Synthesis of hyaluronic acid coated ginsenoside-loaded gold nanoparticles and investigating its potential in cancer treatment

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Abstract

The development of nanoparticles as effective and harmless drug carriers to treat cancer has been widely researched over the world. This study aims at enhancing the effects of gold nanoparticles by using *Panax* ginseng leaves for non-cytotoxic synthesis as well as hyaluronic acid for enhanced specificity of the gold nanoparticles. FTIR spectroscopy tests and Transmission Electron Microscope (TEM) were carried out on the gold nanoparticles to characterize them after synthesis. The motion of *C. elegans* that were fed the different sets of gold nanoparticles were assessed via a stereomicroscope to determine the percentage of alive *C. elegans* after treatment. The results showed that the gold nanoparticles were successfully synthesised with the relevant bonds established and *C. elegans* that have undergone ginsenoside-reduced nanoparticles treatment and HS-HA coated nanoparticles treatment remained healthy whereas those that have undergone trisodium citrate-reduced nanoparticles treatment were found mostly dead.

Introduction

Over the years, chemotherapy, which refers to the use of delivering chemicals or other drugs directly into the body, as well as immunotherapy, have been extensively used for treating cancer. However, such therapies pose side effects and limitations such as toxicity and mutation of tumor cells. As a result, gold nanoparticles (NPs) are often used in drug delivery, as they possess high surface area for easier bioconjugation or modification (Khan, Rashid, Murtaza, & Zahra, 2014), thereby having huge potential to increase its specificity when delivering drugs to target cells such as tumor cells or bacteria. However, the process of creating chemosynthetic NPs usually creates residual amounts of toxic ingredients that may be left behind on the nanoparticles and potentially cause toxicity upon entering the body. According to Dai et al. (2015), mediating the formation of NPs in plant extract solution eliminates the need for potentially harmful reducing and stabilizing agents for the metal ions when they are converted into neutral nanoparticles (Singh *et al.*, 2015). The hydroxyl and carbonyl groups in plants make them and effective reducing and stabilizing agent.

Panax ginseng has shown to have many health benefits and has medicinal uses like anti-aging effects, boosting energy, fighting off a plethora of diseases, anti-inflammatory effects, and preventing cancer (Kang & Min, 2012). Ginsenosides, which are saponins glycosides, are one of the major constituents of ginseng. These ginsenosides are composed mainly of hydrophobic

triterpenes and hydrophilic sugar side-chains (Dai *et al*., 2015). Ginsenosides have also been found to have immunomodulatory effects, induce pro-apoptotic molecules as well as tumor reduction both *in vivo* and *in vitro*, making them an appealing candidate in treating cancer (Wong, Che & Leung, 2014).

A study done by Hurh *et al.* (2017) performed a procedure whereby ginsenosides were loaded onto the gold nanoparticles and tested for its cytotoxicity to healthy cells.In this study, gold nanoparticles were mediated in a solution of only ginsenosides (C-K and Rh2) rather than whole parts of the plant. It was found that this process produced ginsenoside-loaded gold nanoparticles with loading efficiencies of approximately 50-60%. These gold nanoparticles were then added in varying concentrations to cultured cells. It was found that C-K gold nanoparticles caused significantly less damage to healthy cells than an equal concentration of free C-K, indicating that C-K gold nanoparticles may be useful in fighting cancer cell growth while reducing the damage to healthy cells.

To improve the cytotoxic selectivity of the nanoparticles, they will be further modified with hyaluronic acid (HA). According to Mattheolabakis *et al*. (2015), the CD44 receptor is highly overexpressed on a variety of tumor cells. Hyaluronic acid is found to be the predominant binding molecule to the CD44 receptor and hence only those tumor cells that have an overexpression of CD44 receptors can uptake the gold nanoparticles and this would then allow the nanoparticles to specifically target tumor cells and avoid healthy cells, inflicting less cytotoxic damage to them.

