Human Serum Albumin Nanoparticles for the Treatment of Hepatocellular Carcinoma

Mina Hibino
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Human Serum Albumin Nanoparticles for the Treatment of Hepatocellular Carcinoma

Honors thesis presented to the Department of Chemistry of the University of New Orleans

In partial fulfillment of the requirements for the degree of Bachelor of Science with University High Honors and Departmental Honors in Chemistry

Mina Hibino
May 2019
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Abstract

Hepatocellular carcinoma (HCC) is an aggressive and destructive cancer of the liver. Currently available treatment plans are locoregional therapies aimed to decrease tumor burden, resection that surgically removes the tumor, or systemic therapies for advanced stage to extend survival. However, decompensation post-locoregional treatment and recurrence rates for HCC remain high despite the different treatment modalities. There is a great need for alternative and more effective treatment methods that will further dampen tumor burden while decreasing the risk of intra- and extrahepatic spread. The goal of this project was to develop a non-invasive therapeutic treatment to target HCC cells using human serum albumin particles (HSAPs).

In this study, nanoparticles were prepared using human serum albumin (HSA) and synthesized to house gold nanorods (AuNRs). The AuNR-HSAPs were incubated with hepatic cell lines under different stresses of chemotherapy drug, doxorubicin, with near-infrared laser irradiation. Metabolic activity and viability were determined via MTT assay and flow cytometry.

Particle uptake in HCC lines was confirmed. AuNRs were sufficient to cause metabolic stress and reduce viability by 10-25% in a cell line dependent manner. The combination of AuNRs and laser irradiation did not significantly impact viability that differed from AuNRs alone in any of the cell lines tested, doxorubicin doses chosen were aimed to decrease viability by 10%. The combination of AuNRs, laser irradiation, and doxorubicin provided the largest decrease in viability. However, it was not statistically significant from the control.
Introduction

Hepatocellular carcinoma (HCC) is the most common form of liver malignancy, accounting for over 75% of all liver cancers.\(^2\) HCC arises due to underlying cirrhosis mainly caused by hepatitis viral infections, nonalcoholic steatohepatitis (NASH), or alcohol. Each year, over 700,000 patients are diagnosed with HCC globally.\(^1\) As of 2018, HCC is the third leading cause of cancer-related deaths worldwide. As the curative rate of the hepatitis C virus continues to increase with advancements in medicine, HCC will continue to be a threat in the obesity epidemic with it developing in the background of NASH.

The only curative treatment options available for patients with HCC are orthotopic liver transplantation (removal of the cancer and the underlying cirrhosis) and resection (partial removal of the liver reserved only for patients without cirrhosis). Despite advances in medicine, the five-year survival rate for HCC remains low at approximately 18%.\(^{17}\) Recurrence rates for HCC are higher for resected patients, ranging from approximately 50% to 70% for patients undergoing resection, while rates range from 20-40% for transplanted patients.\(^{21}\) Organ shortage is a major issue, as it lengthens the patient waitlist time, allowing for tumor progression. It also limits the number of patients able to receive a transplant. To decrease tumor burden, locoregional therapy (LRT) is used while patients are on the transplant waitlist. The main types of LRT include: microwave/thermal ablation; doxorubicin-eluting bead transarterial chemoembolization (DEB-TACE); or radioembolization. While LRT has proven effective in some patients on eliminating tumors, LRT can also cause decompensation (acute liver failure) and intrahepatic tumor spread, likely from aggressive tumor biology. Lastly, for patients with
advance-stage HCC, palliative systemic treatment remains the only treatment options. The drug, sorafenib, is widely used, but it only increases survival by a few months.\textsuperscript{3}

Thus, the development of alternative, more effective methods of care are necessary to combat HCC and eliminate the negative effects on surrounding non-cancerous tissue. The goal of this research project is to develop a non-invasive therapeutic treatment with diminished side effects impacting the patient.

Human serum albumin nanoparticles (HSAPs) are prepared using the most abundant protein in the blood, human serum albumin (HSA). The albumin is used as a carrier protein to assist in moving molecules into cells. The nanoparticles are synthesized to form globular structures, which may be internally bound with chemotherapeutic drugs and/or gold nanorods (AuNRs) during particle synthesis. The nanocomposites can be injected directly into the tumor site. Enzymatic degradation of the HSAPs allow for the release of encapsulated drugs to attack the tumor. HSAP could also be combined with infrared laser irradiation that heats the AuNRs, thereby inducing apoptosis in cells through hyperthermal effects.

The combinatorial use of the gold-loaded particles (AuNR-HSAPs), laser irradiation, and dosage of the cancer-combative drug provides a trifocal approach to treat HCC. This approach could be performed laparoscopically with direct injection of particles into tumors through probes or catheters, eliminating the need for an invasive surgery. The HSAPs are naturally biocompatible and unlikely to be rejected by the body. The toxicity associated with gold of the AuNRs is low.\textsuperscript{12} The photothermal irradiation from the laser is near-infrared (NIR) radiation readily absorbed by the AuNRs and converted into heat for a selective thermal ablation.\textsuperscript{8} Mammalian tissue allows for
this wavelength of light (808nm) to deeply penetrate it rather than absorbing it.\textsuperscript{10} The targeted treatment of the AuNR-HSAPs combined with the irradiation induces stress to the cells, which allows for the usage of a lesser effective systemic drug dosage. A lowered dosage would reduce unwanted side-effects and improve safety. Furthermore, when a locoregional drug is encapsulated inside of the nanoparticles, the drugs are only released upon particle degradation, typically after cellular ingestion. This allows for the delivery of a higher dosage of drugs to the targeted site with less exposure to non-targeted areas. Remaining particles not ingested could circulate through the bloodstream harmlessly until they are removed by metabolic processes.

