Green Synthesis of Gold Nanoparticles for embedding into a Cellulose Acetate Membrane to kill *Escherichia Coli and Staphylococcus Epidermidis* found in water

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Abstract

The aqueous fraction of *Ananas comosus* (Pineapple extract) has been used as a reducing agent and stabilizer in the green synthesis of AuNPs (AuNPs). UV-Vis spectrophotometry and transmission electron microscopy-selected area electron diffraction (TEM) were used to characterize AuNPs. The AuNPs have a diameter of about 70 nm and have a face centered cubic (fcc) crystal structure and are stable for 14 days. Chlorogenic acid and ferulic acid in aqueous pineapple extract is considered having an important role as reducing agent to reduce Au^{3+} ions to Au^{0}, as well as to stabilize the AuNPs through the process of green synthesis. AuNPs exhibit excellent antibacterial properties against *E. Coli* and *S. Epidermidis* activity for embedding into a cellulose acetate (CA) membrane for water purification. The CA membrane was electrospun at a voltage of 15kV, syringe tip diameter of 0.07mm and collector distance of 100mm. The AuNPs showed good stability in the membrane and displayed the same antibacterial properties.

Introduction

Pollutants in the form of sewage, pesticides and fertilizers have increasingly reduced the supply of usable water in recent years. Although 70% of the Earth's surface is covered by water, only 2.5% is in freshwater lakes and rivers and the United Nations has predicted that 2 billion people may face water scarcity by 2025 (UNDP, 2015). Moreover, in the wake that 502,000 people die each year relating to contaminated water (World Health Organisation, 2018) and $578 billion worth of food is recalled due to contamination by pathogenic bacteria (Ray, Khan, Zhen, Fan & Senapati, 2013), it is now an expectation in most countries for water to be purified using membranes to sift out impurities and harmful bacteria and parasites.

However, as backwashes are done to wash off bacteria stuck onto the membrane itself during water purification processes and not kill them. Not only is this wasting precious water resources, but paradoxically, produces contaminated water. Essentially, it is a waste of scarce natural resources that needs to be improved and the bacteria is never actually killed, but only transferred, which is not a long term solution. Furthermore, these backwashes can be carried

out every 5 minutes (Water Treatability Database, 2007). To solve this problem, this study seeks to embed AuNPs into the filtration membrane to kill bacteria trapped in them.

In recent years, research on the synthesis of nanoparticles using green materials, commonly called green synthesis, has been growing as it is more environmentally friendly. The proposed materials are types of microorganisms, enzymes, plants or plant extracts. The biological resources can be used as a reducing agent and stabilizer in the synthesis of metal nanoparticles. They can reduce Au^{3+} ions to Au^{0}, while capping agents cover the refine surface of nanoparticles and prevent them from coagulation. This study has chosen to make use of this process, producing environmentally benign solvents and renewable materials. Moreover, this methodology improves the biocompatibility of the obtained nanoparticles.

As one of the most abundant renewable polymer resources, cellulose has been widely used for fibers and films. In recent years, the electrospinning of cellulose nanofibers has attracted a great deal of attention due to good thermal stability, chemical resistance, biodegradability, etc. These properties will ensure that they find a broad range of applications in affinity membranes, biosensors, chemosensors and in our study, water filters. Cellulose nanofibers have been prepared by the direct electrospinning of cellulose solution with solvents such as acetone, acetic acid and N,N-dimethylacetamide (DMAc). Interestingly, it was found that the average diameters of the resulting CA nanofibers could be easily controlled by changing the composition of this mixed solvent. In this study, varying concentrations of CA was dissolved in the 3 different solvents before being electrospun at a critical voltage of 15kV, where electrostatic forces acting on the surface of the cone overcome the surface tension of the solution, and a jet is ejected and accelerated toward the grounded collector by the electrical field generated between the electrode and the counter-electrode.

This study hopes to work with water purification companies in Singapore to evaluate if this nanotechnology is effective in purifying water, cost-efficient and scalable. The ultimate aim is to provide accessible and potable water to everyone and an alternative use to expired fruits.

2. Objectives and Hypotheses

This study aims to synthesize AuNPs from commonly found plants, embed stable AuNPs into a CA Membrane, and therefore purifying water contaminated with pathogenic bacteria like *E. Coli* and *S. Epidermidis* using AuNPs such that it is safe for human consumption. Finally, this hope to reduce bacteria found in water in a more environmentally friendly way than backwashes, which includes the green synthesis of AuNPs.