C. elegans have been used as a model for this study as *C.elegans* has been traditionally considered by the scientific community as an organism suitable for use in cancer research. According to Kyriakakis, Markaki and Tavernarakis (2014), *C. elegans* homologs have been identified for 60–80% of human genes and many biological processes, including apoptosis, cell signaling, cell cycle, cell polarity, metabolism, and aging, are conserved between *C. elegans* and mammals, which means *C. elegans* pose as an ideal organisms to investigate the genes and pathways involved in diverse pathologies, including neurodegeneration and cancer.

Objective and Hypotheses

Objective

The objective of this study is to investigate the cytotoxicity of the gold nanoparticles, ginsenoside-loaded gold nanoparticles, HS-HA-coated gold nanoparticles as well as HS-HA-coated ginsenoside-loaded gold nanoparticles in treating cancer in *C. elegans*, determining whether it is suitable for use without killing healthy cells.

Hypotheses

It is hypothesised that gold nanoparticles reduced by *Panax* ginseng extract can successfully be loaded with ginsenosides and coated with end-thiol modified hyaluronic acid (HA). Furthermore, it is hypothesised that healthy C. elegans will not be killed when fed any ginsenoside-loaded gold nanoparticle variant.

Experimental Procedures

Extraction of ginsenosides from ginseng

Dried *Panax ginseng C. A. Mey* leaves were blended into a fine powder using a blender. 10g of the powdered leaves were boiled together with 100 mL of distilled water, and stirred with a glass rod frequently for 30 min. The solution was then filtered and the filtrate was centrifuged at 10000 rpm at 10 min to get rid of any remaining suspended powder. The supernatant was transferred into a stock bottle and kept at 4◦ C for the synthesis of gold nanoparticles.

Synthesis of gold nanoparticles

10.2mg of gold (III) chloride trihydrate was added to 15 ml of 38.8 mM trisodium citrate solution. The solution was then kept at 80◦ C until it changed colour from yellow to purple, signifying reduction of gold (III) ions to gold nanoparticles. The resulting solution was centrifuged at 2000 rpm for 20 min, decanted, and centrifuged again at 16000 rpm for 15 min. Afterwards, they were washed with sterile water to remove impurities on its surface.

Synthesis of ginsenoside-loaded gold nanoparticles

5 mL of the ginseng extract solution was mixed together with 25 mL distilled water and the solution was stirred using a glass rod. 10.2mg of gold (III) chloride trihydrate was subsequently added to this. The solution was then kept at 80◦ C until it changed colour from yellow to purple, signifying reduction of gold (III) ions to gold nanoparticles. The resulting solution was centrifuged at 2000 rpm for 20 min, decanted, and centrifuged again at 16000 rpm for 15 min. Afterwards, they were washed with sterile water to remove impurities on its surface.

Addition of thiol end group to hyaluronic acid molecules

100 mg of hyaluronic acid (10000 Da) and 60mg of cystamine dichloride were dissolved in a 10mL of 0.1 M borate buffer and mixed with 0.234g of sodium chloride. 0.1257g of sodium cyanoborohydride was added to the solution and the solution was left to react at 50° C for 72 hours. The reaction mixture was incubated with 0.1 M DTT solution for 12 h to introduce a free thiol group. The solution was dialyzed against 0.1 M NaCl solution, 25% ethanol, and DI water for one day each to form a resultant solution of HS-HA.

Synthesis of hyaluronic acid-coated ginsenoside-loaded gold nanoparticles

HS-HA was added dropwise to ginsenoside-loaded gold nanoparticles dispersed in DI water at 25° C and subsequently stirred for 24h. The resulting HA-GNP conjugate was purified by centrifuging it twice at 10,000 rpm for 20 min to remove unbounded hyaluronic acid. Afterwards, they were washed with sterile water to remove impurities on its surface.

