antimicrobial agent already established in the literature.[44,47,55] This study will investigate AgNPs synthesised in a DES to be used as an antimicrobial wound dressing, so the DES itself will be included as part of the dressing and hopefully will contribute to the antimicrobial properties.

The antimicrobial activity of different DES's have been studied and reported in the literature, which has shown that some DES have greater antimicrobial activity than current antibiotics.[29] However, upon further investigation some concerns do arise from the methods used. The main concern is that the DES is usually dissolved in either water or DMSO to perform these studies and due to the volume of water used, the DES's may break down into their original components and therefore an antimicrobial study will have been performed on a solution of the DES components and not of the DES themselves, which have distinct properties from the aqueous solution of the DES components.

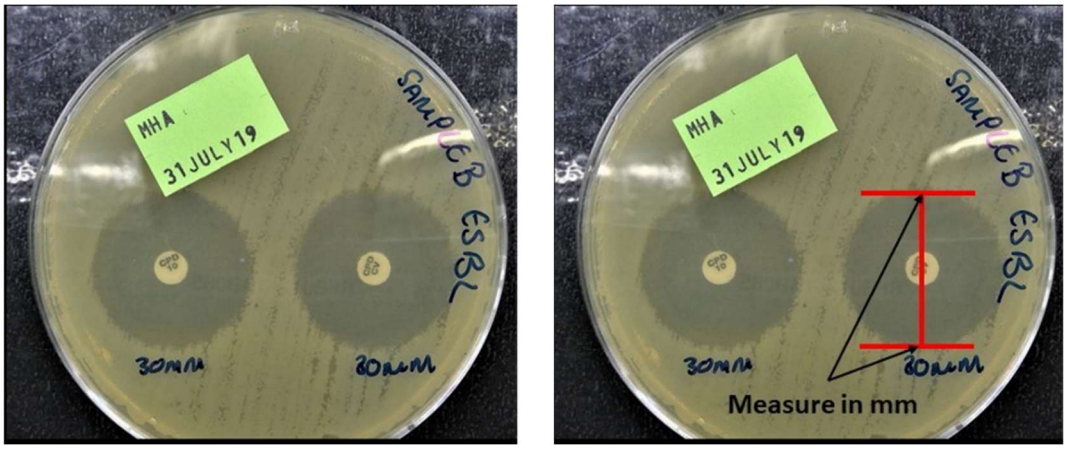


Figure 10: Example of a disk diffusion assay showing the antimicrobial impregnated sterile disk and the area of antimicrobial effect (zone of inhibition) against a strain of *Escherichia coli*. Image supplied by Samantha McLean, NTU.

One such technique found in the literature for testing of DES's for antimicrobial activity is a disk diffusion method, in this method the DES is dissolved in DMSO used to saturate a sterile filter paper disk. The disk is then applied to an agar plate inoculated with a culture of chosen bacteria.[29] Since the DES was dissolved in DMSO, it is very likely the DES has broken down into its original components. This does not accurately reflect the antimicrobial properties of the DES's themselves since the large hydrogen bond structure has not been tested. Inayat *et. al.* also looked at the antimicrobial and antifungal properties of DES's by disk diffusion method and whilst not stating whether the DES's are diluted in water or DMSO, they do explain how serial dilutions of the DES's were tested.[66] However unlike the previous publication, a pure DES was tested as well. The results of the pure DES tested against both *Gram*-positive and *Gram*-negative bacteria in general shows that the pure DES performed better than the diluted versions, this is what would be expected since the DES structurally is different to the solution of components. However, the error shown for the pure DES's are much higher than the solutions which could indicate that the DES does not perform consistently, which would be an issue if used as a commercial wound dressing. However, this could be an artefact of the assay design, rather than performance of the DES due to the viscosity of the DES itself, how it is applied, and how well it can diffuse throughout the agar. A higher viscosity would likely diffuse less throughout the agar resulting in smaller measurements; however, the water content of the agar itself may interact with the DES resulting in a lower viscosity due to the change in water content.[15] With regards to the AgNP-DES wound dressing that this project is focusing on, these results only give an idea of half of the project, whilst some of the data in these papers are questionable, it does show that different DES's

have different antimicrobial properties so developing the right DES for the job is just as critical as development of the AgNPs.

Some of the reviewed papers have already looked at using a DES as part of a wound dressing, however they have taken a different approach and instead synthesised a DES hydrogel. One such example has been already mentioned above in **Section 1.2.2** in which AgNPs were synthesised on a DES-DASH hydrogel.[60] This Ag-hydrogel was tested for antimicrobial activity using a disk diffusion assay instead deciding to use an Oxford cup, which is a small stainless steel cylinder containing the sample to be tested that can be placed in the agar to measure the zone of inhibition. This method works the same as described above but instead used the Oxford cup in the centre of the agar and measures the zone of inhibition around it.

Figure 11 shows the reported results of the antimicrobial inhibition zone of this DES-DASH hydrogel with AgNPs, the zone of inhibition graph (**Figure 11b**) shows that the DES-DASH hydrogel with AgNPs has an increased antimicrobial activity compared to that of just the DES-DASH hydrogel, which would indicate that having a dual action antimicrobial wound dressing with both the DES as well as the AgNP would have an increased effectiveness. Whilst this research looks promising there are a few observations, as discussed above in **Section 1.2.2** this method to synthesise the nanoparticles uses aqueous media and as such the control of the size of the AgNPs is much less than if the AgNPs were synthesised in the DES itself. The size range reported was between 50-100 nm which are outside the range of antimicrobial effectiveness.[30,60]

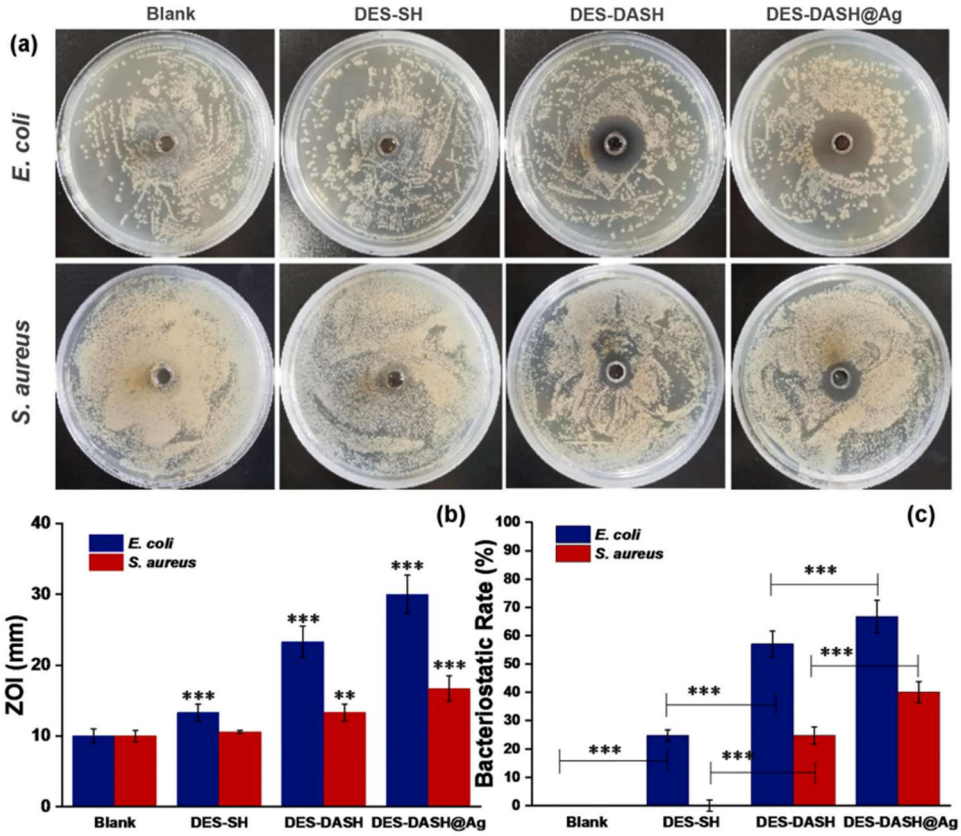


Figure 11: a) Agar plates showing the inhibition zone around the Oxford cup showing the blank as well as the different DES hydrogels tested and the AgNP DES-DASH hydrogel. b) shows the measured diameter of the zone of inhibition on the agar plates. c) shows the bacteriostatic rate of the blank, hydrogels and the Ag DES-DASH hydrogel with statistical significance plotted determined using a one-way ANOVA/Fisher test.[60] *

The work from Wang *et. al.* also looked at a DES hydrogel however without the AgNPs.[67] The results of this study also showed that the DES hydrogel was antimicrobial and focused more on changing the type of hydrogel formed to show the different effectiveness. With an objective of this project to synthesise the AgNPs within the DES to then use as an antimicrobial wound dressing, forming the hydrogel first would not be suitable, however the work on the hydrogel is still useful for showing the difference in antimicrobial activity when the AgNPs are present alongside a DES.

* Reprinted from International Journal of Biological Macromolecules, 191, Wen Li, Xinyao Zhao,Ting Huang,Yu Ren,Wanxin Gong,Yuhui Guo,Jinyi Wang,Qin Tu, Preparation of sodium hyaluronate/dopamine/AgNPs hydrogel based on the natural eutetic solvent as an antibaterial wound dressing, 60-70, Copyright (2021), with permission from Elsevier.

The other tests that were performed on DES's were MIC and MBC assays, an example of which can be seen in **Figure 12**. To perform these tests, the DES's would be added to an equal volume of nutrient broth before a serial dilution is performed. An inoculation of bacteria is then added to the wells including a positive control which shows the nutrient broth can support the growth of bacteria. A negative control is also used to show the broth is not contaminated before or during the assay. After a set time the MIC can be determined (commonly after overnight incubation at the appropriate temperature for growth of the bacterial species), and aliquots of the wells can be spotted onto agar to see if any bacteria survived exposure. If no bacteria grow, then we can determine the MBC.

Already there is an issue with this method with regards to DES analysis, since the DES is added to equal volumes of broth this would break down the DES and instead form an aqueous solution of its components.[15] However, there have been reports of DES MIC assays in the literature, which have reported that the DES shows a greater antimicrobial effect than that of the individual components.[29] This is more likely due to the dual action of two components in solution against the bacteria culture and not to the DES itself. MIC and MBC assays are generally a staple of antimicrobial testing in the literature but would not be a suitable method for testing DES's. However, since these assays are well documented for other antimicrobial agents, these tests would be required in this project as a direct comparison with the current literature but are not to be stated to be a definitive value for the AgNPs DES's developed.

Since the wound dressings being developed will contain AgNPs, looking through the current literature for how previous researchers have analysed the antimicrobial properties of AgNPs-DES's is crucial. The work from Vorobyova *et. al.* was previously reviewed as part of the AgNPs synthesis in DES's, but this was not the only focus of the research, they then went on to test the AgNP-DES solution for antimicrobial properties.[63] Similarly to the previous publications, these solutions were also tested using a disk diffusion assay, however unlike Inayat *et. al.* the AgNP-DES is tested after already having the DES diluted in water since the synthesis steps required used equal parts silver nitrate solution and DES.[30,60] In order to get the most representative antimicrobial activity of the AgNPs DES's synthesised in this project, a new method for antimicrobial testing will have to be developed to reduce the amount of water that would need to be added to the DES for testing.

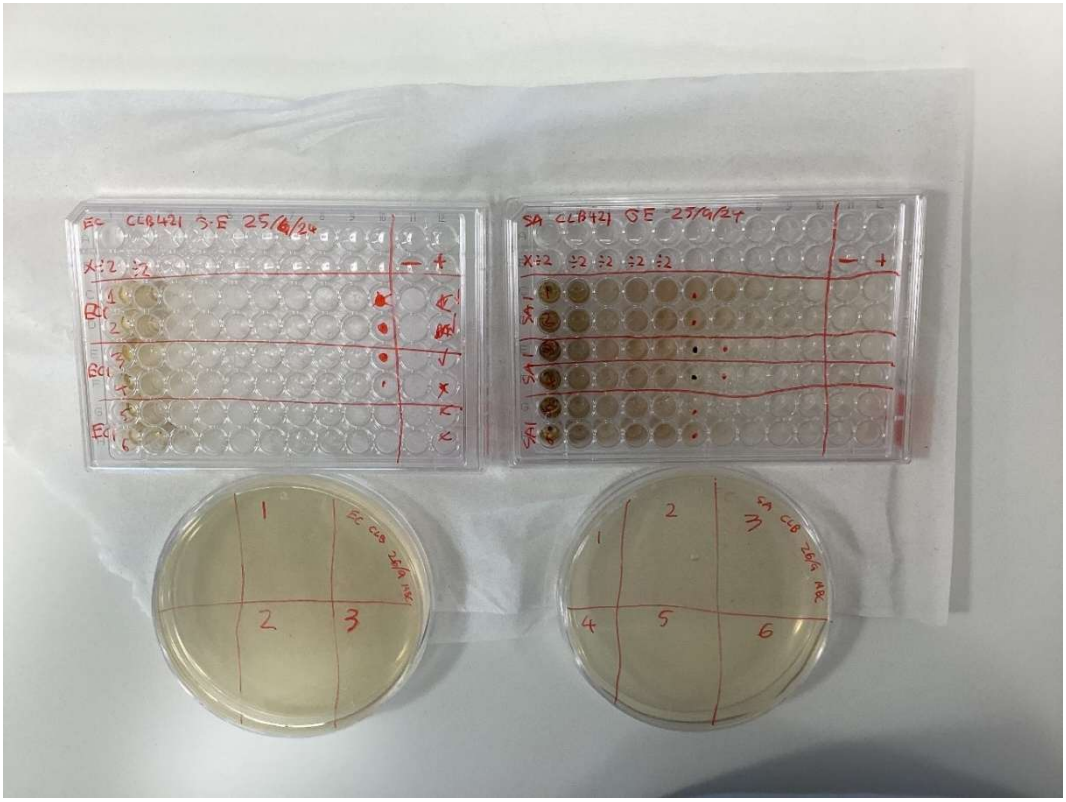


Figure 12: Example of MIC and MBC assays performed on AgNP DES's synthesised in this project. The 96 well plates show the MIC assays including the two control tests, and the agar plates show the MBC assays showing whether or not any bacteria survived to form colonies after the MIC study.

Chapter 2: Methodology

2.1 Materials

2.1.1 Deep Eutectic Solvent (DES) and silver nanoparticle (AgNP) synthesis materials

The following chemicals were used in these experiments: Betaine, 98 % anhydrous ($C_5H_{11}NO_2$) supplied by Acros Organics. Glycerol, 99+ % ($C_3H_8O_3$), choline bitartrate, 98+% ($C_9H_{19}NO_7$), malonic acid, 99% ($C_3H_4O_4$), L-proline, 99% ($C_5H_8NO_2$), glutaric acid, 99% ($C_5H_8O_4$), D-(+)-glucose, anhydrous, 99% ($C_6H_{12}O_6$) was supplied by Alfa Aesar. Silver sulphate, ≥99.5 % (Ag_2SO_4), oleylamine, technical grade 70 %($C_{18}H_{37}N$), decanoic acid, natural ≥98 %, FCC, FG ($C_{10}H_{20}O_2$), levulinic acid, ≥97 %, FG ($C_5H_8O_3$), urea, ≥99.5% (CH_4N_2O), oxalic acid, 98% ($C_2H_2O_4$), citric acid, ≥99.5% ($C_6H_8O_7$), maleic acid, ≥99.0% ($C_4H_4O_4$) was supplied by Sigma-Aldrich. Methanol, ≥99.9 % (CH_3OH), toluene, low in sulphur laboratory reagent grade (C_7H_8), acetone, analytical reagent grade ≥99.8 % (C_3H_6O), lauric acid, 99 % ($C_{12}H_{24}O_2$), nitric acid, 68-70% (HNO_3), ethanol, 99.8% analytical reagent grade (C_2H_6O) was supplied by Fisher Scientific. Malonic acid, 99% ($C_3H_4O_4$) supplied by Thermoscientific. Silver nitrate ($AgNO_3$) was supplied by Fluorochem. All reagents were used as received.

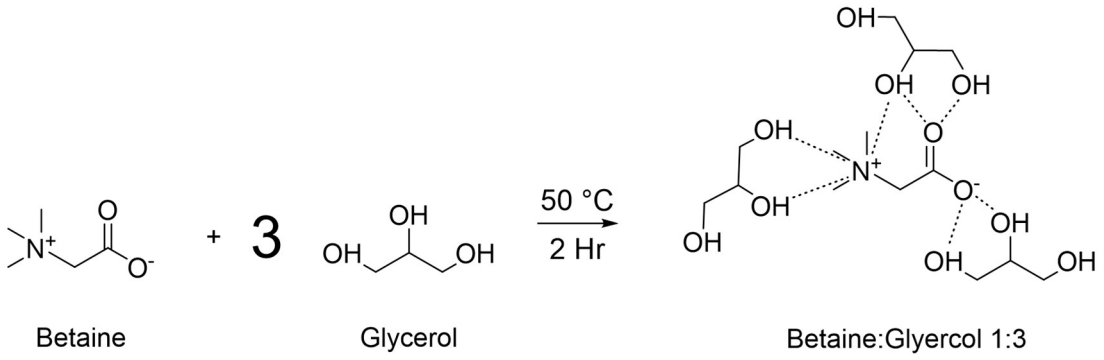
Ultra-high quality water was generated at ≥18.2MΩ

2.1.2 Antimicrobial testing materials

A *Gram*-positive bacterial strain; *Staphylococcus aureus* (*S. aureus*) was supplied by the Nottingham University Hospitals NHS Trust pathogen bank and a *Gram*-negative bacterial strain *Escherichia coli* (*E. coli*) was supplied by the University of Sheffield Poole group were used in this study. These strains were chosen due to the bacteria being isolated from wounds or infections in the body.

Media used to cultivate strains were, Mueller-Hinton Agar (MHA) used for both *S. aureus* and *E. coli growth*, Mannitol Salt Agar (MSA) used for *S. aureus*, MacConkey agar (MCA) used for *E. coli*, Mueller-Hinton Broth (MHB) used with both strains were supplied from ThermoFisher Scientific. Phosphate buffered saline tablets (PBS) were supplied from Sigma-Aldrich. All reagents were used as received.

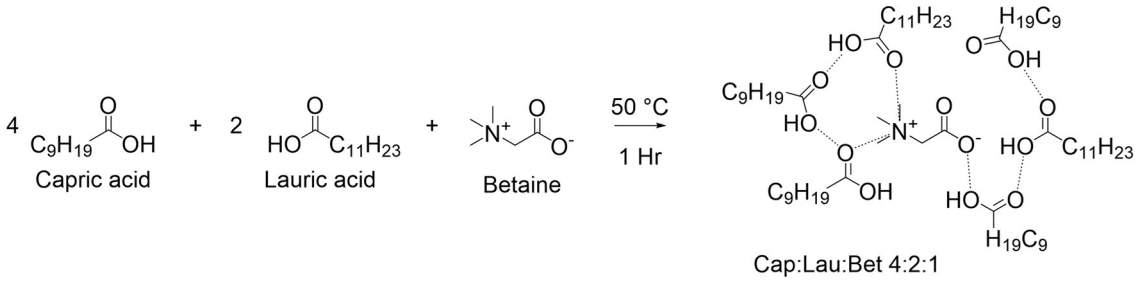
2.2 Preparation of Deep Eutectic Solvent (DES) Inspired Molecular Liquids



Scheme 1: Formation of a Betaine:Glycerol 1:3 DES with one possible structure of the DES (unconfirmed structure).

To synthesise the Betaine:Glycerol 1:3 DES, betaine (Bet) (1 molar equivalent) and glycerol (Gly) (3 molar equivalent) were added to a Duran bottle with a magnetic stirrer bar. The stoppered flask was shaken (*ca.* 1 min) before removing the top and heated (50 °C, *ca.* 3 Hr) with stirring (200 RPM) resulting in a single-phase colourless liquid. When necessary (*ca.* 30 min), the sample was capped and inverted to prevent agglomeration of the betaine. The sample was then capped and allowed to cool to room temperature before further use.

Viscosity (@ 25 °C) = 1556±18.75 mPa.s. Conductivity (@ 25 °C) = 0.14 μS cm^{-1}. Surface tension (@ 21 °C) = 65.17 mN m^{-1}. Density (@ 21 °C) = 1.23 g mL^{-1}. Refractive index (@ 22 °C) = 1.4795.



Scheme 2: Formation of a Capric acid:Lauric acid:Betaine 4:2:1 molecular liquid with one possible structure of the molecular liquid (unconfirmed structure).

To synthesise the Capric:Lauric:X DES inspired molecular liquids (where X= betaine (Bet), levulinic acid (Lev), L-proline (Pro), urea (Ure), glutaric acid (GA), choline bitartrate (ChBit), glycerol (Gly), malonic acid (MA), maleic acid (Mal), oxalic acid (OxA), glucose (Glu), citric acid (CA)), capric acid (Cap), lauric acid (Lau) and X at different molar equivalents (see **Table 10**) were added to a small test vial with a

magnetic stirrer bar (see **Figure 13** for structures). The unstoppered sample vials were heated (50 °C, *ca.* 30 min) with stirring (200 RPM) resulting in a single-phase colourless liquid. When necessary (*ca.* 1 Hr), if a single-phase colourless liquid had not formed, the temperature was increased (*ca.* 10 °C increments), this was repeated until a single-phase colourless liquid formed or the temperature had reached 120 °C, see **Table 10** for the formation time. The samples were then capped and allowed to cool to room temperature.

The maximum temperature (120 °C) was chosen due to the aims of the project to produce a greener and more sustainable synthesis, which ideally would require lower temperatures (50-80 °C) to form the molecular liquids, to reduce the energy usage required to heat the samples over the synthesis time (*ca.* 1 Hr). See **Table 10** for molar ratios that formed.

To synthesise larger quantities of the Capric acid:Lauric acid:betaine 4:2:1 molecular liquid, capric acid (Cap) (4 molar equivalent), lauric acid (Lau) (2 molar equivalent) and betaine (Bet) (1 molar equivalent) were added to a Duran bottle with a magnetic stirrer bar. The stoppered flask was shaken (*ca.* 1 min) before removing the top and heated (50 °C, *ca.* 1 Hr) with stirring (200 RPM) resulting in a single-phase colourless liquid. The sample was then capped and allowed to cool to room temperature before further use.

Viscosity (@ 35 °C) = 65.7±0.75 mPa.s. Conductivity (@ 35 °C) = 0.73 μS cm^{-1}. Surface tension (@ ~35 °C) = 31 mN m^{-1}.

To synthesise larger quantities of the Capric acid:Lauric acid:levulinic acid 2:1:1, 4:2:1 and 8:4:1 molecular liquid, capric acid (Cap), lauric acid (Lau) and levulinic acid (Lev) were added to a Duran bottle with a magnetic stirrer bar. The stoppered flask was shaken (*ca.* 1 min) before removing the top and heated (50 °C, *ca.* 1 Hr) with stirring (200 RPM) resulting in a single-phase colourless liquid. The sample was then capped and allowed to cool to room temperature before further use.

Cap:Lau:Lev 2:1:1 Viscosity (@ 25 °C) = 11.91±0.15 mPa.s. Conductivity (@ 25 °C) = 0.01 μS cm^{-1}. Surface tension (@ 21 °C) = 32.7 mN m^{-1}. Density (@ 21 °C) = 0.925 g mL^{-1}. Refractive index (@ 22 °C) = 1.4370.

Cap:Lau:Lev 4:2:1 Viscosity (@ 25 °C) = 11.22±0.15 mPa.s. Conductivity (@ 25 °C) = 0.00 μS cm^{-1}. Surface tension (@ 21 °C) = 32.2 mN m^{-1}. Density (@ 21 °C) = 0.907 g mL^{-1}. Refractive index (@ 22 °C) = 1.4375.

Cap:Lau:Lev 8:4:1 Viscosity (@ 25 °C) = 10.87 ± 0.15 mPa.s. Conductivity (@ 25 °C) = 0.00 μS cm^{-1}. Surface tension (@ 21 °C) = 33.0 mN m^{-1}. Density (@ 21 °C) = 0.898 g mL^{-1}. Refractive index (@ 22 °C) = 1.4380.

All molecular liquids formed were characterised using available physiochemical property testing methods (viscometry, conductivity, refractive index, density and surface tension) to determine if the molecular liquid had formed. See **Section 2.5** for methods.

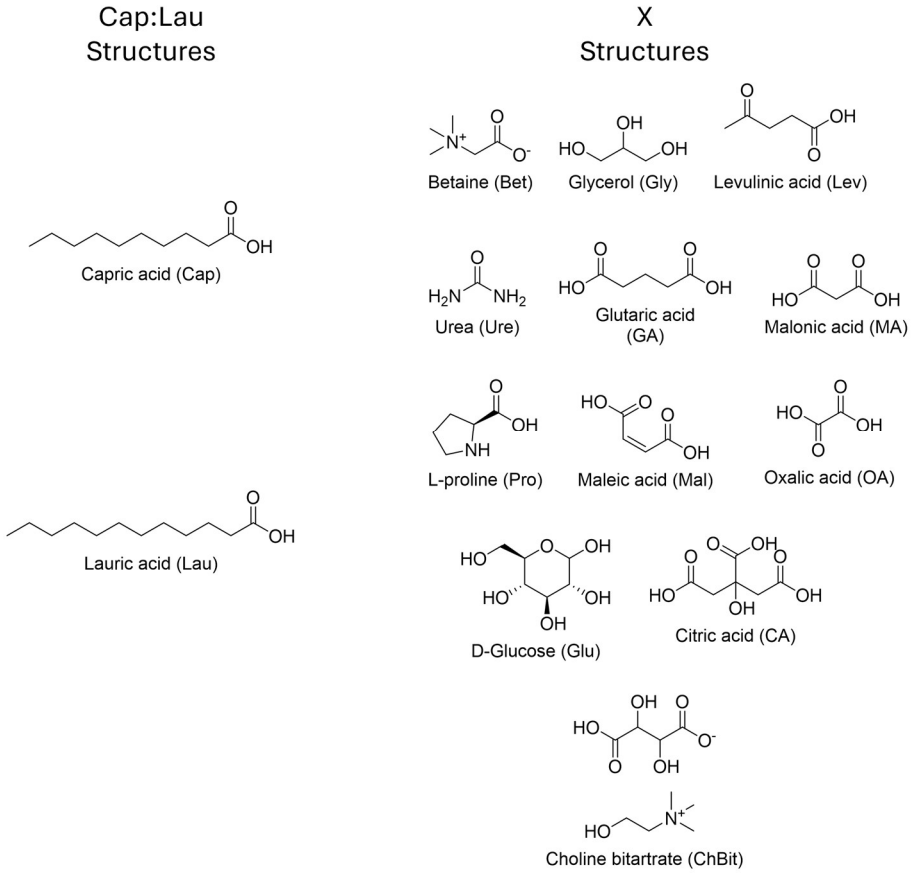


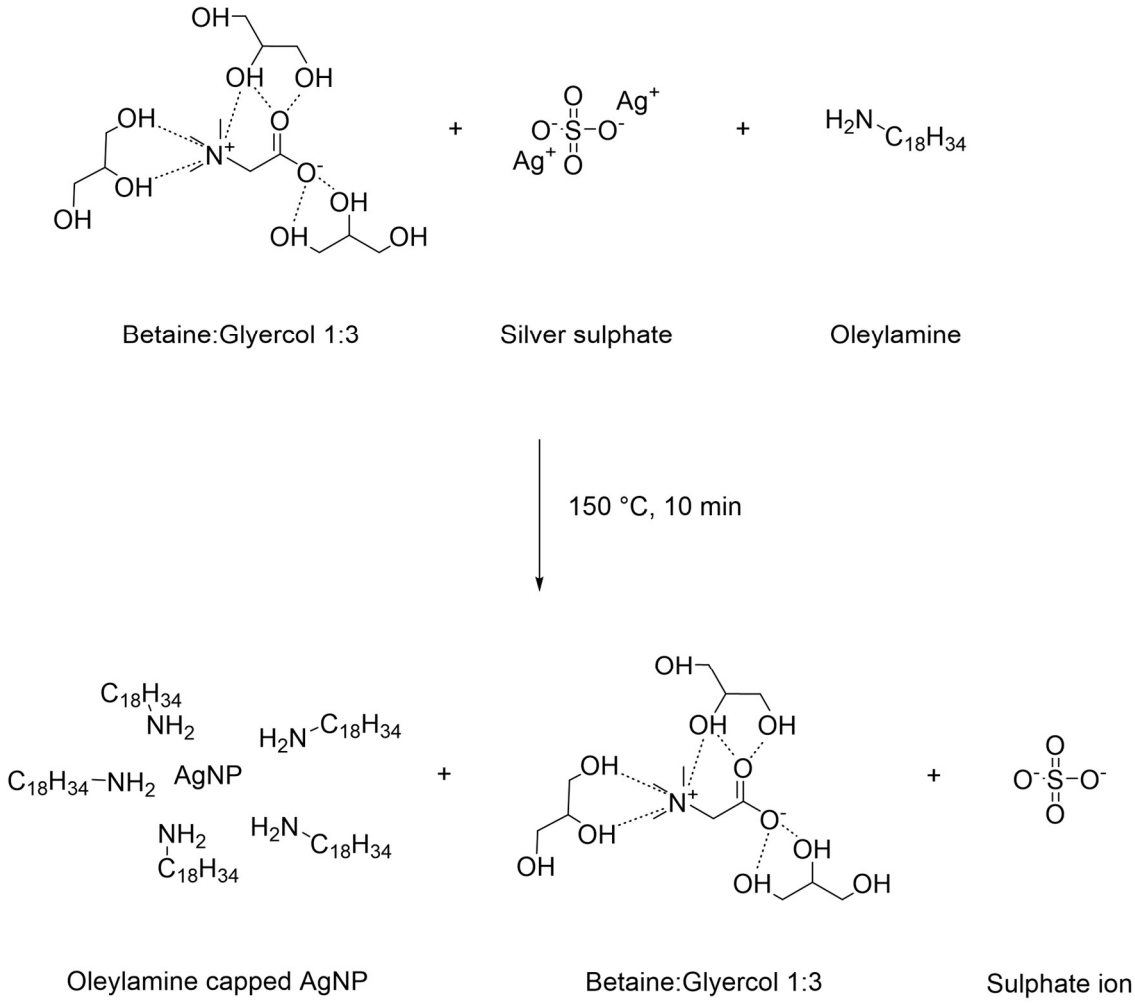
Figure 13: Structures of components tested in synthesising Cap:Lau:X molecular liquids.

Table 10: Cap:Lau:X components use and ratios that successfully formed a molecular liquid with the temperature range and time required at which the molecular liquids formed.

DES Components	Ratios the DES formed	Temperature of formation /°C	Time required for formation /Hr
Cap:Lau:Bet	1:1:1, 2:1:1, 4:2:1, 1:1:2, 1:2:1, 2:2:1	50	1
Cap:Lau:Pro	2:1:1, 4:2:1, 1:2:1, 2:2:1	50-70	1-3
Cap:Lau:ChBit	No ratios formed		
Cap:Lau:Ure	1:1:1, 2:1:1, 4:2:1, 1:2:1, 2:2:1	70-120	3-8
Cap:Lau:Gly	No ratios formed		
Cap:Lau:GA	2:1:1, 4:2:1, 1:2:1, 2:2:1	50-80	1-4
Cap:Lau:MA	No ratios formed		
Cap:Lau:Mal	2:1:1, 4:2:1, 2:2:1,	100-120	6-8
Cap:Lau:OxA	No ratios formed		
Cap:Lau:Glu	No ratios formed		
Cap:Lau:CA	No ratios formed		
Cap:Lau:Lev	1:1:1, 2:1:1, 4:2:1, 1:1:2, 2:2:1, 8:4:1	50	1

2.3 Preparation of AgNPs using a DES

2.3.1 AgNP synthesis in Bet:Gly 1:3 DES with oleylamine (OAm)



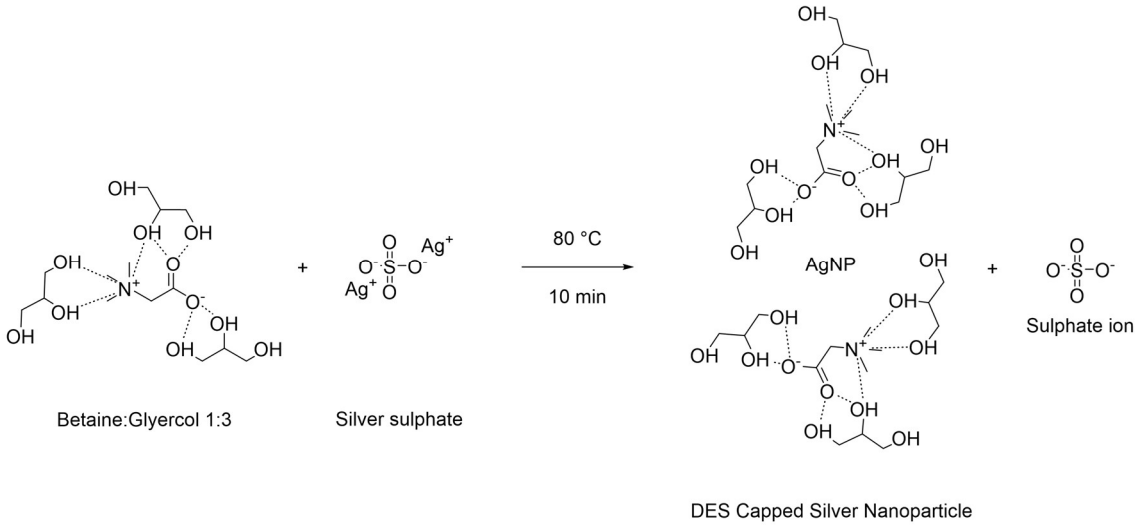
Scheme 3: A potential reaction scheme for the synthesis of silver nanoparticles capped with a Oleylamine. Reaction scheme is unknown.

To synthesise silver nanoparticles (AgNPs) in a Bet:Gly 1:3 DES, Oleylamine (OAm) (2-4 mL) was added, using a Finnpipette ML42083A (Fisher), into a round bottom flask, dried in an oven (*ca.* 16 Hr, 80°C), with a magnetic stirrer, followed by Bet:Gly 1:3 DES (*ca.* 5 mL), measured in a 10 mL plastic syringe. This results in a two-phase system, with the clear yellowish OAm as the upper phase and the clear colourless Bet:Gly 1:3 DES as the bottom phase. A glass coated temperature probe (Radleys) was inserted into the mixture and the sample heated (120-150 °C, *ca.* 10 min) with stirring (300 RPM), resulting in a cloudy cream single-phase liquid.

Once at temperature, silver sulphate (Ag_2SO_4) (*ca.* 25 mg) was added to the round bottomed flask and a timer started (5 min - 7 min 30 sec). Resulting in a change from a cloudy cream liquid to a cloudy orange/brown liquid as the AgNPs were synthesised. The round bottomed flask was removed from the heat (*ca.* 1 min) and MeOH added (*ca.* 10 mL) quenching the reaction. Resulting in a clear brown liquid with some AgNPs precipitated at the bottom of the flask. The sample was used immediately.

λ_{max} = 410.0 nm. DLS diameter size = 16.97±2.99 nm. TEM diameter size = 10±2.4 nm.

2.3.2 AgNP synthesis in Bet:Gly 1:3 without oleylamine (OAm)



***Scheme* 4**: A potential reaction scheme for the synthesis of silver nanoparticles capped with a betaine:glycerol 1:3 DES with one possible structure of the DES capping the AgNP (unknown capping of the AgNP). Reaction scheme is unknown.

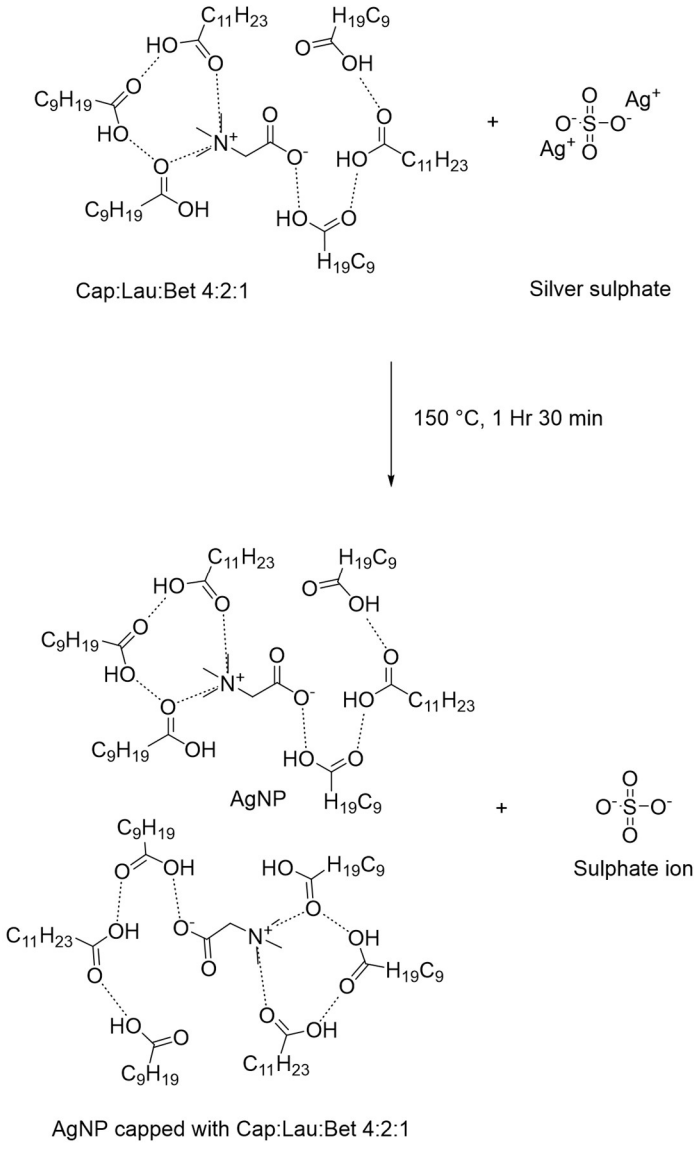
Following the method in **Section 2.3.1** with the alteration of not using OAm at the start of the synthesis.