Over the years, the development of functionalized nanoparticles as a selective therapeutic for treating cancer has been explored. The term nanoparticle is versatile and can refer to any appropriately-sized particle prepared from polymers, inorganic compounds, protein scaffolding, etc. with each type having different advantages and disadvantages. Scientists hoped to exploit exclusive properties of these particles such as their small size, tunable surface chemistry, drug encapsulation abilities, longer duration of circulation as drug-carriers, ease of cellular membrane penetration, and site-specific targeting.\textsuperscript{24} With their dedication, many projects are in progress, while some have reached the stage of clinical trials on various types of cancers including, prostate, breast, lung cancer, and more. The most common trials of nanoparticles used for cancer therapeutics involve encapsulating or conjugating drugs to the surface of the particles. Some trials are testing nanoparticles with ligand functionalized surfaces, which serve to direct the particles to specific cells via receptors. Other trials involve the
exploration of pharmaceuticals previously thought unusable due to being hindered by pharmacokinetic or biocompatibility issues.\textsuperscript{16}

For these nanoparticles encapsulating gold with an albumin scaffold, previous research has established successful synthesis protocols\textsuperscript{19} and their effectiveness in treating breast cancer cell lines\textsuperscript{20} and murine models of renal cell carcinoma\textsuperscript{13}. Research using gold-conjugated albumin particles similar to AuNR-HSAPs have also been conducted on two immortalized HCC cell lines, HepG2 and HepB5. The study showed a measure of success in eliminating cells using the method of enhanced laser thermal ablation, but concluded that further research was necessary.\textsuperscript{14} The same team also tested the gold-conjugated particles paired with the photothermal ablation on \textit{ex vivo}-perfused liver specimens obtained from HCC patients. They were able to show a considerable amount of necrosis in the tissue, which was indicative of treatment success.\textsuperscript{15}

In our own study, we tested the effectiveness of HSAP, AuNRs-HSAPs, and a combination of near-infrared irradiation, chemotherapeutic drug, and AuNR-HSAPs on the \textit{in vitro} treatment of HCC. The patient-derived line would better reflect genomic modifications within patients with HCC compared to immortalized cell lines.
Materials and Methods

Materials

The Human Serum Albumin and 8% aqueous glutaraldehyde were purchased from Sigma Aldrich (St. Louis, MO, USA). The ethanol (200-proof, ACS/USP grade) was obtained from Pharmco-AAPER (Brookfield, CT, USA). All water was distilled, deionized, and nano-purified with the Barnstead Nanopure filtration system from ThermoFischer Scientific Inc. (Marietta, OH, USA). All reagents used were sterile or were sterilized by filtration using a 0.2µm PES filter from EMD Millipore (Burlington, MA, USA). Alexa Fluor™ 647 NHS Ester (Succinimidyl Ester) was purchased from ThermoFisher Scientific Inc. (Marietta, OH, USA).

The unfunctionalized bare gold nanorods were purchased from Nanopartz, Inc. (Loveland, CO, USA). The rods were colloid-shaped, 10nm diameter, 40nm length, surface plasmon resonance (SPR) peak of 808nm, SPR absorbance OD = 1, $6.3 \times 10^{11}$ AuNR/mL in water containing cetyltrimethylammonium bromide (CTAB). CTAB is an antiseptic capping agent toxic to cells and highly sensitive to ethanol, which causes particle aggregation and malformation during synthesis. It appears as silver swirls in the solution at low temperatures in excess, which must be removed. To prepare the AuNR solution for use, the solution was warmed briefly in a water bath to dissolve the CTAB into solution. Once the silver precipitate was no longer visible, it was centrifuged at 15,000RPM for 5 minutes. The supernatant was removed and saved. The pellet was redispersed in nanopure water (H$_2$O-NP). The supernatant was centrifuged to capture remaining AuNRs, and the supernatant was saved once again. Pellets were consolidated. The process of centrifuging supernatants and collecting pellets was
repeated approximately 5 times until a pellet no longer formed. The total volume remained consistent with the original starting volume to avoid diluting the AuNR solution.

Human hepatic adenocarcinoma immortalized cell line SKHep1 and human HCC immortalized cell line Hep3B were acquired from ATCC (Manassas, VA, USA). Patient-derived HCC cell line TS-50.1.1 was obtained from Ochsner Medical Center (Jefferson, LA, USA). Dulbecco Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), QPS, Bovine Serum Albumin (BSA), B27 supplement 50x, Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF), insulin, trypsin-EDTA (0.25%), Phosphate Buffered Saline (PBS) 1x pH7.4 were purchased from ThermoFisher Scientific Inc. (Marietta, OH, USA). PolyHEMA was acquired from Sigma Aldrich (St. Louis, MO, USA).

Complete media (c-DMEM) for the two immortalized hepatic cell lines was made by adding 10% FBS, 1% QPS, and 89% DMEM. The preparation for the tumor-spheroid media (TS-media) for the TS-50.1.1 cell line was adapted from Song et al. protocol. Approximately 10mL of TS-media was prepared fresh at the time of each use and leftover media was discarded. The following components were added: 93.17% of c-DMEM, 2% of BSA at 0.4mg/mL, 4% of B27 diluted to 2x, 0.1% of FGF diluted to 20ng/mL, 0.1% of EGF diluted to 20ng/mL, and 0.63% of insulin diluted to 25µg/mL.

Doxorubicin hydrochloride 98-102% (HPLC) was obtained from Sigma Aldrich (St. Louis, MO, USA). CellTiter 96® Non-Radioactive Cell Proliferation (MTT) Assay was obtained from Promega (Fitchburg, WI, USA). eBioscience™ Fixable Viability Dye eFluor™ 780 was purchased from Life Technologies (Carlsbad, CA, USA).
Synthesis of blank HSAPs, AF647-HSAPs, AuNR-HSAPs

The HSAPs were synthesized via a modified desolvation technique used by Peralta and her team. The overall process is outlined in Figure 1.