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Characterization of gold nanoparticle composite

A Transmitting Electron Microscope was used to take images of the HS-HA coated gold nanoparticles, the ginsenoside-loaded gold nanoparticles as well as the HS-HA-coated ginsenoside-loaded gold nanoparticles. The TEM images were then compared to images of the original gold nanoparticles to establish whether binding between gold nanoparticles and HS-HA and ginsenoside has occurred. FTIR spectroscopy tests were also conducted on the various gold nanoparticle composites on a range of 4000 to 450 cm^{-1} to establish whether cross-linking between the various groups had occurred.

Testing of cytotoxicity of gold nanoparticle composite

Nematode growth agar (NGM) was autoclaved, after which 0.3mL of a 0.0129 mol dm^{-3} cholesterol solution and 7.39mg of magnesium sulfate heptahydrate were then added. 33.29 mg

of calcium chloride and 7.5mL potassium phosphate buffer pH 6.0 (1M) were then added. 0.05mL of *E. coli* OP50 were added to a fresh NGM plate and grown overnight at 37◦ C. A block of agar containing *C. elegans* N2 was placed on the plate and incubated at 20◦ C for 48h. *C. elegans* and *E. coli* were collected in M9 buffer before adding to NGM plate, with 100µl of gold nanoparticle solution spread on it. The control is sterile water. The percentage survival of worms was determined after 24h. Worms were considered dead if they did not move.

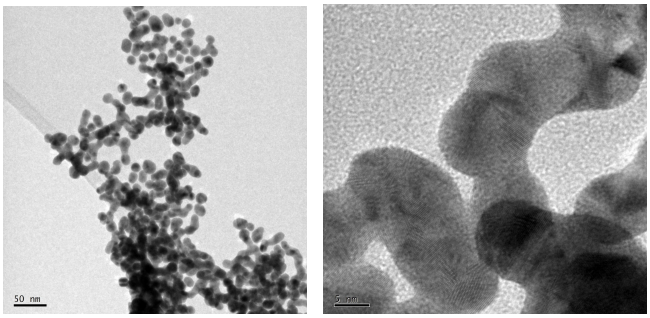
Risk and Safety

To avoid exposure to microorganisms and corrosive chemicals, gloves and lab coats will be worn. Work involving microorganisms is done in the laminar flow hood.

Results and Discussion

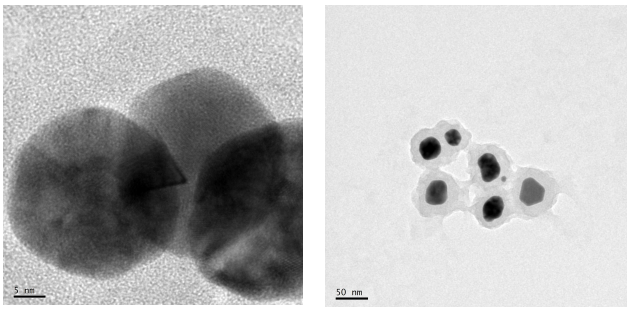
Transmission Electron Microscope Imaging of nanoparticles

Fig 1.1: TEM images showing morphology of gold nanoparticles



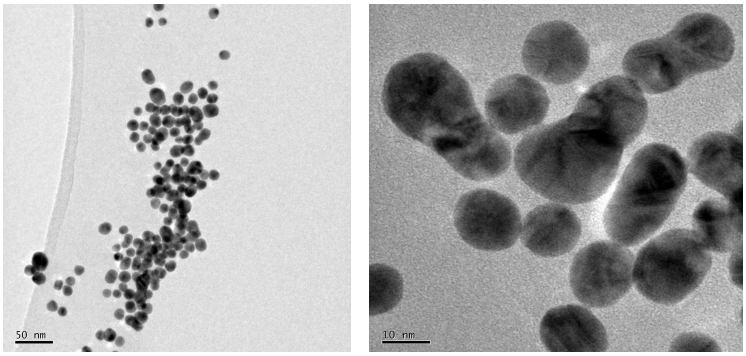
The average diameter of nanoparticles is about 12nm, which is similar to the diameter of AuNPs synthesised in literature (Pan *et al*., 2015) The gold nanoparticles that are synthesised using trisodium citrate are amalgamated because of the absence of a capping agent such as HS-HA that prevents the nanoparticles from being packed closely together.