To synthesise silver nanoparticles (AgNPs) in a Bet:Gly 1:3 DES, Bet:Gly 1:3 DES (*ca.* 5 mL), measured in a 10 mL plastic syringe was added to a dry round bottom flask, dried in an oven (*ca.* 16 Hr, 80°C), with a magnetic stirrer. This results in a single-phase system with a clear colourless Bet:Gly 1:3 DES. A glass coated temperature probe (Radleys) was inserted into the DES and the DES was heated (80-150 °C, *ca.* 10 min) with stirring (300 RPM). Once at temperature, silver sulphate (Ag_2SO_4) (*ca.* 25 mg) was added to the round bottomed flask and a timer started (5-15 min). Resulting in a change from a clear colourless liquid to a clear orange/brown liquid as the AgNPs were synthesised. The round bottomed flask was removed from the heat and allowed to cool by air flow from the fume hood (*ca.* 5 minutes). If

necessary, once cooled, OAm (*ca.* 3 mL) was added to the round bottom flask and stirred (*ca.* 30 min, 100 RPM), resulting in a colour changed from a clear brown liquid to a cloudy brown liquid. MeOH (*ca.* 10 mL) was added to breakdown the DES resulting in a clear brown liquid with AgNPs precipitated at the bottom of the flask.

Yield = 61.4 %. λ_{max} = 421.2 nm. DLS diameter size = 12.78±2.22 nm. TEM diameter size = 13±1.94 nm.

2.3.3 AgNP synthesis in Cap:Lau:Bet 4:2:1



Scheme 5: A potential reaction scheme for the synthesis of silver nanoparticles capped with a capric acid:lauric acid:betaine 4:2:1 molecular liquid with one possible structure of the molecular liquid capping the AgNP (unknown capping of the AgNP). Reaction scheme is unknown.

To synthesise silver nanoparticles (AgNPs) in a Cap:Lau:Bet 4:2:1 molecular liquid, solid waxy Cap:Lau:Bet 4:2:1 was heated (*ca.* 40 °C) in a water bath, resulting in a clear colourless liquid. The melted Cap:Lau:Bet 4:2:1 (5 mL) was added to a dry round bottom flask, dried in an oven (*ca.* 16 Hr, 80°C), with a magnetic stirrer. This results in a single-phase system with a clear colourless Cap:Lau:Bet 4:2:1 molecular liquid. A glass coated temperature probe (Radleys) was inserted into the molecular liquid and the sample was heated (140-160 °C, *ca.* 10 min) with stirring (300 RPM). Once at temperature, silver sulphate (Ag_2SO_4) (*ca.* 25 mg) was added to the round bottomed flask and a timer started (1-3 Hr). Resulting in a change from a clear colourless liquid to a clear orange/brown liquid as the AgNPs were synthesised. The round bottomed flask was removed from the heat and allowed to cool by air flow from the fume hood (*ca.* 5 minutes). If necessary, once cooled, OAm (*ca.* 3 mL) was added to the round bottom flask and stirred (*ca.* 30 min, 100 RPM), resulting in a colour changed from a clear brown liquid to a cloudy brown liquid. MeOH (*ca.* 10 mL) was added to breakdown the DES resulting in a clear brown liquid with AgNPs precipitated at the bottom of the flask.

Yield = 47.0 %. λ_{max} = 431.0 nm. DLS diameter size = 10.58±1.41 nm. TEM diameter size = 6.36±2.89 nm.

2.4 Extraction of AgNPs for Analysis

2.4.1 Extraction of AgNPs from Bet:Gly 1:3

To extract the AgNPs from Bet:Gly 1:3 (see **Section 2.3.2**), the AgNP DES solution in MeOH was transferred to Eppendorf tubes (*ca.* 10-12 tubes). The stoppered tubes were centrifuged (9000 RPM, 10 min) using a Labnet Prism high speed centrifuge. This resulted in a clear pale brown waste solvent with the AgNPs seen adhered to the surface at the bottom of the Eppendorf tube. The waste solvent was decanted off, resulting in the AgNPs at the bottom of the tube. MeOH was added (*ca.* 2 mL per tube) to wash the AgNPs before centrifuging again (9000 RPM, 10 min). The waste solvent was decanted off and the unstoppered tubes with the AgNPs were dried by gently blowing with nitrogen gas (*ca.* 1 min) inside the tubes. Toluene (*ca.* 1 mL per tube, 10 mL total) was added and the stoppered tubes were shaken (*ca.* 2 min). This resulted in a clear brown liquid. The sample was transferred to a 20 mL glass syringe

and filtered, using a PTFE 0.2 μm filter, into a sample vial. The final product was a clear brown liquid.

2.4.2 Extraction of AgNPs from Cap:Lau:Bet 4:2:1

To extract the AgNPs from Cap:Lau:Bet 4:2:1 (see **Section 2.3.3**), the AgNP solution in MeOH was transferred to Eppendorf tubes (*ca.* 10-12 tubes). The stoppered tubes were centrifuged (9000 RPM, 10 min) using a Labnet Prism high speed centrifuge. This resulted in a clear pale brown waste solvent with the AgNPs seen adhered to the surface at the bottom of the Eppendorf tube. The waste solvent was decanted off, resulting in the AgNPs at the bottom of the tube. Acetone was added (*ca.* 2 mL per tube) to wash the AgNPs before centrifuging again (9000 RPM, 10 min). The waste solvent was decanted off and the unstoppered tubes with the AgNPs were dried by gently blowing with nitrogen gas (*ca.* 1 min) inside the tubes. Toluene (*ca.* 1 mL per tube, 10 mL total) was added and the stoppered tubes were shaken (*ca.* 2 min). This resulted in a clear brown liquid. The sample was transferred to a 20 mL glass syringe and filtered, using a PTFE 0.2 μm filter, into a sample vial. The final product was a clear yellow or orange liquid.

2.5 AgNP Characterisation

2.5.1 UV-vis Analysis

UV-vis analysis calculates the absorbance of a sample by measuring the transmittance of light through the sample. In this case AgNPs (*ca.* 10 nm) absorb light at *ca.* 410 nm, which means that when the incident beam of light passes through the sample, the electrons will absorb the light and jump to a higher energy level. The light that is not absorbed will pass through the sample and will be detected. The wavelength at which AgNPs absorb the light depends on the type of NPs, in this case AgNPs, the size of the NP and the shape of the NP, see **Section 3.1** for further discussion. In this project, UV-vis was used quantitatively to determine if the AgNP were successfully synthesised as well as a quantitative comparison of the amount of AgNPs synthesised between the different reaction conditions using the absorbance as an estimate of the concentration. It should be noted that the actual concentration of the AgNPs was not determined by UV-vis and instead was determined by ICP-MS.

UV-vis analysis was performed on a Mettler Toledo UV5Bio Spectrophotometer wavelength range 190 nm-1100 nm. Methods used on the spectrophotometer were used on the direct methods subcategory and were designed based on each intended use. A single sample holder was used with two enhanced chemical resistant quartz cuvettes that were paired to ensure no error between the blank sample and AgNP sample. The AgNP solution (*ca.* 2 mL) was added to the quartz cuvette and stoppered in a fume hood before transferring to the UV-vis. After analysis the AgNP solution was discarded in the waste container and the cuvette carefully cleaned using acetone.

2.5.2 DLS Analysis

DLS analysis calculates the Brownian motion of a suspended colloid in a solvent by firing a laser of a fixed wavelength at a sample. The suspended colloid will scatter the laser and will be detected by 3 different detectors positioned at 15° (forward scattering), 90° (side scattering) and 175°C (back scattering). This scatting is due to the Brownian motion of the colloids which if the viscosity and temperature of the solvent is known, can be used to calculate the hydrodynamic radius of the colloid. The hydrodynamic radius will consider the size of the colloid, in this case a AgNP, as well as the capping agent on the surface of the AgNP and the solvation layer around the AgNP. Whilst this method will not provide an exact size of the AgNP, which is an important characterisation for antimicrobial properties as previously discussed in **Section 1.2**, it will provide a quick approximation of the size of the AgNP as well as a representation of the range of sizes present in the sample. TEM analysis was used to give a more accurate size determination of the AgNPs and compared with the DLS results.

DLS analysis was performed on a Malvern Zetasizer Nano S using a ZEN2112 ultra-micro cell for Nano Series. Methods used on the DLS were designed from the internal SOP creator. Samples were used without dilution in the ZEN2112 cell and ran at room temperature (25 °C). Data was collected from the software directly as a results table copied into excel to be replotted at a later date. When retrieving the data from the DLS, the range of sizes of the AgNPs was noted from the results sheet calculated by the DLS itself and presented.

2.5.3 TEM Analysis

The TEM was used to determine the size and shape of the AgNPs synthesised. By spotting the AgNPs suspended in the solvent onto a TEM grid and allowing to dry, the AgNPs will adhere to the surface of the TEM grid and can be inserted into the TEM for analysis. TEM works by detecting electrons that pass through the sample (transmitted). Electrons that hit the AgNPs can also be scattered and are detected. Using the information collected from the detectors of the transmitted and scattered

electrons, the software can generate an image of the AgNPs. In addition to the electrons being detected, X-rays can also be emitted from the sample which can be detected, and EDS analysis can be performed to determine the elemental composition of the sample.

TEM analysis was performed on a JEOL JEM-2100Plus. Samples were prepared by drop casting AgNPs in toluene (*ca.* 5 µL) onto a carbon film 300 mesh copper and the toluene allowed to evaporate (*ca.* 2 Hr), images captured at a x200 magnitude, unless stated otherwise, to ensure direct comparisons between samples.

TEM images were counted in Image J Fiji by manual counting due to low contrast between the background and AgNPs. The scale bar size was inputted into the program and straight lines from one edge through the centre of the AgNP to the other edge were measured in ImageJ to produce a table of results. If AgNPs were not fully spherical, the largest length was used. Any AgNPs that were not fully on the image were not measured, as well as any overlapping AgNPs where the edge of the NP's could not be seen. If the image was not populated with enough AgNPs, other images taken from the same sample were also sized and a representative of the population of AgNPs were shown in the figures. When plotting the AgNPs a standard deviation of the sample was performed to provide a size range of the AgNPs in the sample.

2.5.4 XRD Analysis

XRD analysis was performed on a Rigaku Smartlab SE Powder X-ray Diffractometer (PXRD) by Hannah Beska, using **CuKα radiation (λ =), kV – 40mA,** incident Soller slit of 2.5°, length limiting slit of 10 mm, receiving Soller slit of 2.5° and a Kβ filter. Data was taken for the 2θ range of 5 to 80° with a step of 0.02° and a 2θ range of 30 to 50° with a step of 0.02° and a speed of 0.1 °/min.

The sample was prepared by adding a thin layer of AgNPs in Bet:Gly 1:3 (*ca.* 0.2 mL) onto the zero-background silicon sample holder. This was inserted into the PXRD with the method developed by Hannah Beska. Measurements were using a 1/8th slit with a duration of 13 Hr and 4 Hr runs. Data was saved in a .raw file and converted to excel using a PowDLL converter.

2.5.5 ICP-MS Analysis

ICP-MS was used to determine the concentration of the AgNPs in the Bet:Gly 1:3 and Cap:Lau:Bet 4:2:1. However, before the samples could be used in the ICP they were first acid digested with conc. nitric acid to break down the DES's and dissolve the AgNPs to form $AgNO_3$. This was done to make it easier to introduce the sample into the ICP. The ICP-MS works by nebulising the sample to be passed through an argon plasma flame, this will ionise the sample. The sample ions are then detected by mass

spectrometry, in this case a quadrupole mass spectrometer that can output the intensity of the selected element, which in this case was Ag. Whilst ICP-MS was used due to its availability, ICP-OES is another method that could be used, which detects the light emitted from the sample after the electrons are excited to the higher energy state and drop back to the previous energy levels.

ICP-MS analysis was performed on a Perkin Elmer NexION 1000 by Richard Akrill. AgNP DES samples were prepared by microwave digestion in an Ethos Up milestone connect high performance microwave digestion system using a method designed by David Edwards. AgNP DES (0.2 mL) and concentrated HNO_3 (10 mL) was added to a digestion chamber. A check standard of 5 mg/mL solution of Ag was added to a different chamber to test the calibration graph. Chambers were sealed and placed in the microwave digestor, temperature (200 °C) was ramped (10 min) and held (30 min). Once cooled (*ca.* 30 min) they were removed from the microwave digestor.

Ultra-pure H_2O was added *(ca*. 40 mL) to create "strong solutions". To a 50 mL volumetric flask, the "strong solution" was added (1 mL) followed by ultra-pure water to the 50 mL volume. This resulted in an expected concentration of 0.4 ppm. Samples were filtered through a 0.45 µm syringe filter before analysis in the ICP-MS. A blank HNO_3 solution (10 mL) was run alongside calibration samples of Ag solutions made from $AgNO_3$ with concentrations ranging from 0.2 ppm to 1 ppm to produce the calibration graph (**Figure 14**). ICP-MS method was designed by Glyssa Dizon

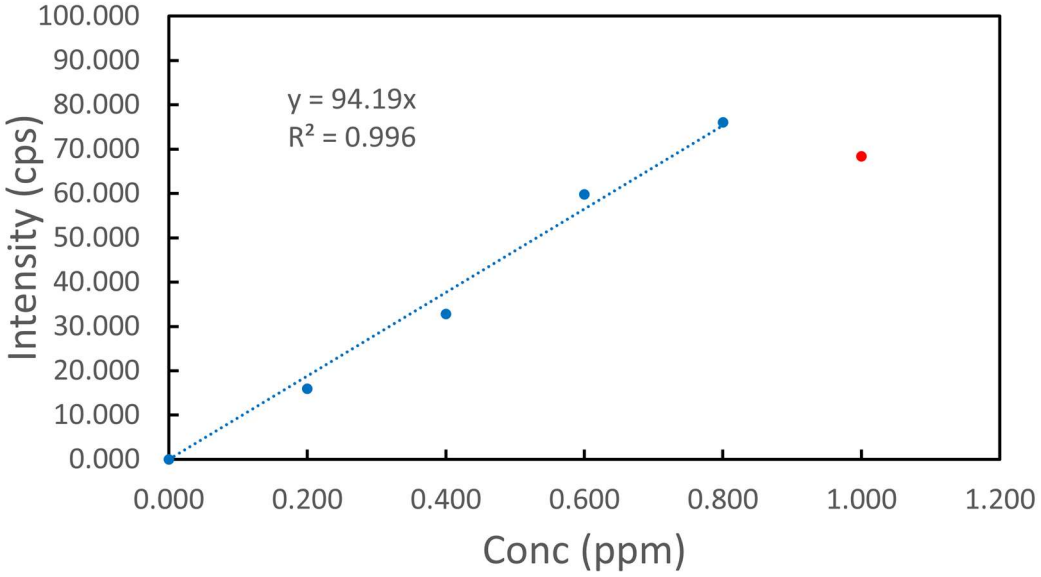


Figure 14: ICP-MS calibration graph including R^2 value, of Ag solutions prepared from 0.2 ppm to 1 ppm in HNO_3. 1 ppm data point was omitted due to being out of the detection range of the instrument (1ppm). Standard error = ±0.035 cps

2.6 Physical Property Testing of DES's

Bet:Gly 1:3, Cap:Lau:Lev 4:2:1, Cap:Lau:Lev 8:4:1, Cap:Lau:Lev 2:1:1 was liquid at room temperature (*ca.* 22 °C) and their density, refractive index, conductivity, viscosity and surface tension was measured. Cap:Lau:Bet 4:2:1 was a waxy solid at room temperature (*ca.* 22 °C) and only had conductivity, viscosity and surface tension measurements taken.

2.6.1 Density testing

The density measurements were carried out using a pyknometer, which was purchased with the exact known volume of the flask. This allows for the pyknometer to be filled with a known volume of the DES to obtain an accurate weight of the sample so that the density can be calculated using **Equation 3**. One downside to using the pyknometer is that measuring higher temperature is not as easy as using a densitometer. Since a densitometer was not available the DES's density were determined at room temperature (*ca.* 22 °C).

Density was calculated at room temperature (*ca.* 22°C) using a 10 cm^3 pyknometer (10.364mL) purchased from Blaubrand. The empty bottle was weighed on an analytical balance to obtain the blank weight. The DES sample was added to the bottle and the lid inserted; excess DES was cleaned away. The overall weight was obtained by reweighing on the same analytical balance used previously. The blank weight was subtracted from the overall weight to obtain the weight of the DES. This was repeated 3 times for an average weight of the DES, this can then be used to calculate the density using **Equation 3**.

$$Density\ (\rho) = \frac{mass\ (m)}{Volume\ (mL)} \qquad (\mathbf{3})$$

2.6.2 Refractive Index

Refractive index is the measure of how much a light beam is refracted when travelling through a material. For these DES's a refractometer was used which when calibrated with an ethanol sample can be used to determine the refractive index of a liquid, by "sandwiching" the sample between two crystals and shining a light source through it. The temperature of the sample could not be controlled, however did show a reading of the temperature of the crystal so an accurate value of temperature was stated. With the addition of a water bath that feeds into the machine higher temperatures could have been measured, however this was not available during the project.

Refractive index was determined using an ABBE 5 refractometer. The instrument was calibrated using ethanol (*ca.* 1 mL) at room temperature (*ca.* 21 °C). The ethanol

was cleaned off using a soft tissue. A DES was added (*ca.* 1 mL) on top of the crystal to form a thin film before the sample holder was closed for analysis. This was performed in triplicate with the temperature of the crystal noted using the built-in temperature probe. The DES was cleaned away using ethanol and a soft tissue.

2.6.3 Conductivity

Conductivity measurements are determined by measuring the potential drop between the probe's electrodes to determine resistivity of the sample. The machine can then calculate the conductivity of the sample and output the conductivity value using **Equation 4**. Before use, the probe is calibrated with a standard that is close to the conductivity of the sample tested to ensure accurate data.

$$Conductivity\ (\sigma) = \frac{Length\ of\ material\ (L)}{Resistance\ (R)\ x\ Cross{-}sectio\quad area\ (A)} \qquad (\mathbf{4})$$

Conductivity was determined using a Hanna Edge^{EC} instrument calibrated with a standard solution (1413 µS/cm). DES's were added (*ca.* 20 mL) to a falcon tube and inserted into a water bath to control the temperature starting at 25 °C and increasing in 5 °C intervals to a max temperature of 60 °C. The probe was inserted into the DES and all air bubbles were removed by gently moving the probe and gently tapping on the side. The probe was left in the solution (*ca.* 10 min) at the desired temperature to ensure DES was acclimatised. Three readings were taken and then averaged for the conductivity value.

2.6.4 Viscosity

The viscosity of the DES's were determined using a Brookfield viscometer DV2T with the small sample adapter. This viscometer works by rotating a spindle at a desired RPM and measuring the torque required to spin the spindle in the sample. The viscometer can measure the shear stress using **Equation 5,** where M is the torque input by the viscometer, R_b is the radius of the spindle and L is the effective length of the spindle. The shear rate of the sample using a small sample adapter can be measured using **Equation 6**, where ω is the angular velocity, R_c is the radius of the container and R_b is the radius of the spindle. This is then used to calculate the viscosity of the samples using **Equation 7**.

$$Shear\ stress\ (\tau) = \frac{M}{2\pi R_b^2 L} \qquad (\mathbf{5})$$

$$Shear\ rate\ (\dot{\gamma}) = \frac{2\omega R_c^2}{R_c^2 - R_b^2} \qquad (\mathbf{6})$$

$$Viscosity\ (\tau) = \frac{Shear\ stress\ (\tau)}{Shear\ rate\ (\dot{\gamma})} \qquad (\mathbf{7})$$

Spindle SC4-29 was used for Bet:Gly 1:3 whilst the other DES's used SC4-18 to ensure accurate viscosity readings. The DES was added to the small sample adapter (*ca.* 13 mL for SC4-29 spindle, 7 mL for SC4-18) and inserted into the holder. The holder was connected to a water bath (the same bath used for conductivity readings) to control the temperature (25-60 °C in 5 °C intervals). The method was inputted manually with spindle speed set (40-200 RPM) and a measurement time (1 min 30 sec) with a single point average time of 30 seconds. The solution was left at the desired temperature to ensure DES was acclimatised (*ca.* 10 min).

2.6.5 Surface Tension

Surface tension was determined using a Kruss tensiometer with a Du Noüy ring. The tensiometer determined the surface tension by lowering the Du Noüy ring until it sits on the surface of the sample and is not submerged. By raising the ring slowly, the force applied for the ring to break the surface tension can be measured by the machine and output the value as mN m^{-1} using **Equation 8**, where F is the maximum force required to break the lamella of the liquid, L is the wetted length of the ring and θ is the contact angle which generally is 0° in liquids (Cos θ = 1). The tensiometer was checked using a water sample to ensure that the instrument was in working order at room temperature (*ca.* 21 °C). A small volume of DES (*ca.* 5 mL) was added to a small crystallising dish and placed on the sample holder, three readings were taken for surface tension and averaged. The temperature of the room was also recorded.

$$Surface\ tension\ (\sigma) = \frac{F}{L * Cos\theta} \qquad (\mathbf{8})$$

2.7 Antimicrobial Studies on AgNP Bet:Gly 1:3 and AgNP Cap:Lau:Bet 4:2:1

2.7.1 Preparation of Agar, Broth and PBS

Following instructions provided by the supplier; the MHA, MCA, MSA, MHB and PBS solutions were prepared in Duran bottles and sterilised in an autoclave (15 min, 121 °C)

For Defined media, dipotassium hydrogen phosphate (4 g), potassium dihydrogen phosphate (1 g), ammonium chloride (1g), calcium chloride (0.01 g) and potassium sulphate (2.6 g) was added to distilled water (500 mL) in a Duran bottle. "Trace elements" (prepared by Adam Varney) (10 mL) was added. The defined media was topped up to 1 L using distilled water and sterilised (15 min, 121 °C). After sterilised 1 mM magnesium chloride solution (prepared by Adam Varney) (1 mL) was added.

Prior to use, defined media (49 mL) and 1M glucose stock (prepared by Adam Varney) (1 mL) was added to a sterile container.

"Trace elements" was prepared by dissolving Na_2EDTA (5 g) in distilled water (800 mL) and the pH adjusted to 7.4. Iron(III) chloride hexahydrate (0.5 g), zinc oxide (50 mg), copper chloride dihydrate (10 mg), cobalt nitrate hexahydrate (10 mg), boric acid (10 mg), ammonium orthomolybate (0.12 mg) and sodium selenate (17 mg) was added to the solution. The "trace elements" solution was then filter sterilised and kept in the fridge for further use.

2.7.2 Colony biofilm assay

All bacterial stocks were made in triplicate to obtain 3 biological repeats.

A bacterial stock was prepared by adding MHB (10 mL) to a sterile container followed by an individual colony of *S. aureus* or *E. coli*. The stoppered sterile container was incubated (37 °C, 24 Hr) whilst shaking. In another sterile container, the bacterial stock was then diluted in PBS to an OD_{600} of 0.5. The diluted bacterial stock was then spotted (10 µL) onto an S-Pak membrane filter (ordered from Merck) placed on MHA and incubated (37 °C, *ca.* 16 Hr) without shaking.

For the wound simulation the diluted bacterial stock of *S. aureus* (1 mL) and *E. coli* (1 mL) were added to another sterile container and mixed using a vortex to obtain a multiculture stock before spotting (10 µL) onto the S-Pak membrane filter.

After incubation, PBS (10 µL), DES (*ca.* 10 µL) and AgNP DES (*ca.* 10 µL) was spotted onto 3 different biofilms with another biofilm left blank to act as the control. These were then incubated (30 °C for *E. coli*, 37 °C for *S. aureus*) for an allotted time (3 Hr, 4 Hr, 24 Hr). After incubation, the membrane was cut with sterilised scissors around the biofilms. PBS (10 mL) was added to a sterilised container followed by the cut membranes with the biofilms. The samples were vortexed to ensure the biofilm was disaggregated in the PBS.

The PBS/biofilm suspension was then serial diluted along a 96 well plate at 10^1 dilution increments before spotting onto MHA agar to be incubated (37 °C for *S. aureus*, 30 °C for *E. coli*, *ca.* 16 Hr). Colonies were then counted to determine

bacterial viability (CFU/biofilm). For the wound simulation, both MSA and MCA were spotted in order to get selective growth of *S. aureus* and *E. coli* to determine bacterial viability (CFU/biofilm)

2.7.3 Minimum inhibition concentration (MIC) and minimum Bactericidal concentration (MBC)

Bacterial stock was made following the method outlined in **Section 2.7.2**, if *E. coli* was used then defined media replaced the MHB at this stage. After incubation, the bacterial stocks were diluted to an OD_{600} of 0.1 using PBS in a sterilised container. In a 96 well plate, the chosen media (MHB for *S. aureus*, Defined media for *E. coli*) was added (100 µL) to each well, AgNP DES (100 µL) was added to the first well and was serial diluted by a factor of 2 down the wells, leaving a negative and a positive control of media at the end, the last aliquot was disposed of to ensure equal volumes across all wells. Bacterial stock (5 µL) was subsequently added to each well including the positive control well, making sure not to introduce bacteria to the negative control well. The well plate was incubated (37 °C, *ca*. 24 Hr), shaking was required for the Cap:Lau:Bet 4:2:1 DES plates. The well plate was checked for any cloudiness indicating bacterial growth and allowing determination of the MIC, making sure that the negative controls were not cloudy to show no contamination of the media. The positive control wells were cloudy indicating the bacteria could grow in the chosen media. After the MIC was determined the wells were spotted (10 µL) onto MHA, including the wells either side of the recorded MIC value and incubated (37 °C, 24 Hr) to determine the MBC. The next morning the MBC plates were read, if there was no growth on the agar then the MBC was determined, if there was significant growth from all wells then MBC was not determined.

Chapter 3: AgNP synthesis in Bet:Gly 1:3

Literature methods for the synthesis of AgNPs were discussed in **Section 1.2.3**, which identified that whilst the majority of the DES methods did not produce AgNPs of the size range required for antimicrobial properties the work by L. Adhikari *et.al.* all produced AgNPs that could exhibit antimicrobial properties[2,27,62] Unfortunately, these particles are capped with OAm which is harmful to mammalian cells, which means they are not suitable for use as a wound dressing. Whilst the other literature methods discussed did not have the correct sized AgNPs, the capping agents used were natural products *e.g.* green tea extract, grape pomace. These would not be hazardous to humans and are more desirable as a capping agent than OAm.

Bet:Gly 1:3 was chosen since it is a halide-free DES which is liquid at room temperature and is synthesised from natural metabolites found in the human body, resulting in a bio-compatible DES for use on humans. Bet and Gly can also be sourced from natural products such as sugar beets for Bet,[68] which is more readily available than choline nitrate, and Gly can be obtained as a byproduct of biodiesel production,[69] which improve upon the greenness of the synthesis by using natural products or waste products from other synthesises.

To analyse the AgNPs synthesised in the Bet:Gly 1:3 DES, they first must be removed from the DES by breaking down the DES with another solvent and resuspending the AgNPs in a volatile solvent once they are extracted, which will allow for sample preparation for the TEM. To achieve this, the addition of a capping agent to the synthesis will ensure that once the DES is broken down by the extraction solvent they will not aggregate in solution. This will allow for the AgNPs synthesised to be analysed on the UV-vis, DES and TEM for characterization. Synthesising the AgNPs in the DES without a capping agent would be required for the antimicrobial testing. To ensure these AgNPs in the Bet:Gly 1:3 DES can be analysed, after the synthesis an aliquot of AgNP DES can be introduced to a capping agent so that the DES on the surface of the AgNP can be displaced, which would allow for the AgNP to be characterised without compromising the stock AgNP DES batch with harmful reagents. This would also align with the green and sustainability goals for the reaction by reducing the waste products from the synthesis. As previously seen in **Section 1.2.3** when the OAm was removed from the calculation the RAE increased, meaning a more efficient reaction, and the E-factor value decreased due to a lower usage of additional solvents in the reaction, reducing the environmental impact from waste extraction solvent disposal. In larger industrial batches only a small sample of the overall reaction would

require extraction for characterization with minimal effect on the environmental impact in comparison to the smaller lab scale. This is because the small aliquot of sample taken for characterization from the industrial synthesis, will be minimal compared to the overall volume of the batch. This ties into an objective of the project to develop new methods of AgNP synthesis without the use of a hazardous capping agent, to allow the AgNP DES to be used as a wound dressing.

The methods used here were inspired from the work of L.Adhikari *et. al.* which used choline nitrate:glyercol 1:2 DES to synthesise AgNPs ~10 nm in size with the use of oleylamine (OAm) as a capping agent, heated to specific temperature testing values over the range of 50-230 °C in a microwave reactor for 30 seconds.[2] This work instead looks at the synthesis of AgNPs using Bet:Gly 1:3 (which is a known DES in the literature [3]) with OAm as well as without OAm, using conventional heating and stirring to see if ~10 nm sized AgNPs can still be observed after changing the reaction method. Details can be found in **Section 2.3.1** and **Section 2.3.2** for AgNP synthesis and **Section 2.4.1** for the extraction method.

3.1 AgNP synthesis with OAm present in Bet:Gly 1:3

To start with a temperature of 180 °C was chosen which was similar to the temperature used in Adhikari's work to see if the reaction could be replicated on a hot plate instead of in a microwave reactor and with a different HBA (Gly).[2] Since the microwave reactor requires less time for the synthesis in comparison to a hotplate, the reaction time of 30 seconds was too short, so the reaction endpoint was monitored by colour change. The colour change of the solution indicates that AgNPs have been synthesised since the AgNPs will scatter light due to their localized surface plasmonic resonance (LSPR). LSPR theory describes how the electron density of the AgNPs oscillate when interacting with light, the LSPR peak occurs when a specific wavelength of light interacts with the oscillations of electrons at the same frequency, scattering the light which can be seen as a specific colour.[70] The LSPR peak will change depending on the size of the NP, the shape of the NP, what the NP is made from, as well as the solvent the NP is in.[71]

To confirm that the AgNPs were synthesised, they were extracted following the method set out in **Section 2.4.1** and suspended in toluene for analysis on a UV-vis. Whilst toluene is a hazardous solvent, it is used in this method due to the fact it is

known to be a successful extraction solvent for this synthesis to analysis AgNPs synthesised in a DES's for analysis by UV-Vis, DLS and TEM. UV-vis spectra can provide valuable information about the AgNP without the need for a TEM or DLS to analyse the sample, which are more time consuming and costly to purchase. Due to the size of the AgNP affecting the LSPR peak, the λ_{max} of the UV-vis spectra can be used to roughly determine AgNP size. For a size of ~10 nm the λ_{max} can be expected to be between 427 nm for 9.5±3.2 nm sized AgNPs and a λ_{max} of 461nm for 10.0±3.8 nm AgNPs.[72] These values are for when the AgNP is suspended in water so may be different in toluene, however, Adhikari reported that a AgNP sized at 10.1±4.7 nm showed a λ_{max} peak at 425 nm (extracted into toluene).[2] The absorbance of the λ_{max} can also give an indication of the concentration of the sample, due to the Beer-Lambert law, but it should be mentioned that this is not a quantitative analysis and instead was used to compare different spectra on the same plot as a qualitive analysis between each sample.[71] A quantitative analysis of the AgNPs was performed using ICP-MS which will be discussed in **Section 3.2**. The UV-vis peak width can also indicate the range of sizes within the solution, since each sized AgNP will have its own LSPR wavelength, the larger the range of sizes of AgNPs in the solution will result in a broader peak. Depending on the size variation in the sample, this peak widening may only occur on the red shifted side of the spectra shown as a shoulder peak, this can be explained either by aggregation or numerous different sizes of AgNPs larger than the average size. The ideal UV-vis spectra peak for this project would be a narrow peak at either 427nm or 461 nm λ_{max},[72] which would indicate the AgNPs are ~10 nm with a small size range, ideal for use in an antimicrobial wound dressing.

To confirm the sizes of the AgNPs, DLS and TEM analysis was also performed to give accurate size and shape determinations. As discussed in **Section 2.5.2**, the DLS analysis will not provide the exact size of the AgNPs as the light will be scattered by the AgNP, the capping agent and the solvation layer around the AgNP resulting in a larger size value. The DLS data is still a quicker indicator of size of the AgNPs, since TEM analysis will take much longer to perform. However, the TEM analysis will only analyse the solid residue of the AgNPs on a TEM grid after the toluene was removed by evaporation in a fume hood at room temperature. The UV-vis, DLS and TEM will be the main analytical techniques used in this project to characterise the AgNPs.

As seen in **Figure 15**, the method successfully synthesised at least two different sized AgNPs in the Bet:Gly 1:3 DES as seen by the two peaks (*ca.* 400 nm, 530 nm), however the change from microwave to hotplate did not translate perfectly into a mono-sized AgNP synthesis as seen in the presence of 2-3 broad peaks in the UV-vis at λ_{max} of 402 nm and 502 nm. It was hypothesized that

the cause of this could be the reaction conditions or the different properties of the DES used which could also influence particle size. Another factor to consider is that the size of the AgNPs could have been affected by the extraction process into the toluene, resulting in aggregation to larger sized AgNPs. From this initial synthesis, the method was refined by changing a single parameter of the reaction (including time, temperature and volume of OAm) whilst keeping the remaining parameters fixed. These conditions were determined as the most likely to affect the size of the AgNPs.

The refinement of the reaction conditions was an ongoing process, since once one ideal condition was found then that may have affected previous conditions deemed to be ideal. To begin with the volume of OAm was altered to see if the amount could be reduced to lower the overall hazard of the reaction. Ideally the volume would be less than 50% than that of the DES as this is likely to be the limit where DES's break down when adding water as seen in the literature for aqueous synthesises in **Section 1.2.1.1**,[15] however OAm does not mix well with the Bet:Gly 1:3 due to its hydrophobicity and is unlikely to break down the DES at 50% volume in the same way, but may still effect the properties of the Bet:Gly 1:3 so a range of volumes was tested to see which is the ideal volume to use.

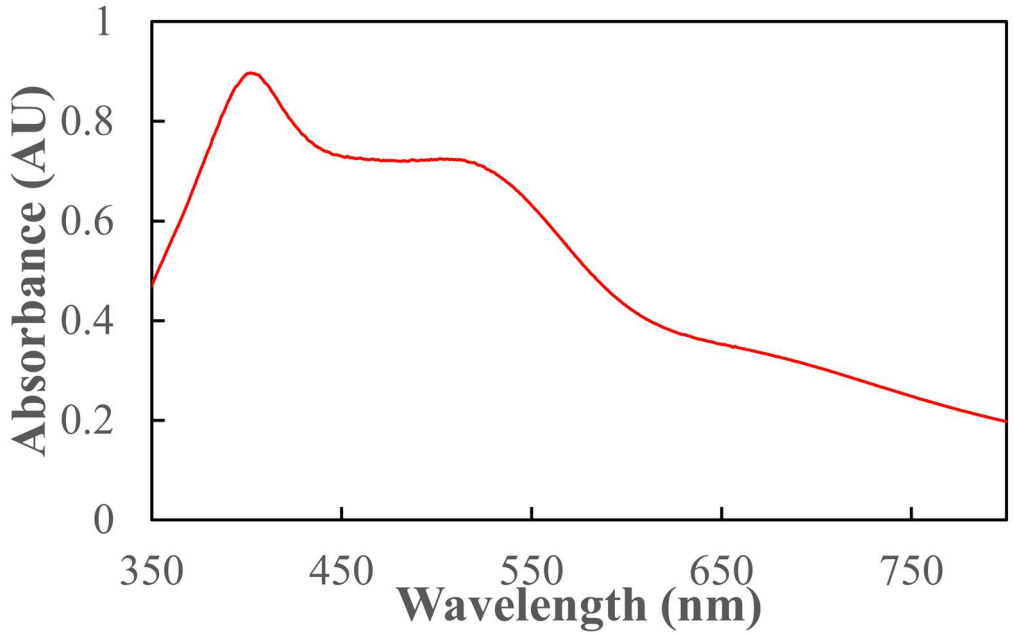


Figure 15: UV-vis spectra of AgNPs synthesised in 5 mL Bet:Gly 1:3 with OAm (4 mL), 25 mg Ag_2SO_4 at 180 °C for 15 minutes. Extracted by centrifuging twice with 20 mL MeOH before drying with N_2 gas and suspending in toluene.

As seen in **Figure 16**, as the volume of OAm increases from 2.0 mL to 2.5 mL, the AgNP peak becomes more defined and less broad, indicating a narrower range of

AgNP sizes synthesised. When the volume of OAm is increased to 3.0 mL, the absorbance value increases indicating more AgNPs have been successfully synthesised in that specific size range. This increase volume would lower the stoichiometry between the DES and the OAm potentially causing more interference with the DES, however since the OAm is not miscible with the DES it would be expected that if in quantities above a certain volume the OAm would not have any further disruption to the DES. However, it would be expected that the larger the quantity of OAm the more it would affect the synthesis of AgNPs if the OAm is indeed disrupting the DES, however this seems to be the opposite since the AgNP synthesis was improving with larger quantities of OAm. The general trend is at lower volumes of OAm the AgNPs synthesised when λ_{max} is 400nm are less than at higher volumes as demonstrated by the qualitative analysis of the max absorbance of the λ_{max} peaks, with the exception of the 2.0 mL OAm peak which has a far broader peak, this would suggest a large range of sizes of AgNPs which is harder to compare to the other narrow peaked samples. An explanation for this would be that a certain amount of OAm is required to fully cap the AgNPs. From this 3 mL OAm was chosen as the minimum quantity required to cap the particles.