Figure 1. HSAP synthesis process.

After dissolving 2mg of solid HSA in 20µL of nanopure water, varying components were added to the mixture to functionalize the particles (Table 1).

<table>
<thead>
<tr>
<th>HSAP Type</th>
<th>H₂O</th>
<th>Additional Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>2mL</td>
<td>N/A</td>
</tr>
<tr>
<td>AF647</td>
<td>1.99mL</td>
<td>10µg AF647 dye dissolved in 10µL of DMSO</td>
</tr>
<tr>
<td>AuNR</td>
<td>N/A</td>
<td>2mL of the pre-prepared AuNR solution</td>
</tr>
</tbody>
</table>

Table 1. Variable components added to produce different HSAPs.

For all types of HSAPs, 5mL of 100% ethanol was slowly added at an approximate rate of 1mL/min., using a variable flow peristaltic pump to denature the HSA protein. Lastly, 4.7µL of 8% aqueous glutaraldehyde was added to crosslink the HSA within the
particles. The solution was allowed to react for at least 18 hours at room temperature in a closed vial to prevent evaporation before undergoing a washing process.

To wash the particles, the solution was centrifuged at 13,000 RPM for 25 minutes. The supernatant was discarded and 1mL of a wash solution containing a 1:3 ratio of water and ethanol (respectively) was added. The pellet was resuspended in solution via ultrasonication. This washing process was repeated twice. The final pellet was redispersed in 1mL of c-DMEM and stored at 4°C until use.

**Microscopy**

Brightfield and fluorescence images were obtained using an Olympus IX71 Microscope (Olympus Corporation, Tokyo, Japan) at 200x or 400x magnification.

**Dynamic light scattering (DLS)**

The diameter of the HSAPs were obtained using a Mobius dynamic light scattering instrument (Wyatt Technology Corporation, Santa Barbara, CA). 10µL of HSAP solution was diluted 1:100 with H₂O-NP before injecting into the instrument.

**Transmission electron microscopy (TEM)**

Images of the HSAPs were obtained by a JEOL transmission electron microscope (FEI, Hillsboro, OR). Samples were prepared on a thin film of gold lattice mesh to facilitate imaging.

**Ultraviolet-visible spectroscopy (UV-Vis)**

Absorbance measurements of the HSAPs were acquired with a Cary 60 absorbance spectrometer (Agilent Technologies, Santa Clara, CA).
**Culturing cell lines: SkHep1, Hep3B, TS-50.1.1**

The SkHep1 and Hep3B cell lines were cultured in c-DMEM at 37°C humidified 5% CO₂ atmosphere. Cells were cultured in T-75 tissue culture flasks and kept in a logarithmic growth pattern by routine passages, splitting upon reaching confluency.

The TS-50.1.1 cell line was derived from a primary tumor of a patient that underwent a resection at Ochsner Health System. The tumor was digested and grown in c-DMEM. Once a cell line was established, cells were cultured in TS-media at 37°C humidified 5% CO₂ atmosphere adapting the protocol developed by Song et al.23 A 24-well tissue culture plate was treated with 100µL/well of polyHEMA and left to dry overnight in sterile conditions. Cells were plated in the polyHEMA-treated non-adherent plate. The media was refreshed after 1 week, and cells were strained though 30µm strainers to enrich the population for tumor spheroid forming cells. Strained cells were passed to a new non-adherent plate. After another week, spheroids were strained again and transferred to grow in a T-25 tissue culture flask in c-DMEM. Once reaching confluency, cells were passed into a T-75 tissue culture flask and kept in a logarithmic growth pattern by routine passages, splitting upon reaching confluency.

**General experimental procedures**

The experiments of this study followed a general protocol with some experiments requiring additional steps (Figure 2). The general protocol can be summarized as a 4-day plan. Each experiment was repeated in triplicate or more with the exception of experiments involving the TS-50.1.1 cell line.
Figure 2. General protocol of experiments.

On day 1, cells are seeded into either 24- or 96-well tissue culture plates according to Table 2. On day 2, media was removed and replaced with either complete media or experiment specific HSAP. For TS-50.1.1, similar confluency was not obtained until 4 days later, delaying the process. For day 3, media was removed, cells were washed with 1x PBS, and fresh media was replaced. For some experiments, cells were treated with laser irradiation treatment and/or doxorubicin drug. Day 4 was the endpoint in which an MTT assay or flow cytometry was performed. All experiments throughout were performed in triplicate with exception to experiments involving TS 50.1.1 cell line.

| 96-Well Tissue Culture Plate (0.32cm²/well) |
|------------------|-----------------|
| Cell Line       | Seeding Density Cells/Well |
| SkHep1          | 8,500            |
| Hep3B           | 5,000            |
| TS-50           | N/A              |
Near-infrared (NIR) photothermal laser irradiation

Cells with and without AuNR-HSAPs were treated with an NIR photothermal irradiation by laser to induce and enhance cytotoxicity. Modelled after protocols used by Liu et al.\textsuperscript{13}, the treatment was administered by a LabSpec 808nm collimated diode laser system (Laserglow Technology, Toronto, Canada). The power was set to approximately 2.1W. The laser was vertically positioned 7cm above the cells. The size of the laser beam was approximately 0.5cm × 1cm.

Laser treatment occurred on day 3 of experimental protocols. Briefly, after cells were washed with 1x PBS and 200µL of c-DMEM was added. Cells were exposed to the thermal laser for differing amounts of time with exposure covering the entire wells in 3 iterative sessions. Upon thermal irradiation completion, media was added to wells for a final volume of 1mL.