Fig 1.2: TEM images showing the morphology of ginsenoside loaded gold nanoparticles



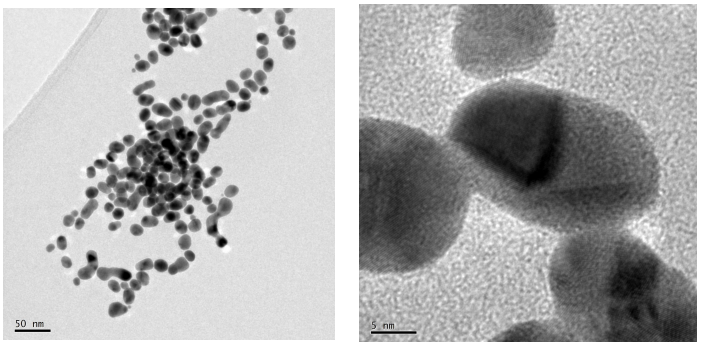
Average diameter of ginsenoside-loaded gold nanoparticles are around 25nm, which is significantly bigger than the trisodium citrate gold nanoparticles. This could explain why ginsenoside gold nanoparticles are less cytotoxic than trisodium citrate gold nanoparticles because the size of the nanoparticles is inversely related to its cytotoxicity hence it shows that the ginsenoside has successfully been loaded onto the nanoparticles. (Gonzalez-Moragas et al., 2017)

Fig 1.3: TEM images showing morphology of hyaluronic coated gold nanoparticles



Average diameter of HS-HA gold nanoparticles is approximately 14.4nm, which is within expectations because the coating of HS-HA makes it slightly larger than the gold nanoparticles without the coating. The TEM imaging also shows that HS-HA has bonded to the gold nanoparticles because the spacing between the nanoparticles indicates the presence of a capping agent such as HS-HA that prevents gold nanoparticles from clustering together has bonded to the gold nanoparticles.

Fig 1.4: TEM images showing morphology of ginsenoside loaded hyaluronic acid coated gold nanoparticles



Average diameter of HS-HA coated ginsenoside-loaded gold nanoparticles is approximately 14.7nm and the slight increase from the gold nanoparticle diameter corresponds to the coating of HS-HA around the nanoparticles. The TEM imaging also shows that HS-HA has bonded to the gold nanoparticles because the spacing between the nanoparticles indicates the presence of a capping agent such as HS-HA that prevents gold nanoparticles from clustering together has bonded to the gold nanoparticles. The slight increase in diameter from that of the HS-HA coated

gold nanoparticles shows that ginsenoside has been loaded successfully as the size of the nanoparticles is inversely related to its cytotoxicity.

FTIR Spectroscopy tests

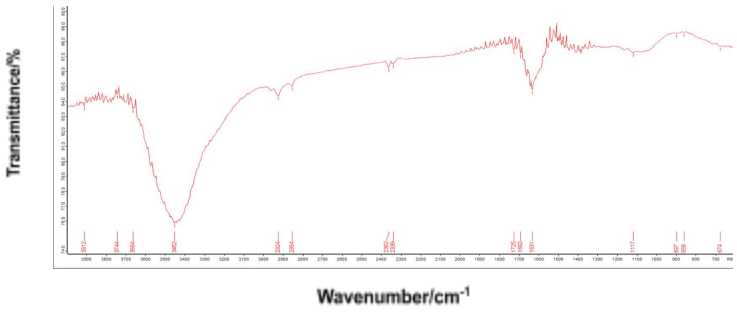


Fig 2.1: FTIR Spectroscopy results for gold nanoparticles (synthesised by trisodium citrate)