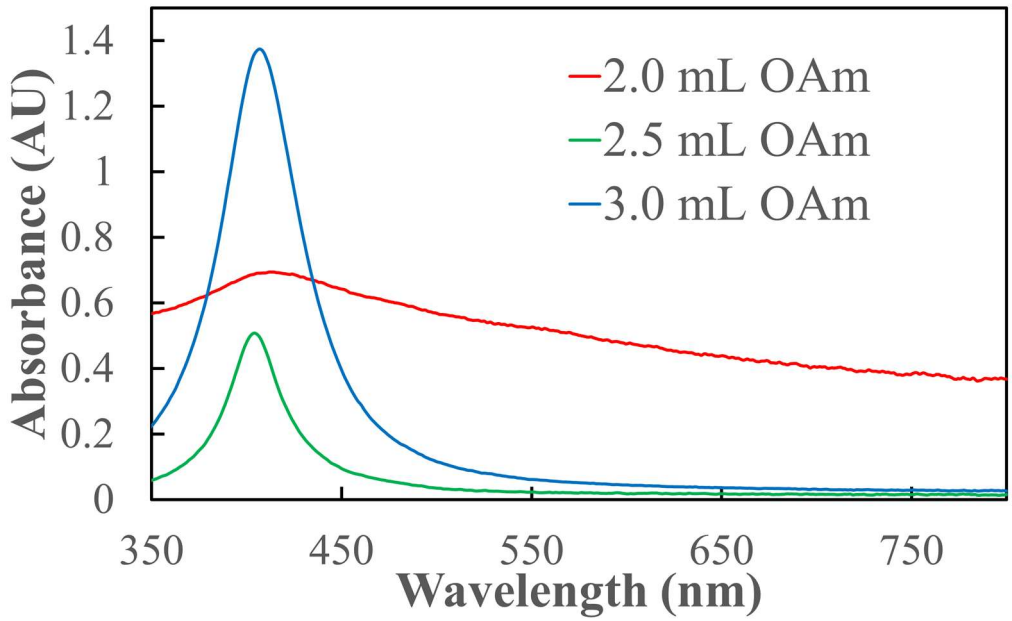


Figure 16: UV-vis spectra of AgNPs synthesised in 5 mL Bet:Gly 1:3 with OAm (2.5-3.0 mL), 25 mg Ag_2SO_4, 120 °C for 30 minutes. Extracted by quenching with 10 mL MeOH and centrifuging at 9000 RPM for 10 minutes, followed by another wash with MeOH before drying with N_2 gas and resuspending in toluene.

After the minimum volume of OAm needed for the reaction to proceed was established, a full study on reaction conditions was performed to see the effect on AgNP particle size. For use in an antimicrobial wound dressing the particle size would

have to be between 5-10 nm,[30] so this was the target size for the AgNPs. The main conditions that were tested were temperature, time, and volume of OAm. **Figure 17A** shows the effect of temperature on the AgNP size, Adhikari *et. al.* chose the temperature to be 180 °C but in the interest of having a more sustainable synthesis it would be better if this could be reduced.

The aim was to find the lowest temperature which would still produce acceptable AgNPs. The temperature range of this study was between 120 °C and 150 °C in **Figure 17A**. At 120 °C the AgNPs are in such a small quantity that when diluted to compare to the rest of the data set, they do not appear on the spectra. As temperature increases, the concentrations of the AgNPs increase up to 150 °C with the highest absorbance of the set. As well as the increase in concentration, the λ_{max} peak width in the UV-vis spectra also decreases which signifies less aggregation in the sample and a more concise size range. A temperature of 150 °C was chosen as the optimum conditions for the rest of this study.

Another condition that was studied was the time of reaction, **Figure 17B** shows the effect of time on the synthesis of AgNPs, the general rule is that the longer a reaction goes for, the more particles, as well as larger sizes, are observed due to an longer time for nucleation to occur, as well as more time for nucleation sites to grow, increasing the AgNP size. In this case, the reaction time allowed for a longer nucleation period which increased AgNP size beyond the nano range, as suggested by the drop in absorbance between the 5 minute and 7 minute 30 seconds samples. A time of 5 minutes was established as the optimum time of reaction at 150°C.

Figure 17C shows how the volume of OAm affects the AgNPs when testing the new temperature and time (150 °C and 5 minutes) previously established in **Figure 17A** and **Figure 17B**. The outcome is that 2.5 mL OAm produced the most AgNPs after extraction with 3 mL and 3.5 mL producing less AgNPs, but all the peaks have similar λ_{max} of 408-410 nm with only minor variation, as seen in **Table 11**, suggesting that the sizes of the AgNPs are all similar at approximately 4-9 nm in size.[72] An approximate size can only be determined since the data in the publication used for reference was analysed in water with a different capping agent, which will both affect the LSPR peak. The sizes of the AgNPs were more accurately determined by TEM and DLS which can be seen in **Table 11**. A volume of 4 mL was also tested which shows a shoulder peak occurring which can indicate larger AgNPs forming due to the red shift to higher wavelengths, or aggregation of the AgNPs synthesised resulting in a larger particle size which would show the same red shift.

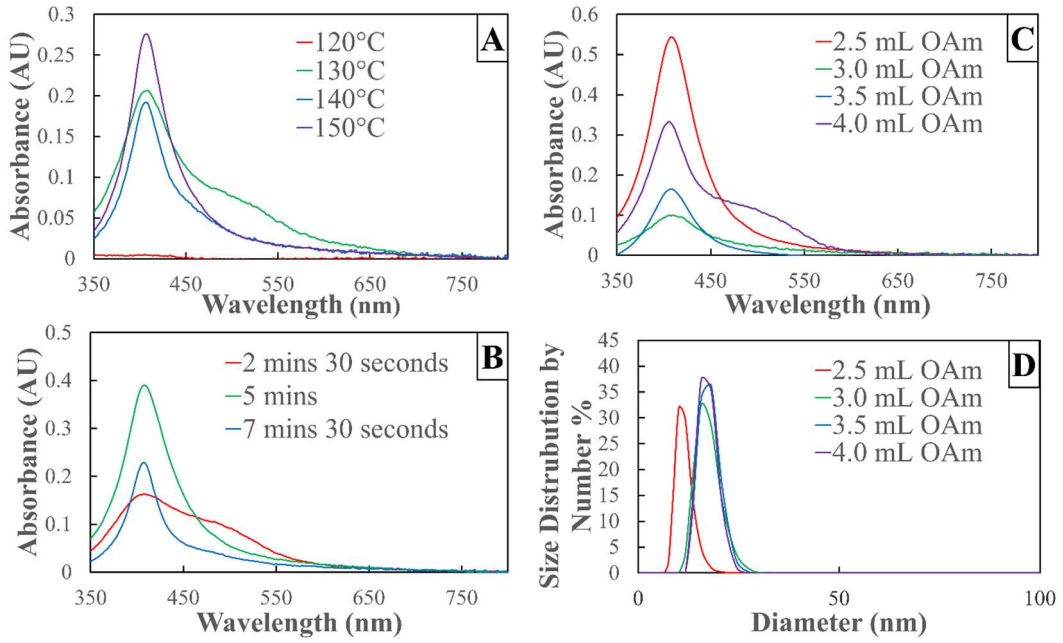


Figure 17: AgNPs extracted into toluene after synthesised in 5 mL Bet:Gly 1:3, 25 mg Ag_2SO_4, 3 mL OAm at 150°C for 5 minutes (unless otherwise stated).UV-vis spectra is baseline corrected and samples diluted by 100-fold. A) Temperature 120 °C-150 °C. B) Time 2 mins 30 seconds to 7 mins 30 seconds. C) Volume of OAm 2.5 mL to 4.0 mL. D) DLS analysis of **Figure 17C**.

The volume of OAm does seem to have the most impact on AgNPs size compared to the temperature of reaction and the reaction time. Although the difference in size is only 5 nm across the samples this is a large variation if the target AgNP size is 10 nm and will determine whether the AgNPs will exhibit a higher potency of antimicrobial behaviour or become toxic to humans. As seen in **Figure 17D**, the DLS readings show that the 2.5 mL OAm sample have a smaller particle size than the others. However, sizing AgNPs using the DLS also requires factoring into account the solvent the AgNPs are suspended in, using toluene can affect the scattering of the AgNPs resulting in an increased size being displayed from the actual size value.[73] As a result to determine the most accurate sizing of the AgNPs was performed using a TEM.

Table 11: Comparison of AgNP size from TEM and DLS data in comparison to λ_{max} taken from UV-vis readings. Reaction conditions are as follows; 25 mg Ag_2SO_4, 5 minutes, 150 °C and 2.5-4.0 mL OAm with 5 mL Bet:Gly 1:3.

Volume of oleylamine added / mL	λmax / nm	Diameter AgNPs / nm	
		TEM data (determined by largest count)	DLS data (determined by highest number %)
2.5	408.4	11±2.6	11.23±2.18
3.0	410.0	10±2.4	16.97±2.99
3.5	408.2	15±2.4	17.42±2.50
4.0	405.6	14±2.5	17.05±2.34

For use as an antimicrobial wound dressing the AgNP size must be between 5-10 nm, **Table 11** shows the difference between the sizes of AgNPs by comparing the UV-vis in **Figure 17C** with the sizes of AgNPs gathered from TEM images (see **Figure 18**) as well as DLS data. The DLS data would suggest that the 2.5 mL OAm sample will be suitable for antimicrobial use with a size range of 11.23±2.18 nm, which means some of the synthesised AgNPs are ~10 nm.[30] The rest of the DLS data suggesting that the sizes of the AgNPs all are ≥15 nm, however the more accurate TEM analysis shows that both the 2.5 mL and 3.0 mL samples have the correct size range for antimicrobial properties, with the 3.0 mL OAm sample being the smallest at 10±2.4 nm, compared to the 11±2.6 nm. It can be observed however, that the λ_{max} peaks do not correlate with the TEM data, it would be expected that the smallest λ_{max} value (405.6 nm, for the 4.0 mL OAm sample) would correspond to the smallest AgNP size, this is not the case. This is likely due to a small difference in the environmental conditions on the day, shifting the LSPR peak. This highlights the importance of the additional characterization techniques of TEM and DLS to ensure an accurate size of the AgNPs to ensure antimicrobial activity.

In **Figure 18**, the TEM images show the AgNPs are similar in size with the AgNPs spreading out across the TEM grid with minimal interactions between the AgNPs which suggests they are fully capped and are not aggregating in solution. Out of these

samples, only 3 mL OAm have the majority of the particles at a suitable size for antimicrobial properties. However, these images do show that depending on the reaction conditions, different size particles can be achieved. These sizes are between 10-14 nm which is not a huge difference in size when compared to the varying sizes from the aqueous methods discussed in **Section 1.2.1.1**, in which the variation between sizes can be up to 20 nm difference.[38]

Whilst these AgNPs would be suitable for antimicrobial property testing, the main problem is the use of OAm as the capping agent which is harmful to humans, and the solvents used to extract the AgNPs, however extraction of the AgNPs from the DES will not be needed for the wound dressing itself since the AgNP can be used directly in the DES which as previously discussed in the introduction to **Chapter 3**, is biocompatible with humans. The most logical next step would be to see if the AgNPs can be synthesised in the DES without including the OAm, and then after the AgNP synthesis is completed, introducing the OAm to see if the DES can be displaced on the surface of the AgNPs with OAm for extracting into toluene whilst still maintaining enough AgNP's in toluene for a definitive analysis. This method was described in **Section 2.3.2** for synthesis of AgNPs in Bet:Gly 1:3 without OAm.

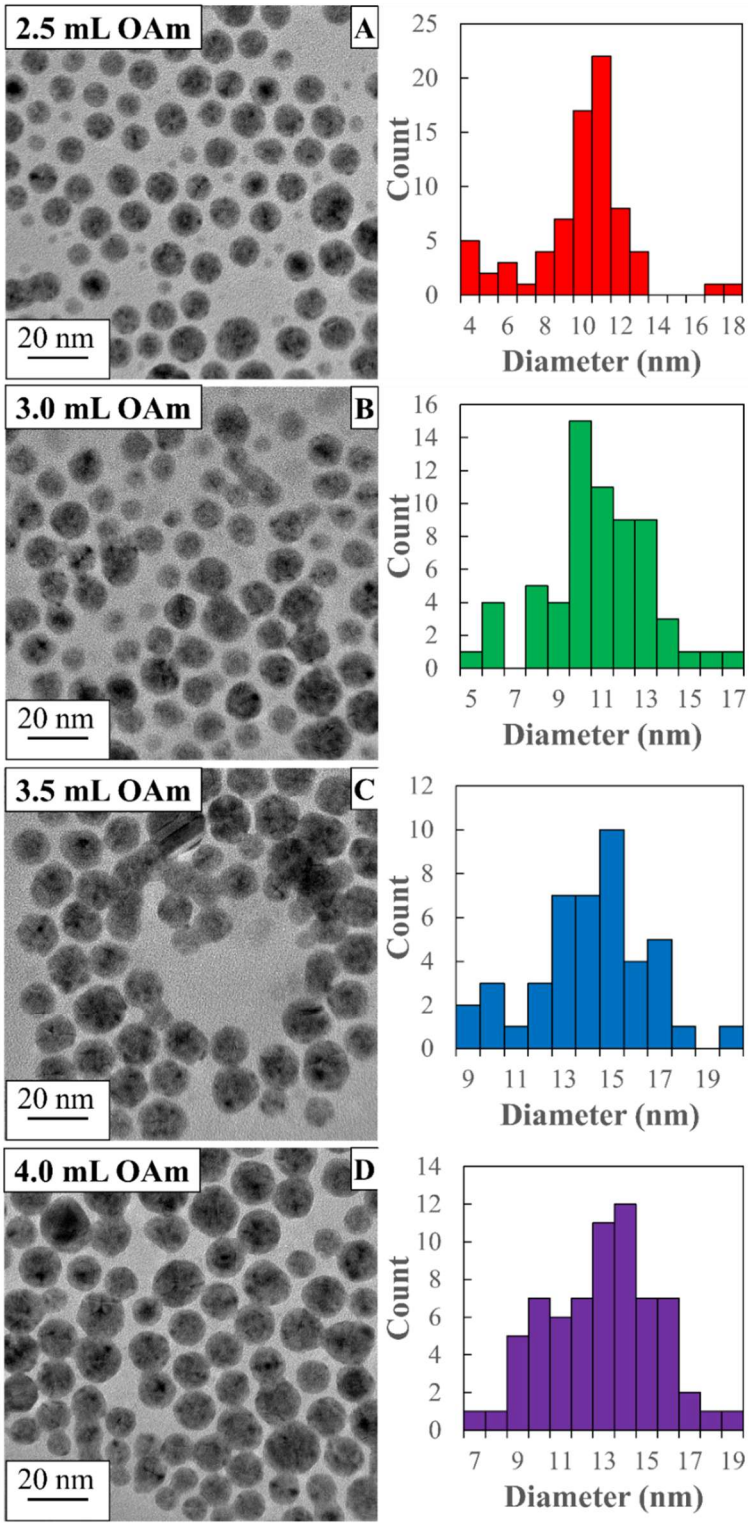


Figure 18: TEM analysis of AgNPs synthesised using 5 mL Bet:Gly 1:3, 150 °C for 5 minutes, OAm volume used A) 2.5 mL B) 3.0 mL C) 3.5 mL D) 4.0 mL. Number of AgNPs counted between 44-75 NPs with an average count of 63 NPs.

3.2 AgNP synthesis without OAm in Bet:Gly 1:3

Initially, a test sample was ran using the optimised reaction conditions for the synthesis with OAm (5 mL Bet:Gly 1:3, 25 mg Ag_2SO_4, 150 °C, 5 minutes). This however did not work as intended since without the OAm, the reaction was much faster. In the reaction series in **Section 3.1** the OAm appears to inhibit the synthesis of AgNPs which required the higher temperature to overcome; without OAm, the reaction temperature can be lowered which would make the synthesis greener. A new extraction protocol was developed (**Section 2.3.2**) whereby OAm was introduced post-synthesis to stabilise the AgNPs in toluene. Whilst the AgNPs without OAm are stable in the DES, they aggregate instantly on extraction into conventional solvents.

Due to the change in reaction conditions, a small study on changing the time and temperature of the reaction was conducted to determine the new optimum conditions for the AgNP synthesis in the Bet:Gly 1:3 DES without the addition of OAm. As seen in **Figure 19**, both the 120 °C and 150 °C samples have a lower absorbance due to the amount of aggregation occurring from overreacting the samples, it can be determined that the optimum temperature for the Bet:Gly 1:3 DES synthesis without OAm is 100 °C or lower. A further study looking at temperature down to 80 °C was performed. It was found that after 5 minutes at 80 °C the reaction had not progressed far enough to extract the AgNPs, so was left for longer to determine a suitable end point. The end point chosen was at 15 minutes reaction time.

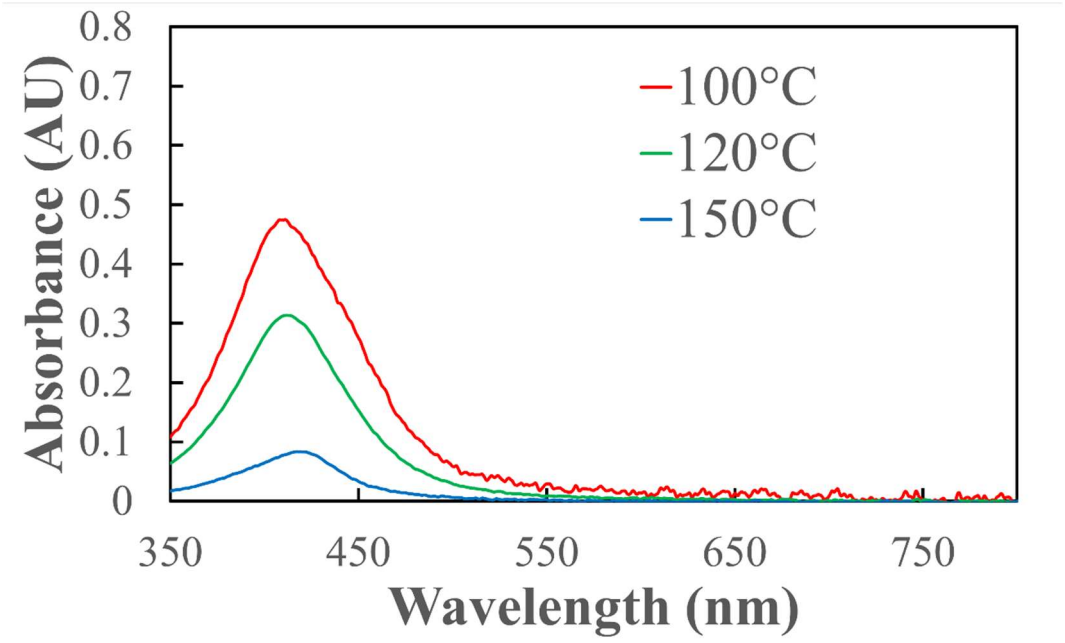


Figure 19: UV-vis spectra of AgNPs extracted using OAm after synthesis in DES. Reaction conditions, 5 mL Bet:Gly 1:3, 25 mg Ag_2SO_4, 100-150 °C, 5 minutes. Then extracted into toluene by allowing to cool to room temperature and adding 3 mL OAm and stirring slowly for 30 minutes.

As seen in **Figure 20a**, the reaction at 80 °C for 15 minutes has the largest absorbance compared to the other samples which means comparatively 80 °C produced the most AgNPs during the synthesis after extracted into the toluene, although there could be due to variations caused by the extraction step. As seen in **Table 12** the λ_{max} values seen in the UV-vis spectra are larger values compared to the λ_{max} values in **Table 11**, this would suggest that larger AgNPs have been synthesised, which is to be expected since the capping agent of OAm is not present, so size is controlled by the Bet:Gly 1:3 without the interference from the OAm. Whilst the λ_{max} values range from 415.0-423.2 nm, these are still close to the target of 427-461 nm established previously in this section.[72] Once extracted, the DLS analysis shows that the sizes of the AgNPs extracted into toluene for the 90 °C sample and the 100 °C sample are 16.84±4.34 nm and 17.57±5.91 nm as seen in **Table 12**, which are a similar size to the optimum conditions of the previous synthesis (5 mL Bet:Gly 1:3, 25 mg Ag_2SO_4, 150 °C, 5 min) at 16.97±2.99 nm (see **Table 11**). The TEM imaging allows for a direct size comparison between the AgNP synthesis with OAm and without OAm. The sizes obtained for the synthesis without OAm (13±1.94 nm), are slightly larger than that of the synthesis with OAm (10±2.40 nm) which may cause some issues with antimicrobial properties. The 80 °C sample was the only TEM sample with enough AgNPs on the grid to be able to count the size with the average size of the particles being 13 nm.

The problem with this method is that many of the AgNPs are lost during the extraction process as seen by the drop in absorbance in the UV-vis spectra, after taking into account the dilutions, from **Figure 17a** where OAm is present during the reaction and **Figure 20a** where there is no OAm present during the reaction. This extraction process could be improved with further study, if there was time, but for the purposes of this project, it is an acceptable extraction for analysis since it will give a characterization of the AgNPs in the DES. But one consideration progressing forward, was if the AgNPs could be analysed in the DES themselves rather than extracting them, there would be no need for this second extraction method.

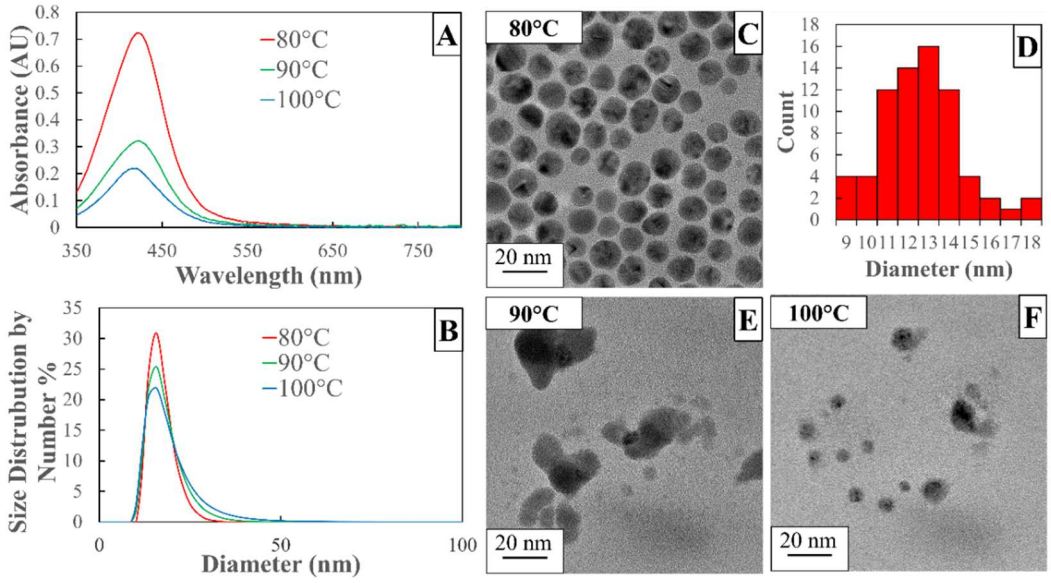


Figure 20: AgNPs synthesised in 5 mL Bet:Gly 1:3 with 25 mg Ag_2SO_4, for 15 minutes at a temperature of 80-100 °C. A) UV-vis spectra suspended in toluene, 80 °C sample was diluted two fold, all other samples not diluted. B) DLS in toluene. C) TEM of AgNPs after toluene evaporated. D) Counts of AgNPs show in **Figure 20C**. Number of counted AgNPs = 72. E) TEM of 90 °C sample. F) TEM of 100 °C sample.

Table 12: Comparison of AgNP size from TEM and DLS data in comparison to λ_{max} taken from UV-vis readings. Reaction conditions are as follows; 25 mg Ag_2SO_4, 5 minutes, 80-100 °C and with 5 mL Bet:Gly 1:3. * Insufficient population of AgNPs for analysis.

Temperature of reaction / °C	**λ_{max} / nm**	**Diameter AgNPs / nm**	
		TEM data (determined by largest count)	**DLS data (determined by highest number %)**
80	421.2	13±1.94	12.78±2.22
90	423.2	*	16.84±4.34
100	415.0	*	17.57±5.91

The issue of AgNP concentration dropping in the analysis for this synthesis without OAm, is most likely due to the extraction out of the DES. Since OAm has a long chain unsaturated alkyl chain, it is not miscible with the Bet:Gly 1:3, so during the 30 minutes of stirring, the OAm may not fully cap the AgNPs in the Bet:Gly 1:3, so when the MeOH is added the partially capped AgNPs will aggregate and fall out of the suspension. With the aim of this project to use these AgNP DES's in a wound dressing, the exact concentration of the AgNPs in solution is necessary for the antimicrobial testing. To determine the concentration, the AgNPs synthesised without OAm were microwave digested and ran through an ICP-MS, the method can be found in **Section 2.5.5**. **Table 13** shows the concentration of the AgNPs synthesised in 5 mL Bet:Gly 1:3, 25 mg Ag_2SO_4, 80 °C for 15 minutes, should be 5 mg/mL (based on the quantity of Ag_2SO_4 added to the DES) if the reaction has gone to completion, the concentration calculated for this sample was 1.73 mg/mL. This method was checked with a known concentration of AgNPs in Bet:Gly 1:3 (commercial AgNP DES), by dissolving a known value of commercially purchased 20 nm AgNPs at 5 mg/mL, as well as a standard of $AgNO_3$ in water with the same concentration at 5 mg/mL, to check the ICP-MS for any calibration errors. The commercial AgNP DES was calculated to have an original concentration of 2.51 mg/mL, and the $AgNO_3$ standard was calculated to be 4.53 mg/mL.

Table 13: Concentration of AgNPs capped by Bet:Gly 1:3, synthesised by using 5 mL Bet:Gly 1:3, 80 °C for 15 minutes, commercially brought AgNPs (20 nm) dissolved in Bet:Gly 1:3, and a check standard of $AgNO_3$ solution, microwave digested in concentrated nitric acid before diluting with ultra-pure water and analysed with an ICP-MS.

Sample	Calculated concentration excluding recovery / mg/mL	Calculated initial concentration from the check standard recovery data / mg/mL	Calculated initial concentration (after including sample loss) / mg/mL
$AgNO_3$ Standard (5 mg/mL)	4.41	5.00	-
Commercial AgNPs in Bet:Gly 1:3 (5 mg/mL)	2.31	2.62	4.74
Synthesised AgNPs without OAm in Bet:Gly 1:3	1.50	1.70	3.07

The check standard had a recovery of Ag in the microwave digestor of 88.3% of the initial value which can be calculated since the $AgNO_3$ standard was prepared at a concentration of 5.0 mg/mL (see **Table 13**). Assuming this is the same recovery for the other samples, the commercial AgNPs in Bet:Gly 1:3 had an initial concentration of 2.62 mg/mL and the synthesised AgNPs in Bet:Gly 1:3 to be 1.70 mg/mL, when considering the loss of sample from microwave digestion. Since the commercial AgNPs sample was prepared to be 5 mg/mL that shows a loss of 53.7% of the sample when microwaved digested, which is an unacceptable loss for this analysis, and shows that this method does need to be improved to work with a DES.

One issue with this method could be that the standard is a solution of $AgNO_3$ in water rather than AgNPs suspended in a DES, $AgNO_3$ was used since it was unknown how the different capping agent present on the commercial AgNPs would affect the digestion process. It is hypothesised that the DES is potentially causing some unknown interaction during the digestion process that is potentially inhibiting the digestion of the AgNPs and requires further analysis. The concentration of the

synthesised AgNPs in Bet:Gly 1:3 was calculated to be 3.07 mg/mL taking into account the recovery of the check standard, as well as recovery of the known AgNP in Bet:Gly 1:3 sample. This is a lower concentration than would be expected and is an indication that the AgNP synthesis did not go to completion.

Since ICP-MS was performed an accurate RAE and E-factor can be calculated for the synthesis without OAm as it is known how much product and waste has been generated (see **Table 14**). Since this reaction is a one-pot synthesis of AgNPs in Bet:Gly 1:3 and the Bet:Gly 1:3 will also be used in the antimicrobial wound dressing, the Bet:Gly 1:3 is also classed as a product.

Table 14: Mass of reactants and products for green metric calculations for the synthesis of AgNPs in Bet:Gly 1:3. Calculations performed in Excel without rounding values.

Reactants	**Mass /g**	**Products**	**Mass /g**
Ag_2SO_4	0.025	**AgNPs**	0.015
Bet:Gly 1:3	6.13	**Bet:Gly 1:3**	6.13
		Waste solids	0.010

$$Real\ Atom\ Economy\ \ (\%) = \frac{0.015 + 6.13\ g}{0.025 + 6.13\ g} x100 = 99.8\%$$

$$Environmental\ factor\ (AU) = \frac{0.01\ (g)}{0.025 + 6.13\ \ (g)} = 0.002$$

$$Process\ mass\ intensity\ (AU) = \frac{0.01 + 0.015 + 6.13\ \ (g)}{0.025 + 6.13\ (g)} = 1.002$$

Considering the mass of AgNPs synthesised in **Table 13** and the mass of the reactants and products in **Table 14**, the atom economy for this synthesis was calculated to be 99.8%, since the exact mass of unreacted Ag_2SO_4 and SO_4 ions can be calculated by deducting the AgNP mass from the starting material. This shows that the AgNP synthesis without using OAm in Bet:Gly 1:3 is a very efficient reaction to produce AgNPs with minimal waste products, as seen by the calculated PMI value of 1.002. It can also be argued due to the spontaneous nature of this reaction at room temperature, the unreacted Ag_2SO_4 will continue to form AgNPs which will increase the atom economy further. Since this reaction is all performed in a one-pot synthesis, with the Bet:Gly 1:3 acting as a capping agent, reducing agent and solvent, the calculated E-factor was found to be 0.002, which results in this reaction

having a minimal impact on the environment in terms of waste generated. The RAE and E-factor for this reaction, when compared to the calculations performed in **Section 1.2**, shows that is just as efficient as the Adhikari *et.al.* paper,[2] whilst having a lower environmental impact due to the changes in the synthesis. This E-factor can be improved by ensuring the reaction goes to completion through further study on optimum reaction conditions. However, there was a concern that over time the size of the AgNPs would change, from the unreacted Ag_2SO_4 in the DES, which may affect the RAE and E-factor due to AgNPs increasing in size beyond the antimicrobial range to the point that the AgNPs no longer are a viable for use in an antimicrobial wound dressing.

If the reaction does not go to completion, there are some concerns regarding spontaneous nucleation of the AgNPs which could affect the potency of an antimicrobial wound dressing over a longer period of time. It was decided that a long-term stability study was required to monitor if the AgNPs change over time when suspended in a DES. This analysis is important for commercialising an antimicrobial wound dressing as a shorter shelf life is more problematic ideally the AgNPs would be stable over a longer period, than previously seen for AgNPs produced using aqueous synthesis methods.

3.3 Stability studies on AgNPs in Bet:Gly 1:3 DES

The main application of this project was to develop an antimicrobial wound dressing for commercial use, this would require that the product made had as long a shelf life as possible. As such a long-term stability study was performed to determine how the AgNPs interact with each other and the DES over the course of 140 days. The reaction method from **Section 2.3.2** was scaled up to a Bet:Gly 1:3 DES volume of 120 mL, 0.60 mg Ag_2SO_4, at 80 °C for 5-15 min.

Large batches of AgNP DES were synthesised using different reaction times (5 min, 10 min and 15 min) and stored in duran flasks in a dark cupboard drawer which was temperature monitored using a sensor blue smart hygrometer (19.81-22.56 °C) with aliquots removed periodically for testing by first extracting the AgNPs into toluene and subsequent characterization by UV-vis, DLS and TEM. These duran flasks were laid on their sides during the storage which was the only way they would fit into the cupboard drawer.

Figure 21a,c,e shows the UV-vis data for the first 28 days of the stability studies which showed the largest variation, primarily on the concentration of the AgNPs. **Figure 21a** and **Figure 21c** both show the absorbance increasing as the days increase, this is likely due to unreacted Ag_2SO_4 undergoing spontaneous reduction forming more AgNPs in the solution. Additional spontaneous reactions may be an issue if this causes the AgNPs to undergo additional growth resulting in a final size that is above the antimicrobial properties limit. However, this additional growth could also be an advantage if this allows new AgNPs to be synthesised during the wound healing process, this of course all depends on the toxicity of the AgNPs as well as any unreacted Ag_2SO_4 still in solution and whether this additional synthesis would go beyond the upper limit for antimicrobial activity. In the 5-minute and 10-minute samples it seems that more AgNPs were synthesised after the heat was removed over the course of the 28 days. The same cannot be said for the 15-minute reaction, **Figure 21e** shows a large peak at day 0 from the initial reaction, and a big drop in absorbance over 1 day. This is likely due to an overreaction occurring on day 0 and overnight the larger AgNPs aggregated and separating from the DES and sedimenting in the bottle. Having said this, since the dilution factor is 5-fold, the concentration of the AgNPs would be similar to that of the 10-minute sample which had a 2-fold dilution and the 5-minute sample which for days 0-7 was not diluted.

Figure 22a,c,e shows the UV-vis data for the final 98 days of the study in which the concentration of AgNPs decrease to a fixed stable concentration, as seen from the absorbance values of λ_{max}. Out of the three, the 15 min reaction time is the sample that seems to stabilise sooner since days 1-28 all have overlapping UV-vis spectra, it is noted however that the original drop in concentration of the day 0 to day 1 samples is too large to ignore. Looking at the other samples and taking into account the dilution factors, it seems that the 10 min sample has the least variation in concentration over the first 28 days, most likely due to the reaction almost going to completion without over-reacting, which is the most ideal situation for a wound dressing due to having a more consistent concentration. The most important factor for the stability study however is the size of the AgNPs which the UV-vis can provide an insight into how the size is changing over time, **Figure 23** shows that the variation of the λ_{max} for each sample over the 140 days is 5-6 nm, which is not a huge variation. The sizes obtained from UV-vis are approximately between 4-9 nm in size,[72] however, DLS and TEM were also performed to give a more accurate size determination.

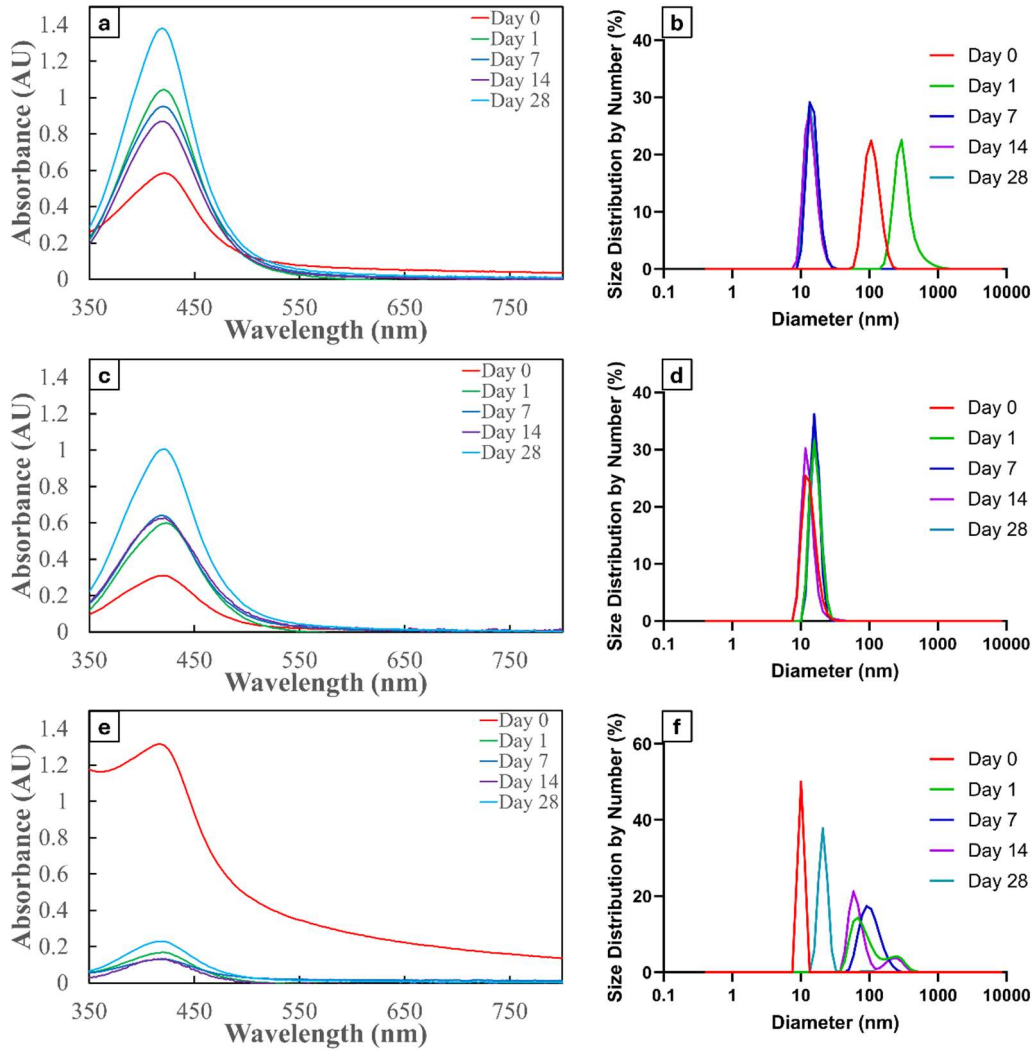


Figure 21: UV-vis and DLS spectra showing AgNPs in Bet:Gly 1:3 and how they are affected over a period of time (Days 0-28). Reaction conditions 120 mL Bet:Gly 1:3, 0.6 g Ag_2SO_4, 80 °C, 5-15 min. a) UV-vis spectra of 5 min sample days 0-7 were not diluted, days 7-140 were diluted 2-fold. b) DLS of 5 min sample. c) UV-vis spectra of 10 min sample, 2-fold dilution. d) DLS of 10 min sample. e) UV-vis spectra of 15 min sample, 5-fold dilution. e) DLS of 15 min sample.

The DLS measurements taken from day 0 to day 28 can be seen in **Figure 21b,d,f** in which out of the three, only the 10 min sample has a stable size of AgNPs at approximately 10 nm consistently throughout the time frame. The 5 min sample shows large fluctuations of size from days 0-7 before stabilising at 10 nm. Out of the three, the 15 min sample was the worst for stability with a constant increase in size of the AgNPs over the 28 days. After 28 days all samples appear to stabilize as seen in **Figure 21b,d,f**, with the 10 min sample having the least amount of overall change in the size of the AgNPs. The sizing's of the DLS readings can be seen in **Table 15**, which confirm that the 10-minute sample has the least variation in AgNP size

(approximately 13-17 nm), up to day 112 when the sizes increase to ~26 nm before showing large aggregation at day 140 with a size of ~170 nm.