Inhibitory concentration of doxorubicin

The SkHep1 and Hep3B cell lines were tested to determine the half maximal inhibitory concentration (IC\textsubscript{50}) of the doxorubicin drug. General experimental protocol described above was used. The range of drug dosages differed for each line and well size (Table 3). Cell metabolic activity and viability was determined by MTT assay and flow cytometry, respectively.

### Table 2. Seeding densities according to cell line and well size.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Seeding Density Cells/Well</th>
</tr>
</thead>
<tbody>
<tr>
<td>SkHep1</td>
<td>50,000</td>
</tr>
<tr>
<td>Hep3B</td>
<td>30,000</td>
</tr>
<tr>
<td>TS-50</td>
<td>30,000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>24-Well Tissue Culture Plate (1.9cm\textsuperscript{2}/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell Line</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>SkHep1</td>
</tr>
<tr>
<td>Hep3B</td>
</tr>
<tr>
<td>TS-50</td>
</tr>
</tbody>
</table>
Combinatorial treatment

The combinatorial experiments were performed on all cell lines to determine the synergistic effects of the treatments. The following conditions were tested on cells in each experiment: zero control; heat-kill control; AuNR-HSAP addition only; doxorubicin addition only; laser treatment only; AuNR-HSAP and doxorubicin additions; AuNR-HSAP addition with laser treatment; doxorubicin addition with laser treatment; and AuNR-HSAP and doxorubicin additions with laser treatment. Each experiment was repeated in triplicate with the exception of experiments involving the TS-50.1.1 cell line.

The experimental protocol described above was used with doxorubicin concentration of 0.025µM for SkHep1, 0.05µM for Hep3B, and 0.25µM for TS-50.1.1.

Flow cytometry

Cellular viability and cytotoxicity were measured using a Attune NXT Flow Cytometer (Thermofisher, Carlsbad, CA) and staining cells using the fixable viability eFluor™ 780 dye according to manufacture protocol.
Cell viability was determined by selecting the number of cells based on a size-gating strategy and normalizing to the control for each experiment.

**MTT assay**

The metabolic activity of the cells was measured using an MTT assay. MTT assay was performed according to manufacturer protocol. Dye solution was allowed to incubate for 1 hour in SkHep1 and 1 hour 20 minutes in Hep3B. Absorbance of the formazan product was detected at 570nm and 650nm and recorded via xMark Microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, CA). The readings at 650nm were subtracted from the readings at 570nm to remove background interference. Metabolic activity was normalized against the control.

**Statistical analysis**

An ANOVA Test (statistical mean analysis) and a Dunnet's Multiple Comparisons Test (statistical comparison against control) were performed on the data to produce the graphs. Results were expressed as mean and its SEM (standard error of mean) relative to the control. A p < 0.05 was indicative of a one-asterisk difference of statistical significance when compared to the control. A p < 0.01 signified a difference of two or more asterisks. Graphs were composed with GraphPad Prism 8 (GraphPad Software, La Jolla, CA).
**Results and Discussion**

**Blank HSAP, AF647-HSAP, AuNR-HSAP**

Each type of nanoparticles synthesized were distinguishable by their appearance, size, and certain properties. Empty, unloaded, blank HSAPs were ivory-colored, while AF647-HSAPs were blue-green-colored particles, and AuNR-HSAPs were pink in color.

The sizes of HSAPs were determined using DLS and outlined in **Table 4**. Blank HSAPs measured an average 93.6nm in radius, AF647-HSAP radius measured 47.0nm, and the AuNR-HSAP radius averaged 49.7nm. The sizes of the HSAPs were affected by the addition of compounds to be encapsulated.

<table>
<thead>
<tr>
<th>HSAP Type</th>
<th>Radius (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>93.6</td>
</tr>
<tr>
<td>AF647</td>
<td>47.0</td>
</tr>
<tr>
<td>AuNR</td>
<td>49.7</td>
</tr>
</tbody>
</table>

**Table 4.** Comparison of HSAP sizes by DLS.

A hypothesis for the decrease in size after the addition of the AF647 fluorescent dye compound is that the compound is interfering with the crosslinking of particles during synthesis. Glutaraldehyde uses amine groups to crosslink HSA proteins into larger nanocomposites. However, the Alexa Fluor 647 is also an amine-reactive compound. The reaction of the dye may be leaving less free amines for the glutaraldehyde to use for binding, which could explain the decrease in particle size compared to blank HSAP. Additionally, because the final particle size is dependent on numerous factors including the capping agent, trace amounts of the CTAB remaining in...
the AuNR solution could affect the size of the AuNR-HSAPs. CTAB is also a cationic detergent which could be causing crosslinking interference as well.

The findings on the size of the particles is an important factor to consider for several reasons. One reason is the size difference would affect housing capacities of certain compounds, such as a chemotherapeutic drug, in which dosage and its limitations would be important. Another reason lies in the delivery method of the HSAPs. Tumors can retain nanoparticles released arterially in fenestrations between endothelial cells of blood vessels through a response called enhanced permeation and retention (EPR). This allows long-circulating particles to accumulate in tumor tissues. If the HSAPs are sent intravenously, the size of the particles necessary to be long-circulating and reach the targeted site must be a diameter between 30nm and 200nm.

The AuNR-HSAPs were imaged by TEM to confirm successful encapsulation of the gold nanorods (Figure 3).