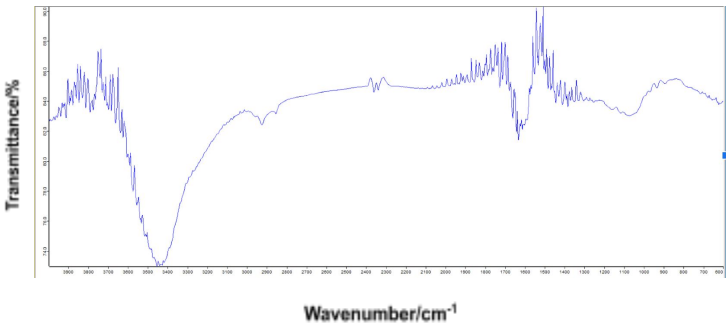


Fig 2.2: FTIR spectroscopy results for ginsenoside loaded gold nanoparticles

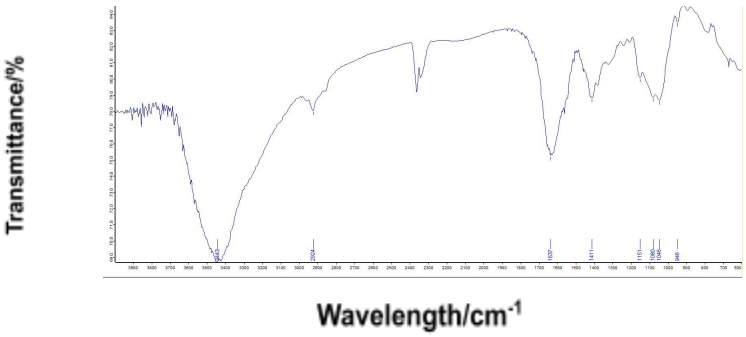


Fig 2.3 : FTIR spectrum of hyaluronic acid-coated gold nanoparticles

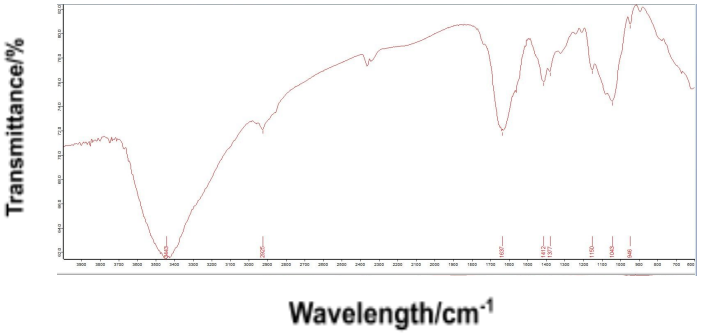


Fig 2.4: FTIR spectroscopy results for ginsenoside loaded hyaluronic acid coated gold nanoparticles

The peak at 1630cm^{-1} in Figure 2.2 shows the cross-linking between Au and the ginsenoside, which weakens the surrounding C=O bonds and gives rise to the peak. The peak at 3450cm^{-1} shows the stretching of the O-H bond in ginsenoside, confirming its presence in the nanoparticle (Lee, Kim, Lim, Huang & Choi, 2017)

The peak at 1043 cm^{-1} in Figure 2.3 indicates the presence of ether functional group in hyaluronic acid. The peak at 2400cm^{-1} proves cross-linking between HS-HA and Au as the decrease in bond wavenumber from 2550cm^{-1} is indicative of the lower level of energy needed for the bond to vibrate due to the HS forming bonds with Au. (Kumar, Raja, Sundar, Antoniraj & Ruckmani, 2015).

In Figure 2.4 , the peak at 1046cm^{-1} corresponds to the presence of ether functional group in HA. The peak at 2400cm^{-1} gives evidence for cross-linking between HS-HA and Au as the decrease in wavenumber from 2550cm^{-1} is indicative of the lower level of energy needed for the bond to vibrate due to the HS forming bonds with Au. The peak at 3443cm^{-1} gives evidence for the O-H bond in ginsenoside.

Testing of cytotoxicity of nanoparticles

After one day, the dish of *C. elegans* with the NGM was analysed under a stereomicroscope. A video of each of the 5 replicates was recorded using the software to determine whether the *C. elegans* are alive and motile.