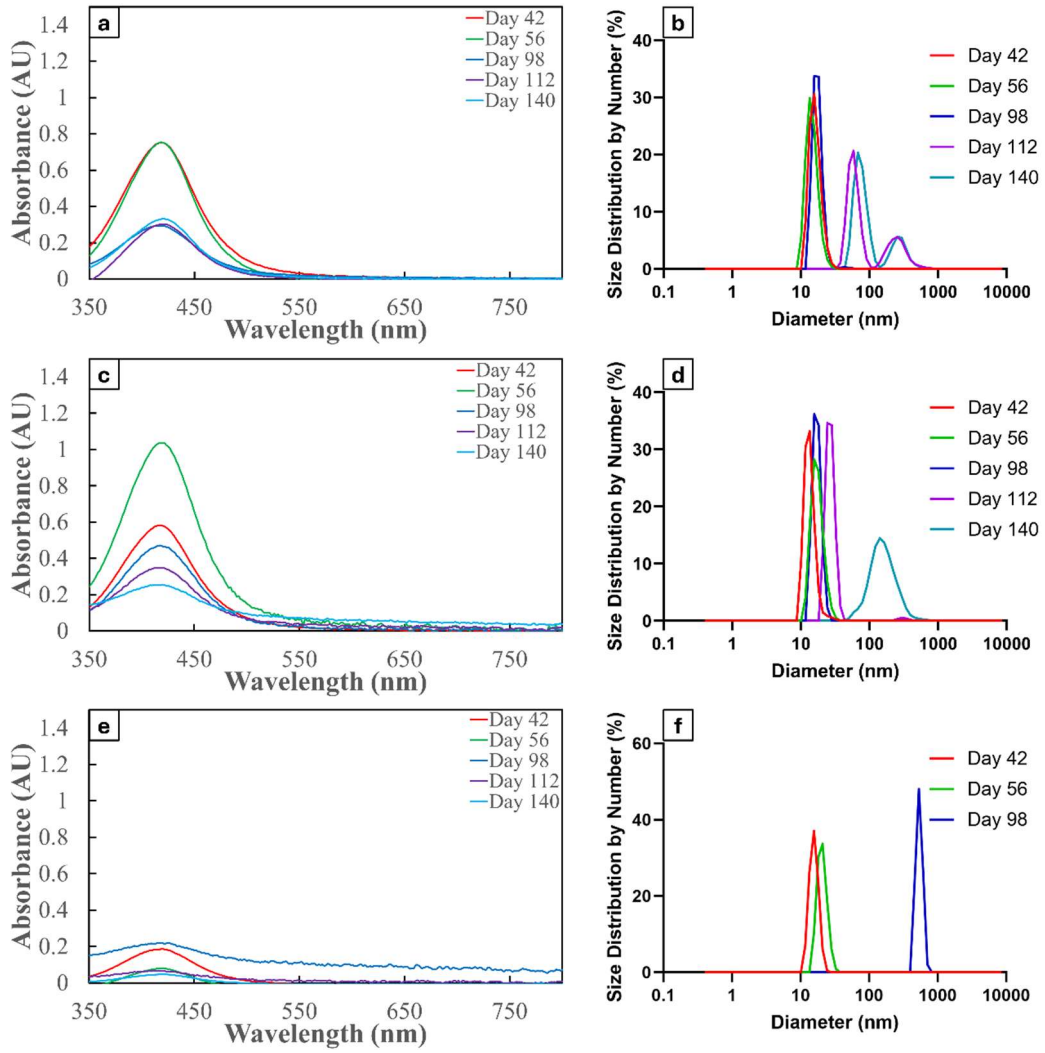


Figure 22: UV-vis and DLS spectra showing AgNPs in Bet:Gly 1:3 and how they are affected over a period of time (Days 42-140). Reaction conditions 120 mL Bet:Gly 1:3, 0.6 g Ag_2SO_4, 80 °C, 5-15 min. a) UV-vis spectra of 5 min sample 2-fold dilution. b) DLS of 5 min sample. c) UV-vis spectra of 10 min sample, 2-fold dilution. d) DLS of 10 min sample. e) UV-vis spectra of 15 min sample, 5-fold dilution. e) DLS of 15 min sample.

The 5-minute sample starts off at sizes <100 nm which is not considered to be a nanoparticle, but then drastically drop in size to 15-17 nm at day 7, this decrease in size is most likely to the larger particles falling out of suspension and no longer available in solution to grow further, possibly allowing the smaller AgNPs to start to form in the empty nucleation sites, which then aggregate at the same time frame as the 10-minute sample at day 112. The 15-minute sample is the least stable according to the DLS data with the size fluctuating through the experiment between 10-100

nm. Looking at both the UV-vis and DLS data it shows that the 10 min sample overall has the better stability with regards to having a consistent size over a long period of time as well as the least amount of change in concentration of the AgNPs as well. The size stability of each sample can be further studied with TEM which will give a more accurate size range for analysis.

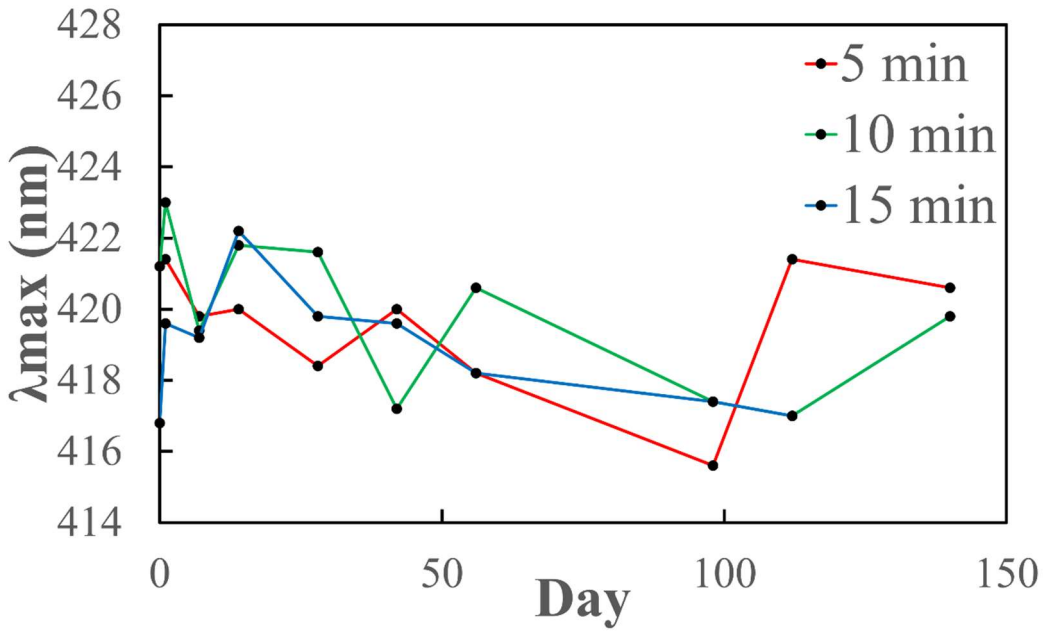


Figure 23: Comparison of the AgNPs suspended in toluene λ_{max} of each sample for the stability studies after extracting out of Bet:Gly 1:3 over the course of 140 days.

Table 15 shows that for each sample the size of the AgNPs is >10 nm from TEM data for each sample (see **Appendix A**). This is to be expected since the DLS size data will also include any size variations from the toluene as previously discussed in **Section 3.1**. Comparing all three samples, the size range does not vary through the experiment with both the 5 min and 10 min sample all having average TEM size ranges of ~<10 nm, with the 15 min samples however showing sizes that reach up to 20 nm. Another issue arose with the 15 minutes samples where the population of the AgNPs on the TEM grid were sometimes too low to generate sufficient data for analysis, this could be due to the extraction process removing all the larger sizes AgNPs which would otherwise be detected, or the larger AgNPs were sedimenting in the toluene so were not suspended when drop cast onto the TEM grid.

Considering that the target size for these AgNPs was 10 nm for the antimicrobial properties, the 5-minutes and 10-minutes samples show sizes that can be too small and potentially would be toxic to humans as well. As a part of the extraction process the larger AgNPs might have been removed which would not be counted during the TEM analysis which may mean that the average AgNP size of the population in the

DES, may include AgNPs much larger than 10 nm, without analysis of the AgNPs in a DES this cannot be confirmed and further analysis on these samples assumes that the sizes from the TEM are representative to the AgNP in DES population. Looking at **Table 15** it would show that the AgNPs in the DES are stable, however when taking into account the data from **Figure 21** and **Figure 22**, the only data set that can be considered to be stable is the 10 min sample. This is due to the fact that the 15 min sample drops in concentration immediately after 1 day by approximately 5-fold, which would indicate a lot of AgNPs falling out of solution, potentially due to aggregation. The 5 min sample is more stable than the 15 min sample however in the first 7 days of the study, more AgNPs were seen to be spontaneously synthesising in the Bet:Gly 1:3 as seen from the dilutions required for the UV-vis analysis, starting at no dilution required for the first 7 days but on day 14, a 2 fold dilution was required to collect data, which indicates that the original reaction on day 0 was not done to completion and there was unreacted Ag_2SO_4 still in the DES. The DLS data for the 5-minutes sample and the 15-minutes sample would indicate that the AgNPs are drastically changing over time, which is not comparative to the TEM sizes (see **Table 15**), however this the 10-minute sample does follow the same sizing trend in both the DLS and the TEM data, where sizing is consistent up to day 112. The 10-minute sample was the only sample to remain consistent through the UV-vis, DLS and TEM analysis resulting in it being the most stable of the three tested. A timeline of the three studies can be seen in **Figure 24** showing that the variation of sizes across a 140-day period.

Table 15: Average AgNP size calculated from TEM images from day 0-140. AgNPs synthesised in Bet:Gly 1:3 (120 mL) at 80 °C over three different timeframes (5 min, 10 min, 15 min). AgNPs in Bet:Gly 1:3 stored in a room temperature (*ca.* 22 °C) dark cupboard. *Data unable to be collected due to sample quality. ** Concentration not high enough for DLS analysis.

Day	Average AgNP size range /nm					
	TEM Analysis			DLS Analysis		
	5 min	10 min	15 min	5 min	10 min	15 min
0	3.81±2.18	5.02±4.06	6.82±4.82	112.2±28.68	13.57±3.31	10.15±1.05
1	4.50±1.73	8.00±3.46	4.53±1.22	329.8±134.2	16.51±3.10	82.17±29.87
7	5.90±1.45	5.43±1.82	6.04±3.64	15.19±3.10	16.14±2.56	108.1±37.84
14	3.67±1.07	4.09±0.92	*	14.13±3.24	13.05±3.49	65.44±15.69
28	7.13±4.00	5.35±3.62	11.12±7.42	14.54±3.22	16.87±3.04	21.27±3.13
42	4.56±2.77	4.87±2.72	4.43±3.82	16.36±3.33	13.60±2.94	15.79±2.41
56	5.95±3.08	4.74±2.95	6.03±5.81	14.75±3.10	17.60±3.74	20.78±3.44
98	7.57±5.89	8.00±3.51	9.07±8.24	17.47±2.72	17.19±2.48	540.80±60.63
112	8.09±2.36	6.23±2.13	*	59.21±11.53	26.86±3.91	**
140	8.16±3.79	9.21±3.72	*	75.18±16.26	170.00±83.17	**
Average size	**5.93**	**6.09**	**6.86**	**66.88**	**32.14**	**108.06**

Thinking of the commercial application of the wound dressings, the 10 min sample even though it is the most stable of the three tested, is not suitable as a commercial wound dressing regarding the shelf life of the product. From synthesis on day 0, the changes in the concentration are too varied to accurately give the quantities of the AgNPs, furthermore, AgNPs are only stable up to day 42 after which there is a significant change in the AgNPs in solution. However, these studies do suggest that if the excess Ag_2SO_4 is removed, or the reaction goes to completion without overreacting, then the synthesised AgNPs would be expected to be stable in the Bet:Gly 1:3 DES. One issue that was found with the analysis was that any sedimented AgNPs in the storage bottle, would constantly be agitated during the sample collections for analysis due to the shift of the bottle from a horizonal storage position to a vertical position to remove the sample aliquots. This meant that visual inspection of any sedimentation on the bottom of the bottle would be difficult due to this agitation and the fact then when the bottle is returned to storage, it is not guaranteed to return to the same position since the bottle is cylindrical. A larger cupboard would remove this agitation since the samples in the bottles can be stored vertically and any sedimentation can be documented more accurately.

Further studies would be required to show if the AgNPs are stable in other DES's however, as discussed in this section previously, the extraction process may be altering the perceptions of whether or not the AgNPs are stable in the DES itself, due to the fact that these AgNPs are removed from the "stable solvent". Developing methods to be able to characterise the size and shape of AgNPs in a DES itself would allow for more accurate stability studies to be performed as well as understanding how the AgNPs are interacting within a DES itself.

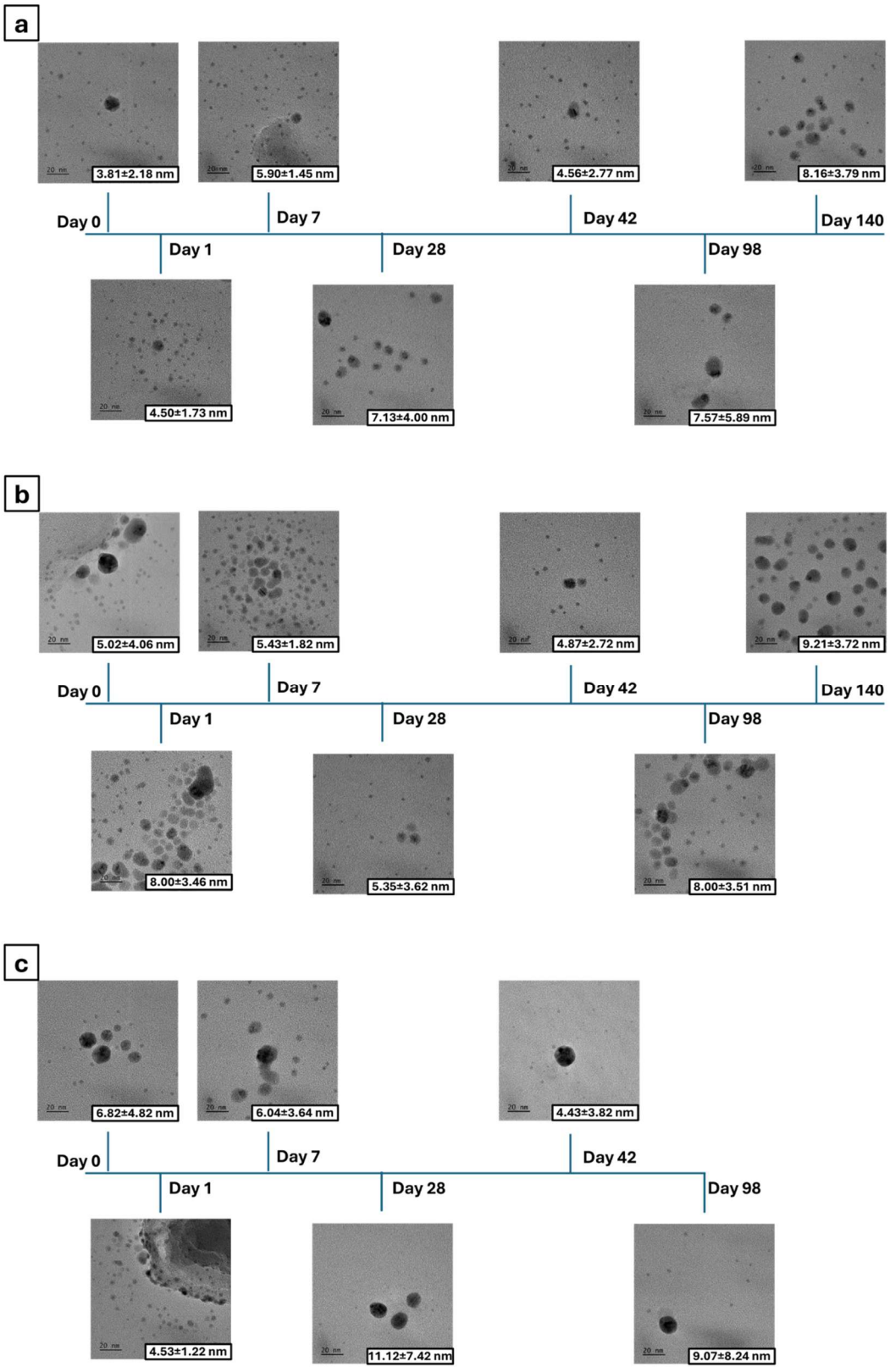


Figure 24: Timeline of stability studies of AgNPs synthesised in Bet:Gly 1:3 showing TEM and average size range at various intervals. Reaction conditions, 120 mL Bet:Gly 1:3, 0.6 g Ag_2SO_4, 80 °C. a) 5-minute sample. b) 10-minute sample. c)15-minute sample.

3.4 Analysis of AgNPs in Bet:Gly 1:3

Since the application of this project is to use the AgNPs in DES in a wound dressing, the extraction of the AgNPs is not needed. However, during method development extraction is required in order to analyse the AgNPs to see if they are suitable for antimicrobial use. In an industrial setting, analysis of the AgNPs is vital for quality assurance on the wound dressings that are being produced. Unfortunately, in the methods used for **Section 3.2** the use of OAm is needed in order to extract the AgNPs out of the DES, with one of the goals being a greener sustainable synthesis, this is not desirable. Developing methods of analysis so that the AgNPs can be analysed in the DES quickly and accurately would help to improve the green credentials of the synthesis by removing the use of hazardous capping agents, as well as quickly analysing the AgNP DES's which also reduces time and energy consumption of the synthesis by eliminating the need for extraction making the reaction a true 'one pot' synthesis.

3.4.1 UV-vis analysis of AgNPs in a Bet:Gly 1:3 DES

Using UV-vis to analyse AgNPs is the quickest method of analysis that was performed as part of this project. As discussed previously in **Section 3.1**, the spectra obtained can give a variety of information about the AgNPs, such as size (λ_{max} value), size range (peak width), concentration (absorbance) and even the shape of the AgNP (shape of the curve).[70,71] This is all valuable information when characterizing the AgNPs, however, the accuracy of this analysis can be brought into question since additional factors, such as the capping agents used can affect the data due to the changes in the LSPR of the AgNPs, and aggregation can affect the data by overlapping peaks resulting in a wider peak width.[70]

In **Section 3.1-3.2**, UV-vis analysis primarily was used to compared different samples in toluene to one another with the size and morphology analysis primarily performed using TEM. However, as a DES is not a conventional solvent for UV-vis analysis there was a concern that this would affect the spectra obtained in comparison to that of AgNPs suspended in the toluene. **Figure 25** shows the UV-vis spectra of AgNPs suspended in a DES which are the same AgNPs synthesised in **Figure 20a**. During the synthesis, before the OAm was added for extraction, a small volume was removed for UV-vis analysis to compare the two UV-vis spectra, in DES and in toluene. The idea being if there aren't any major differences then the AgNP DES spectra can be used qualitatively to compared to a known AgNP size spectrum.

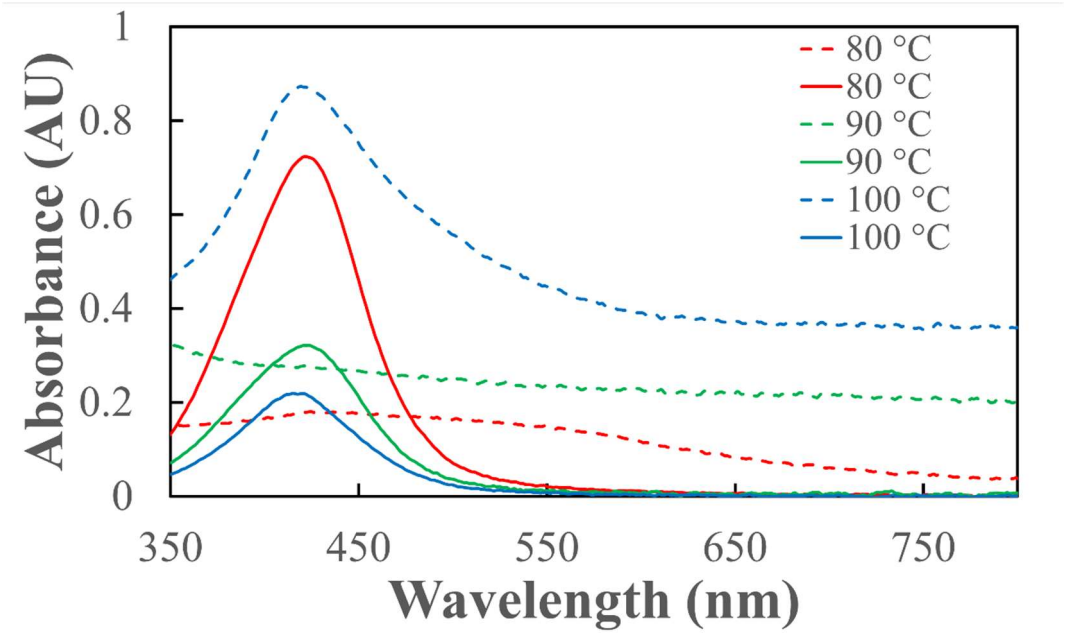


Figure 25: Comparison of UV-vis analysis of AgNPs suspended in Bet:Gly 1:3 (dotted lines) and in toluene (solid lines). Reaction conditions, 5 mL Bet:Gly 1:3, 25 mg Ag_2SO_4, 80-100 °C, 15 minutes. AgNP DES samples were diluted by 12-fold. 80 °C sample suspended in toluene was diluted 2-fold, whilst 90-100 °C samples suspended in toluene were not diluted.

The dashed spectra in **Figure 25** are the AgNPs suspended in DES and are vastly different to that of the AgNP suspended in toluene as seen by the lack of identifiable peaks in the 80 and 90 °C samples of AgNPs in Bet:Gly 1:3, the 100 °C sample of AgNPs in Bet:Gly 1:3 does show a peak however it is unknown what had caused this spectra to be more identifiable than the other samples in DES, especially since it has previously been established in **Section 3.2** that the 80 °C samples were the optimised reaction condition for temperature. **Figure 26** shows the UV-vis spectra of Bet:Gly 1:3 as well as toluene. Whilst these solvents were used as the blanks in the UV-vis analysis, you can also see that within the wavelength range for the AgNPs at approximately 10 nm (427-461 nm) is not going to be affected by these solvents since the solvents do not show any peaks in this range.[72] Even though it is known that the peaks of the AgNPs will be affected by the solvent, the vast difference in the Bet:Gly 1:3 and the toluene spectra means that this method is not viable for future AgNP analysis due to the lack of λ_{max} peaks in some of the spectra.

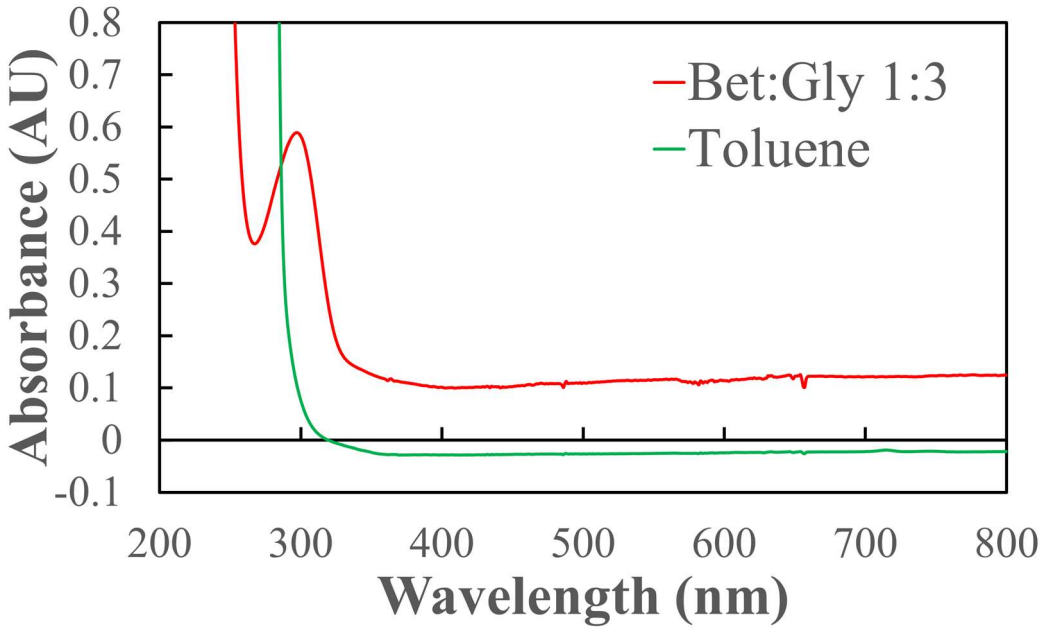


Figure 26: UV-vis of the pure solvents used to suspend the AgNPs including Bet:Gly 1:3 DES and toluene.

However, these results do give insight into the extraction process as the dilution needed for the DES samples were much larger than that of the AgNPs in toluene. This means that the extraction methods designed for this project do not fully extract all the AgNPs synthesised due to the drop in concentration seen in the lower absorbance values when taking into account the dilution factors. Another issue in the extraction that arises from this analysis, as seen in the 100 °C samples, is the peak width in the DES is larger than in toluene, this could be due to the fact that the DES is a poor solvent for UV-vis analysis but could also indicate that the extraction process also removes any larger AgNPs or aggregates. In other publications, such as the Adhikari papers, the solvent they used for the analysis of the AgNPs was also toluene.[2,27,62] The other DES methods reviewed in **Section 1.2.3**, the solvent that used more biological capping agents like GTE, the solvent was water.[26]

The difference between the DES and the conventional UV-vis solvents, in this case toluene, is an issue since if the AgNPs in a DES are to be used in a wound dressing, then an accurate size analysis is needed to ensure the NPs are ~10 nm to exhibit antimicrobial properties. This supports the objective of this chapter of the project to develop analysis methods in a DES. UV-vis providing a qualitive analysis during the project would not be enough to give quantifiable results for an accurate size range, further work was conducted to try to develop methods which could give a more accurate size analysis in a DES.

3.4.2 X-Ray Diffraction Analysis of AgNPs in a Bet:Gly 1:3 DES

Powder X-Ray Diffraction (PXRD) has previously been used to analyse solid powder NPs of different metals, such as CoS, Ni and Fe_2O_3.[74,75] PXRD on metal NPs can be performed similarly to that of the bulk material, if there is enough present for the instrument to detect, to determine the size, shape and purity of the NPs. The size of the NPs can be determined from the broadening of the intensity peaks as long as the NPs are uniformly spherical, with minimal other morphologies present, by using the Scherrer equation (**Equation 9)** which quantifies the broadening of the peak, where D is crystalline size, κ is the Scherrer constant (0.9), β is the width of peak at half height, λ is the X-ray wavelength and θ is the diffraction angle in radians.[75]

$$D = \frac{\kappa\lambda}{\beta cos\theta} \qquad (\mathbf{9})$$

The peak broadening required for the Scherrer equation tends to work best at nanoparticles sizes below 25 nm since the peak broadening is more observable and is less likely to be caused by instrumental effects, however particles below 5 nm become much more difficult to analyse due to the much larger peak broadening as well as to the low signal to noise ratio at these sizes.[75] This should not be a problem for the analyses required for the antimicrobial AgNPs since the targeted size range is 5-10 nm.

As discussed previously, for the Scherrer equation, the shape of the NPs needs to be uniformly spherical, this can also be determined using PXRD by looking at the peak intensities in particular which crystalline directions produce the highest intensities. If the NP is spherical then the relative peak intensities for one crystalline plane will be different than if the NP elongated along the same crystalline plane to form a nanorod. This can be difficult since the NPs need to be analysed in a preferred orientation which is easier for shapes such as nanorods, which tend to lay flat, rather than that of spheres which can be in lots of different orientations. For shape analysis, of antimicrobial spherical AgNPs like the ones synthesised in **Section 3.1** and **Section 3.2**, PXRD might not be the most preferred method.

The final analysis that can be performed on PXRD is the purity of the NPs, this is determined by comparing the spectra obtained and comparing the peak diffraction angles with that of a literature database value. For example, AgNP PXRD diffraction spectra can be compared with the results obtained from the synthesised AgNPs to

determine if they are pure silver or if they also contain silver halide NPs. This is important information since as discussed in **Section 1.2.3**, if the DES contains a halide, then that could introduce impurity into the AgNPs. For the synthesises performed in this project the purity of the AgNPs should be less questionable since the DES is halide-free, but for industrial scale production the quality insurance that the AgNPs are pure is an important step in the production process. PXRD is a good analysis technique for non-suspended AgNPs, however the goal of this part of the work is to analyse the AgNPs in a DES which is not as simple.

Liquid cell XRD has been previously performed to analysis gold NPs in an aqueous solution,[76] however, this was not to size the NPs but to confirm that they were synthesised in the first place. The analysis of AgNPs in a DES from what has been reviewed in the literature, has not been studied previously. One potential issue is that if the Bet:Gly 1:3 DES does have some organisation within the DES, such as the large H-Bond network, then this may produce a peak on the XRD spectra.[77,78] If this peak is within the range of the AgNPs peak, then the AgNP peak can be overlooked due to the interference from the Bet:Gly 1:3 DES, this was easily remedied by running a blank DES sample to determine where the DES may show a peak on the AgNP DES sample.

As seen in **Figure 27a**, the DES was run across the full range of the possible diffraction angles to collect data for the blank in order to see what peaks would occur from the DES itself and which can be determined comes from the AgNPs. The Bet:Gly 1:3 DES does have a very broad and intense peak at 20° 2θ which could show some indication of order within the H-bond network of the DES, this peak does spread across to the peaks that would be expected in Ag metals as seen in **Figure 28**. This is confirmed by the AgNP DES peak on the same graph, where only a very small peak can be seen when 2θ is around 38°.

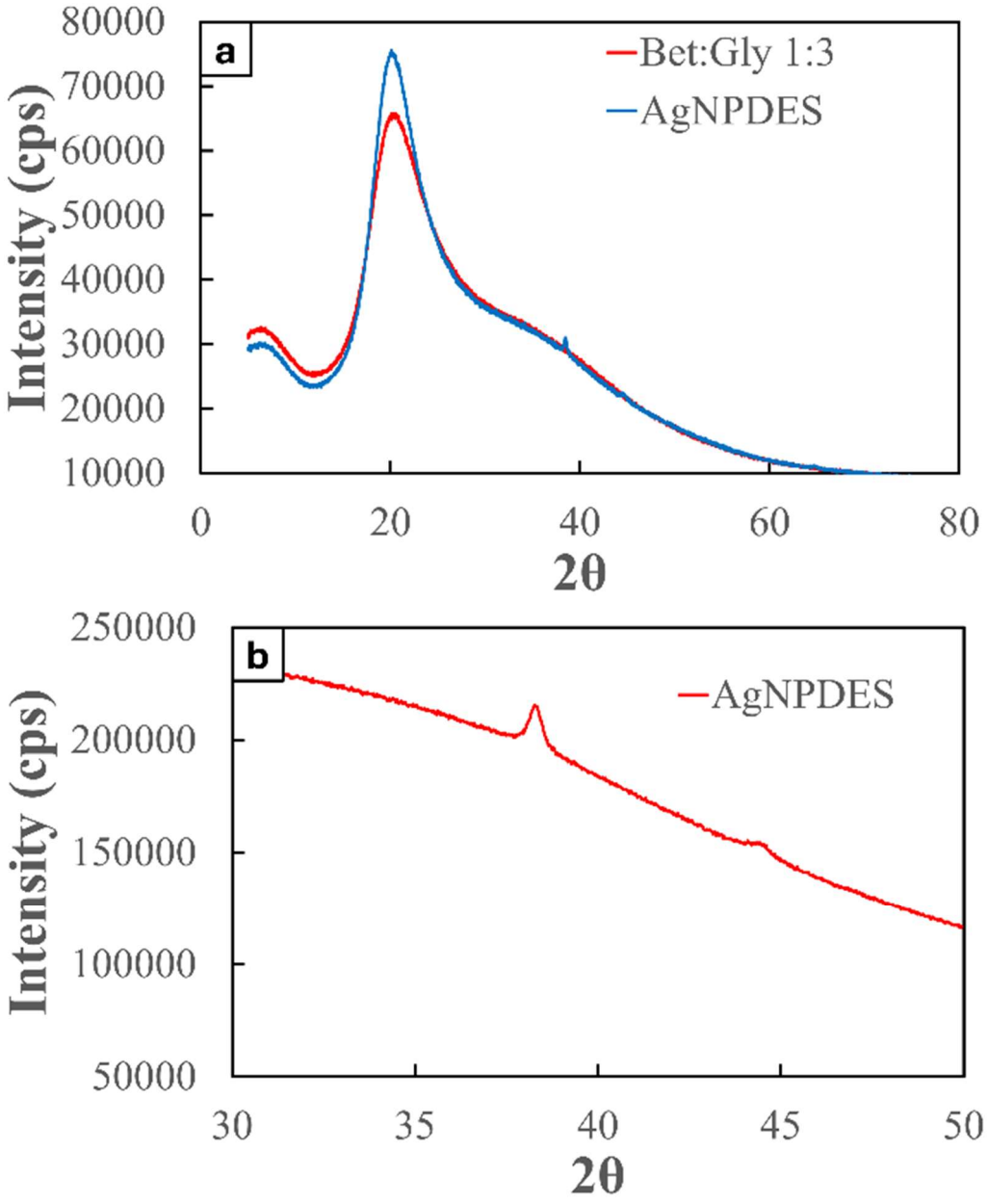


Figure 27: Thin film XRD on approximately 5 mg/mL concentration of AgNPs in Bet:Gly 1:3 DES a) AgNPs in Bet:Gly 1:3 compared to the blank Bet:Gly 1:3, run over 13 Hr. b) AgNPs in Bet:Gly 1:3 run over 4 Hr across a 2θ range of 30-50°. XRD samples ran by Hannah Beska (NTU).

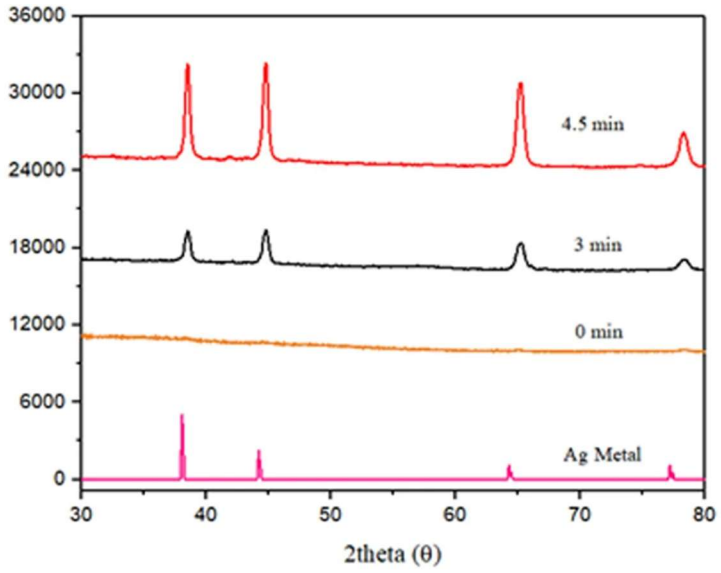


Figure 28: XRD comparison of Ag Metal as well as AgNPs synthesised over time to be used as a reference to the literature.[79]

After the full diffraction angle was ran, a smaller range was tested between 30-50° 2θ since those were the only peaks that were observed on the spectra, this also brought down the time of the scan to 4 Hr as seen in **Figure 27b**. This spectrum compared to **Figure 27a** shows the peaks that would be expected for Ag metal when 2θ is approximately 38° and 44°.[79] Unfortunately, the other key markers that can be seen above 60° were not measured, however, it can be determined that the NPs made were in fact pure silver. A size analysis could not be determined however due to the baseline not being flat resulting in an unknown peak width. Interestingly, the cps for the intensity on **Figure 27b**, were much higher count than those observed in **Figure 27a** even though it was the same concentration of AgNPs being tested. It can be speculated that due to the length of time the XRD is ran for to collect the data, during that time the AgNPs could have been mobile in the DES due to the visible brown line appearing across the middle of the AgNP DES at the end of the XRD analysis. It is unknown why this occurred and further work on the method development would be needed to account for these mobile AgNPs if this is the case.

A better spectrum could be obtained with a higher concentration, therefore the amount of AgNPs in solution were doubled to 10 mg/mL to try and clear up the signal-to-noise ratio. **Figure 29** shows the data collected compared to the DES (**29a**) and a closer look at the peaks observed without the DES data (**29b**), the DES blank appears at a higher cps intensity than that observed in **Figure 27a**, which should not occur since it is the same sample that was ran previously. This is not ideal since it shows that the method used does not produce consistent repeatable results. Another observation that was noted was that the peaks were located on different

diffraction angles than previously observed in **Figure 27** and do not compared to a literature value in **Figure 28**, of 38° and 44°.[79] This could indicate that these are not AgNPs even though the same method was used. Since this is unlikely, and due to the change in the data when comparing the blank DES plots, the methods developed as part of this project were not suitable for analysing AgNPs in a DES.