It was hypothesized that the plants tested are capable of reducing Au^{3+} ions to Au^{0}, producing AuNPs showing good stability in solution, where plant extracts act both as reducing agents and stabilizers in the synthesis of nanoparticles and that stable gold-disk nanoparticles embedded in a CA membrane can effectively kill *E.Coli* and *S. Epidermidis* found in water.

3. Methods and Materials

3.1.1 Materials needed for synthesis of AuNPs from Pineapple Extract

Pineapples (*Ananas comosus)* were purchased from local supermarkets in Singapore, for the preparation of *Ananas comosus* extract. For the synthesis of AuNPs, gold (III) chloride was used.

3.1.1 Synthesis of AuNPs from pineapple extract

Ananas comosus flesh was washed and cleaned, of which 20g was weighed using the electronic scale and cut into small pieces. Small pieces of *Ananas comosus* was blended using the blender until a juice was obtained, which was passed through visking tubing and the filtered juices was poured into centrifuge tubes. Each centrifuge tube of 12㎖ was centrifuged for 12 min at 8000 rpm before dilution with 20㎖ of deionised water and poured into a glass container. 0.031g of gold (III) chloride was added into diluted solution then mixed with a glass rod and heated at 80.0 °C for 10 min. The UV-Vis Spectrophotometer was used to check the wavelength of samples before being sent for TEM. The above was repeated for *Zingiber officinale*.

3.1.2 Materials needed for the preparation of Mueller-Hinton agar plates and the culturing of *Staphylococcus Epidermidis* and *Escherichia Coli*

For the culturing of *S. Epidermidis* and *E. Coli,* 9㎖ of Lysogeny broth, *S. Epidermidis* and *E. Coli*, Orbital Shaker, Mueller-Hinton Agar powder as well as agar plates were used. The whole process was done in a laminar flow hood with a flame.

3.1.2 Preparation of Mueller-Hinton agar plates and the culturing of *Staphylococcus Epidermidis* and *Escherichia Coli*

For the preparation of agar plates, 200g of Mueller-Hinton agar powder was added to 500㎖ of water and autoclaved. It was then poured onto 20 agar plates and allowed to solidify overnight. To 9㎖ of Lysogeny Broth, a sterile inoculating loop was used to swab E. *Coli* and *S. Epidermidis* and inserted for 5 seconds, repeated 4 times. The Lysogeny broth was then put into the Orbital Shaker for 24 h at 35.0 °C for the mixing and growth of bacteria in the broth.

3.1.3 Materials needed for the Testing of antibacterial properties of AuNPs

For the testing of antibacterial properties of AuNPs, a micropipette set to 800µl, 10% bleach, sterile water, Mueller-Hinton agar plates, laminar flow hood, flame, forceps and sterilised cotton swabs were used.

3.1.3 Wells Diffusion Test of AuNPs

The Mueller-Hinton agar plates were labelled according to positive control, negative control, and the solution we are testing for antibacterial properties which is the AuNP Solution. Using a sterile cotton swab, the E. *Coli* and *S. Epidermidis* culture was evenly spread onto the petri dishes with Mueller-Hinton agar. Indents were then made on the Mueller-Hinton agar plates according to labelled spots, which were subsequently removed using forceps. The positive control was filled with 800 µl of bleach, negative control with 800 µl of sterile water and 800 µl of AuNPs for the solution indent. The Mueller-Hinton agar plates were then left overnight in the incubator. The diameters of the inhibition zone were then measured after 24h.

3.2 Materials needed for electrospinning of CA membrane

For the electrospinning of the CA membrane, three different solvent systems were used in this study. The three solvent systems used to generate mixtures for electrospinning CA membrane were acetone, glacial acetic acid and dimethylacetamide (DMAc). CA from Sigma Aldrich with a degree of substitution (DS) of 2.45 and a number-average molecular weight (*M*n) of 30,000 Da was used.

3.2 Electrospinning of CA membrane

To prepare each solvent systems, CA was dissolved in acetone, acetic acid, DMAc, and their mixtures under constant stirring at room temperature. Each solvent system was then placed in a 5㎖ Terumo Syringe. As for the electrospinning, each polymer solution was placed in a glass capillary with a stainless steel electrode immersed in the solution and connected to a power supply (Gamma High Voltage Supply, ES 30-0.1 P). A grounded counter-electrode was connected to collectors that included aluminum foil wrapped around a piece of corrugated cardboard. The electrospinning conditions of voltages between 15 and 20 kV and distance of 100mm were used to deform the pendent drop at the tip of the capillary into a conical shape called the “Taylor cone.” 30 minutes after the electrospinning has begun, a sample was taken. The membrane was then collected from the aluminium foil after about 4h of electrospinning.