Figure 3. TEM image of AuNR-HSAP.
Further evidence to support the successful encapsulation of the AuNRs in the nanoparticles was collected via UV-Vis spectrometer. In Figure 4, a comparison of the absorbance measurements between the free AuNR solution, blank particles, and AuNR particles is shown. A small peak was observed near 500nm wavelength for the free AuNR solution. This peak is not present in the line representing the blank HSAPs. However, although the slope of the AuNR-HSAP line is similar to the blank HSAP line, the AuNR-HSAP line shows a small peak corresponding to that observed in the free AuNR solution peak. This indicates the presence of gold nanorods in the particles of the AuNR-HSAPs.

![Graph](image)

**Figure 4.** Absorbance graph of blank HSAP, AuNR-HSAP, and AuNR solution.

**Effects of HSAPs on cellular metabolic activity and viability**

The metabolic activity and cellular viability of the cells after the addition of the different types of HSAP were determined via MTT assay and flow cytometer, respectively. These were important steps to establish the baseline cytotoxicity of the HSAPs in HCC cell lines.
In the graphs of Figure 5, the effects of the addition of blank HSAPs on SkHep1 and Hep3B cell lines are shown. Each bar represents wells with a percentage of its total media volume replaced by the particle-containing solution. The range of particle amounts added to a well included 25, 50, and 100%. The maximum volume, 100%, meant the complete replacement of media with HSAP solution. Most of the data showed no difference or only a slight decrease in both metabolic activity and cellular viability when compared to the control – no statistical difference from the control (NS – no significance). Although, with the maximum addition of particles to the Hep3B cell line, the graph indicated a degree of statistically significant difference to the control for its viability. However, over 85% of cells were still viable at this most cytotoxic point.
Figure 5. Metabolic activity and cellular viability of SkHep1 and Hep3B with the addition of blank HSAP.

The effects of the dye-loaded AF647-HSAPs on the cell viability of SkHep1 and Hep3B cell lines were also explored. When the experiments were performed in tandem with blank HSAPs, the observed effects of the AF647-HSAPs were nearly identical to those of the blank HSAPs (Figure 6) for both cell lines. Neither had a negative effect on the cell viability. Blank HSAP or dye-loaded HSAP do not cause any cytotoxic effects to the cells.
Lastly, the effects on metabolic activity and cytotoxicity of SkHep1 and Hep3B cell lines with the addition of gold-loaded AuNR-HSAPs were determined. For cytotoxicity, the AuNR-HSAPs appeared to have a similar statistically insignificant effect as the blank HSAPs with less than 15% cellular death observed with the maximum amount of particle loading (Figures 17 and 18). However, for the metabolic activity, a significant toll was observed (Figure 7). With 25% of the media in the well replaced with the AuNR particle solution (25% of the maximum loading amount), the metabolic activity dropped to approximately 75% of the control for both cell lines. As the AuNR-HSAP loading amount increased, cellular activity decreased as well. At the maximum amount of particle addition to the wells, the metabolic activity decreased to hover approximately between 55% and 65% for both cell lines. Every datapoint collected in these experiments showed there were statistically significant differences between them and the control. A cause for the decrease in metabolic activity with addition of the AuNR-HSAPs which is unobserved in the blank HSAPs could be due to the presence of the CTAB capping agent. It is an antiseptic reagent and trace amounts could affect the
cells. However, it was determined the addition of the AuNR-HSAPs had negatively impacted the metabolic activity of the cell without causing a significant cytotoxic effect.

Figure 7. Metabolic activity of SkHep1 and Hep3B with AuNR-HSAPs.

In summation, all types of the nanocomposites by themselves were found to cause a statistically insignificant amount of cellular death in both SkHep1 and Hep3B cell lines. Although the AuNR-HSAPs do appear to induce enough stress to the cells to negatively affect their metabolism, the cytotoxicity was still less than 15% of the control. Thus, moving forward, the maximum amount of particles was used in each experiment for utmost efficacy.

Cellular uptake or adherence of AF647-HSAPs

For the AuNR-HSAPs to function effectively in conjunction with the photothermal effects of the laser irradiation, they must be present in or on the target cells. If the nanoparticles are not ingested nor bound to the cell surface, they may be swept away from the treatment area, missing the target site, but also missing the photothermal effects of the irradiation. Therefore, obtaining a confirmation of the uptake or adherence of the particles to the cells were vital. By adding AF647-HSAPs to the wells, the cells
were essentially labelled with a bright far-red fluorescent dye if the particles were taken up into the cells or adhered onto cell surfaces. Then, the fluorescence of the cells was measured by flow cytometry using a channel suited for detecting the AF647 dye.

Initially, varying amounts of dye-loaded AF647-HSAPs were added to the wells to determine if a saturation point of the particles taken up into the cell or bound to the cell surface was reached before the maximum amount of particles were added. The range of particle amounts added to a well included 25, 50, and 100%. Again, the maximum volume, 100%, meant the complete replacement of media with HSAP solution. In the graph of Figure 8a, the fluorescence intensity (MFI units) of the cells is depicted, with each bar shown as a fold over the control (0%, no particle addition). With 25% of the media in the well replaced with the AF647-HSAP solution, the fluorescence readings saw a one-thousand-fold increase compared to the control. With the 50% amount particle addition, the fluorescence readings had a two-thousand-fold increase from the control. The maximum amount of particle addition produced a four-thousand-fold increase compared to the control. From the linear progression of fluorescence measurements, it was concluded the increase in added particle amounts cause the SkHep1 cells to uptake or bind to its cell surface more particles. It can also be concluded the saturation point for the uptake or binding of particles was not reached by the SkHep1 cells before the maximum amount of HSAPs were added.