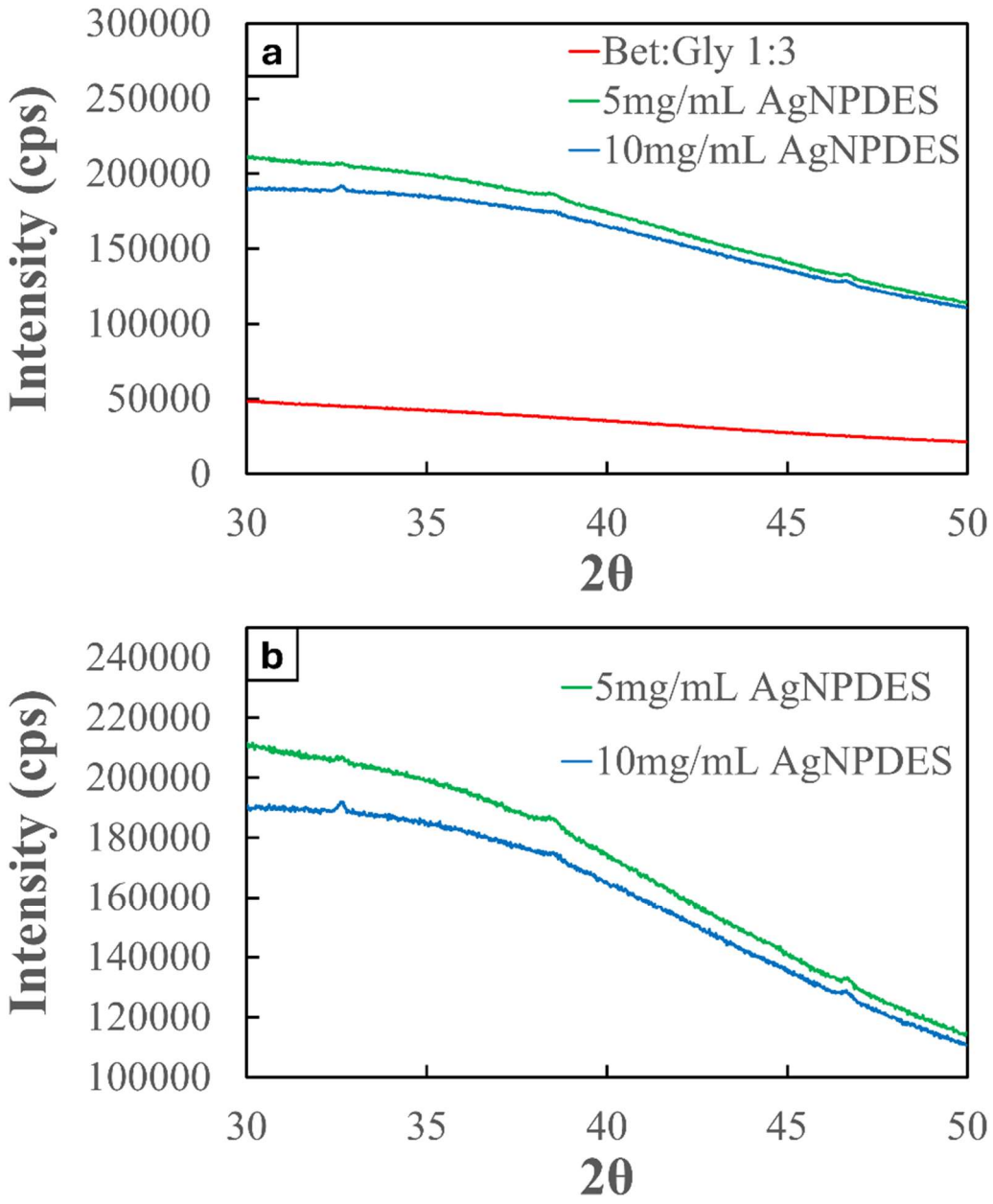


Figure 29: Thin film XRD on AgNPs in Bet:Gly 1:3 DES, ran for 4 Hr across a 2θ range of 30-50° a) 5 mg/mL and 10 mg/mL AgNPs in Bet:Gly 1:3 compared to the blank Bet:Gly 1:3. b) 5 mg/mL and 10 mg/mL AgNPs in Bet:Gly 1:3. XRD samples ran by Hannah Beska (NTU).

Given more time in the project, this method could be altered to see if more consistent data can be obtained, as well as using AgNPs of a set size that was purchased online,

these would be useful since the purity of the AgNPs would be known and any peak shifts would be due to the method or the DES interference rather than the synthesis. Whilst the data observed was not to a standard that would be required for an industrial scale analysis for quality testing, it was a good first attempt at an analysis of AgNPs within a DES that has not been performed in the literature to date. Given more time, a new XRD method could have been developed to improve the baseline of the spectra so that this analysis could be used to characterise AgNPs in a DES.

3.4.3 DLS and TEM analysis of AgNPs in Bet:Gly 1:3 DES

Whilst no data was collected for DLS or TEM analysis within a DES, it is worth mentioning that these analyses can be attempted. The SOP creator in the DLS instrument used as part of this project, can input custom solvents at a maximum viscosity of 10000 mPa.s, which the Bet:Gly 1:3 DES is within this range (see **Section 4.1**). It is unknown whether the pockets of density within the DES due to hole theory,[80,81] may affect these readings due to the change in density potentially interfering with the DES size analysis. Unfortunately, due to time restraints this study of AgNPs in a DES analysed in a DLS was not attempted. On the other hand, liquid TEM samples can be run with a liquid cell holder, these holders are expensive and was not within the budget that was provided for the project. An alternative liquid cell was tested which could be mounted in the currently available holders. Unfortunately, these liquid cells that were purchased were difficult to use and when tested by the MTIF Imaging Suite technicians with a AuNP sample, did not clearly capture any images. It was determined that using a DES in these holders would most likely give the same results, not taking into account how the DES would affect the liquid cell. A standard liquid TEM holder may provide much clearer images which would be useful and would be something worth researching since TEM would give the best analysis of size and shape. Liquid TEM have been previously performed on aqueous AgNPs in the literature, with size and shape analysis.[82] Whilst the image quality was not at the highest resolution, which may be an issue for smaller AgNPs, due to the silicon windows scattering the electron-beam, but by observing the AgNPs in a liquid phase, other analysis such as how the AgNPs grow and move through the solution over time can be monitored.[82]

Out of the methods tested for AgNP analysis in a DES, UV-vis does have the most potential for quality assurance for industrial production, whilst the liquid TEM would provide the best information, the cost of the holder and TEM itself would not be ideal as a start-up cost, whilst the UV-vis instrument would cost a fraction of the price. For this project, unfortunately none of these methods were used in the future work due

to the lack of consistency with the UV-vis spectra as well as running out of time to perform more research into improving the method.

With the successful development of a synthesis of AgNPs in Bet:Gly 1:3 without the use of OAm and calculating the RAE and E-factor to compare to literature methods. It was established that going forward, synthesising the AgNPs in a DES without the use of a capping agent is the optimum way to improve the RAE and E-Factor. However, OAm was required to characterise the AgNPs to determine size and shape to ensure that they are suitable for an antimicrobial wound dressing. This led to the idea of introducing a capping agent into the DES itself, which would allow for the E-factor to be kept to a minimum by reducing to use of further reagents in the synthesis which will be disposed of as waste material. The design of new DES's, whose components were specifically chosen for their likely suitability for AgNP synthesis, that are yet to be seen in the literature were considered as a replacement for the Bet:Gly 1:3.

This chapter's work was based on the previous studies performed by Adhikari *et. al.* which uses OAm as a capping agent alongside a DES to synthesise AgNPs.[2,27,62] Since these AgNPs used a microwave reactor to synthesise the AgNPs, method development was performed to determine new ideal conditions for synthesise by heating and stirring on a hotplate (3 mL OAm, 5 min, 150 °C). Further study was performed on a synthesis which did not include the OAm, as this is hazardous to humans, so that the AgNPs in the Bet:Gly 1:3 can be tested for antimicrobial properties. This resulted in new "ideal" conditions for synthesis (15 min, 80 °C), in which the AgNPs in the Bet:Gly 1:3 can be added to OAm after synthesis to extract the AgNPs for characterisation. Once the extraction methods were developed, a stability study was performed on AgNPs synthesised in Bet:Gly 1:3 at 80 °C over 5 min, 10 min and 15 min, to determine the effect on AgNP stability in the DES. It was determined that out of the 3 samples, the 10 min reaction time was the most stable over the 140 days tested, with the size of the AgNPs were fluctuating between ~4-9 nm. It was noted that if the AgNPs could be analysed within the DES for characterization, the use of a hazardous capping agent could be removed from the reaction completely, resulting in a true "one-pot" synthesis of AgNPs. UV-vis, DLS, XRD and TEM methods were tested for AgNP characterisation with promising results for XRD analysis. However, the baseline for the XRD study meant that identifying the peaks proved difficult due to the silver peaks being masked by the DES baseline, but with further method development this could prove to be a viable method for AgNP characterisation in a DES.

Chapter 4: New Three Component Deep Eutectic Solvent (DES) Based Molecular Liquid Discovery, Characterization and AgNP Synthesis

The ability to tailor DES's to suit a specific application is one of the advantages of choosing a DES over more conventional solvents. Specifically in the synthesis of AgNPs, reaction temperature and reaction time may be affected by the properties of the DES, as well as the AgNP size due to the hypothesis that the "holes" in a DES are the nucleation sites, these "holes" can be attributed to fluctuations of lower density voids throughout the liquid as described by Hole Theory, which will be covered further into this chapter.[80,81] To tailor a new DES for the purpose of this project, three properties were established as targets for the new DES. Firstly, it was determined that a low viscosity liquid would be more ideal, since these DES's are more easily transferred from the media bottle to the reaction flask, reducing errors in the quantity of the DES's used in both the AgNP synthesis and the antimicrobial testing.

Secondly, a higher conductivity would also be ideal as this ensures that the Ag^+ metal ions can move more easily in the solution to the reduction and nucleation sites for AgNP growth. This can also be achieved with a low viscosity liquid, therefore combining the two properties, low viscosity and high conductivity, a molecular liquid can be designed for the purpose of AgNP synthesis. This movement of ions in a DES can be described by Hole Theory, in which the DES can be considered to have theoretical holes throughout the liquid in which the ions can pass through, if the hole is larger than the ions then the movement of these ions is unhindered and if the holes are smaller than the ions, then the movement is hindered and conductivity decreases.[80,81] The cause of these holes is due to constantly changing pockets of local density in the DES, these pockets create holes of random size and are constantly in flux, a DES with a higher viscosity will have smaller holes, which may not facilitate the movement of the Ag^+ ions.[80,81]

Thirdly, whilst it was important to consider the physiochemical properties of the DES during the process of designing a new DES, the composition of the DES itself was also an important consideration. To obtain a greener more sustainable synthesis of AgNPs, removing the hazardous capping agent and incorporating a non-hazardous capping agent into the DES would improve upon the green credentials and sustainability of the synthesis which aligns with the objectives of the project in **Section 1.1**, to improve sustainability by removing harmful reagents.

Whilst the synthesises in **Chapter 3** was successful in producing AgNPs, the use of OAm, which is a hazardous capping agent, is not optimum. Whilst the OAm is not needed for the synthesis of the AgNPs it is however required for the extraction and characterization. Due to OAm's hazardous nature, the idea of including a non-hazardous capping agent as part of the new DES's would allow for successful extractions without the need for additional capping agents. However, there will still be waste produced from the extraction solvents which will impact the E-factor, unless methods can be developed to analyse the AgNPs in the DES, as previously discussed in **Section 3.4**.

The components used in the synthesis of the new DES's were first researched to determine their antimicrobial behaviour with the aim that a DES synthesised from antimicrobial components would also be antimicrobial. Previously, DES's formed from fatty acids have been tested for antimicrobial properties using the disk diffusion method, including mainly capric acid (Cap) which itself is also anti-bacterial and anti-inflammatory.[29,83] Other studies have looked at DES's that include ChCl and Bet as the HBA with different HBD's, including some three component DES, however water was also introduced; which this synthesis aims to avoid so that the DES is not affected by high water content, which will break down the DES's. It was decided based on this literature work, that a DES that was formed from fatty acids would be the ideal DES to use to fulfil the criteria of forming a DES from inherently antimicrobial components. These three targets: physiochemical properties; incorporating a capping agent into the DES itself and the antimicrobial properties of the components, were considered in order to design new DES's not seen in the literature.

Early on in the designing process Cap and lauric acid (Lau) (to form Cap:Lau 2:1) were chosen as the fatty acid components of the new DES's as they can be used as a capping agent due to their hydrophobic tail, which is similar to that of OAm. However, Cap and Lau do have much shorter carbon chains which may require a larger quantity of each component to cap the AgNPs compared to OAm, but this should not be an issue since these components are in abundance in the DES during the synthesis. Cap:Lau 2:1 was synthesised using the same conditions as the Bet:Gly 1:3, so can be considered equal in terms of RAE for the formation of the DES.

A small sample of Cap:Lau 2:1 was synthesised, as this is a known DES in the literature,[84] and was used to try to synthesise AgNPs by heating at 150 °C and monitoring the reaction over a 5 Hr period to see if there was any colour change indicating AgNP synthesis. After approximately 5 hours, no colour change was observed. If reactions did occur, the timeframe and intense heating would mean that the reaction would not be viable as an alternative to the Bet:Gly 1:3 synthesis, as

well as being unsuitable for industrial synthesis. Due to the long reaction times and the high temperatures required, the sustainability of the reaction would be lower than that of the Bet:Gly 1:3 DES. It was also observed that the Ag salt was not very soluble in the Cap:Lau 2:1 most likely because of the DES's hydrophobicity, this would mean that the Ag^+ ions were not available for reduction into AgNPs. Moving forward with designing of the DES's a third component was added to potentially reduce the hydrophobicity and assist in the dissolution of the Ag salt. The method for forming the new three component DES's of Cap:Lau:X can be found in **Section 2.2**, the methods for physiochemical property testing can be found in **Section 2.6**.

4.1 Capric acid:Lauric acid:X based Molecular Liquids and their Properties

Since the eutectic point is a unique property of a DES and these new liquids have not been tested to see if they are at the eutectic point, the Cap:Lau:X liquids will be referred to as a DES inspired molecular liquid, or molecular liquid (ML) for short. A variety of three component composition of Cap:Lau:X molecular liquids were successfully synthesised (see **Table 10**), however, only the Cap:Lau:Bet and Cap:Lau:Lev ML's were chosen for further study due to their low synthesis temperatures as well as the ML forming a liquid at approximately room temperature (~22 °C). Since these ML's have yet to be published in the literature, a series of physiochemical property testing was performed, including viscosity, conductivity, surface tension, density and refractive index to compare with a Bet:Gly 1:3 DES, which was also characterised to compare to known literature values. As Bet:Gly 1:3 is hydrophilic in nature, its physiochemical properties are dependent on both temperature and water content,[85] however, as the Cap:Lau:X ML's should have a more hydrophobic behaviour, it was assumed that their properties would be more dependent on temperature than water content as water would not incorporate into the ML's as readily as the Bet:Gly 1:3 DES. Development of hydrophobic ML's is important for synthesises where water in the reaction can be detrimental to the product, which is the case for this project due to possible changes in the nucleation of the AgNPs with water present.

Figure 30 shows that the Bet:Gly 1:3 DES used in the previous synthesis of AgNPs in **Chapter 3**, has a much higher viscosity (1556 mPa.s at 25.3 °C) than the new

three component Cap:Lau:X ML's (where X= Bet, Lev), with the lowest viscosities being the Cap:Lau:Lev based ML's. Out of the three different ratios of the Cap:Lau:Lev ML's, the lowest viscosity (10.86 mPa.s at 24.9 °C) was seen in the 8:4:1 ratio and the highest viscosity (11.91 mPa.s at 24.8 °C) was seen in the 2:1:1 ratio. As the temperature increased it was observed that the viscosity of all the ratios of Cap:Lau:Lev would be very similar in value as seen in **Figure 30c**. **Figure 30** also shows that there was a big drop in viscosity (65.7 to 36.9 mPa.s) from 308.5 K to 313.5 K (35 °C to 40 °C) for the Cap:Lau:Bet 4:2:1 ML, which is due to the ML not fully melting to form a liquid. With the new ML's having a much lower viscosity this fulfils the first objective of designing these new ML's. However, this objective also requires the conductivity of the ML to be at a level where there can be ions moving throughout the H-Bond network. **Figure 30d** confirms that the Cap:Lau:Lev ML's have a lower conductivity than the Bet:Gly 1:3 DES, with all ratios of the ML not registering on the conductivity probe, which would likely result in a synthesis that would take longer than the already established synthesises in **Chapter 3**, however, the Cap:Lau:Bet 4:2:1 ML has a higher conductivity than the Bet:Gly 1:3 DES's which theoretically should improve upon the synthesis of the AgNPs by speeding up the reaction due to easier transport of the Ag^+ ions to reaction sites.

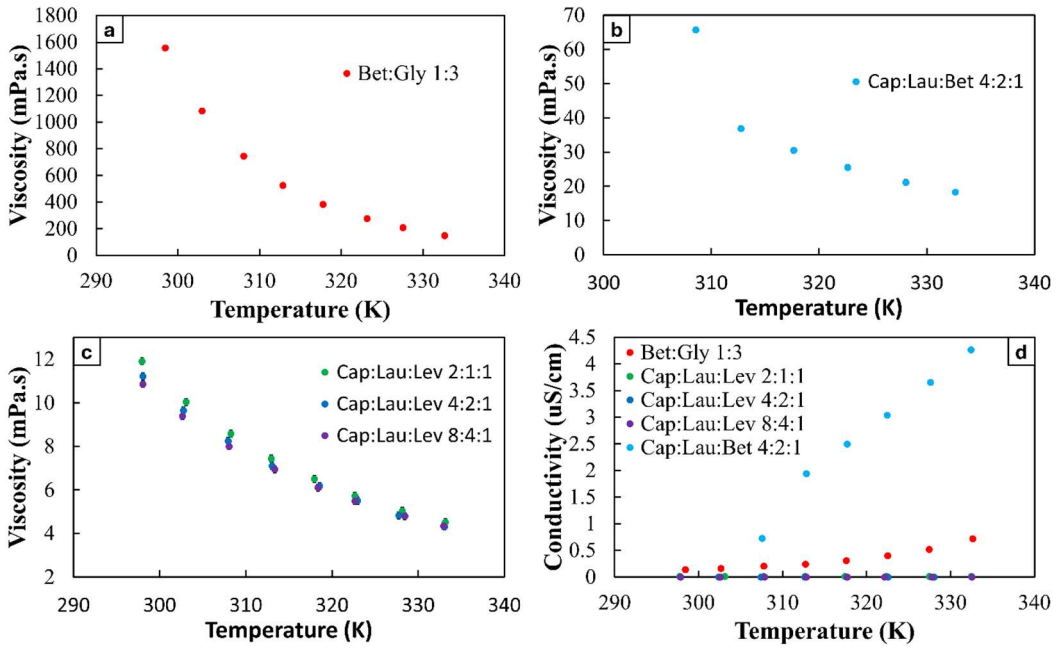


Figure 30: Viscosity and conductivity graphs for new three component Cap:Lau:X based ML's (where X= Bet,Lev) compared to Bet:Gly 1:3. a) viscosity over temperature plot for Bet:Gly 1:3. Error = ±18.75 mPa.s b) viscosity over temperature plot for Cap:Lau:Bet 4:2:1. Error = ±0.75 mPa.s c) viscosity over temperature plot for Cap:Lau:Lev ML's. Error = ±0.15 mPa.s d) conductivity over temperature plot for Bet:Gly 1:3 and Cap:Lau:X ML's

Looking at the data acquired and applying the Arrhenius equation for viscosity (see **Equation 10**) and conductivity (see **Equation 11**), the activation energy for the viscosity and conductivity can be calculated for each DES.

$$ln\eta = ln\eta_0 - \frac{E_\eta}{RT} \qquad (\mathbf{10})$$

$$ln\sigma = ln\sigma_0 - \frac{E_\sigma}{RT} \qquad (\mathbf{11})$$

Where η is the viscosity, η_0 is a constant, E_η is the activation energy for flow, R is the ideal gas constant, T is the temperature in Kelvin, σ is conductivity, σ_0 is a constant and E_σ is the activation energy for electrical conduction. This will allow the data set to be plotted with a linear gradient ($\frac{E_Y}{R}$, where Y= η, σ) in which the activation energy for flow and electrical conductance can be calculated. **Figure 31a,b,c**, show the new three component DES's plotted against the Bet:Gly 1:3 DES and the activation energies presented in **Table 16**. Due to the Cap:Lau:Lev based ML's not registering on the conductivity probe, the E_σ could only be calculated for the Bet:Gly 1:3 and the Cap:Lau:Bet 4:2:1 ML. As seen in **Table 16**, the E_σ for the Bet:Gly 1:3 is larger than the E_σ for the Cap:Lau:Bet 4:2:1, showing that electrical conduction requires less energy in the Bet:Gly 1:3 than in the Cap:Lau:Bet 4:2:1. This will potentially require a higher energy requirement in terms of temperature of the reaction, to synthesise AgNPs in the Cap:Lau:Bet 4:2:1 to overcome this energy barrier.

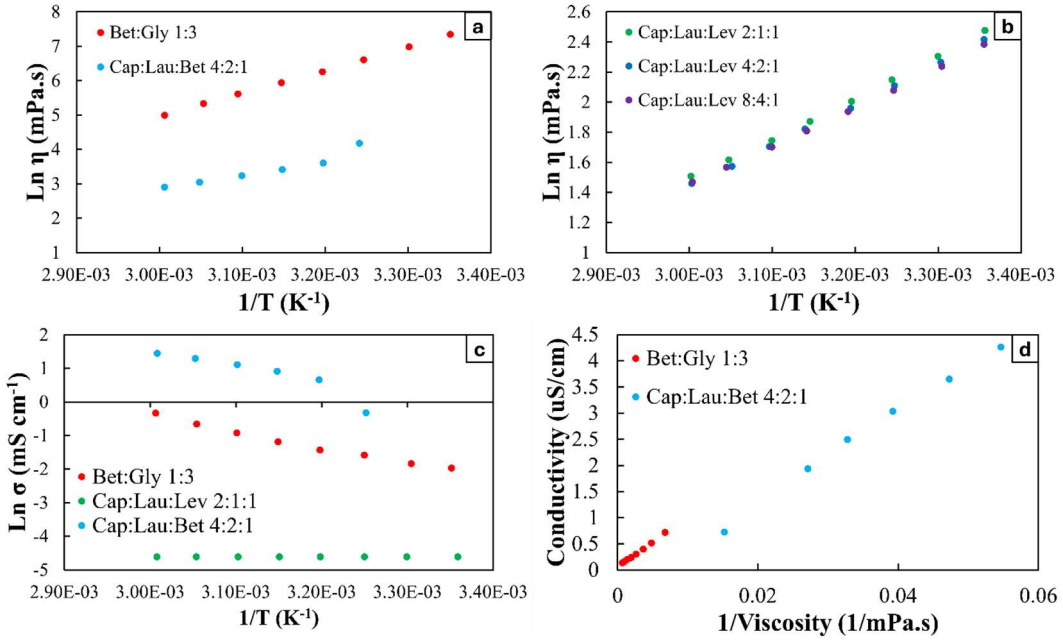


Figure 31: Arrhenius plots the new three component Cap:Lau:X based ML's (where X= Bet,Lev) compared to Bet:Gly 1:3, to determine activation energy. a) ln viscosity over 1/temperature plot for Bet:Gly 1:3 and Cap:Lau:Bet 4:2:1. b) ln viscosity over 1/temperature plot for Cap:Lau:Lev DES's. c) ln conductivity over 1/temperature plot for Bet:Gly 1:3, Cap:Lau:Bet 4:2:1 and Cap:Lau:Lev 2:1:1 ML's. d) conductivity over 1/viscosity plot for Bet:Gly 1:3 and Cap:Lau:Bet 4:2:1 ML's.

Due to a full collection of viscosity data, the E_η could be calculated for all the ML's, which shows an opposite trend, where the Bet:Gly 1:3, due to the larger viscosity of the DES, has a larger E_η than the Cap:Lau:X based ML's (see **Table 16**). The Cap:Lau:Bet 4:2:1 ML has the larger E_η of the hydrophobic DES's with the Cap:Lau:Lev ML's increasing in E_η as the concentration of Lev increases in the ML. In terms of using these DES's for AgNP synthesis, as discussed previously, a ML that requires a lower E_η would be easier to transfer the AgNPs in DES from the reaction vessel to other vials, resulting in less wasted product and easier scale-up, which increases the sustainability of the synthesis by reducing E-factor. Due to the collection of both viscosity and conductivity data only available for the Bet:Gly 1:3 and the Cap:Lau:Bet 4:2:1 ML's, only these ML's could be used to determine if the conductivity is limited by the ionic mobility of the ML by Walden's rule.[86,87]

Table 16: Activation energy of electrical conduction and flow data for Cap:Lau:X (where X= Bet, Lev) ML's compared to Bet:Gly 1:3 DES. * data unavailable due to conductivity not registering on the scale.

ML	Activation Energy for Electrical Conduction E_σ / kJ mol^{-1}	Activation Energy for Flow E_η / kJ mol^{-1}
Bet:Gly 1:3	3.90x10^4	5.61x10^4
Cap:Lau:Lev 2:1:1	*	2.28x10^4
Cap:Lau:Lev 4:2:1	*	2.26x10^4
Cap:Lau:Lev 8:4:1	*	2.16x10^4
Cap:Lau:Bet 4:2:1	5.42x10^4	4.09x10^4

Figure 31d shows the conductivity plotted against the reciprocal of the viscosity at different temperatures, for the Bet:Gly 1:3 and Cap:Lau:Bet 4:2:1 ML's. The Cap:Lau:Bet 4:2:1 ML was chosen since it is the only three component composition ML which has a visible change in conductivity readings, as seen in **Figure 30d**. This plot serves to show that the Bet:Gly 1:3 and the Cap:Lau:Bet 4:2:1 DES's both follow the Walden rule, which links the relationship between viscosity and ionic conductivity.[86,87] As mentioned at the start of this chapter, the objective for these new DES's are to have a conductivity similar or greater to that of Bet:Gly 1:3 to facilitate the Ag^+ ion movement through the DES to the reduction and nucleation sites. In this case the Bet:Gly 1:3 and Cap:Lau:Bet 4:2:1 ML's have gradients that are similar in terms of linear correlation which means that the ML should function alike with regards to charge carrying species within the ML, in this case the Bet. Since the Cap:Lau:Lev ML's did not register a value for conductivity on the instrument, the same plot cannot be done accurately to see if there is a difference in correlation, since there isn't a charged carrying species in the Cap:Lau:Lev ML. Other physical properties were also measured at room temperature with the ML's that were liquid including surface tension, refractive index and density readings (see **Table 17**).

Due to Cap:Lau:Bet 4:2:1 ML being a waxy solid at room temperature, it was excluded from further physical property testing. **Table 17** shows the density, surface tension and refractive index of the remaining DES's. Since Bet:Gly 1:3 is a known DES in the literature, these values can be compared to already published results. Research by Meredith *et. al.* have stated the Bet:Gly 1:3 DES density at <1 wt% water content to be 1.24± 0.01 g mL^{-1}, the surface tension to be 90±6.1 and the refractive index to be 1.479±0.003.[3] Compared to the values calculated in this study,

the density and the refractive index are acceptable when taking into account the errors associated from the instruments used. This is assuming that the Bet:Gly 1:3 that was synthesised for this project had a water content of < 1%, however this would need to be measured in a larger commercial scale-up to ensure each batch of DES's are equal in terms of physiochemical properties.

In comparison to the Bet:Gly 1:3 DES, the Cap:Lau:Lev ML's have a lower density, and a reduced surface tension. The density of the ML's decreases from Cap:Lau:Lev 2:1:1 to 8:4:1, which is to be expected as the ratio of the Lev in the ML's decreases. This is not the same for the surface tension however, as the surface tension decreases from the 2:1:1 ML to the 4:2:1 ML, to then increase to the largest value seen in the 8:4:1 ML. The irregularity of these results could be due to contamination on the Du Noüy ring, or the sample being contaminated from the testing dish. Repeating these results would have confirmed what the trend should be, however this was not performed due to time constraints. The decreasing concentration of Lev in the ML's from the 2:1:1 ML to the 8:4:1 ML is the potential cause of the refractive index to increase across the ratios of ML's, as seen in **Table 17**.

The reduced surface tension could be an issue with regards to AgNP synthesis due to the fact that a lower surface tension in larger alkyl chain HBD means that there are larger "holes" in the ML,[86] this could mean that the AgNP particle size could be larger than in the Bet:Gly 1:3 and increase in size beyond the antimicrobial range. However, the size of AgNPs compared to the physical properties of the ML has not been studied, if the larger "holes" in the ML do not affect AgNP size then having larger holes for mobility of the AgNPs is more desirable so they can diffuse through the ML to the wound site increasing potency of the wound dressing.

Out of the new three component ML's synthesised, the Cap:Lau:Bet 4:2:1 ML was chosen to proceed further with the study due to its higher conductivity and lower viscosity to that of Bet:Gly 1:3. Whilst the other physical properties were not tested due to the Cap:Lau:Bet 4:2:1 not being fully liquid at room temperature, and a lack of heated stages for the density, surface tension and refractive index testing, it was still decided it would be the best candidate for AgNP synthesis and potential antimicrobial testing.

Table 17: Physical property values for density, surface tension and refractive index for Cap:Lau:Lev ML's compared to Bet:Gly 1:3.

ML	Density ρ / g mL^{-1}	Surface tension γ / mN m^{-1}	Refractive index μ / AU (±0.00005)
Bet:Gly 1:3	1.226±0.0005	65.2±0.5	1.4795
Cap:Lau:Lev 2:1:1	0.925±0.0005	32.7±0.5	1.4370
Cap:Lau:Lev 4:2:1	0.907±0.0005	32.2±0.5	1.4375
Cap:Lau:Lev 8:4:1	0.898±0.0005	33.0±0.5	1.4380

4.2 AgNP synthesis in Cap:Lau:Bet 4:2:1

Following a similar protocol that was discussed in **Section 3.1** and **Section 3.2**, the ideal conditions for synthesis were first established, see **Section 2.3.3** for the method. Since this reaction does not include the addition of OAm during the synthesis, the reaction conditions tested included the temperature of the reaction and the time of reaction.

Firstly, as a starting point to determine the rough reaction conditions needed to synthesise AgNPs, a sample of Cap:Lau:Bet 4:2:1 ML was tested using the same conditions established in **Chapter 3**. A temperature of 150 °C was chosen at this point since a higher temperature would likely be needed, since previous reactions (in **Section 4.1**) including OAm required higher temperature and the Cap and the Lau have similarly long alkyl chains. The reaction was monitored every 30 minutes until a colour change was observed, after 2 Hrs the reaction had changed colour from colourless to a dark brown which would suggest AgNPs in solution and the reaction was removed from the heat. The extraction technique used for the Bet:Gly 1:3 DES was unsuccessful when used with the Cap:Lau:Bet 4:2:1.

Since the Cap:Lau:Bet 4:2:1 ML contains more hydrophobic components, the MeOH was not enough to dissolve the ML and instead caused the ML to collect at the bottom of the centrifuge tube alongside the AgNPs. Further testing was therefore performed on different extraction methods which could remove the AgNPs from the ML. A modified extraction method was developed, see **Section 2.4.2** which included acetone as an intermediary solvent to assist in the removal of the Cap:Lau:Bet 4:2:1

ML. Once the extraction technique was established, the ideal reaction conditions could be determined.

Figure 32a shows that the different reaction times do cause a large shift in the λ_{max}, with the difference between the maximum and the minimum λ_{max} being roughly 20 nm (see **Table 18**), but the estimate of sizes is still around ~10 nm, which is the desired range for the antimicrobial properties.[72] This approximation of size is comparable to the TEM characterization but is not comparable to the DLS data (see **Table 18**). This difference in λ_{max} is higher than the difference observed in Bet:Gly 1:3 (see **Table 12**), this can be explained by the reaction having a slower nucleation time, which means that the changes in time are having a greater effect on the sizes of the AgNPs produced. As expected, the longer the reaction, the more AgNPs were synthesised, as seen in the increase of the dilution needed for the UV-vis analysis to be under 1 AU as the time increased. Although this concentration analysis is qualitative, an ICP-MS measurement to determine concentration of the optimum reaction conditions will be discussed at the end of this section.

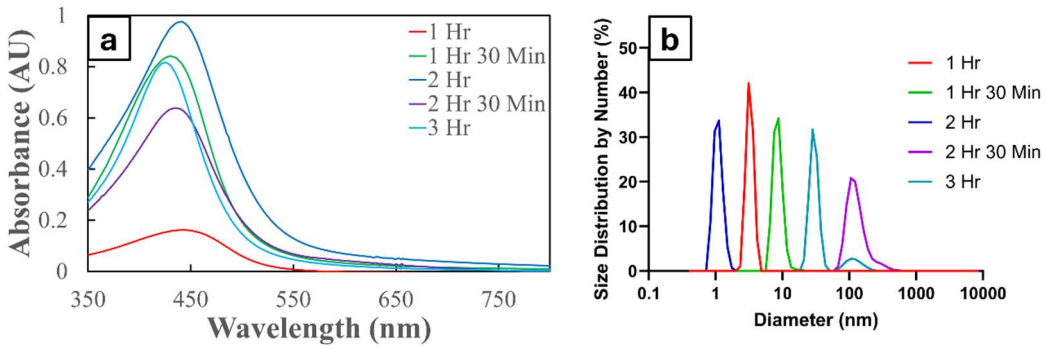


Figure 32: UV-vis and DLS data for synthesis of AgNPs in Cap:Lau:Bet 4:2:1 looking at the effect of reaction time. Reaction conditions, 5 mL Cap:Lau:Bet 4:2:1, 25 mg Ag_2SO_4, 150 °C, 1-3 Hr. a) UV-vis spectra of AgNPs suspended in toluene, 1-2 Hr samples diluted 8-fold, 2.5 Hr sample diluted 16-fold, 3 Hr sample diluted 36-fold. b) DLS spectra, no samples diluted.

The effect of time on the synthesis of the AgNPs can be seen by comparing this data with that measured by the DLS in **Figure 32b** and in **Table 18**. Each different reaction time produced different sizes AgNPs, the 1-2 Hr reaction times produced the smallest sized AgNPs all under 10 nm, whilst the 2.5-3 Hr reaction times produced much larger AgNPs beyond the size range for antimicrobial properties, with the 2.5 Hr synthesis producing particles above the 100 nm limit, so they cannot be classed as nanoparticles.

TEM analysis was also performed alongside the UV-vis and DLS analysis to give accurate size ranges of the AgNPs, as seen in **Figure 33** and **Table 18**. The TEM

sizing does contradict the DLS data for some of the reaction conditions. The 1-2 Hr samples have small AgNPs which also correspond with the UV-vis and the DLS data, but unfortunately the more accurate sizing of these AgNPs show that they are approximately 5 nm which could potentially be harmful to mammalian cells. However, most of the AgNPs are >5 nm and a toxicity study would be required to fully determine if these AgNPs are toxic to humans.

Table 18: Comparison of how the reaction time effects the AgNP size using TEM data in comparison to λ_{max} taken from UV-vis readings. Reaction conditions are as follows; 1-3 Hr, 150°C and with 5 mL Cap:Lau:Bet 4:2:1.

Time / Hr	λ_{max} /nm	Average Size of AgNPs /nm	
		TEM analysis	DLS analysis
1.0	442	4.99±2.48	3.26±1.58
1.5	431	6.36±2.89	8.61±1.58
2.0	441	5.46±2.31	1.10±0.18
2.5	435	10.64±7.97	135.4±58.93
3.0	425	6.09±2.93	29.88±4.60

The 2.5 Hr TEM image does not agree with the DLS data in **Figure 32b**, since it shows that the AgNPs have an average sizing of 10.64±7.97 nm (from the AgNPs that could be sized) instead of >100 nm. These AgNPs have a large range of size, which is not ideal since the majority of the AgNPs are too large for antimicrobial properties. It should be noted that the scale of the 2.5 Hr TEM is not the same as the other images due to a lack of populated AgNPs on the grid to provide an image with enough population to collect an average sizing of the AgNPs. A different scale was also used to image the larger AgNPs which would otherwise be too large to perform a size analysis using the standard scale of imaging throughout the project (x200 k magnification). The difference between the DLS data and the TEM data may be due to some aggregation during the time it took from preparing the sample to running the DLS, since the TEM grids were prepared straight away, there was less chance for aggregation due to the quick evaporation of the toluene on the TEM grids.

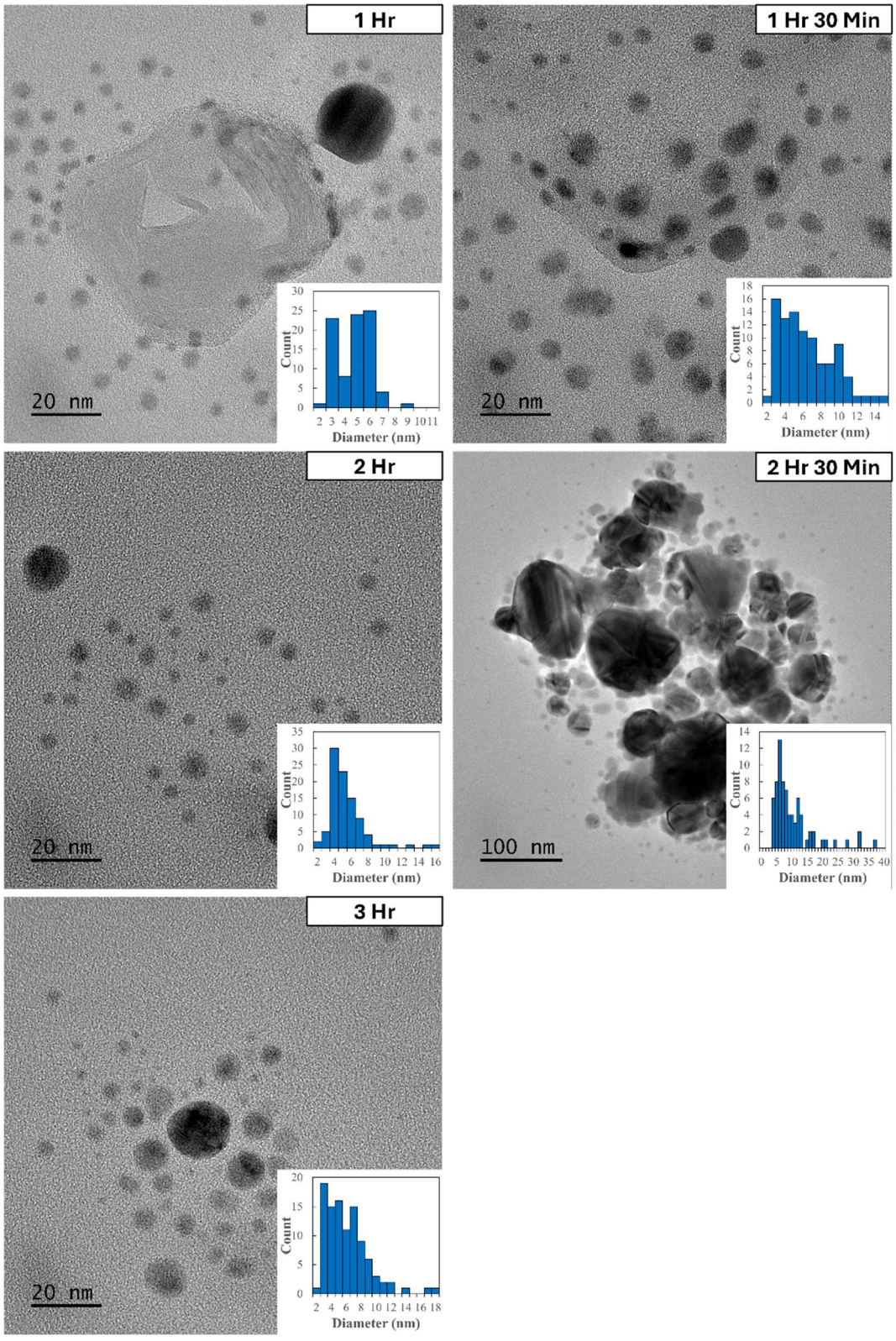


Figure 33: Representations of the TEM samples analysed to determine the effect of reaction time on AgNP size, synthesised using 5 mL Cap:Lau:Bet 4:2:1 DES, 25 mg Ag_2SO_4, 150 °C for 1-3 Hr.