3.3 Materials needed for Testing of antibacterial properties of AuNPs embedded onto CA membrane

For the testing of antibacterial properties of AuNPs embedded onto CA membrane, m-TEC agar powder, Bantex Colony Counter 920A, sterile syringe, deionized water, plastic bags and an incubator were used.

3.3 Testing of antibacterial properties of AuNPs embedded onto CA membrane

After determining the best concentration of CA and the ratio of the solvent system (15% CA, 2:1 Acetone - DMAc), centrifuged AuNPs were electrospun together with the CA solution, allowing the AuNPs to be successfully embedded into the membrane. Alternatively, AuNPs-embedded membrane could be prepared by filter using a syringe unit by washing the CA membrane (supported by a filter unit with deionized water (10㎖) three times. Withdraw 10㎖ of AuNPs synthesized from pineapple extract with a sterile syringe (20㎖) and add it slowly into a pre-washed CA membrane. Wash the filter unit with 10㎖ of deionized water three times to remove non-immobilized AuNPs. In order to test the antibacterial properties of the AuNPs embedded in the CA membrane, the colony count was used to examine the effect of the AuNPs on *E. Coli* and *S. Epidermidis* Bacteria. Specimens of *E. Coli* and *S. Epidermidis* were cultured and spread onto using a sterile cotton swab onto Modified m-TEC Agar Plates. A control and a test set-up were set up for the colony count test.

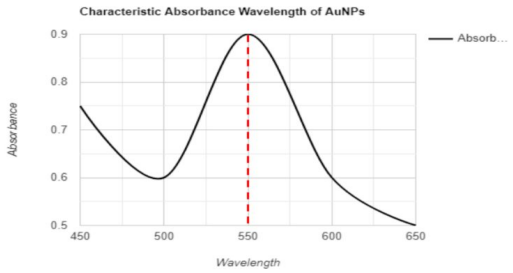
- Control Set-Up: CA membrane without gold nanoparticles embedded is carefully placed onto the agar plate with cultured *E. Coli* or *S. Epidermidis,* ensure there are no air bubbles between agar plate and membrane.
- Test Set-Up: CA membrane with embedded gold nanoparticles is carefully placed onto the agar plate with cultured *E. Coli* or *S. Epidermidis,* ensure there are no air bubbles between agar plate and membrane.

Both plates were then covered with the lid and inverted. Then, they are sealed in a plastic bag and placed in the incubator for 22 h at 44.5°C. The number of colonies were then determined using the Bantex Colony Counter 920A after incubation.

4. Results and Discussion

4.1 Characterising of AuNPs synthesized from pineapple extract

Characteristic Absorbance Wavelength



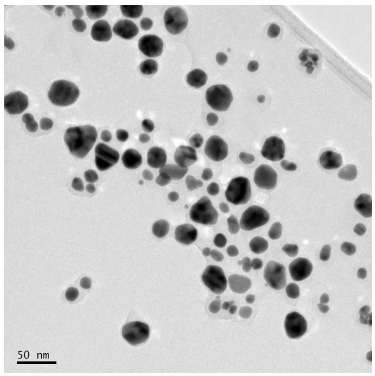


Figure 1: UV-Vis absorption spectra of AuNPs synthesized from *Ananas Comosus* extract

Figure 2: TEM images of AuNPs

From the results obtained from the UV-Vis Spectrophotometer, AuNPs synthesised from *Ananas Comosus* Extract have a peak plasmon resonance of 547nm. The AuNPs showed good stability in solution when first synthesized, producing a homogeneous purple solution.

From the TEM images (Figure 2), the average diameter of the AuNPswas found to be 70.65nm, showing that AuNPs have indeed been successfully synthesised from *Ananas Comosus* extract. However, the attempt to synthesise AuNPs using ginger did not yield viable AuNPs, resulting in a cloudy and scattered solution. This is because the role of plants in the synthesis of metal nanoparticles depends on the content of secondary metabolites. Specific plants contain specific chemical compounds which can act as active substances in reducing and stabilizing. These compounds are alternative environmentally friendly materials in nanoparticle production. Biomolecules in plant extracts that can reduce metal ions into nanoparticles include proteins, polysaccharides, alkaloids, flavonoids, terpenoids, and phenolic acids. However, ginger is found to be short of such biomolecules, especially essential monosaccharides and disaccharides, making it unfavourable for the synthesis of AuNPs.