In the graph of Figure 8b, the fluorescence intensity of SkHep1 cells with no particle addition (0%, control), blank HSAP addition (100%, maximum amount of particle loading), and AF647-HSAP particle addition (100%) were compared. Without the addition of any type of HSAPs, the graph of the cells show a peak at low fluorescence.
It also sets a baseline for the other datasets as the control group. The cells with the addition of the blank HSAPs show a peak shifted to the right from the control with approximately a one-fold logarithmic increase in fluorescence measurements. The graph of the cells with the dye-loaded AF647-HSAPs show a peak at higher fluorescence shifted even farther to the right with approximately a two-fold logarithmic increase in fluorescence reading compared to the control. Interpreting the data of the graph, it was determined that the particles were present in or on the SkHep1 cells due to the increased fluorescence measurements caused by the dye in the AF647-HSAP. These results do not confirm uptake of particles by the cells (or lack thereof). However, they do suggest the likelihood of uptake or surface binding of HSAPs.

![Graph showing fluorescence intensity of SkHep1 with various amounts of AF647-HSAP added.](image)

**Figure 8a.** (Left) Fluorescence intensity of SkHep1 with various amounts of AF647-HSAP added.

**Figure 8b.** (Right) Fluorescence intensity of SkHep1 with no particles, blank particles, and AF647 particles added.

A point to note in the data represented in **Figure 8b** was the peak shift observed for the cells with the addition of the blank HSAPs. There was an increase in
fluorescence readings although the blank nanoparticles contain no dyes to produce any fluorescence. This is likely caused by interference from background fluorescence. It was determined the particles themselves produce a bright green fluorescence similar to fluorescein (FITC), which has an absorption maximum at 490nm and emission maximum of 525nm\textsuperscript{25}. This is visualized with a fluorescence microscope at 200x magnification in Figure 9. The particles were dispersed in an acidic buffer, which caused particles aggregation for better visualization. Although the channel of the flow cytometer uses a filter set specific for red fluorescent dyes (AF647), it is possible for the green background fluorescence to spill over into this channel and capture the data.

![Fluorescence microscopy image of blank HSAP dispersed in acidic buffer.](image)

**Figure 9.** Fluorescence microscopy image of blank HSAP dispersed in acidic buffer.

The uptake/adherence experiments were repeated for the Hep3B cell line. As shown in Figure 10a, the addition of increasing amounts of AF647-HSAPs to the wells resulted in a similar linear progression of fluorescence measurements. However, Hep3B cells with the maximum amount of particle addition saw only an approximately five-hundred-fold increase compared to the control, rather than the four-thousand-fold increase the SkHep1 cells showed. In the graph of Figure 10b, similar peak shifts to the previous experiment with SkHep1 indicative of the presence of particles in or on the
Hep3B cells were observed. However, for Hep3B, the shifts were more minor with the AF647-HSAP peak only showing approximately a one-fold logarithmic increase in fluorescence measurements as opposed to the two-fold logarithmic increase seen of the SkHep1.

**Figure 10a.** (Left) Fluorescence intensity of Hep3B with various amounts of AF647-HSAP added.

**Figure 10b.** (Right) Fluorescence intensity of Hep3B with no particles, blank particles, and AF647 particles added.

For the Hep3B cell line, the overall conclusion was the same as the SkHep1 – particle uptake/adherence has a positive relationship with the amount of HSAPs added, and the particle uptake/adherence does not reach a point of saturation before the maximum amount of HSAPs were added. However, it was also determined that the cells of the Hep3B cell line had much less interaction (whether by ingestion or adherence) than the SkHep1 cells. This conclusion is depicted clearly in **Figure 11** showing the difference in MFI units detected during the experiments. The combinatorial treatment is likely to be more effective with a greater amount of AuNR-HSAP uptake or
adherence to absorb the photothermal irradiation. Therefore, moving forward, the maximum amount of particles was used in all experiments.

**Figure 11.** Comparison of the fluorescence intensity of SkHep1 and Hep3B with various amounts AF647-HSAP added.

For the patient-derived TS-50.1.1 cell line, the experiment was repeated, but only testing the maximum amount of particle addition as decided after the uptake/adherence experiments on the two immortalized cell lines. In **Figure 12**, the graph shows a slight shift in the peak of the cells with the AF647-HSAPs added compared to the control peak. However, the shift in fluorescence measurements is even more minor than the shift seen in the Hep3B line, as this peak shows an approximately half-fold increase rather than a one- or two-fold increase.
Figure 12. Fluorescence intensity of TS-50.1.1 with no particles, blank particles, and AF647 particles added.

In the graph of Figure 13, the fluorescence intensity data of SkHep1, Hep3B, and TS-50.1.1 with maximal AF647-HSAP addition are compared to each other in MFI units. While the SkHep1 hovers approximately at 4,500 MFI units, Hep3B is at around 600 MFI, and TS-50.1.1 is at about 200 MFI. Although much less than the immortalized lines, the TS-50.1.1 line does show indication of the presence of particles in or on the cells. It can be concluded the patient-derived line has even less uptake/adherence of the HSAPs than the other lines. This difference in the interaction between HSAPs and cell lines may be due to the difference in the characteristics between the cell lines.
The three cell lines used for this study are all hepatic lines. However, they display differences in cell morphology and functionality. In terms of morphology, the differences can be observed through a microscope. The images of the cells were visualized with a brightfield microscope at 400x magnification. The SkHep1 cells typically have an elongated shape and may also have semi-long slender tendrils extending from the cell body (Figure 14a). The Hep3B is rounder and more circular in shape with short tendrils that may extend from the body (Figure 14b). The TS-50.1.1 patient-derived cells have two types of cells: one type with a very stretched and elongated shape with long tendrils extending from the body and another cell type (tumor spheroid) unadhered to the flask surface and circular in shape (Figure 14c). The difference in morphologies of the cell lines may affect the available surface area of the cells, which could potentially account for differences in particle adherence, if any.
Figure 14a. (Left) Microscopy image of SkHep1.

Figure 14b. (Center) Microscopy image of Hep3B.