The overall increase in size across the samples can be explained since at longer reaction times the nucleation of the AgNPs occurs over a longer time resulting in larger particles. The drop in size at 3 Hr is potentially due to larger particles reaching a size where they cannot be suspended in the toluene or were removed during the syringe filter step. Out of the different reaction times, it was decided that the 1.5 Hr synthesis was the optimum reaction time since the size range is the closest to 10 nm, to have antimicrobial properties whilst minimising the smaller sizes that are potentially hazardous to humans. Using this reaction time further studies on the optimum temperature of the reaction was performed.

The temperature of reaction was revisited to see if using a 1.5 Hr reaction time would alter the optimum reaction temperature. **Figure 34a** shows the UV-vis spectra and how it changes depending on the different reaction temperatures, unlike **Figure 32a** the λ_{max} is not as affected by the change in temperature (as seen in **Table 19**) with a range of 8 nm with an estimated size of 9-10 nm.[72] This is a smaller range than that of the reaction time, which means that the time of the reaction has more impact on size than that of the reaction temperature.

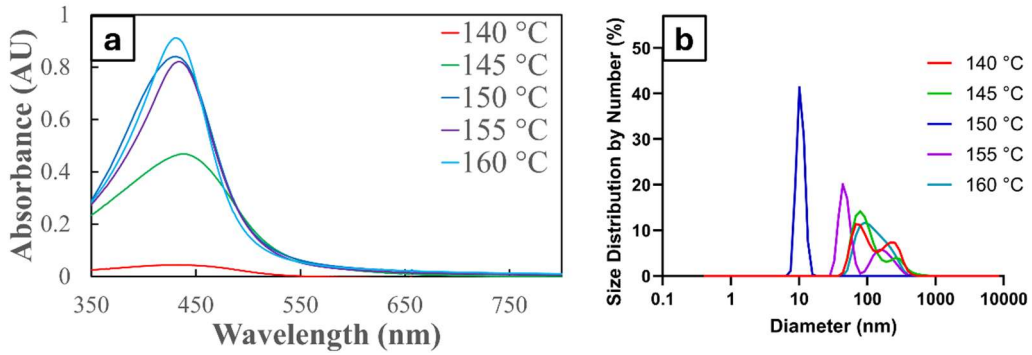


Figure 34: UV-vis and DLS data for synthesis of AgNPs in Cap:Lau:Bet 4:2:1 looking at the effect of reaction temperature. Reaction conditions, 5 mL Cap:Lau:Bet 4:2:1, 25 mg Ag_2SO_4, 140-160 °C, 1.5 Hr. a) UV-vis spectra of AgNPs suspended in toluene, 145 °C sample was not diluted, the rest of the samples were diluted 8-fold. b) DLS spectra, no samples diluted.

The change in the temperature did not affect the rate at which the AgNPs were synthesised as seen by the dilution factors required for each sample. The majority of the samples required an 8-fold dilution, except the 145 °C sample which was not diluted. Comparing the max absorbance of each sample after 150 °C to 160 °C there was not a great deal of variance between each absorbance value. It can be speculated that at higher temperature it would be expected to follow this trend to a point, before dropping in absorbance where an overreaction occurs. This is supported by the DLS data in **Figure 34b** in which most of the different temperatures analysed are

overlapping at sizes > 40 nm. With only the 150 °C sample showing the AgNPs within the desired 10 nm size range at 10.58±1.41 nm.

Table 19: Comparison of how the reaction temperature effects the AgNP size using TEM data in comparison to λ_{max} taken from UV-vis readings. Reaction conditions are as follows; 1.5 Hr, 140-160 °C and with 5 mL Cap:Lau:Bet 4:2:1.

Temperature / °C	λ_{max} /nm	Average Size of AgNPs /nm	
		TEM analysis	DLS analysis
140	433	4.60±2.01	86.19±26.19
145	438	16.84±8.44	93.93±34.14
150	431	6.36±2.89	10.58±1.41
155	435	6.55±1.52	47.16±8.90
160	430	6.35±2.43	134.2±65.87

A TEM study was also conducted to determine an accurate size range of the AgNPs compared to the results of the DLS analysis. Similar to the reaction time, the change in temperature does not show a large change in size of the average AgNP with all samples having the majority of the AgNPs at <10 nm, seen in **Table 19**, except the 145 °C sample which was the largest size at 16.84±8.44 nm. The 145 °C AgNPs do not follow the trend as they are much larger than expected. It would be expected that these particles would be much smaller around 5-6 nm in size, which means this result should be repeated to confirm the analysis. However, this was not possible due to the time constraints of the project and the recommendation would be that these reaction conditions be repeated. This is in contrast to the DLS data which showed the sizes of the AgNPs to be much larger than the TEM analysis. The only sample that had a similar size for both the DLS and TEM data was the 150 °C sample. The DLS data for the different reaction conditions does not show a general trend for AgNP size based on time of the reaction, which further reinforces the need to repeat this data as further work to be performed.

Figure 35 shows a representation of the TEM images collected for each sample, and shows similar sized AgNPs for each sample, supporting the fact that the temperature of the reaction has little effect on the size of the AgNPs. The 145 °C TEM analysis was the only sample to show 2 distinct different size ranges, with one range being 11-12 nm, which could be used for antimicrobial synthesis, and the other size range being 19-24 nm (see **Figure 35**), which are too large for antimicrobial properties,

this is most likely the factor which increased the average size of the AgNPs as seen in **Table 19**, resulting in an average size above 10 nm.

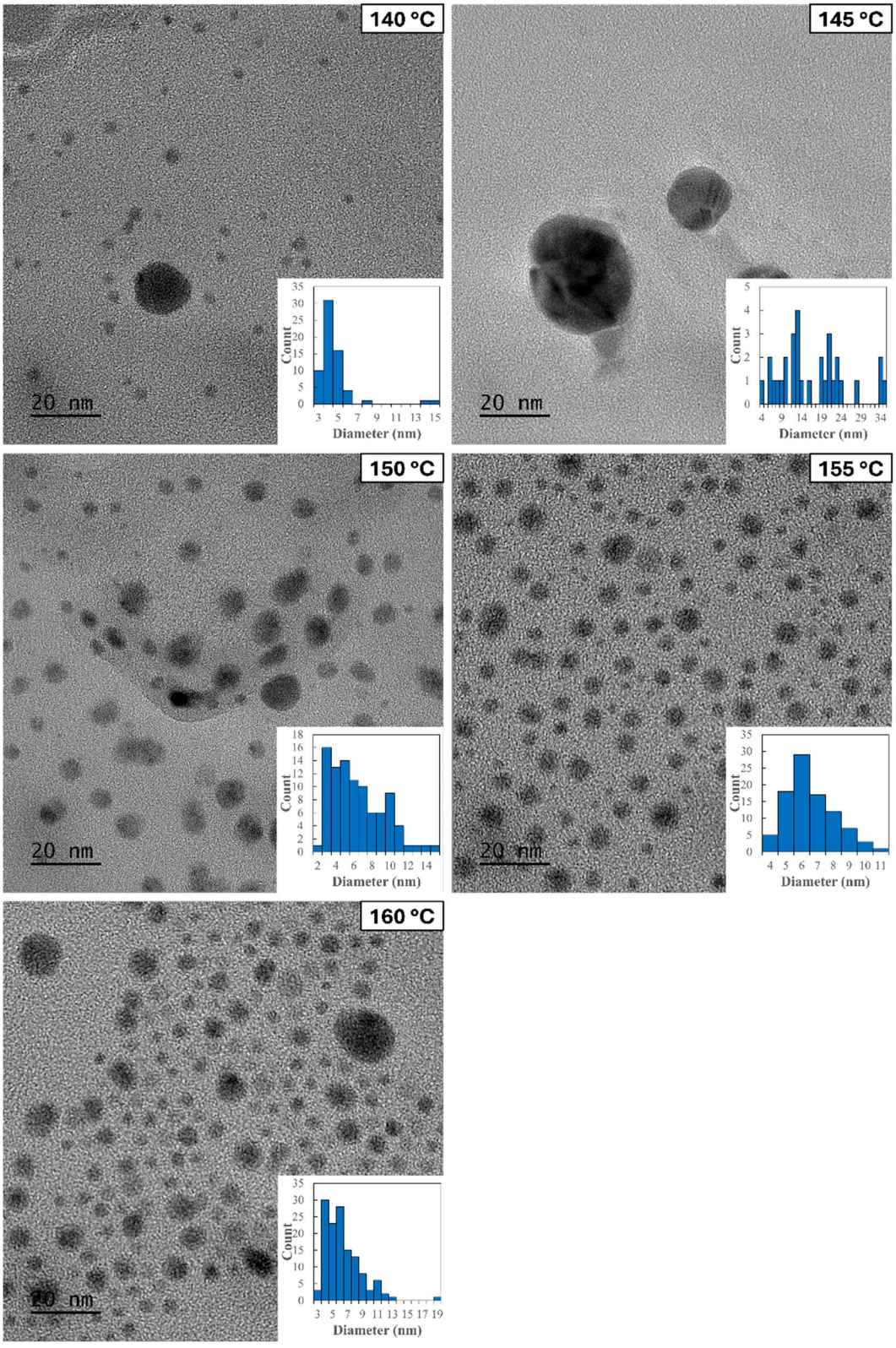


Figure 35: Representations of the TEM samples analysed to determine the effect of reaction temperature on AgNP size, synthesised using 5 mL Cap:Lau:Bet 4:2:1, 25 mg Ag_2SO_4, 140-160 °C for 1.5 Hr.

With the optimum reaction conditions established, the concentration of the AgNPs in the Cap:Lau:Bet 4:2:1 ML after synthesis was also studied alongside the Bet:Gly 1:3 DES, previously discussed in **Section 3.2**. Using the same methods of calculating the recovery and taking into account the sample loss, the concentration of the AgNPs in the Cap:Lau:Bet 4:2:1 was calculated to be 2.48 mg/mL as seen in **Table 20**. This is lower than the calculated values for the AgNPs in the Bet:Gly 1:3 DES which was 3.07 mg/mL as seen in **Table 13**, which could be an indication that the Cap:Lau:Bet 4:2:1 optimum conditions could be altered slightly to see if a higher yield can be synthesised. However, this was not possible in the timeframe of the project.

Table 20: Concentration of AgNPs in Cap:Lau:Bet 4:2:1, synthesised by using 5 mL Cap:Lau:Bet 4:2:1, 150 °C for 1 Hr 30 min, microwave digested in concentrated nitric acid before diluting with ultra-pure water and analysed with an ICP-MS.

Sample name	Calculated concentration excluding recovery / mg/mL	Calculated initial concentration from the check standard recovery data / mg/mL	Calculated initial concentration (after including sample loss) / mg/mL
Synthesised AgNPs Cap:Lau:Bet 4:2:1	1.15	1.30	2.48

The reduction in yield from the Bet:Gly 1:3 synthesis seen in **Section 3.2** would have an effect on the RAE and E-factor of this reaction. Since the change in the quantity of AgNPs synthesised in Cap:Lau:Bet 4:2:1 is minimal compared to the overall mass of the reaction, the effect on RAE was minimal with a decrease to 99.4% down from 99.8% in the Bet:Gly 1:3. This shows that this reaction is just as efficient in terms of conversion of reactants to products. Likewise with the RAE, the E-factor was minimally affected with a calculated value of 0.003, which also indicates a minimal effect on the environment as with the Bet:Gly 1:3 synthesis and a lower environmental impact when compared to the literature methods in **Section 1.2**. The calculated PMI value of 1.006 indicates that in this reactions the majority of the raw material reacts to form the product making it an efficient process, similarly to that of the Bet:Gly 1:3 synthesis of AgNPs in **Section 3.2**.

Table 21: Mass of reactants and products for green metric calculations for the synthesis of AgNPs in Cap:Lau:Bet 4:2:1. Calculations performed in Excel without rounding values.

Reactants	Mass /g	Products	Mass /g
Ag_2SO_4	0.025	**AgNPs**	0.0124
Cap:Lau:Bet 4:2:1	4.1718	**Cap:Lau:Bet 4:2:1**	4.1718
		Waste solids	0.0126

$$Real\ Atom\ Economy\ \ (\%) = \frac{0.0124 + 4.1718\ g}{0.025 + 4.1718\ g} x100 = 99.4\%$$

$$Environmental\ factor\ (AU) = \frac{0.0126\ (g)}{0.025 + 4.1718\ \ (g)} = 0.003$$

$$Process\ mass\ intensity\ (AU) = \frac{0.0126 + 0.0124 + 4.1718\ (g)}{0.025 + 4.1718\ (g)} = 1.006$$

With the goal of using these new three component ML's for synthesising AgNPs for use as a wound dressing, it was decided that the optimum conditions for this reaction would be 5 mL Cap:Lau:Bet 4:2:1, 25 mg Ag_2SO_4, 150 °C for 1 Hr 30 min. These were chosen because they were the AgNP's synthesised within an acceptable sized range for antimicrobial properties. These optimum conditions also minimise the energy required for heating the reaction flask whilst reacting for shortest time frame possible. The efficiency of the reaction was similar to the Bet:Gly 1:3 reaction with regards to the RAE whilst maintaining a low environmental impact as seen in the comparable E-factor calculations. Given time, a stability study should have been performed on these AgNPs in Cap:Lau:Bet 4:2:1 for a comparison with the AgNPs in Bet:Gly 1:3, however, since this is a long term analysis there was not enough time in the project. The stability study would need to be performed before commercialising the AgNPs in Cap:Lau:Bet 4:2:1 to ensure a long shelf life. With the methodology of each synthesis of each AgNP defined, both Bet:Gly 1:3 and Cap:Lau:Bet 4:2:1 were then used in antimicrobial testing to determine which was most suitable for use as a wound dressing.

In this chapter a new ML was developed which incorporated the fatty acids Cap and Lau to potentially act as a capping agent during an AgNp synthesis. Upon testing Cap:Lau 2:1, which is a known DES in the literature,[88] for AgNP synthesis, it was observed that no reaction had occurred over a significant period of time. To facilitate

the synthesis of the AgNPs, a variety of different third components was introduced form a new ML. Out of the new ML's formed, Cap:Lau:Bet 4:2:1 and Cap:Lau:Lev 2:1:1, 4:2:1 and 8:4:1 was tested for physicochemical properties to confirm the synthesis of a ML. The Cap:Lau:Bet 4:2:1 ML was determined to be the most viable option for AgNP synthesis due to its similar values for activation energy for electrical conductance ($5.42x10^4$ kJ mol^{-1}) and activation energy for flow ($4.09x10^4$ kJ mol^{-1}) to that of Bet:Gly 1:3. The Cap:Lau:Bet 4:2:1 ML was then used to synthesise AgNPs with the following conditions: 5 mL Cap:Lau:Bet 4:2:1, 25 mg Ag_2SO_4, 150 °C for 1 Hr 30 min, which produced spherical AgNPs with an average size of 6.36±2.89 nm analysed on the TEM. These AgNPs were then studied for their antimicrobial properties which will be discussed in the next chapter.

Chapter 5: Antimicrobial Studies on AgNPs in a Bet:Gly 1:3 DES and a Cap:Lau:Bet 4:2:1 ML for Development of a Prototype Wound Dressing

An antimicrobial study was performed, with the optimum conditions established for AgNP synthesis in both the Bet:Gly 1:3 DES and the Cap:Lau:Bet 4:2:1 ML from what has been studied in this project. As discussed in **Section 1.2.4**, antimicrobial testing has previously been performed on DES's/ML's in the literature but involved the DES/ML breaking down due to the additional solvent required (DMSO or water).[28,29] Avoiding using water in a microbiological assay is difficult since an aqueous broth is required to grow the bacterial stock (method found in **Section 2.7.1)** used in MIC and MBC assays. One solution to avoid the DES mixing with water, is to run a biofilm assay to determine the antimicrobial activity of the AgNP DES's. Using a DES to break down biofilms have previously been discussed in the literature, especially with the use of natural deep eutectic solvents (NADES), which are composed of naturally occurring compounds and therefore should be biocompatible with humans and theoretically have little to no toxicity.[89] However, it was noted in the publication that if the DES contains acidic components in this case the (Cap and Lau), the toxicity on human tissue may be an issue when used as an antimicrobial agent,[89] however, standard pH probes are not designed for use with a DES so an accurate reading on the pH from the probe is not possible. Human tissue toxicology was planned as part of the project, however due to time constraints and external factors this was not performed but remains an important study before any potential commercialisation of the wound dressings.

One of the objectives of this project to produce a prototype antimicrobial wound dressing with the AgNP DES's, using a biofilm assay is the logical testing choice since the DES's contact with water is minimised allowing for the antimicrobial properties of the DES (pure or containing AgNPs) to be determined, rather than a solution of the components. Another advantage of this test is that biofilms are known to be disruptive to a wound healing process, by causing inflammation as well as having resistances to antimicrobial agents, which occurs in chronic wounds, so the assay is similar to what would be expected when the wound dressing is used.[89] Therefore, developing an AgNP DES that is effective against biofilms would fulfil an objective of this project, whilst introducing a new way to combat chronic wound infections. The designing of new antimicrobial testing methods for use with a DES was one of the

goals established in **Section 1.1**, to attempt to determine the antimicrobial properties of a DES using a colony biofilm assay minimising contact with water.

The Bet:Gly 1:3 DES and the Cap:Lau:Bet 4:2:1 ML were tested on *Gram*-positive (*S. aureus*) and *Gram*-negative (*E. coli*) bacterial biofilms, both as a "pure" DES/ML as well as containing the synthesised AgNPs to determine the antimicrobial effects of the DES/ML and the AgNP's as well. These biofilms were tested against controls with either no addition of solvent or with application of PBS, to compare how a liquid would spread the biofilm across the surface. MIC and MBC assays were also performed, which required the DES/ML to be dissolved in a broth, whilst these are not the best way of analysing the antimicrobial properties of the DES/ML, it allowed for a comparison with the literature. Knowing the concentration of the AgNPs in the DES for the MIC/MBC studies will also allow for a comparison with currently used silver wound dressings that have been studied in the literature. One publication determined that the concentration of silver incorporated in four different burn dressings was between 0.03 mg/cm^2 and 1.39 mg/cm^2,[90] which will not be a direct comparison to the AgNPs in DES/ML in this project but will give an indication if the DES/ML's antimicrobial properties allow for a lower the concentration of silver required for antimicrobial activity compared to the burn dressings currently on the market. It is important that the probability of the DES/ML breaking down in the broth is considered when analysing the MIC and MBC data. These methods can be found in **Section 2.7.2**, for colony biofilm assays, as well as **Section 2.7.3** for the MIC and MBC assays.

5.1 Colony Biofilm Assays on AgNPs in Bet:Gly 1:3 and Cap:Lau:Bet 4:2:1

As previously mentioned, colony biofilm assays would allow for the DES's to be tested for their antimicrobial properties, whilst minimising contact with water. The biofilm assays also allow for the AgNP DES to be tested in a way that would simulate their real-world function as a wound dressing, by applying the AgNP DES to a surface just like with the wound dressing onto the skin. To fully understand to what extent the AgNP contribute to the antimicrobial activity, the DES's were tested separately, to demonstrate that AgNPs enhance the antimicrobial potency of the product. Whilst the

DES could potentially also be inherently antimicrobial, the design of the wound dressing would also incorporate AgNPs to achieve the highest potency possible.

5.1.1 AgNPs in Bet:Gly 1:3

As a starting point, *S. aureus* was selected for preliminary testing of the AgNPs in Bet:Gly 1:3, and the colony biofilm assay was performed with a 24 Hr incubation time. **Figure 36** shows that the Bet:Gly 1:3 and the AgNPs in Bet:Gly 1:3 samples both had an increased bacterial viable count (CFU/biofilm) compared to the control which would indicate that the biofilm was not inhibited during the incubation period and grew in size. This means that for this test, the AgNPs in Bet:Gly 1:3 are not antimicrobial, this is most likely due to the DES itself containing primary metabolites such as glycerol which could have aided the growth of the biofilm rather than inhibit it. A one-way ANOVA test was performed on the data to show any significant statistical change between the values which resulted in no significance shown between each of the samples and the blank, indicating no significant change in the biofilm. To confirm whether the Bet:Gly 1:3 was aiding in the growth of the biofilm, the assay was repeated with 3 Hr and 4 Hr incubation times, which would show whether or not the AgNPs are acting as an antimicrobial agent, before the growth of the biofilm is aided by the Bet:Gly 1:3.

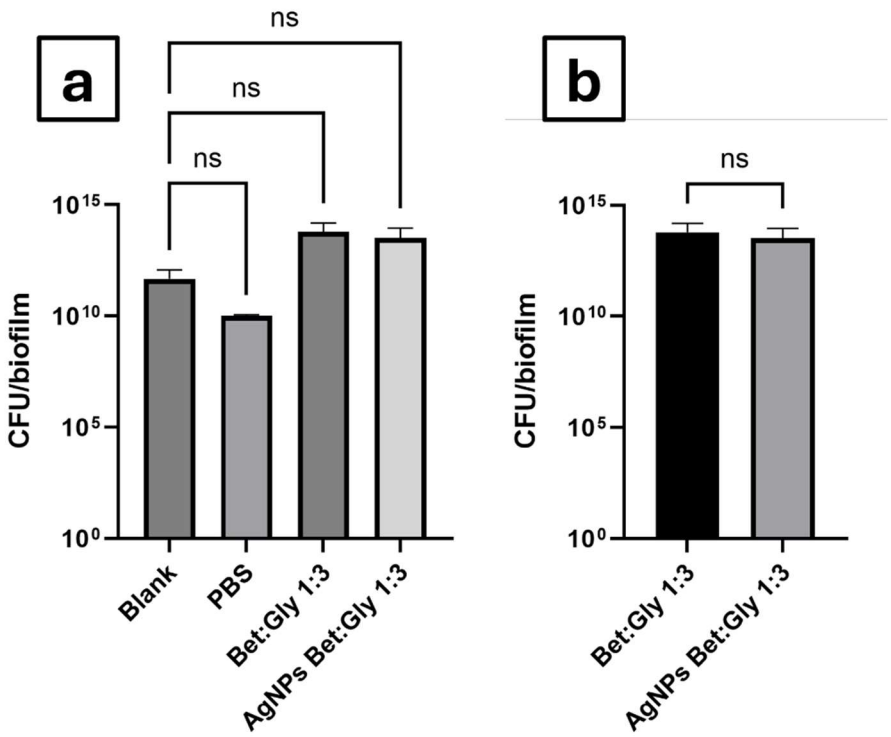


Figure 36: Bacterial viability (CFU/biofilm) from a colony biofilm assay using *S. aureus*, performed on PBS, Bet:Gly 1:3 and AgNPs in Bet:Gly 1:3 compared against

a blank biofilm with an incubation time of 24 Hr. a) ANOVA testing comparing samples to the blank control for statistical significance. b) Welch's t-test comparing the Bet:Gly 1:3 to the AgNPs Bet:Gly 1:3 for statistical significance between the samples. No significance in p-value (ns). Viability determined from 3 biological repeats.

Incubating the biofilms for a shorter time, meant the effect of growth from the Bet:Gly 1:3 should be minimised to see the antimicrobial effect of the AgNPs in the DES. This can be seen in **Figure 37a** where the bacterial viability count for the Bet:Gly 1:3 show less growth over 3-4 Hr than 24 Hr, however, the AgNPs in Bet:Gly 1:3 also show a minimal decrease in viability on the graphs for both 3-4 Hr, this is supported by the one-way ANOVA test which also shows no significant statistical change. In comparison, it can be seen in **Figure 37b**, that the AgNPs in Bet:Gly 1:3 over a 4 Hr incubation does show that the presence of the AgNPs is showing a significant statistical change to that of the Bet:Gly 1:3 sample. This is an indication that the AgNPs are acting in a antimicrobial way, but after an allotted time the growth of the biofilm aided by the Bet:Gly 1:3 is overcoming the antimicrobial effect as seen in **Figure 36** with a 24 Hr incubation time.

From the data in **Figure 36** and **Figure 37**, it can be determined that the AgNPs in Bet:Gly 1:3 show some antimicrobial effects on *S. aureus* over 3-4 Hr, but cannot be considered to be antimicrobial due to the lack of significant change in the bacterial viability. Over a longer period of time, the Bet:Gly 1:3 assists in the growth of the *S. aureus* biofilm, which would make it illogical to use as an antimicrobial dressing. Colony biofilm assays were then performed on *E. coli* biofilms to determine the antimicrobial effects on a *Gram*-negative bacterium.

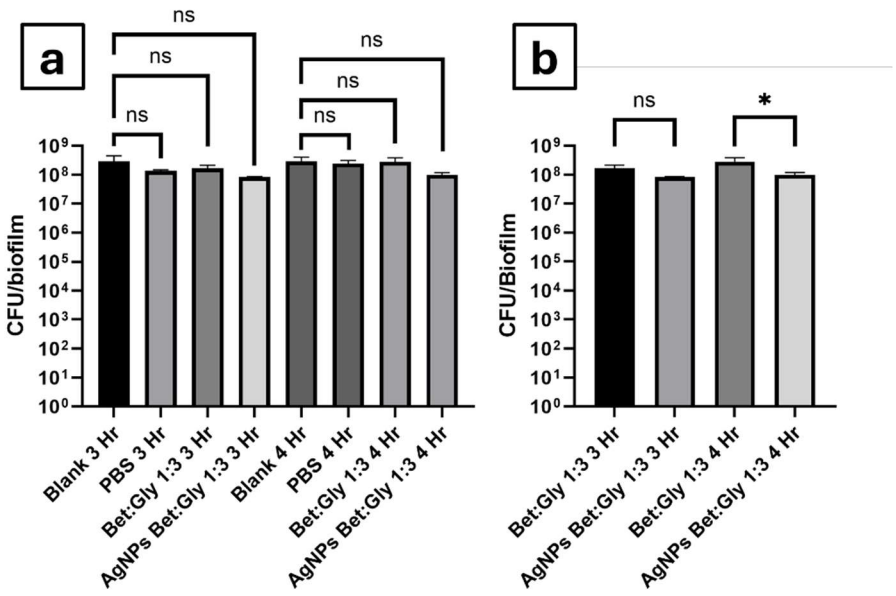


Figure 37: Bacterial viability (CFU/biofilm) from a colony biofilm assay using *S. aureus*, performed on PBS, Bet:Gly 1:3 and AgNPs in Bet:Gly 1:3 compared against a blank biofilm with an incubation time of 3-4 Hr. a) ANOVA testing comparing samples to the blank control for statistical significance. b) Welch's t-test comparing the Bet:Gly 1:3 to the AgNPs Bet:Gly 1:3 for statistical significance between the samples. No significance in p-value (ns), p-value <0.05 (*). Viability determined from 2 biological repeats for PBS 3 Hr and AgNPs DES 3 Hr, and 3 biological repeats for the other samples.

Figure 38 shows the results from the *E. coli* colony biofilm assay, which like the *S. aureus* assay in **Figure 37**, shows no significant change in bacterial viability, with some significant change observed between the Bet:Gly 1:3 sample and the AgNPs Bet:Gly 1:3 sample, with further incubation times not studied due to a time constraint on the project. These results conclude that the use of the AgNPs in Bet:Gly 1:3 is not antimicrobial, however as discussed previously, breaking down a biofilm is much more difficult than if the bacteria were growing planktonically (free floating in a liquid environment),[89] Since these tests were performed on fully formed biofilms, the antimicrobial effects of the AgNPs may not be at a high enough potency to break down the biofilms, however for a short period of time, it could possibly be inhibiting growth to a certain extent. This hypothesis could be tested with more regular testing over different incubation periods to determine at what point the viability increases due to the DES, unfortunately there was not enough time left in the project to do multiple tests on the incubation time. Another test could be to spot the bacterial stock onto the membrane and then add the DES's to see how they would inhibit growth. Without these hypothesised tests however, it was decided to proceed with the testing

on the Cap:Lau:Bet 4:2:1 as the preferred option for the prototype wound dressing material.

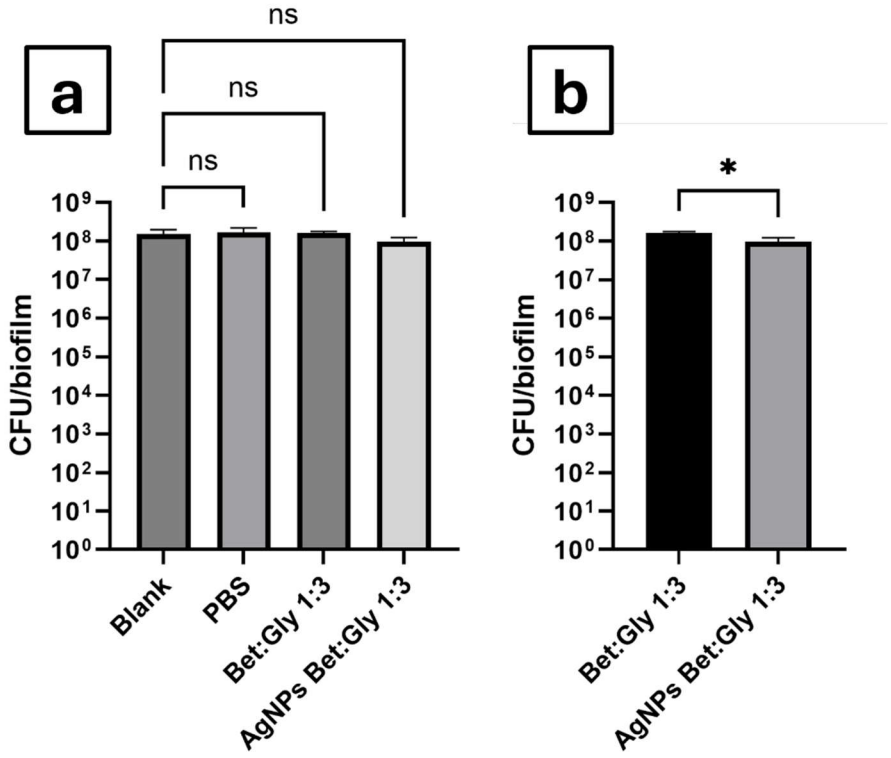


Figure 38: Bacterial viability (CFU/biofilm) from a colony biofilm assay using *E. coli*, performed on PBS, Bet:Gly 1:3 and AgNPs in Bet:Gly 1:3 compared against a blank biofilm with an incubation time of 4 Hr. a) ANOVA testing comparing samples to the blank control for statistical significance. b) Welch's t-test comparing the Bet:Gly 1:3 to the AgNPs Bet:Gly 1:3 for statistical significance between the samples. No significance in p-value (ns), p-value <0.05 (*). Viability determined from 3 biological repeats.

6.1.2 AgNPs in Cap:Lau:Bet 4:2:1

The Cap:Lau:Bet 4:2:1 and AgNPs in Cap:Lau:Bet 4:2:1 were tested with *S. aureus* with a 4 Hr and a 24 Hr incubation time to compare to the Bet:Gly 1:3 samples, one issue with the Cap:Lau:Bet 4:2:1 samples however, was that the ML is not a liquid at room temperature and had to be heated to approximately 40-50 °C prior to spotting on the biofilm. Care was taken to ensure that the ML was sufficiently cooled to a useable temperature so that the effect of the hotter temperature when spotting was not a contributing factor to the assay.

Figure 39 shows the bacterial viability results from the 4 Hr and 24 Hr assay, with the 4 Hr assay showing a more visible drop in viability for both the Cap:Lau:Bet 4:2:1

and the AgNPs Cap:Lau:Bet 4:2:1 samples, highlighting a greater antimicrobial effect present in these samples. The one-way ANOVA test performed on the 4 Hr samples show that the p-values were <0.0001, which shows a significant change in the viability compared to the controls. Whilst these samples show an antimicrobial effect, due to the viability decreasing by less than 2-fold, they cannot be classified as an antimicrobial agent. However, these results do show that the new three component ML designed for use as an antimicrobial agent is promising and ties back to the goals set out at the start of **Chapter 4**, as well as the overall goal of the project to design an inherently antimicrobial DES.

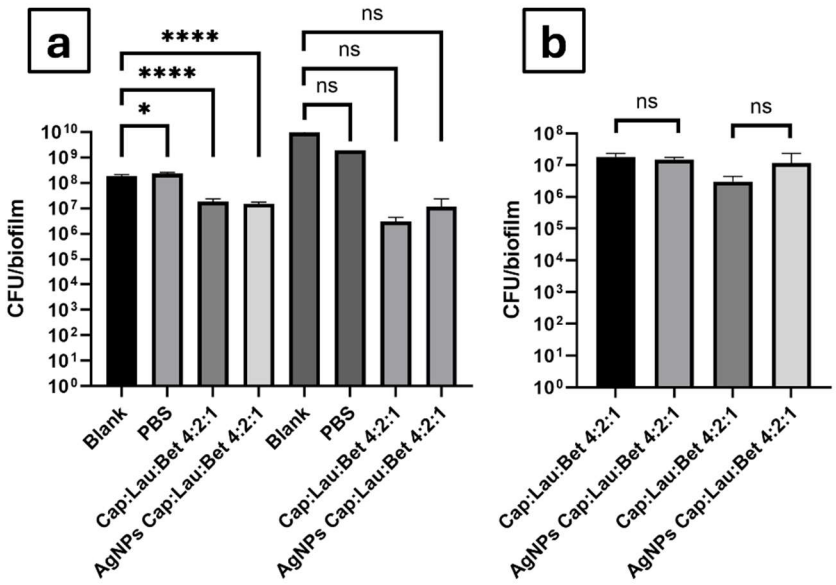


Figure 39: Bacterial viability (CFU/biofilm) from a colony biofilm assay using *S. aureus*, performed on PBS, Cap:Lau:Bet 4:2:1 and AgNPs in Cap:Lau:Bet 4:2:1 compared against a blank biofilm with an incubation time of 4 Hr and 24 Hr. a) ANOVA testing comparing samples to the blank control for statistical significance. b) Welch's t-test comparing the Cap:Lau:Bet 4:2:1 to the AgNPs Cap:Lau:Bet 4:2:1 for statistical significance between the samples. No significance in p-value (ns), p-value <0.05 (*), p-value <0.0001 (****). Viability determined from 3 biological repeats for the 4 Hr samples, 2 biological repeats were used for the 24 Hr blank and 24 Hr AgNP ML samples, 1 biological repeat was used for the 24 Hr PBS sample

The results for the 24 Hr study also support this conclusion since a greater drop in bacterial viability can be seen in the Cap:Lau:Bet 4:2:1 and AgNPs in Cap:Lau:Bet 4:2:1 samples, which would be expected if the samples were antimicrobial. Unfortunately, due to time constrains, the 24 Hr study could only be performed once and as such there were not enough biological repeats analysed to perform a suitable ANOVA test to determine statistical significance, which is why the analysis shows that

there was no significant statistical change between the samples and the controls. If more time was available, more biological repeats could be performed, and a more accurate p-value determined from the ANOVA test. Unlike the Bet:Gly 1:3 samples seen in **Figure 38b**, there was no significance observed between the Cap:Lau:Bet 4:2:1 and the AgNPs Cap:Lau:Bet 4:2:1 sample for both 4 Hr and 24 Hr incubation times (see **Figure 39b**). For the 24 Hr samples, this could be because the comparison was drawn from only 2 biological repeats and therefore was not enough data for a throughout comparison. This could be further study with additional biological repeats, however, due to the time constraints at the end of the project this was not feasible. For the 4 Hr samples, the lack of significant change between the samples may be an indication that only the Cap:Lau:Bet 4:2:1 is having an antimicrobial effect due to the reduction in bacterial viability being present in both samples. On the whole, the assays performed on *S. aureus* was successful in determining that the new three component Cap:Lau:Bet 4:2:1 shows an antimicrobial effect against a *Gram*-positive bacteria.