Figure 3: Table showing the effect of AuNPs on *E. Coli* and *S. Epidermidis* through the measurement of the diameter of inhibition zones/mm

	Inhibition Zone of *S. Epidermidis* /mm			Inhibition zone of *E. Coli* /mm		
	1st Reading	2nd Reading	3rd Reading	1st Reading	2nd Reading	3rd Reading
AuNPs (S)	17.2	16.7	16.7	16.7	17.6	16.5
AuNPs (S)	16.6	15.4	17.3	16.6	17.4	16.5
AuNPs (S)	16.8	16.6	17.8	15.9	17.9	16.6
AuNPs (S)	16.5	17.1	16.5	16.3	16.8	16.4
AuNPs (S)	17.7	16.7	16.3	17.7	16.5	16.8
Mean	17.0	16.5	17.0	16.6	17.2	16.6

Figure 4: Table showing the effect of Bleach on AuNPs on *E. Coli* and *S. Epidermidis* through the measurement of the diameter of inhibition zones/mm

	Inhibition Zone of *S. Epidermidis*/mm			Inhibition zone of *E. Coli* /mm		
	1st Reading	2nd Reading	3rd Reading	1st Reading	2nd Reading	3rd Reading
Bleach (+)	17.7	16.6	15.9	17.5	16.6	18.0
Bleach (+)	16.4	18.7	17.2	18.1	17.5	16.7
Bleach (+)	17.6	18.1	16.6	18.0	18.3	18.0
Bleach (+)	17.9	16.8	15.8	18.3	18.2	17.7
Bleach (+)	17.5	16.5	17.3	17.6	17.6	16.9
Mean	17.4	17.3	16.6	17.9	17.6	17.5

Figure 5: Mann Whitney U Test

Null Hypothesis: AuNPs have weaker or equal antibacterial properties as Bleach

Alternative Hypothesis: AuNPs have stronger antibacterial properties than Bleach

α value= 0.05

Sample Comparison	p-value	Conclusion
AuNPs and Bleach against *S. Epidermis*	0.862 (>0.05)	AuNPs are weaker or have equal antibacterial properties as bleach against *S. Epidermidis*
AuNPs and Bleach against *E. Coli*	0.985 (>0.05)	AuNPs are weaker or have equal antibacterial properties as bleach against *E. Coli*

From Figures 3 and 4, the average diameter of the inhibition zones of AuNPs on *E. Coli* is 16.8mm and 16.8mm on *S. Epidermidis*, while the average diameter of the inhibition zones of Bleach on *E. Coli* is 17.7mm and 17.1mm on *S. Epidermidis*, this means that the average diameter of inhibition zones only differ by 0.9mm and 0.3mm respectively, therefore AuNPs have similar antibacterial properties with Bleach against *E. Coli* and *S. Epidermidis*. Although in Figure 6, it is shown that AuNPs are less effective in killing *S. Epidermidis* and *E. Coli*, the diameters of inhibition zones differ by insignificant amounts, and thus, AuNPs are proved to be effective against *S. Epidermidis* and *E. Coli.*

4.2 Electrospinning of CA membrane

In this study, multiple tests with varying concentrations of CA and different ratios of solvent systems were done to find the best conditions for electrospinning the membrane. However, only tests with conclusive data will be shown. Electrospinning of 10-15 wt % CA in acetone generates short fibers with diameters around 1nm and a “beads on string” morphology. The morphology appears to be due to the low viscosity of acetone. Because the low boiling point of acetone also causes fast evaporation and gelation of the polymer solution, better solvent systems are needed to sustain continuous fiber formation. At 13% CA concentration, 3:1 acetic acid/DMAc supported continuous electrospinning and membrane formation, however 3:1 acetic acid/acetone with the same CA concentration produced loosely packed fibers of 1–2 nm in diameter (Figure 6). In the Acetone: Glacial Acetic acid solvent tests, the slow exchange of solvent from the solution to the water phase and resulting coalescence of fibers was significant and led to the formation of dense films rather than fibrous structures. In 10:1 acetone:DMAc, 15% CA solution fibers with very smooth surfaces and uniform diameters (Figure 8). Electrospinning of 10% CA in this mixture produced long fibers which showed good stability. At 5:1 or higher acetone/DMAc ratios, the capillary tip could be readily blocked after a few minutes.

Our study concludes that 15% CA dissolved in solvent system 10:1 Acetone/DMAc is able to be electrospun into fibrous membrane, with no beads.