Figure 14c. (Right) Microscopy image of TS-50.1.1.

The cell lines differ in terms of characteristics and functionality as well. SkHep1 is a hepatic adenocarcinoma line. Although it was obtained from the fluid of a patient liver and considered a human hepatoma cell line, it does not have the typical properties of a hepatocyte, it does not express liver genes, nor does it exhibit expected liver functions. One major point to note is SkHep1 does not produce albumin\(^7,9\).

Alternatively, the Hep3B cell line is an HCC line which does produce albumin as expected of a hepatocyte\(^22\). Little information is known about the TS-50.1.1 HCC cell line as it is a new line expanded recently. The results of the experiment in terms of particle uptake for SkHep1 and Hep3B align with the information on albumin production (unknown for TS-50.1.1). If Hep3B cells produce albumin and SkHep1 cells do not, then having less uptake of particle composed of albumin by Hep3B due to inhibitory effects is logically reasonable.

As mentioned, while the Hep3B line and the TS-50.1.1 line are HCC cell lines, the SkHep1 is an adenocarcinoma cell line. There are differences in the doubling times
and growths between the lines. While SkHep1 cells grow rapidly and double within 30 hours\textsuperscript{6}, the Hep3B cells have a longer doubling time between 40-50 hours\textsuperscript{5}. The TS-50.1.1 line has an even longer doubling period than the immortalized cell lines that may exceed a week. For SkHep1, cells would require a more rapid ingestion rate of metabolites from the surrounding media to facilitate aggressive growth. The rates of ingestion for Hep3B would be lower due to its longer doubling time, and even more so for TS-50.1.1. This could account for differences in HSAP uptake by ingestion. The higher levels of fluorescence possibly indicating increased particle uptake for SkHep1 support this hypothesis. The lower fluorescence readings indicating less HSAP presence in Hep3B and TS-50.1.1 align with this hypothesis as well.

**Effects of photothermal laser irradiation treatment on cells**

The metabolic activity and cellular viability of the cells after different durations of photothermal laser irradiation were determined by MTT assay and cytometry. These experiments were conducted on cells without any particle addition. The results of these experiments were essential steps to determine an appropriate treatment time with maximum duration while still causing little cytotoxic effect. The irradiation should not be harmful to the cells by themselves without the combination of the AuNR-HSAP addition.

In Figure 15, the photothermal effects on both SkHep1 and Hep3B cells for 2.5, 5, 10, and 20 minutes are graphed as comparisons to the control (no irradiation). For SkHep1, the data showed no significant difference from the control in metabolic activity with no more than a 20% decrease in activity for any treatment duration. Therefore, the data of the cell viability should reflect similar results without significant difference. However, a degree of statistical difference was detected for the 20-minute treatment
period, the maximum irradiation duration. Logically, the cytotoxicity should gradually increase with the increase in treatment duration. However, a fluctuation is observed for the viability graph, which could be due to experimental error. The incongruity between the 20-minute data of the metabolic activity and viability could also be due to experimental error. Regardless, the cytotoxicity for SkHep1 is still relatively low at 25% with the maximum treatment duration. For the Hep3B cells, the data showed significant degrees of statistical difference for the photothermal treatment’s effects on metabolic activity. The activity decreased to approximately 70% for the 2.5-minute and 10-minute treatment periods, then to 60% for the maximum duration. Although even the minimum treatment duration had negatively impacted the metabolic activity of the cell, the cell viability remained hardly affected. With the cytotoxicity at a very low 5%, even at the maximum 20-minute irradiation time, there was no significant difference of cellular viability from the control. For Hep3B, it was determined the photothermal laser treatment induced sufficient stress to the cells to inhibit metabolism without causing significant cellular death. Moving forward, cells were exposed to the maximum treatment duration of 20 minutes for each experiment as it had relatively low levels of cytotoxicity for both cell lines.
Figure 15. Metabolic activity and cellular viability of SkHep1 and Hep3B with the laser irradiation treatment.

Inhibitory concentration of doxorubicin

The inhibitory concentration (IC\textsubscript{50}) of doxorubicin was explored for both SKHep1 and Hep3B cell lines by determining cellular viability using flow cytometry. A range of dosages up to 1\textmu M was tested on cells without any particle addition. The results of these IC\textsubscript{50} experiments were used to determine an appropriate drug dosage to cause minimal cytotoxic effects by themselves, as the minimal dosage is safer for the patient recipient of the treatment.
Graphs of cellular viability of SkHep1 and Hep3B cell lines with the doxorubicin dosages compared to the control (no drug addition) are shown in Figure 16. The cytotoxicity was not statistically significant initially. Then, it increased to show a difference as the dosages increased for both lines. However, the point in which a significant difference from the control was observed was different for the two cell lines indicating approximately 45% cellular death at 0.05µM in SkHep1 and 0.1µM in Hep3B. From that point until 1µM, the cytotoxicity increased to reach approximately 75% for both cell lines. Moving forward, cells were exposed to the maximum dosage of doxorubicin that causes no significant cytotoxicity when compared to the control. These were determined to be 0.025µM in SkHep1 and 0.05µM in Hep3B cells.

**Figure 16.** Cellular viability of SkHep1 and Hep3B with doxorubicin.

**Combinatorial treatment**

Finally, the combinatorial treatment experiments were performed on all cell lines to determine the effectiveness of the concerted treatments, and the results were measured in terms of cell viability with the flow cytometer.