Just like the Bet:Gly 1:3 samples, the *E. coli* study was only performed at an incubation time of 4 Hr due to time constraints. **Figure 40a** shows that the AgNP Cap:Lau:Bet 4:2:1 sample does show a decrease in the bacterial viability, but the DES itself appears less antimicrobial than with the *S. aureus* seen in **Figure 39**. Unlike with *S. aureus*, it seems that the three component ML has less of an antimicrobial effect on the *E. coli* due to the ANOVA test showing no significant statistical change. Similarly to **Figure 39b**, the comparison between the Cap:Lau:Bet 4:2:1 and the AgNPs Cap:Lau:Bet 4:2:1 sample shows no significant change between the samples, although the graph shows that the AgNPs Cap:Lau:Bet 4:2:1 samples has a lower viability. This could indicate that the AgNPs are more antimicrobial active in this sample, which may be the results of how the biofilm inoculated with the sample. Since this is a newly developed method for testing DES/ML's, these results are promising for an accurate determination of the antimicrobial properties of DES/ML's with further development required to improve the process.

Overall, the Cap:Lau:Bet 4:2:1 samples showed more of an antimicrobial effect, than that of the Bet:Gly 1:3 samples, whilst not encouraging further biofilm growth as seen with the Bet:Gly 1:3 24 Hr incubation tests. With these assays showing an antimicrobial effect on both a *Gram*-positive and a *Gram*-negative bacterium, a "wound simulation" was prepared following the method in **Section 2.7.2** to test the AgNPs Cap:Lau:Bet 4:2:1.

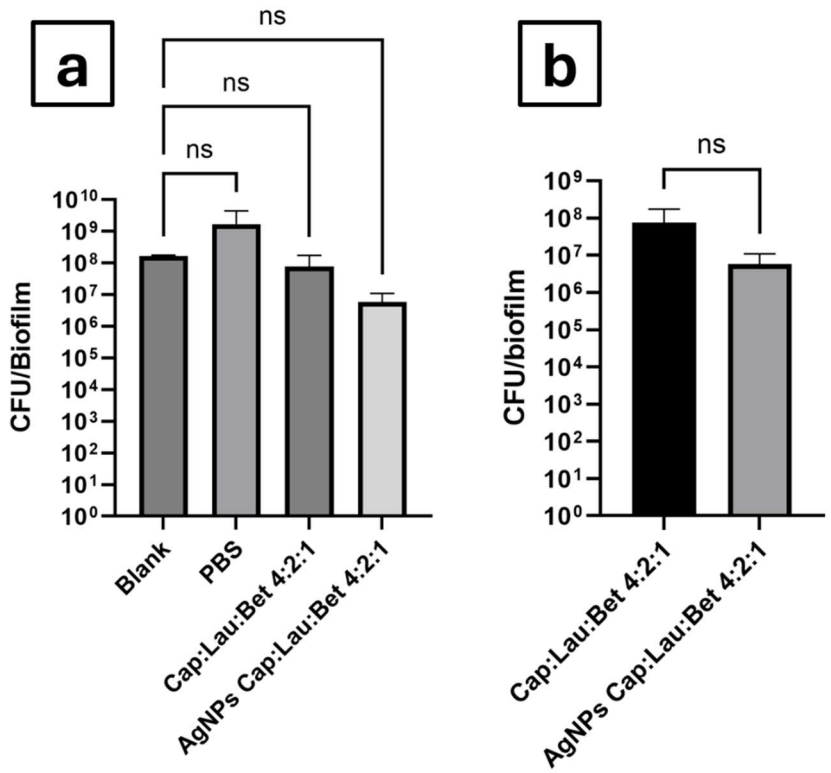


Figure 40: Bacterial viability (CFU/biofilm) from a colony biofilm assay using *E. coli*, performed on PBS, Cap:Lau:Bet 4:2:1 and AgNPs in Cap:Lau:Bet 4:2:1 compared against a blank biofilm with an incubation time of 4 Hr. a) ANOVA testing comparing samples to the blank control for statistical significance. b) Welch's t-test comparing the Cap:Lau:Bet 4:2:1 to the AgNPs Cap:Lau:Bet 4:2:1 for statistical significance between the samples. No significance in p-value (ns). Viability determined from 3 biological repeats.

5.1.3 Wound Simulation to develop a Prototype Wound Dressing

To prepare the "wound simulation" a bacterial broth containing both *S. aureus* and *E. coli* were made using MHB before spotting onto the membrane filter and incubated overnight (see **Section 2.7.2**). The "wound simulation" testing was unsuccessful due to *E. coli* outcompeting *S. aureus* when incubated to form the biofilm, as such the results from this test are not representative of a real-world situation and further testing should be performed. It is known that the *S. aureus* was outcompeted as seen in **Figure 41a** where there was no growth observed on MSA, which is selective for *S. aureus*, in contrast **Figure 41b** shows that *E. coli* was present on MCA. Whilst this was not the intended result for this assay, the *E. coli* count allowed for another set of data to be considered to determine the antimicrobial potency for the Cap:Lau:Bet 4:2:1 samples (see **Figure 41c**). These results are similar to **Figure 40a** but with the 3 biological repeats the ANOVA test can show that the change in viability is

statistically significant, reinforcing that the Cap:Lau:Bet 4:2:1 samples had a greater antimicrobial effect than the Bet:Gly 1:3 samples. **Figure 41d**, also shows a significant change in the Cap:Lau:Bet 4:2:1 and the AgNPs Cap:Lau:Bet 4:2:1 sample which could be an indication that the AgNPs are having an antimicrobial effect. This change from the previous data in **Figure 39b** and **Figure 40b** could be attributed to the fact that this was the final samples ran and over the course of the project with the development of the method and techniques used, the data collected was at a higher standard which allowed for the antimicrobial effect to be observed during the bacterial viability counting. A multispecies biofilm is difficult to establish in laboratory conditions due to the competing bacteria and since these results were collected near the end of the project there was not enough time to achieve a stable multispecies biofilm for this study.

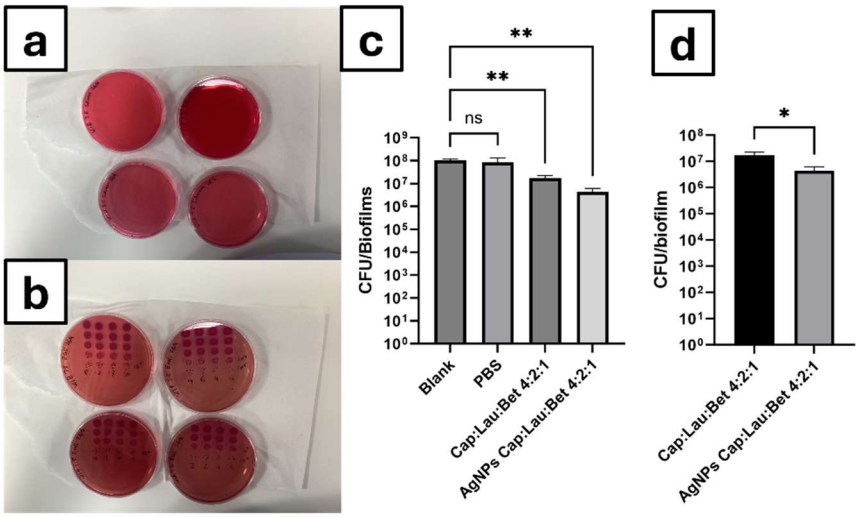


Figure 41: "Wound simulation" to obtain bacterial viability (CFU/biofilm) from a colony biofilm assay using a multicultural stock of *S. aureus* and *E. coli*, performed on PBS, Cap:Lau:Bet 4:2:1 and AgNPs in Cap:Lau:Bet 4:2:1 compared against a blank biofilm with an incubation time of 4 Hr. a) colony count for *S. aureus*, b) colony count for *E. coli*, b) viability plot with ANOVA testing comparing samples to the blank control for statistical significance. b) viability plot with Welch's t-test comparing the Cap:Lau:Bet 4:2:1 to the AgNPs Cap:Lau:Bet 4:2:1 for statistical significance between the samples.No significance in p-value (ns), p-value <0.05 (*), p-value <0.01 (**). Viability determined from 3 biological repeats.

These colony biofilm assays could be improved, since only 3 biological repeats were performed for each sample, with some samples having fewer biological repeats due to user error when performing the tests, resulting in unviable samples to analyse. With further biological repeats the ANOVA testing could be improved to determine whether the statistical difference between the samples is significant. Given more

time, additional bacterial species present in chronic wounds could be tested to further show any antimicrobial effect across numerous pathogens to justify the use of these AgNPs in the new three component ML's as antimicrobial agents. Furthermore, only one of the three component ML's was selected for testing, however there were more potential ML's that could have been tested for AgNP synthesis and then for antimicrobial testing. These results are a good indicator that with further study a new three component ML could be designed that would be inherently antimicrobial and a good candidate for an antimicrobial wound dressing. To compare with the literature, MIC and MBC assays were also performed, see **Section 2.7.3** for the method, however as previously discussed these are not an ideal test for the antimicrobial properties of DES's and are to be analysed with this in mind.

5.2 MIC and MBC studies on AgNPs in Bet:Gly 1:3 and Cap:Lau:Bet 4:2:1

With the aim of producing an antimicrobial wound dressing for use as a commercial product, MIC and MBC assays are an important study since the minimum concentration of AgNPs in the DES can be determined. These values are important, since the aim is to produce antimicrobial wound dressings as cost effectively as possible as well as minimise any chemical waste. By synthesising the lowest concentration of AgNPs required, less starting product would be used, which would lower the costs, as well as reducing the amount of AgNPs not required for antimicrobial effects by having a concentration larger than required.

A few issues with these tests however are that the AgNP's will be introduced to a broth solution that will be 50 % of the overall volume. Adding a DES/ML to 50 % volume of water, will potentially break down the DES/ML,[15] also when the AgNPs are suspended in the DES/ML they are stable in solution, but as seen in **Section 3.3**, when the DES is removed the AgNPs could aggregate and fall out of solution. This is an issue for the MIC and MBC assays since the concentration of the starting solution, may not be the same as the actual concentration due to these losses.

At the time of the first MIC and MBC assays performed, the ICPMS method was yet to be developed, this was an issue since the known concentration of the AgNPs in the DES/ML was required. To ensure that there was a known concentration of AgNPs, commercially purchased 20 nm AgNPs were suspended in the Bet:Gly 1:3. Whilst

these AgNPs were larger than the target size for antimicrobial properties of ~10 nm, they were the only AgNPs found commercially that were closest to the size range without the AgNPs being suspended in water.

Table 22 shows that the AgNP's in Bet:Gly 1:3 required a much higher concentration of sample to inhibit *S. aureus* than what was required to inhibit *E. coli* for both the MIC and the MBC assays. For the *S. aureus* testing however, the MBC could not be determined due to the concentration being > 2562 µg/mL. Due to time constraints on the project further study was not performed on higher concentrations of AgNPs in Bet:Gly 1:3, however this itself could prove to be difficult due to the potential aggregation of the AgNPs in the broth, which is more likely to occur at higher concentrations due to more availability of AgNPs in close proximity to each other for aggregation. It is also a possibility that the MIC and the MBC may be lower than stated, since it is unknown how many AgNPs aggregated during the incubation time, resulting in a lower concentration of AgNPs suspended in the broth, available for antimicrobial activity. This would be the same for the *E. coli* assays, however due to the greater antimicrobial effect against the *E. coli*, both the MIC and MBC was determined. The MBC data would need to be verified due to the large standard deviation calculated, but this can be explained by having a 2-fold dilution in mM across each well, which means that the true value of the MBC may lie between the well dilutions, in this case between 11.5mM (1240 µg/mL) and 0.72mM (77.6 µg/mL). These concentrations however, may not be taking into account the effect of the Bet:Gly 1:3, as seen in **Table 22** the Bet:Gly 1:3 aided the growth of the *S. aureus* biofilm, and now that the Bet:Gly 1:3 is readily available in a broth, the effect of the DES will not be seen in these tests. This will not be the case for the Cap:Lau:Bet 4:2:1 ML however, since this ML does not mix well with water and will remain as a ML in a separate layer in the well.

The separation of layers in the Cap:Lau:Bet 4:2:1 ML and the broths does raise another issue, if the layers are separated the AgNPs might not be available for antimicrobial activity. One solution to this problem was to incubate the samples whilst agitating with a shaking incubator, this introduced another issue however, since when the samples were shaken, the Cap:Lau:Bet 4:2:1 would form a cloudy mixture in the broth, which made it difficult to read the MIC due to the bacteria also forming a cloudy mixture as well when growing. Therefore, after shaking the wells were allowed to settle to allow the Cap:Lau:Bet 4:2:1 to separate from the broth to increase the accuracy of the collected data.

Table 22: MIC and MBC of commercially purchased 20 nm AgNPs in Bet:Gly 1:3 and synthesised 6.36±2.89 nm AgNPs in Cap:Lau:Bet 4:2:1. See **Appendix B** for images of the MIC/MBC assays.

Bacteria	MIC/MBC Concentration (µg/mL) (StdDev.S)	
	AgNPs Bet:Gly 1:3	**AgNPs Cap:Lau:Bet 4:2:1**
Staphylococcus aureus	2562 (0) / -	38.74 (0) / 38.74 (0)
Escherichia coli	90.64 (31.72) / 458.9 (605.9)	≤2.42 (0) / 2.42 (0)

Table 22 shows that the MIC and MBC concentrations for the AgNPs in Cap:Lau:Bet 4:2:1 are much lower than the 20 nm commercially purchased AgNPs in Bet:Gly 1:3. This is most likely due to the size of the AgNPs in the Cap:Lau:Bet 4:2:1 having a greater antimicrobial potency, due to being a smaller size than that of the commercially purchased 20 nm AgNPs, which would lie within the range required for the greater antimicrobial effect (~ 10 nm).[30] Another explanation is that the Cap:Lau:Bet 4:2:1 itself is also contributing to the antimicrobial activity, since the DES was designed with antimicrobial components, which has demonstrated antimicrobial activity against the biofilms (see **Figure 39a** and **Figure 41c**) or if the DES was broken down in the broths, the individual components that are antimicrobial are readily available in solution.[83] The MIC data set for the Cap:Lau:Bet 4:2:1 AgNPs was difficult to determine due to the cloudiness of the Cap:Lau:Bet 4:2:1 in the both, even after allowing to settle, so the MBC data values were used to determine a value for the MIC which would be ≤2.42 µg/mL.

Compared with the results for the Bet:Gly 1:3, the AgNPs in Cap:Lau:Bet 4:2:1 showed greater antimicrobial activity at much lower concentrations making them a promising candidate to be used to create an antimicrobial wound dressing.

Looking at the data in **Table 22** compared to literature values of AgNPs synthesised through other green alternatives, the values obtained for the MIC of AgNPs in Cap:Lau:Bet 4:2:1 were lower when compared to AgNPs synthesised through an aqueous green method, using *C. arabica* husks. The *C. arabica* husk AgNPs were stated to have an MIC of 0.75 mg/mL for *S. aureus* and 1.1 mg/mL for *E. coli*,[91] these values are interesting since they show a greater antimicrobial effect on the *S. aureus* compared to the *E. coli*, which is the opposite to the AgNPs synthesised in this project. The MBC data for both *S. aureus* and *E. coli* was found to be 2.42 µg/mL, which

means the AgNPs in the Cap:Lau:Bet 4:2:1 has a higher antimicrobial potency. This literature value only considers the effect of the AgNPs, but the Cap:Lau:Bet 4:2:1 will also have a possible effect on the antimicrobial activity.

Since the Cap:Lau:Bet 4:2:1 is a newly discovered ML, antimicrobial data is not available in the literature, however similar DES's (Cap:Menthol and Cap:Lau) have been studied.[29,84] The Cap:Lau DES in the literature was tested against *S. aureus* and was stated to have a MIC/MBC of 625/1250 µg/mL which is a higher concentration than the AgNPs in Cap:Lau:Bet 4:2:1 in this study, with a concentration of 38.74 µg/mL for both MIC and MBC.[84] Since the DES's are similar, it can be said that the AgNPs do improve on the antimicrobial activity of the Cap:Lau:Bet 4:2:1 resulting in a more potent effect. One issue with this comparison however is that the concentration of the Cap:Lau:Bet 4:2:1 is not known in the well since unlike in the publication, the DES's in this study were not diluted in DMSO, so this is not a direct comparison. The other DES in the literature (Cap:Menthol) was tested against both *S. aureus* and *E. coli*, the *E. coli* MIC and MBC's showed no effect in the literature, whilst the MIC/MBC for the *S. aureus* was stated to be 0.2/0.4 µg/mL.[29] These MIC/MBC values for *S. aureus* are a lower concentration than found in this project with the AgNP's in Cap:Lau:Bet 4:2:1 however, showing no effect against *E. coli*. Both these literature studies also tested additional bacteria that weren't tested in this project, due to time constraints, the additional testing for this project may allow for a greater comparison for the antimicrobial activity against multiple species. The overall trend, however, is that including the AgNPs with the DES improves the antimicrobial activity of both, and that the AgNPs are essential to include in the DES for a wound dressing prototype.

Comparing the MIC/MBC values for the AgNPs in Cap:Lau:Bet 4:2:1 (see **Table 22**) to the currently used concentrations of Ag in burn dressings previously mentioned at the start of **Chapter 5**, the values for the MIC's are similar to the concentration of Ag in the burn dressings. Whilst a direct comparison cannot be performed due to the burn dressings in the literature being evaluated based on an area of contact rather than as a concentration in a liquid, it can allow for a rough estimation on whether the AgNPs synthesised in this project are performing similarly to those currently used as wound dressings. The lowest concentration of silver in the burn dressings was 0.03 mg cm^{-2} (30 µg cm^{-2}) and the maximum concentration was 1.39 mg cm^{-2},[90] when compared with the Cap:Lau:Bet 4:2:1 MIC/MBC value for *S. aureus*, the concentrations determined in this project are close to the lowest concentration in the burn dressings. However, when comparing with the MIC/MBC value for *E. coli* the lowest concentration in the burn dressings is a factor of 10 higher than the MIC/MBC

values determined in **Table 22**, which shows the potential that AgNPs in a ML could improve upon currently used dressings. Further study would be required to confirm whether this is the case but from these preliminary results are promising. The MIC and MBC tests in this project were performed on different sized AgNPs (6.36±2.89 nm, 20 nm) in different DES's which means that the comparisons between the samples are not ideal. Given more time, a further study of Bet:Gly 1:3 using synthesised AgNPs would allow for a more direct comparison. The main issue with the MIC and MBC assays is that the broth required to grow the bacteria is also disrupting the DES's, which means that the MIC/MBC values may be different when used with a none disrupted DES.

An objective of this project was to develop methods for antimicrobial testing of DES's without contact with water which was achieved with the development of the modified method of the colony biofilm assay. This was the first attempt, from what is available in the literature, to characterise the antimicrobial properties of a DES using a colony biofilm assay. Due to time constrictions, a method was not developed for the MIC/MBC assays which would minimise contact with water, but due to the nature of these assays requiring an aqueous broth mixing with the sample, it is unlikely that a method would be developed. This highlights the importance of developing the water-minimising methods like the colony biofilm assay to allow for the antimicrobial properties of DES's to be accurately determined.

Further study into testing DES's for antimicrobial properties will be required to obtain accurate data if these AgNPs DES's are to be used commercially. Overall, the AgNPs in Cap:Lau:Bet 4:2:1 was successfully showed to have antimicrobial activity against a biofilm and against bacteria in solution, which makes it a good candidate for a prototype wound dressing. Other new three component DES's can also be tested, which may improve the potency, so this will be on ongoing study for future work in order to supplement and improve upon the discoveries of this project.

In this chapter Bet:Gly 1:3 and Cap:Lau:Bet 4:2:1 was tested for their antimicrobial properties as a pure DES/ML as well as containing AgNPs synthesised on the same day. Colony biofilm assays were chosen as this would have a minimal interaction with the DES/ML resulting in a lower chance the DES/ML would break down due to water content. The Bet:Gly 1:3 DES was shown to aid in the growth of the *S. aureus* bacteria over 24 Hr as both the "pure" DES and the AgNPs in the DES had a larger bacterial viability than that of the blank control. When the incubation time was changed to 3 or 4 Hr then it could be seen that the AgNPs in the DES did lower bacterial viability, but no significant change was determined compared to the control. Similar results were seen in the 4 Hr incubation time with *E. coli* and the Bet:Gly 1:3

DES was determined to be unsuitable for an antimicrobial purpose. The Cap:Lau:Bet 4:2:1 ML was also tested for its antimicrobial properties with a significant drop in bacterial viability over 4 Hr with *S. aureus* for both the "pure" ML and the AgNPs in the ML, however due to the lack of biological repeats the same cannot be said for the 24 Hr incubation time due to the lack of data for statistical analysis, however it can be seen on the graph that bacterial viability has also reduced in the "pure" ML and the AgNPs in the ML. Cap:Lau:Bet 4:2:1 was only tested for a 4 Hr incubation with *E. coli* and even though a drop in bacterial viability can be seen, it was not a significant change from the control. A "wound simulation" was prepared to test the Cap:Lau:Bet 4:2:1 ML and AgNPs in the ML, however the *E. coli* outcompeted the *S. aureus* in the biofilm and only a set of data for *E. coli* was collected which did show a statistical significant drop in bacterial viability. It cannot be confirmed that these samples are antimicrobial due to the lack of a large from in bacterial viability, however it was determined that they are having an antimicrobial effect. MIC and MBC assays were performed on the AgNPs in Bet:Gly 1:3 and in Cap:Lau:Bet 4:2:1 which confirmed that the Cap:Lau:Bet 4:2:1 sample had a larger antimicrobial effect than the Bet:Gly 1:3 sample due to the lower concentration of AgNPs required for the MIC and MBC. This however was not taking into account the Bet:Gly 1:3 or Cap:Lau:Bet 4:2:1 due to the breaking down of the Bet:Gly 1:3 during the study as well as the Cap:Lau:Bet 4:2:1 being immiscible with the broth during the MIC.

Chapter 6: Conclusion and Further Work

6.1 Conclusion

In line with the aims and objectives of this project, taking inspiration from Adhikari *et. al.* AgNPs with a discreet size of 10±2.4 nm were successfully synthesised in Bet:Gly 1:3 using OAm as a capping agent with optimised conditions of 150 °C for 5 min. This method was then altered to align with the goal of synthesising AgNPs in a DES for use as an antimicrobial wound dressing, by removing the OAm from the synthesis, which also changed the conditions of the reaction to 80 °C for 15 min, improving the sustainability of the synthesis by reducing the energy required as well as removing the hazardous OAm from the reaction. Sustainability was quantified for the AgNP synthesis without OAm by calculating an RAE of 99.7% with an E-factor of 0.001. An extraction method was developed using OAm after the synthesis to extract the AgNPs from the DES into toluene for characterization and analysis, which showed that the AgNPs synthesised without OAm in Bet:Gly 1:3 were 13±1.94 nm, it was noted that this resulted in a minimal change in size from the synthesis with OAm. The AgNPs in Bet:Gly 1:3 without OAm were tested for their stability over 140 days in order to achieve the goal of proving that the AgNPs in Bet:Gly 1:3 would have a long shelf life as an antimicrobial wound dressing. The AgNPs synthesised in Bet:Gly 1:3 at 80 °C for 10 minutes showed to be the most stable over the 140 days with minimal change in size up to day 112. The 5 min and 15 min samples were less stable than the 10 min sample, with the 15 min sample showing immediate changes after 1 day to then not be stable over the remaining 98 days, compared to the 5 min sample which stabilised after 7 days.

A new three component ML's were designed to incorporate a capping agent as well as the intent of being inherently antimicrobial to increase the potency of the antimicrobial effect of the AgNPs in a ML. Two new ML's Cap:Lau:Bet and Cap:Lau:Lev were successfully synthesised and characterised, to compare to the Bet:Gly 1:3 DES which is a known DES in the literature.[3] The Cap:Lau:Bet 4:2:1 ML was chosen to synthesise AgNPs due to its lower viscosity and higher conductivity at 25 °C compared to Bet:Gly 1:3. AgNPs at a size range of 6.36±2.89 nm were successfully synthesised in the Cap:Lau:Bet 4:2:1 with optimum reaction conditions of 150 °C for 1 Hr 30 min, AgNPs were characterised by editing the extraction method developed for the AgNPs in Bet:Gly 1:3 without OAm synthesis, to include a wash with acetone. The calculation RAE for this new synthetic method was determined to be 99.5% which is a minimal change from the RAE value determined for the Bet:Gly 1:3, and this is the

same for the E-factor which for the Cap:Lau:Bet 4:2:1 synthesis was calculated to be 0.003. The concentration of the AgNPs was determined to be 4.74 mg/mL in the Bet:Gly 1:3 DES and 2.48 mg/mL in the Cap:Lau:Bet 4:2:1 ML, using a new method developed for ICP-MS which was one of the goals of the project, to allow for accurate determination of MIC/MBC values during the antimicrobial testing.

Antimicrobial studies were conducted on the AgNPs in Bet:Gly 1:3 and AgNPs in Cap:Lau:Bet 4:2:1 by colony biofilm assays as well as MIC/MBC assays. Colony biofilm assays were chosen against the usual disk diffusion assay's seen in the literature to minimise the contact the DES's had with water, which would break down the H-bond network.[15,29] The colony biofilm assays showed that the Bet:Gly 1:3 DES was aiding the growth of the biofilms over 24 Hr whilst the Cap:Lau:Bet 4:2:1 was showing antimicrobial effects at 4 Hr incubation time. In both DES's the AgNPs were having an antimicrobial effect, with an increase of antimicrobial potency with the Cap:Lau:Bet 4:2:1 ML. MIC/MBC assays were performed to compare with literature values, whilst commenting on that the method was not the ideal method to use with a DES, showing that 20 nm commercially purchased AgNP's in Bet:Gly 1:3 had MIC values of 2560 µg/mL and 90.6 µg/mL for *S. aureus* and *E. coli*, with an MBC value of 459.0 µg/mL for *E. coli* only. The synthesised 6.36±2.89 nm AgNPs in Cap:Lau:Bet 4:2:1 had MIC and MBC values of 38.74 µg/mL and 2.42 µg/mL for *S. aureus* and *E. coli*, which has a more potent antimicrobial effect than the AgNPs in Bet:Gly 1:3 DES and as a result was used in a simulated wound test. The wound test was performed with a biofilm of both *S. aureus* and *E. coli,* which aligns with the goal of the project to carry out a wound simulation on the prototype wound dressing, which was unsuccessful due to the *E. coli* outcompeting the *S. aureus* on the biofilm. The AgNPs in Cap:Lau:Bet 4:2:1 were determined to be the idea candidate out of the ML's tested in this project to be used as a prototype antimicrobial wound dressing fulfilling the aim of this project to develop a new antimicrobial DES that can synthesise AgNPs by acting as the solvent, reducing agent and capping agent, by synthesising spherical AgNPs at sizes 6.36±2.89 nm.

This work successfully designed and synthesised a new three component ML Cap:Lau:Bet 4:2:1 which was inherently microbial as well as acting as the solvent, reducing agent and capping agent to synthesise AgNPs. These AgNPs synthesised in the DES was greener than currently used methods in the literature, as quantified by the atom economy calculations and environmental impact factor whilst maintaining an acceptable concentration of AgNPs synthesised with a size of 6.36±2.89 nm.

6.2 Further Work

The mechanism of AgNP synthesis in a DES is not fully understood and an unifying theory to determine how DES properties may affect size and shape of AgNPs would be a valuable research subject. Understanding this would allow for DES's to be more easily designed, either by computational calculations or physiochemical property testing, to synthesise any metal NP for a specific size and shape for a variety of different functions. This has previously been studied in the literature using choline chloride and urea DES using molecular dynamic simulations,[92] and density functional theory.[93] Since capric acid (Cap) and lauric acid (Lau) could both act as a capping agent, the computational study would predict which of these DES components have the most impact on the AgNP size and whether a different ratio would be more suitable for the synthesis.

An extensive study of new three component ML's was not performed. Due to time constraints, the determination of whether the ML's synthesised in this project were at the eutectic point was not performed. This means that the ML's cannot be classified as a DES until this study is performed. Therefore, it would be suggested that the ML's melting points are determined either by a melting point apparatus, or preferably a Differential Scanning Calorimeter (DSC). This could also be expanded by testing other components to see if they form a DES at room temperature, such as using larger chain fatty acids such as oleic acid or linoleic acid. One DES that was characterised but not used for AgNP synthesis was the Cap:Lau:Lev DES's which may be an improvement on the Cap:Lau:Bet 4:2:1 DES, both in terms of AgNP synthesis and antimicrobial effects, but since it was not tested due to time constraints, it is unknown how useful this DES would be.

It was mentioned that the characterization of the AgNPs in a DES using XRD, DLS and liquid-cell TEM was a possibility in **Section 3.4**, given more development time with the XRD and the DLS as well as having access to a liquid cell holder for the TEM. These characterization methods may eliminate the need for an extraction process for the AgNPs in a DES, to determine the exact size and stability of the AgNPs in a DES. This would be vital research for developing the commercialization of the AgNPs in a DES for a wound dressing since quality control on the product would be easier if a sample can be used in an instrument, such as a DLS, without extraction.

Lastly, additional toxicology testing could be performed on the AgNPs in a DES to determine if they are safe to use on human skin. Theoretically, since the components are natural primary metabolites the DES's should be non-toxic, but the remaining sulphate ions in the DES after the AgNP synthesis may prove to be harmful to humans which makes this test vital before a wound dressing can be commercialised.

This could be achieved by performing an MTT assay to determine living cells viability via mitochondrial activity when in contact with the AgNPs in a DES. The living cells will convert the MTT into formazan crystals which can be detected by spectrochemical methods at an absorbance of 570 nm. This would be an ideal assay since the AgNPs absorb light at ~410 nm so would not interfere with the MTT assay. Additionally, to the MTT assay, a permeability study on human tissue would also be required to identify if the AgNPs or DES will permeate into the skin and how far into the body it will travel. This could be achieved using a Franz cell diffusion study, which looks at how substances move across a skin barrier. Since the wound dressing would be applied directly on the skin, for the full chemotherapeutic effect, it would be expected that the AgNPs and DES would not pass through the skin barrier and into human tissues and stay on the surface to prevent bacterial infection on a wound. It would be expected that some of the AgNPs and DES's would enter the bloodstream through the wound itself so a full pharmacokinetic study on how the body would process the AgNPs and DES into waste streams would be required.

Additional bacterium can also be tested with a biofilm assay and MIC/MBC assays to prove a multi-bacteria antimicrobial potency since a wound may not just contain *S. aureus* or *E. coli* but a variety of different bacteria, such as *Pseudomonas. aeruginosa*, which is a common infection in humans which can also be resistant to antibiotics. New methods for antimicrobial studies can be used to improve upon currently existing assays to be able to test a DES without water breaking down the H-Bonds,[15] to further characterise the antimicrobial effects of the AgNPs in a DES. Understanding how a DES will diffuse through agar would be a good starting point to allow for the potential use of a disk diffusion assay as a viable method for antimicrobial testing. Mainly, whether the DES itself diffuses through the agar as a DES, or if the components of the DES diffuse through separately to each other. This is an important factor to determine whether or not a disk diffusion assay can be performed since if the components travel through the agar separately then it is not an antimicrobial study on a DES and instead of its components. One method for this which could work is to take samples of the agar after diffusion has occurred and analyse how far each component has travelled. If the values are different then it can be assumed the components are travelling separately to each other, however if they are similar then it can be assumed they are travelling together through the agar.

Appendices

Appendix A: TEM of AgNPs in DES for Stability Study

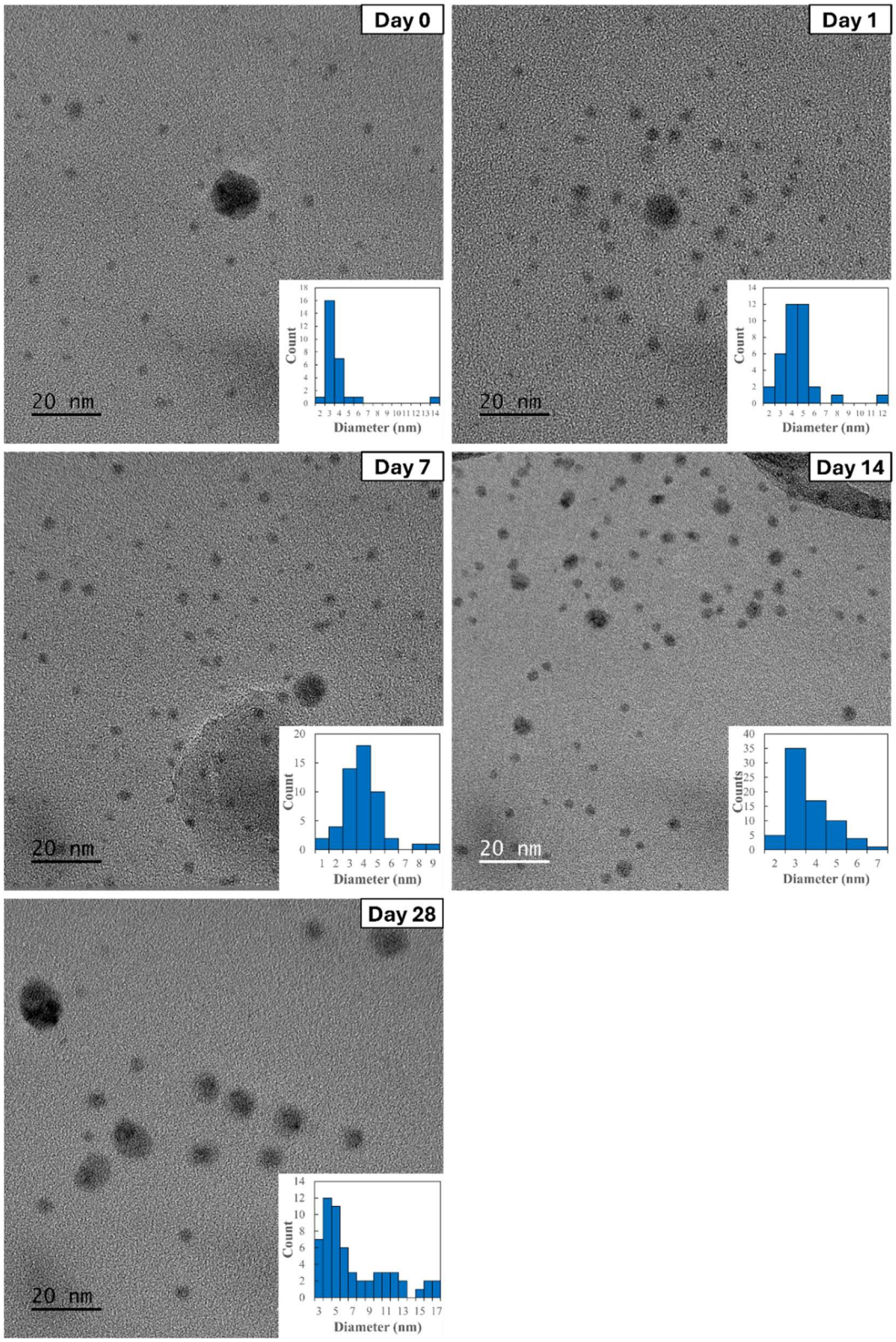


Figure A1: Stability study TEMs of AgNPs synthesised in a Bet:Gly 1:3 DES left in a dark cupboard, day 0-28 samples. Reaction conditions 120 mL Bet:Gly 1:3, 0.6 g Ag_2SO_4, 80 °C, 5 min.

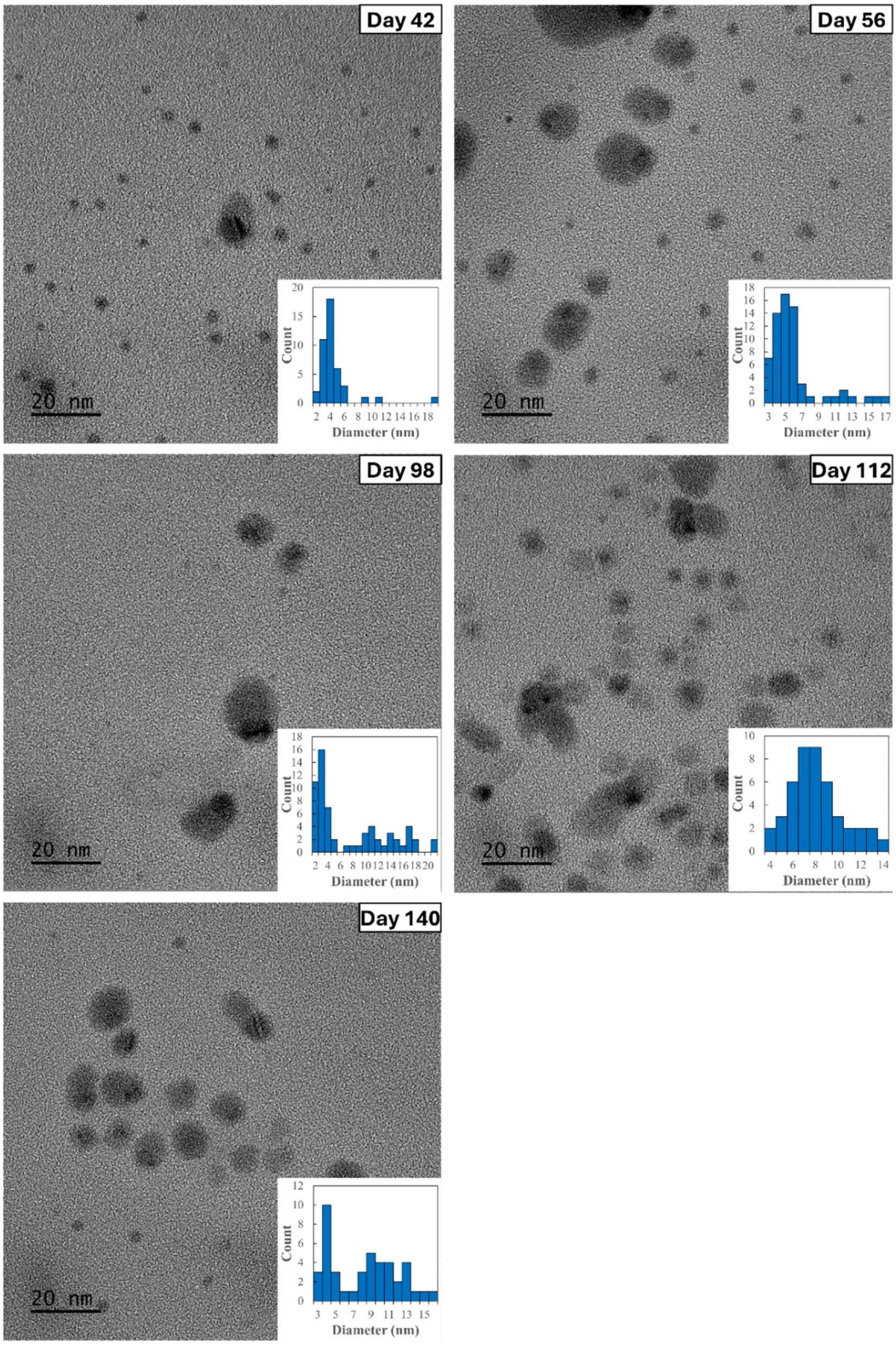


Figure A2: Stability study TEMs of AgNPs synthesised in a Bet:Gly 1:3 DES left in a dark cupboard, day 42-140 samples. Reaction conditions 120 mL Bet:Gly 1:3, 0.6 g Ag_2SO_4, 80 °C, 5 min.