Fig 3.1: Images of *C. elegans* after treatment with nanoparticles

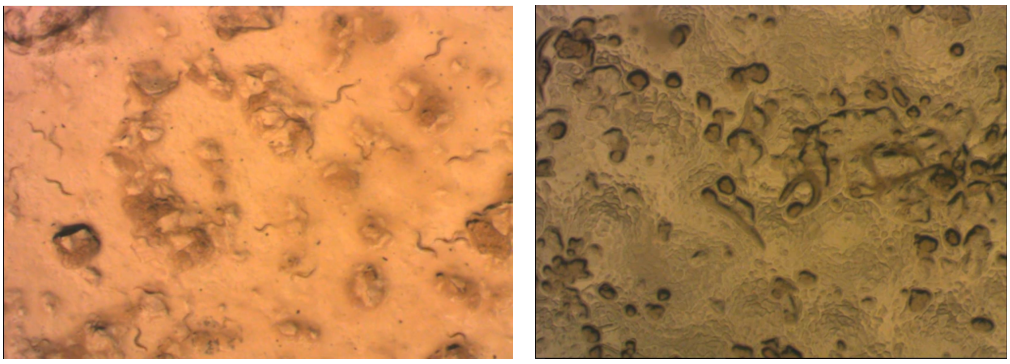
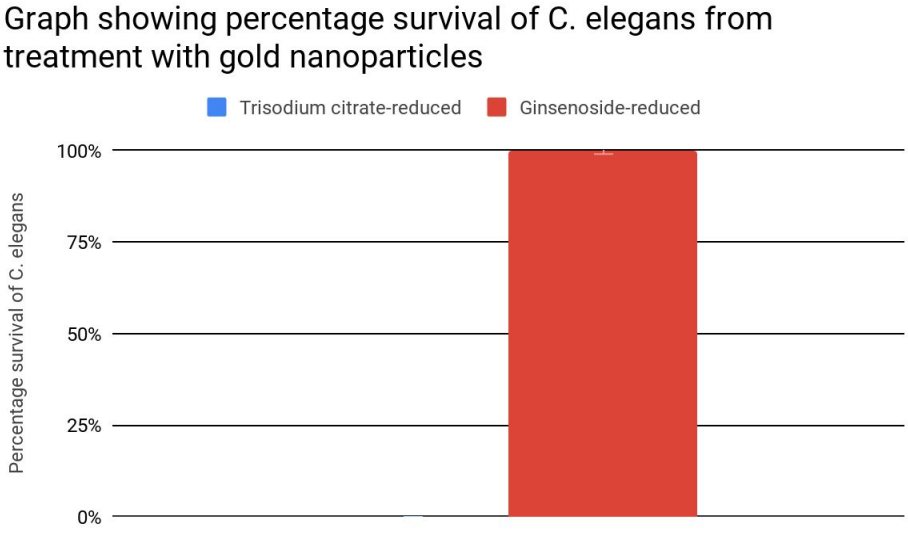


Fig 3.2: Graph showing percentage survival of *C. elegans* from treatment with gold nanoparticles



None of the *C. elegans* in the five trials survived the treatment of sodium citrate gold nanoparticle solution. The toxic byproducts of the gold nanoparticle synthesis are cytotoxic to *C. elegans* therefore resulting in the extremely low survival rate of *C. elegans*. The survival rate of *C. elegans* that are treated with ginsenoside-loaded gold nanoparticle solution is very close to

100%. Reduction of gold nanoparticles using ginsenosides produced little to no toxic byproducts that results in the death of *C. elegans*, accounting of the low cytotoxicity of nanoparticles synthesised using ginsenosides as a reducing agent.