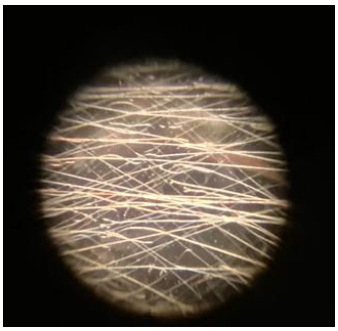


Figure 6:Membrane from 13% CA, 3:1 acetic acid/acetone with beads

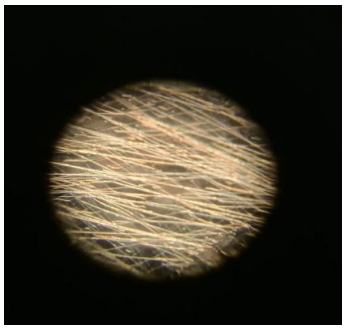
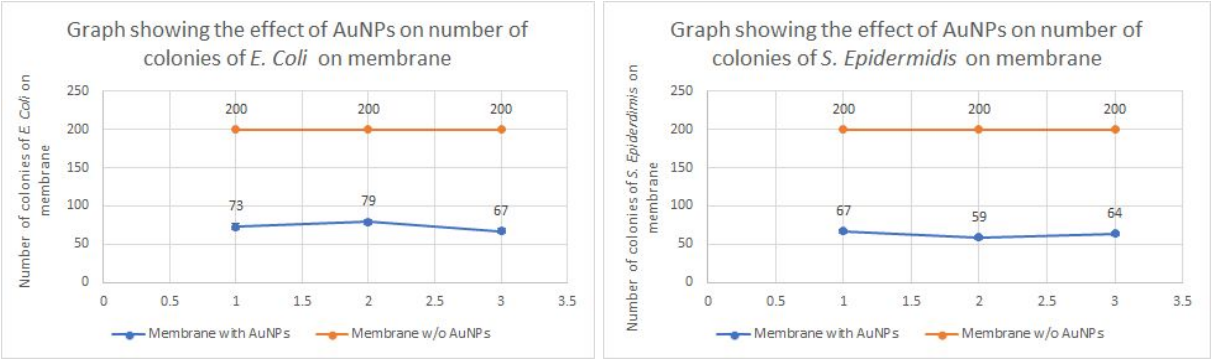


Figure 7: Membrane from 15% CA, 10:1 acetone:DMAc

Figure 8: Solvent and Concentration Effects on Electrospinning of CA (DS=2.45)

Solvent	Ratio	CA concentration (wt%)	Voltage (kV)	Distance (mm)	Collector	Fibrous Membrane	Beads on string
Acetone	-	15, 20	15	100	Al Foil	Few	Small Beads
Acetic acid	-	13	15	100	Al Foil	Few	None
DMAc	-	15-25	15	100	Al Foil	None	None
Acetone: DMAc	10:1	15	15	100	Al Foil	Fibrous	None
Acetic acid: DMAC	3:1	13	15	100	Al Foil	Fibrous	None
Acetic acid: acetone	3:1	12.5-20	15	100	Al Foil	Few	Small beads

4.3 Colony count carried out on bacteria cultured in CA membrane



Figures 9 and 10: Error Bars for the graphs showing the effect of AuNPs embedded on CA membrane through colony count

From Figures 9 and 10, it can be seen that AuNPs exhibit antibacterial properties as it reduces the number of colonies of *E. Coli* and *S. Epidermidis* to 63 and 71 on average, showing its effective antibacterial properties.

5. Conclusion and Recommendations for future work

In conclusion, this study has been able to obtain accessible fruits such as *Ananas Comosus,* which has been proven to be successful to be used in green synthesis of AuNPs. Furthermore, biomolecules found in *Ananas Comosus* are found to be able to stabilise synthesised AuNPs, sustaining their antibacterial properties and thus improving their effectiveness against pathogenic bacteria such as E. *Coli* and *S. Epidermidis*, having antibacterial properties comparable to 10% bleach. With the uprising dilemma of global warming, it has become increasingly important to adopt environmentally friendly methods to solve daily problems, including water purification and food wastage. Making use of food waste to combat pathogenic bacteria which can cause contagious bacterial infections is what our study has set out to do, and the goal has been reached. Since AuNPs can be synthesised from such commonly found fruits, people in rural areas who commonly suffer from bacterial infections due to pathogenic bacteria found in water can exploit our methods to help their people in combating such bacterial infections. In the future, the results of the study makes it possible for cooperation with water purification companies and provide them with our innovative solutions to combat water pollution.

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