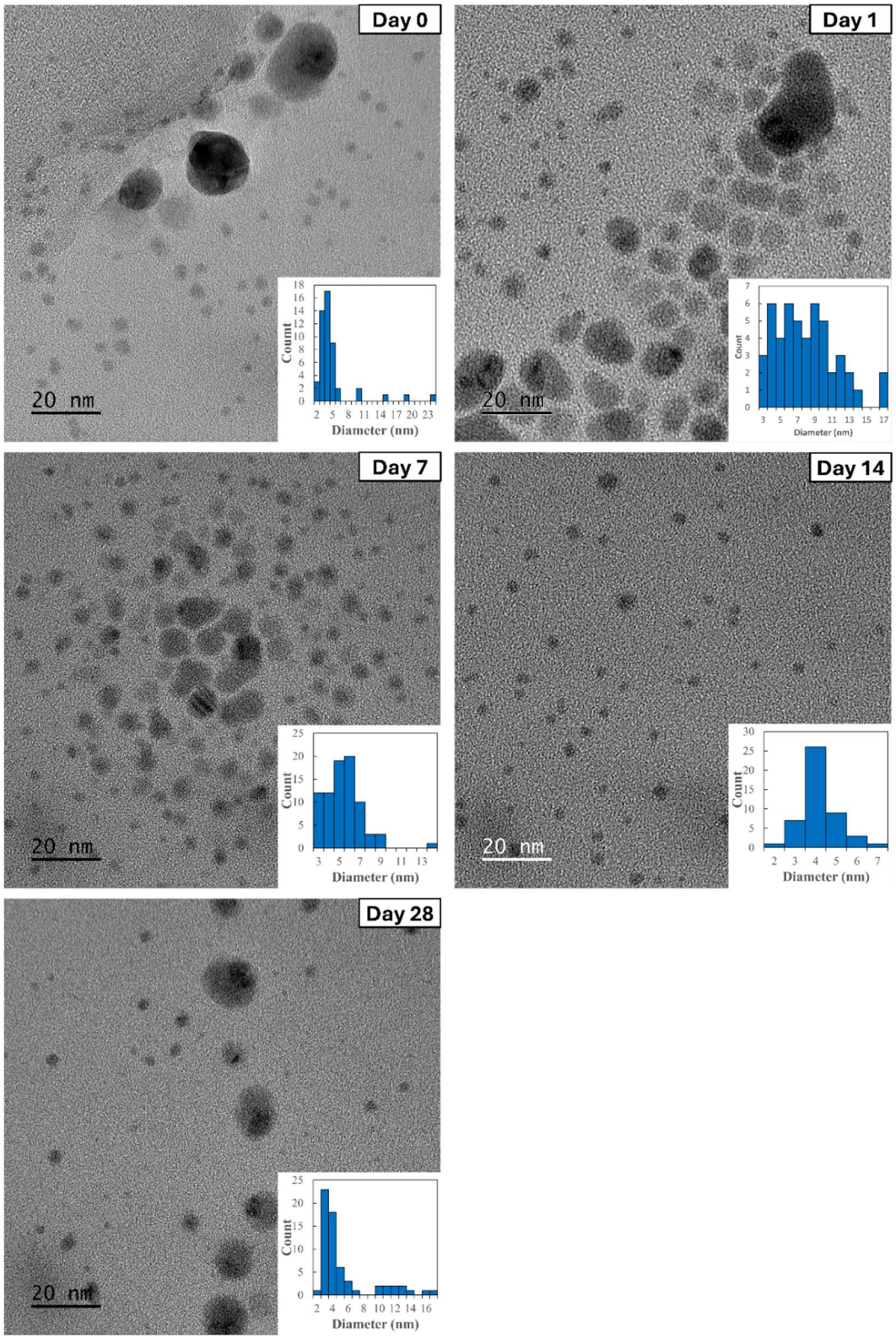


Figure A3: Stability study TEMs of AgNPs synthesised in a Bet:Gly 1:3 DES left in a dark cupboard, day 0-28 samples. Reaction conditions 120 mL Bet:Gly 1:3, 0.6 g Ag_2SO_4, 80 °C, 10 min.

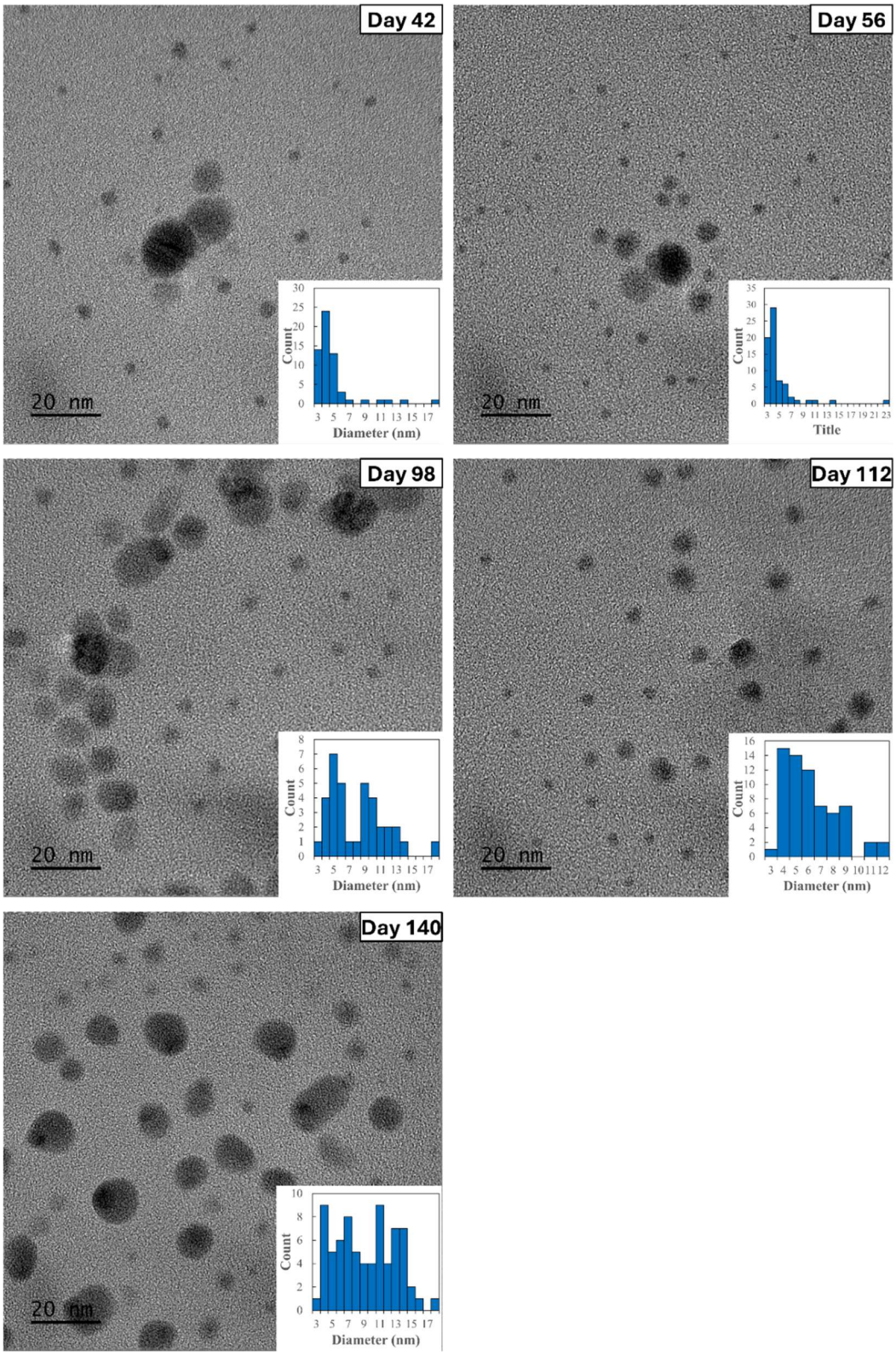


Figure A4: Stability study TEMs of AgNPs synthesised in a Bet:Gly 1:3 DES left in a dark cupboard, day 42-140 samples. Reaction conditions 120 mL Bet:Gly 1:3, 0.6 g Ag_2SO_4, 80 °C, 10 min.

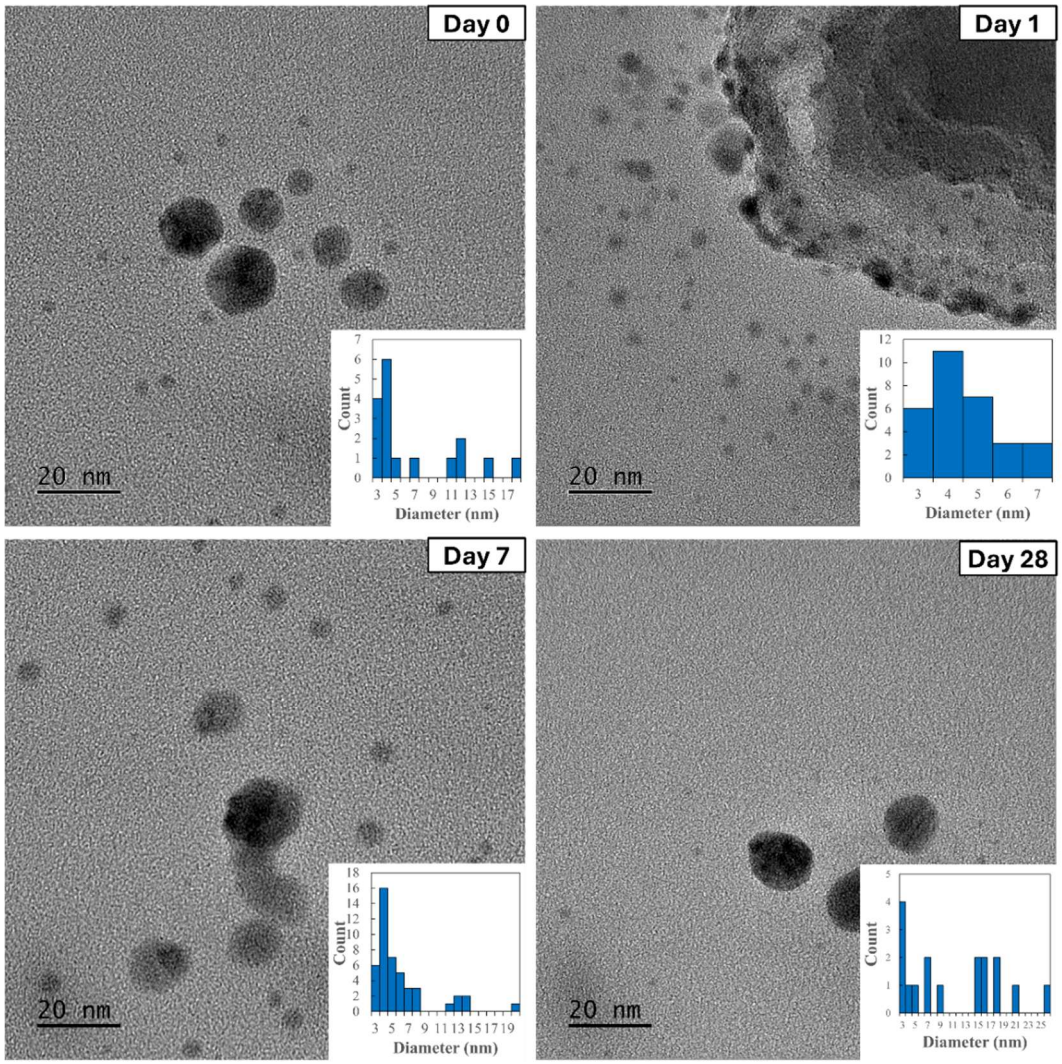


Figure A5: Stability study TEMs of AgNPs synthesised in a Bet:Gly 1:3 DES left in a dark cupboard, day 0-28 samples. Reaction conditions 120 mL Bet:Gly 1:3, 0.6 g Ag_2SO_4, 80 °C, 15 min.

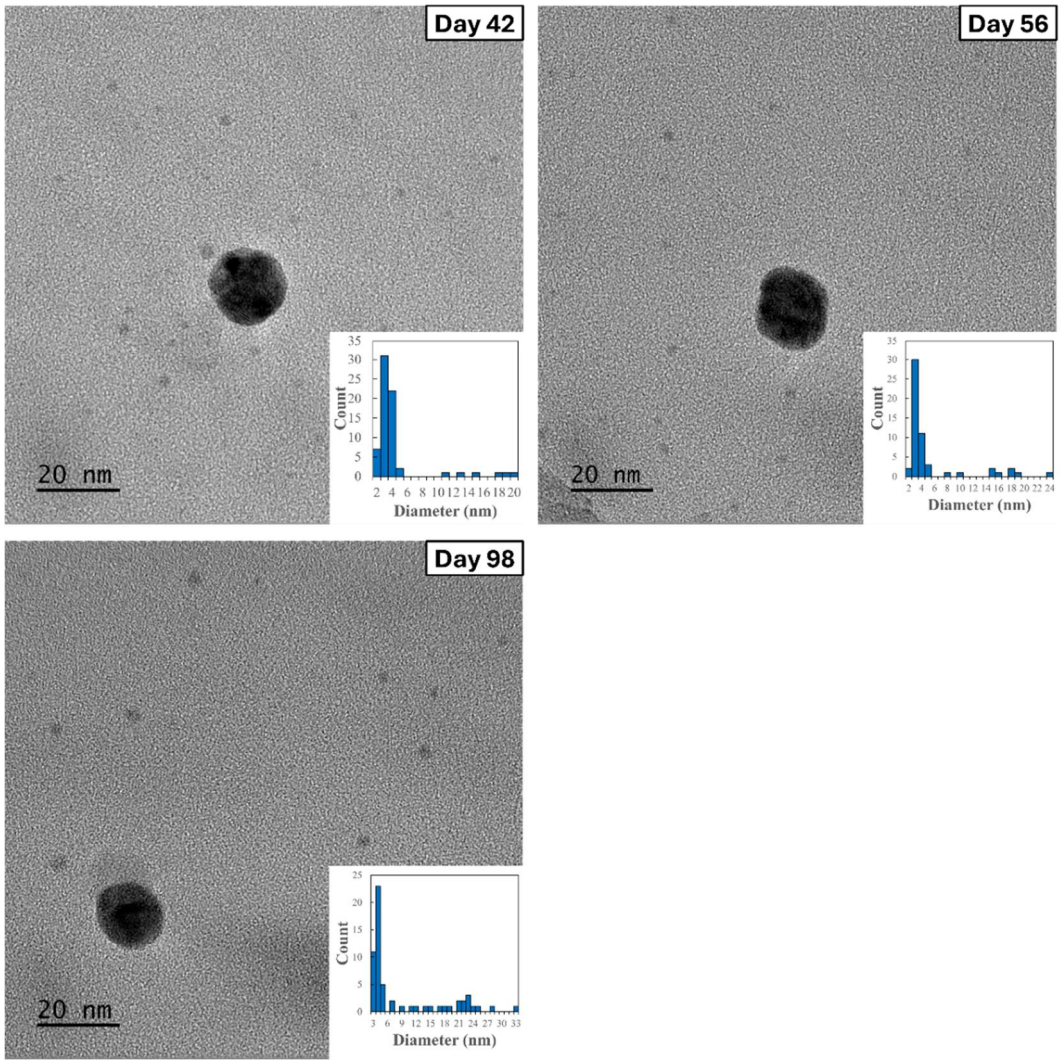


Figure A6: Stability study TEMs of AgNPs synthesised in a Bet:Gly 1:3 DES left in a dark cupboard, day 42-98 samples. Reaction conditions 120 mL Bet:Gly 1:3, 0.6 g Ag_2SO_4, 80 °C, 15 min.

Appendix B: MIC and MBC assays

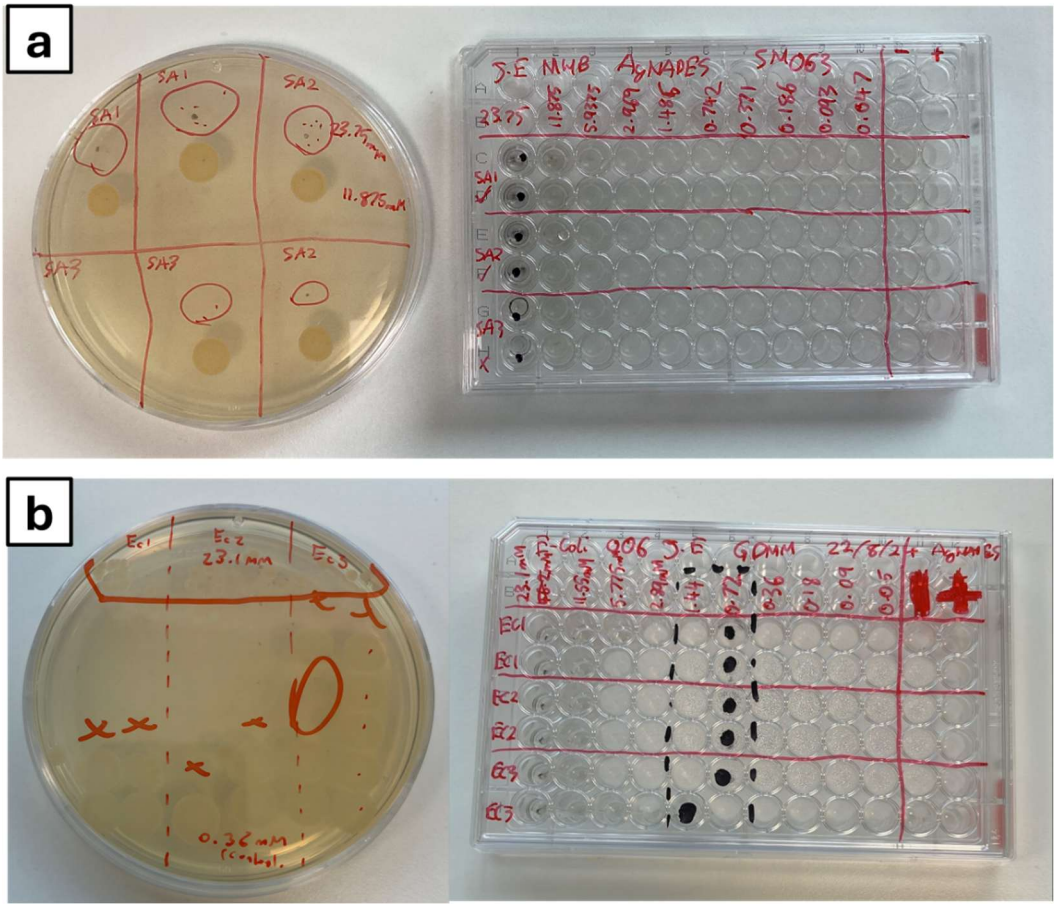


Figure B1: MIC and MBC assay for 20 nm AgNPs suspended in Bet:Gly 1:3 with three biological repeats. a) *S. aureus* in MHB. b) *E. coli* in defined media.

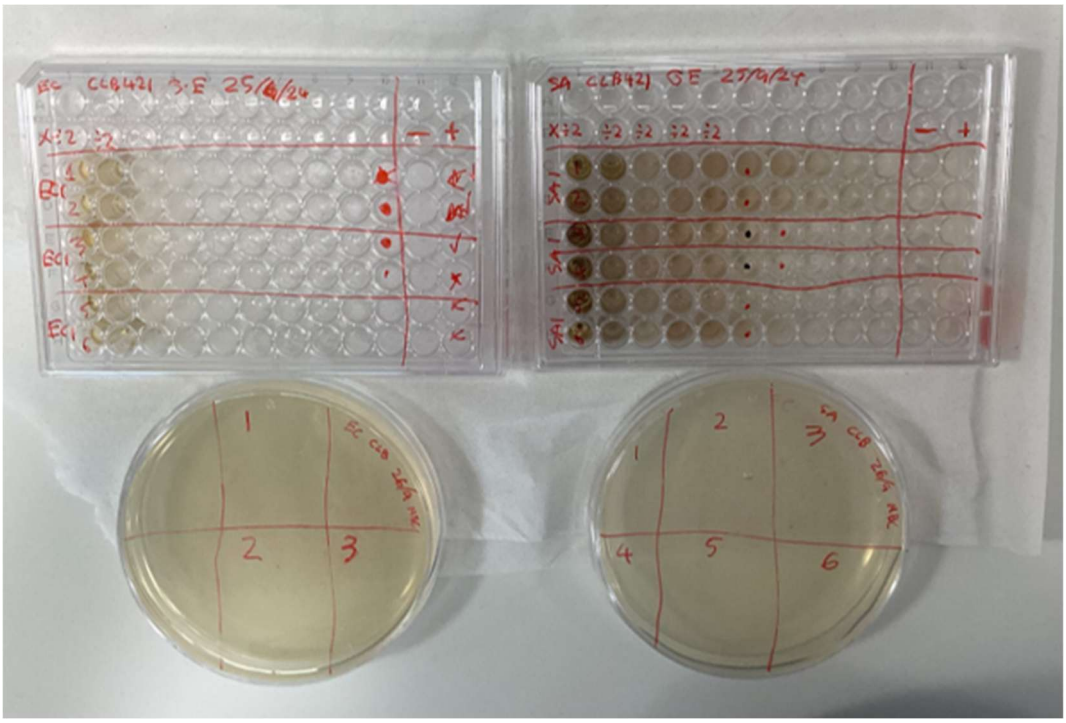


Figure B2: MIC and MBC assay for *S. aureus* and *E. coli* with synthesised AgNPs suspended in Cap:Lau:Bet 4:2:1 with three biological repeats.

References

1 M. E. A. de Kraker, A. J. Stewardson and S. Harbarth, *PLoS Med*, 2016, **13**, e1002184.

2 L. Adhikari, N. E. Larm, N. Bhawawet and G. A. Baker, *ACS Sustain Chem Eng*, 2018, **6**, 5725–5731.

3 L. Meredith, A. Elbourne, T. L. Greaves, G. Bryant and S. J. Bryant, *J Mol Liq*, 2024, **394**, 123777.

4 C. Daruich De Souza, B. Ribeiro Nogueira and M. E. C. M. Rostelato, *J Alloys Compd*, 2019, **798**, 714–740.

5 N. Abid, A. M. Khan, S. Shujait, K. Chaudhary, M. Ikram, M. Imran, J. Haider, M. Khan, Q. Khan and M. Maqbool, *Adv Colloid Interface Sci*, 2022, **300**, 102597.

6 B. Nowack, H. F. Krug and M. Height, *Environ Sci Technol*, 2011, **45**, 1177–1183.

7 J. Lin, W. Zhou and C. J. O'Connor, *Mater Lett*, 2001, **49**, 282–286.

8 A. Bardow, J. Pérez-Ramírez, S. Sala and L. Vaccaro, *Green Chemistry*, 2024, **26**, 11016–11018.

9 N. Fantozzi, J.-N. Volle, A. Porcheddu, D. Virieux, F. García and E. Colacino, *Chem Soc Rev*, 2023, **52**, 6680–6714.

10 A. Rai, A. Prabhune and C. C. Perry, *J Mater Chem*, 2010, **20**, 6789.

11 S. B. Maddinedi, B. K. Mandal and K. K. Anna, *Environ Toxicol Pharmacol*, 2017, **51**, 23–29.

12 Z. Zaheer and Rafiuddin, *Colloids Surf B Biointerfaces*, 2012, **89**, 211–215.

13 C. Florindo, F. Lima, B. D. Ribeiro and I. M. Marrucho, *Curr Opin Green Sustain Chem*, 2019, **18**, 31–36.

14 E. L. Smith, A. P. Abbott and K. S. Ryder, *Chem Rev*, 2014, **114**, 11060–11082.

15 Y. Dai, G.-J. Witkamp, R. Verpoorte and Y. H. Choi, *Food Chem*, 2015, **187**, 14–19.

16 X. Wang, M. Sun, S. Xiang, M. Waqas, Y. Fan, J. Zhong, K. Huang, W. Chen, L. Liu and J. Yang, *Electrochim Acta*, 2020, **337**, 135742.

17 A. Di Crescenzo, M. Tiecco, R. Zappacosta, S. Boncompagni, P. Di Profio, V. Ettorre, A. Fontana, R. Germani and G. Siani, *J Mol Liq*, 2018, **268**, 371–375.

18 S. P. Sakthi Sri, J. Taj and M. George, *Surfaces and Interfaces*, 2020, **20**, 100609.

19 L. Das, L. D. Koonathan, A. Kunwar, S. Neogy, A. K. Debnath and S. Adhikari, *Mater Adv*, 2021, **2**, 4303–4315.

20 L. Wei, Y.-J. Fan, H.-H. Wang, N. Tian, Z.-Y. Zhou and S.-G. Sun, *Electrochim Acta*, 2012, **76**, 468–474.

21 L. Wei, B. Lu, M. Sun, N. Tian, Z. Zhou, B. Xu, X. Zhao and S. Sun, *Nano Res*, 2016, **9**, 3547–3557.

22 J. A. Hammons, J. Ustarroz, T. Muselle, A. A. J. Torriero, H. Terryn, K. Suthar and J. Ilavsky, *The Journal of Physical Chemistry C*, 2016, **120**, 1534–1545.

23 R. Wang, Y. Hua and Q. Zhang, *ECS Trans*, 2014, **59**, 505–511.

24 A. Bazzaz Dilmaghani, F. Monajjemzadeh, M. R. Afshar Mogaddam and M. A. Farajzadeh, *Microchemical Journal*, 2022, **181**, 107665.

25 H. Yao, H. Ma, R. Mao, J. Qiu, C. Chen and D. Ju, *Journal of Wuhan University of Technology-Mater. Sci. Ed.*, 2022, **37**, 32–37.

26 Y. Liu, S. Kang, K. Li, J. Chen, B. Bae, I. Hwang, E.-Y. Ahn, Y. Park, K.-H. Chun and J. Lee, *J Clean Prod*, 2022, **379**, 134655.

27 L. Adhikari, N. E. Larm and G. A. Baker, *ACS Sustain Chem Eng*, 2019, **7**, 11036–11043.

28 K. Radošević, I. Čanak, M. Panić, K. Markov, M. C. Bubalo, J. Frece, V. G. Srček and I. R. Redovniković, *Environ Sci Pollut Res Int*, 2018, **25**, 14188–14196.

29 F. Al-Akayleh, R. M. Khalid, D. Hawash, E. Al-Kaissi, I. S. I. Al-Adham, N. Al-Muhtaseb, N. Jaber, M. Al-Remawi and P. J. Collier, *Lett Appl Microbiol*, 2022, **75**, 607–615.

30 L. Wang, C. Hu and L. Shao, *Int J Nanomedicine*, 2017, **Volume 12**, 1227–1249.

31 K. Z. Kučan, M. Perković, K. Cmrk, D. Načinović and M. Rogošić, *ChemistrySelect*, 2018, **3**, 12582–12590.

32 M. Fahim, A. Shahzaib, N. Nishat, A. Jahan, T. A. Bhat and A. Inam, *JCIS Open*, 2024, **16**, 100125.

33 A. Menichetti, A. Mavridi-Printezi, D. Mordini and M. Montalti, *J Funct Biomater*, 2023, **14**, 244.

34 A. Reznickova, H. Y. Nguyenova, K. Zaruba, J. Strasakova, Z. Kolska, A. Michalcova, F. Prusa, O. Kvitek, P. Slepicka, P. Sajdl and V. Svorcik, *Vacuum*, 2022, **203**, 111268.

35 A. Ivanković, *International Journal of Sustainable and Green Energy*, 2017, **6**, 39.

36 R. Desai, V. Mankad, SanjeevK. Gupta and PrafullaK. Jha, *Nanoscience and Nanotechnology Letters*, 2012, **4**, 30–34.

37 A. Mirzaei, K. Janghorban, B. Hashemi, M. Bonyani, S. G. Leonardi and G. Neri, *J Nanostructure Chem*, 2017, **7**, 37–46.

38 E. Panzarini, S. Mariano, C. Vergallo, E. Carata, G. M. Fimia, F. Mura, M. Rossi, V. Vergaro, G. Ciccarella, M. Corazzari and L. Dini, *Toxicology in Vitro*, 2017, **41**, 64–74.

39 X. Dong, X. Ji, H. Wu, L. Zhao, J. Li and W. Yang, *The Journal of Physical Chemistry C*, 2009, **113**, 6573–6576.

40 S. Wojtysiak and A. Kudelski, *Colloids Surf A Physicochem Eng Asp*, 2012, **410**, 45–51.

41 R. Javed, M. Zia, S. Naz, S. O. Aisida, N. ul Ain and Q. Ao, *J Nanobiotechnology*, 2020, **18**, 172.

42 S. Panigrahi, S. Kundu, S. Ghosh, S. Nath and T. Pal, *Journal of Nanoparticle Research*, 2004, **6**, 411–414.

43 R. A. Sheldon, *Green Chemistry*, 2023, **25**, 1704–1728.

44 J. K. Mining, M. J. Moloto, W. N. Omwoyo, D. Menge and W. K. Maboya, *Journal of Food Chemistry & Nanotechnology*, 2023, **9**, 88–93.

45 H. Hiramatsu and F. E. Osterloh, *Chemistry of Materials*, 2004, **16**, 2509–2511.

46 M. Distaso and W. Peukert, *React Chem Eng*, 2023, **8**, 1855–1867.

47 T. V Mathew and S. Kuriakose, *Colloids Surf B Biointerfaces*, 2013, **101**, 14–18.

48 O. Taher Mohammed, R. Jamal Abdulkhaliq and S. Taher Mohammed, *J Phys Conf Ser*, 2019, **1294**, 62075.

49 M. M. Ramos, E. dos S. Morais, I. da S. Sena, A. L. Lima, F. R. de Oliveira, C. M. de Freitas, C. P. Fernandes, J. C. T. de Carvalho and I. M. Ferreira, *Biotechnol Lett*, 2020, **42**, 833–843.

50 N. Vigneshwaran, N. M. Ashtaputre, P. V Varadarajan, R. P. Nachane, K. M. Paralikar and R. H. Balasubramanya, *Mater Lett*, 2007, **61**, 1413–1418.

51 F. E. Meva, A. A. Ntoumba, P. B. E. Kedi, E. Tchoumbi, A. Schmitz, L. Schmolke, M. Klopotowski, B. Moll, Ü. Kökcam-Demir, E. A. M. Mpondo, L. G. Lehman and C. Janiak, *Journal of Materials Research and Technology*, 2019, **8**, 1991–2000.

52 H. Peng, A. Yang and J. Xiong, *Carbohydr Polym*, 2013, **91**, 348–355.

53 O. Tavukcuoglu, N. Evcimen Duygulu, A. Altinbay and F. Ciftci, *Ind Crops Prod*, 2024, **222**, 119825.

54 M. Ohiduzzaman, M. N. I. Khan and K. A. Khan, *J Mol Struct*, 2024, **1317**, 139141.

55 S. Alam, Md. A. Aziz, Md. R. Karim, Md. H. Rahman, M. Khatun, M. A. Rabbi and Md. R. Habib, *J Mol Struct*, 2025, **1321**, 140264.

56 H. C. A. Murthy, T. Desalegn Zeleke, C. R. Ravikumar, M. R. Anil Kumar and H. P. Nagaswarupa, *Mater Res Express*, 2020, **7**, 55016.

57 S. K. Sahu, A. Mansoori, S. K. Jana, A. Kumar and T. K. Ghorai, *J Mol Struct*, 2025, **1320**, 139690.

58 G. Devendrapandi, D. Padmanaban, R. Thanikasalam, A. Panneerselvam, R. Palraj, J. R. Rajabathar, N. Rajendiran, R. Balu, T. H. Oh and S. Ramasundaram, *Int J Biol Macromol*, 2023, **252**, 126205.

59 L. H. Tho, B. X. Khuyen, N. X. D. Mai and N. H. T. Tran, *Beilstein Journal of Nanotechnology*, 2024, **15**, 426–434.

60 W. Li, X. Zhao, T. Huang, Y. Ren, W. Gong, Y. Guo, J. Wang and Q. Tu, *Int J Biol Macromol*, 2021, **191**, 60–70.

61 M. S. Rahman, R. Roy, B. Jadhav, M. N. Hossain, M. A. Halim and D. E. Raynie, *J Mol Liq*, 2021, **321**, 114745.

62 L. Adhikari, N. E. Larm and G. A. Baker, *ACS Sustain Chem Eng*, 2020, **8**, 14679–14689.

63 V. Vorobyova, M. Skiba, O. Horodniuk, L. Khrokalo and G. Vasyliev, *Bionanoscience*, 2023, **13**, 1726–1740.

64 M. Skiba and V. Vorobyova, *Chemical Papers*, 2022, **76**, 5789–5806.

65 T. El Achkar, S. Fourmentin and H. Greige-Gerges, *J Mol Liq*, 2019, **288**, 111028.

66 S. Inayat, S. Rashid Ahmad, S. Javaid Awan and N. Muhammad, *J Mol Liq*, 2023, **388**, 122735.

67 Y. Wang, Y. Zhang, J. Su, X. Zhang, J. Wang and Q. Tu, *ACS Sustain Chem Eng*, 2020, **8**, 14243–14252.

68 S. Altinisik, H. Zeidan, M. D. Yilmaz and M. E. Marti, *ACS Omega*, 2023, **8**, 11029–11038.

69 Z. Pirzadi and F. Meshkani, *Fuel*, 2022, **329**, 125044.

70 F. Y. Alzoubi, A. A. Ahmad, I. A. Aljarrah, A. B. Migdadi and Q. M. Al-Bataineh, *Journal of Materials Science: Materials in Electronics*, 2023, **34**, 2128.

71 D. Titus, E. James Jebaseelan Samuel and S. M. Roopan, in *Green Synthesis, Characterization and Applications of Nanoparticles*, Elsevier, 2019, pp. 303–319.

72 T. Huang and X.-H. N. Xu, *J Mater Chem*, 2010, **20**, 9867.

73 K. Wong, C. Chen, K. Wei, V. A. L. Roy and S. M. Chathoth, *Journal of Nanoparticle Research*, 2015, **17**, 153.

74 G. A. Dorofeev, A. N. Streletskii, I. V Povstugar, A. V Protasov and E. P. Elsukov, *Colloid Journal*, 2012, **74**, 675–685.

75 C. F. Holder and R. E. Schaak, *ACS Nano*, 2019, **13**, 7359–7365.

76 B. Fritsch, T. S. Zech, M. P. Bruns, A. Körner, S. Khadivianazar, M. Wu, N. Zargar Talebi, S. Virtanen, T. Unruh, M. P. M. Jank, E. Spiecker and A. Hutzler, *Advanced Science*, DOI:10.1002/advs.202202803.

77 G. W. Stewart, *Physical Review*, 1928, **32**, 558–563.

78 J. A. Greenberg and J. Carpenter, in *Counterterrorist Detection Techniques of Explosives*, Elsevier, 2022, pp. 315–338.

79 S. V. Kumar, A. P. Bafana, P. Pawar, A. Rahman, S. A. Dahoumane and C. S. Jeffryes, *Sci Rep*, 2018, **8**, 5106.

80 A. P. Abbott, *ChemPhysChem*, 2004, **5**, 1242–1246.

81 A. P. Abbott, G. Capper and S. Gray, *ChemPhysChem*, 2006, **7**, 803–806.

82 B. H. Kim, J. Yang, D. Lee, B. K. Choi, T. Hyeon and J. Park, *Advanced Materials*, 2018, **30**, 1703316.

83 W.-C. Huang, T.-H. Tsai, L.-T. Chuang, Y.-Y. Li, C. C. Zouboulis and P.-J. Tsai, *J Dermatol Sci*, 2014, **73**, 232–240.

84 J. M. Silva, E. Silva, R. L. Reis and A. R. C. Duarte, *Sustain Chem Pharm*, 2019, **14**, 100192.

85 I. M. Aroso, A. Paiva, R. L. Reis and A. R. C. Duarte, *J Mol Liq*, 2017, **241**, 654–661.

86 K. A. Omar and R. Sadeghi, *J Mol Liq*, 2023, **384**, 121899.

87 A. P. Abbott, D. Boothby, G. Capper, D. L. Davies and R. K. Rasheed, *J Am Chem Soc*, 2004, **126**, 9142–9147.

88 C. Zeng, Y. Liu, Z. Ding, H. Xia and S. Guo, *J Mol Liq*, 2021, **338**, 116950.

89 R. K. Thapa, J. O. Kim and J. Kim, *J Pharm Investig*, 2023, **53**, 627–641.

90 C. Rigo, M. Roman, I. Munivrana, V. Vindigni, B. Azzena, C. Barbante and W. R. L. Cairns, *Burns*, 2012, **38**, 1131–1142.

91 T. Kavin, V. Murugaiyah, J. K. Tan, M. N. I. Kassim, S. Ramakrishna and S. Vigneswari, *Biomass Bioenergy*, 2025, **194**, 107625.

92 M. Atilhan and S. Aparicio, *The Journal of Physical Chemistry C*, 2018, **122**, 18029–18039.

93 H. R. Ghenaatian, M. Shakourian-Fard, V. Alizadeh and G. Kamath, *J Mol Graph Model*, 2022, **110**, 108072.