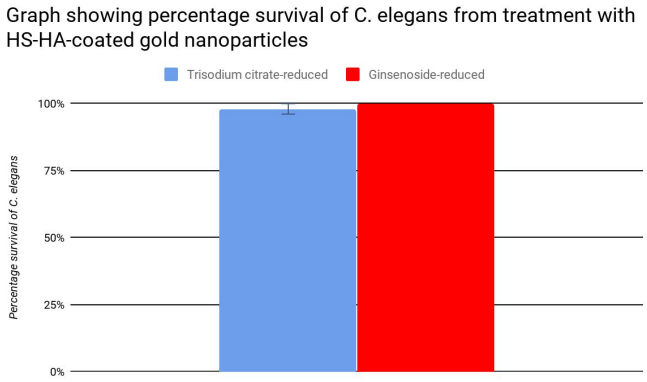


Fig 3.2: Graph showing percentage survival of *C. elegans* from treatment with HS-HA coated gold nanoparticles

The survival rate of C. elegans that are treated with HS-HA-coated trisodium citrate-reduced and ginsenoside-reduced gold nanoparticles is extremely close to 100%. The results show that HS-HA has successfully bound to the gold nanoparticles in solution as the percentage survival of *C. elegans* treated with the HS-HA-coated variant of the sodium-citrate reduced nanoparticles is 98%, significantly greater than the 0% survival rate of *C. elegans* treated with sodium-citrate reduced nanoparticles. This demonstrates the nature of HS-HA as a bonding molecule to the CD44 receptor present in human cells, as it renders the originally toxic composite non-cytotoxic to the cells of *C. elegans* as the receptors present in the cell membranes are not recognised by the HS-HA molecule.

Data Analysis

The Kruskal-Wallis test was carried out to determine significant differences in the percentage of mean survival of *C. elegans* after treatment with the different types of gold nanoparticles.

Nanoparticle	GNPs and Ginsenoside GNPs	GNPs and HS-HA GNPs	Ginsenoside GNPs and HS-HA Ginsenoside GNPs	HS-HA GNPs and HS-HA Ginsenoside GNPs
p-value	0.046	0.046	0.617	0.617

The p-value of 0.046 for trisodium citrate-reduced gold nanoparticles and ginsenoside-reduced gold nanoparticles indicates a significant statistical difference in the 2 sets of data, due to the absence of cytotoxic substances in the latter that enabled the *C. elegans* to survive

The p-value of 0.046 for trisodium citrate-reduced gold nanoparticles and HS-HA coated trisodium citrate-reduced gold nanoparticles indicates a significant difference in the 2 sets of data, as the addition of HSHA made the nanoparticles more receptive to only cancer cells.

The p-value of 0.617 for ginsenoside-reduced gold nanoparticles and HS-HA ginsenoside-reduced gold nanoparticles indicates no significant statistical difference in the 2 sets of data, as the effect of both nanoparticles were not negative towards the *C. elegans*

The p-value of 0.617 for HS-HA trisodium citrate-reduced gold nanoparticles and HS-HA ginsenoside-reduced gold nanoparticles indicates no significant statistical difference in the 2 sets of data, as the presence of HS-HA on both nanoparticles made them more receptive to only cancer cells

Conclusion

From the size and morphological analyses of the gold nanoparticles done in the FTIR and TEM imaging characterization tests, it can be concluded that ginsenosides were successfully loaded onto the gold nanoparticles due to the significant increase in size of the nanoparticles as well as the corresponding bonds observed in the FTIR spectrum. Hyaluronic acid was successfully coated onto the gold nanoparticles which is illustrated in the additional peaks in the FTIR spectrum. From the cytotoxicity tests that were carried out on the *C. elegans,* it can be concluded that the synthesis of gold nanoparticles using ginsenosides as reducing agent produces nanoparticles that are less cytotoxic to organisms compared to synthesis using trisodium citrate. The coating of the hyaluronic acid made the nanoparticles much less receptive to non-desired cells, such as healthy cells from *C. elegans*, hence the *C. elegans* did not intake much cytotoxic substance and showed a near full survival rate.

Future Work

These nanoparticles can be tested on A549 lung carcinoma cells, which carry a CD44 receptor in its cell membrane, to which the hyaluronic acid is a primary bonding molecule to. These nanoparticles can also be tested on healthy human cells to ascertain that it is non-cytotoxic towards any non-lung carcinoma cells.

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