In Figure 17, the results of the experiment for the SkHep1 cell line are graphed. Unfortunately, there was no difference statistically significant from the control (AuNR-
HSAP addition only, listed as “Au only” in bar graph) for any of the conditions tested. There was little cytotoxicity observed for wells with only the individual addition of the AuNR-HSAP, doxorubicin (“Dox only” in bar graph), and laser irradiation treatment (“Laser” in bar graph). Those results, with individual treatment methods were expected, as conditions optimized for efficacy without causing significant cytotoxicity were selected. A 15% decrease in cellular viability from the control was observed testing the combination of the doxorubicin and the laser irradiation (“Dox-laser” in bar graph). The 11% of cellular death shown in the drug-only mostly accounts for the death seen in the combination of the two conditions. These two methods operate independently of each other and a combination treatment was not expected to produce enhanced results. Similarly, for the combination testing of AuNR-HSAPs and doxorubicin (“Au-dox” in bar graph), 19% cellular death was observed. The 9% death seen in the AuNR-HSAP only condition and the 11% death seen in the doxorubicin only condition mathematically accounts for the approximately 20% cellular death observed in the combination. Again, these methods operate independently of one another and the results were expected. However, for the combination of the AuNR-HSAPs and the laser treatment (“Au-laser” in bar graph), anticipated results included an exponential increase in cellular death because the two methods combined should have operated synergistically to induce greater effects. Instead, the observed result was a lackluster cell death of only 13%. For the combination of all three conditions (“Au-dox-laser” in bar graph), our hypothesis was that the combination of metabolic stress attributed by the AuNR-HSAPs, combined with the cytotoxic effects of doxorubicin and heating of the AuNR-HSAPs would be large enough to cause massive cellular death. However, with the AuNR-HSAP and laser
combination condition was unsuccessful in causing a significant level of cytotoxicity as a loss in viability was only at 19%, similar to the combination of the AuNR-HSAP and doxorubicin.

![Comparison of cellular viability of SkHep1 with controls and combinatorial treatments.](image)

**Figure 17.** Comparison of cellular viability of SkHep1 with controls and combinatorial treatments.

The data from the same combinatorial experiment performed on the Hep3B cell line was graphed (**Figure 18**). As with the previous experiment, there was no statistical difference from the control in the testing of any condition. In the combination of the doxorubicin and laser treatment tested, a 29% cellular death was observed. However, much of the damage is attributed to doxorubicin (testing the doxorubicin by itself caused 18% cell death), but the laser does appear to add to the stress on the cell to enhance cytotoxicity (testing the irradiation treatment by itself caused no cell death). The results of the combination of the aforementioned AuNR-HSAP with laser treatment was similar to those obtained for SkHep1; it was not as effective as anticipated with only 9% cellular death. The cytotoxicity of the AuNR-HSAP/irradiation combination was mostly attributed to the addition of the AuNR-HSAPs (testing the particles by themselves...
yielded 5% cell death), and it was enhanced only by 4% from the combined photothermal treatment. This likely reduced the synergistic effectiveness of the final three-pronged combinatorial test, which showed a 36% cellular death.

![Graph showing cellular viability](image)

**Figure 18.** Comparison of cellular viability of Hep3B with controls and combinatorial treatments.

The combinatorial experiment was performed on a patient-derived cell line, TS-50.1.1 (**Figure 19**). However, this experiment could not be performed in triplicate as the growth rate of these cells were slow and a sufficient number of cells for the experiments could not be cultured in time. Without repetition, the results of the experiment could neither be averaged nor checked for consistency. Consequently, the results must be treated as preliminary.

Preliminary findings show no statistical difference from the control for any of the conditions tested. The TS-50.1.1 cell line may show a greater cytotoxicity reaction (20% cell death) to the addition of AuNR-HSAPs than the immortalized cell lines. However, that level of cytotoxic effect is not observed in any of the other tests involving the addition of AuNR-HSAPs. In fact, the data was found to be conflicting with the
AuNR-HSAP/doxorubicin showing 0% cell death, AuNR-HSAP/laser treatment showing 7% death, and the AuNR-HSAP/doxorubicin/laser treatment showing only 3% cellular death. The 20% cell death found in the testing of the AuNR-HSAP addition by itself could be attributed to an experimental error or anomaly. Repeat experiments must be conducted to confirm. Again, the results of the combination of the AuNR-HSAP addition, with the photothermal treatment to the cells exhibited no synergistic effects as anticipated. The final combinatorial test involving all three variables showed an even less efficient 3% cellular death. Overall, the data from the single TS-50.1.1 combinatorial experiment was conflicting, inconsistent, and likely unreliable. The experiment must be repeated.

**Figure 19.** Comparison of cellular viability of TS-50.1.1 with controls and combinatorial treatments.
Conclusion

After repeating the experiments of this study to produce consistent results in triplicate, the inefficacy of the combination of the AuNR-HSAPs treated with the photothermal laser irradiation must be addressed. According to the research of Liu et. al\textsuperscript{13}, renal carcinoma epithelial cells did show indications of uptake and the AuNR-HSAPs combined with the photothermal treatment were effective in treating a tumor of the kidney. From the study outlined in this thesis, the results from the MTT assay and flow cytometry suggest a difference in the level of interaction with the particles, whether by surface binding or uptake, for each cell line. An overall low adherence or uptake of the HSAPs for the hepatocytes could be possible, and it may be the issue causing the inefficacy. This hypothesis could be explored with the expansion to different types of cells. It could be that this alternative treatment using HSAPs is more effective for certain organs than others and will be used for those specific cancers rather than a broad overall treatment encompassing all cancers. However, another direction of the research could move towards using specific antibodies affixed to the particles to bind to cell surface receptors for targeted binding specific to hepatocytes. Many pathways are available for exploration.

If the limitation of the AuNR-HSAPs combined with the photothermal treatment could be overcome, the next steps for this study would be to load the particles with both AuNR and the doxorubicin drug. The experiments should be tested \textit{in vitro} once again, but then eventually moving on towards \textit{in vivo} models.
References


