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In Vitro Studies of Gold and Gold Silica Nanoparticle Radiosensitization with Kilovoltage X-Rays

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IN VITRO STUDIES OF GOLD AND GOLD SILICA NANOPARTICLE
RADIOSENSITIZATION WITH KILOVOLTAGE X-RAYS

by

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A thesis submitted in partial fulfillment of
the requirements for the

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**Master of Science - Health Physics**
Department of Health Physics and Diagnostic Sciences

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ABSTRACT

Technological advances in the ability to construct and manipulate nanoscale particles have opened up the possibility of using solid metallic nanoparticles and mixed metal nanoshells as a means to increase dose enhancement and treatment efficacy to tumors. In order for nanoparticles to be an effective form of treatment, they must be delivered to tumors in sufficient concentrations so that there is a dose enhancement factor due to ionizing radiation, as well as being essentially non-toxic to healthy cells. Gold nanoparticles and silica-gold nanoshells fit these requirements. Gold has a high atomic number (Z=79), which gives a larger cross section for the photoelectric effect vs. tissue with regards to kilovoltage x-rays. Both gold and silica are also relatively inert and biocompatible.

The investigation of dose enhancement to cells that have been incubated with nanoparticles and nanoshells is the focus of this thesis. The effectiveness of the treatment was determined by measuring the size of multicellular hybrid spheroids consisting of human glioma cells and murine lymphocytic monocytes. Dose enhancement effects was also examined in murine lymphocytic monocytes using an MTS assay, which measures metabolic activity in cells.

A clear dose response was observed for spheroids consisting of human glioma cells only: increasing doses resulted in decreased spheroid growth. With a few exceptions, this trend was also observed in hybrid spheroids consisting of glioma cells and nanoparticle or nanoshell loaded monocytes. Contrary to the premise of utilizing the photoelectric effect, the most pronounced dose effect was observed in the pure glioma irradiated spheroids which showed greater growth suppression compared to the
nanoparticle and nanoshell loaded hybrid spheroids at each dose investigated. A similar trend was found when comparing the viability of bare and nanoparticle/nanoshell loaded monocytes exposed to kilovoltage x-rays. These results are considered anomalous since kilovoltage x-rays are expected to be more damaging to cells and spheroids containing nanoparticles/nanoshells due to enhanced photoelectric absorption. The anomalous results were attributed to inaccuracies in x-ray tube output.

Optimization of MTS parameters required for accurate determination of monocyte viability represents the most significant finding of this work. It was found that 50,000 cells per well yielded an accurate MTS signal. Furthermore, the MTS assay should not be performed less than 96 hours from the time of irradiation. As long as this 96 hour criterion is satisfied, any of the investigated MTS incubation times (1 – 4 hours) can be used. Finally, at the concentrations used in these studies, neither nanoparticles nor nanoshells were toxic to murine lymphocytes.
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This thesis is dedicated to the memory of my aunts, Francis and Sylvia, and my uncle John.
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CHAPTER 1: INTRODUCTION

1.1: NANOPARTICLES

Technological advances in the ability to construct and manipulate nanoscale particles has opened up the possibility of using solid metallic nanoparticles and mixed metal nanoshells as a means to enhance treatments to cancerous tissue (malignant tumors). Different modalities of treatment utilizing nanoparticles exist such as radiosensitization, photothermal therapy, photodynamic therapy, and as a delivery mechanism for site-specific compounds. The purpose of radiosensitization is to increase dose deposition in tumors through the use of high atomic number nanoparticles which have an increased probability of interacting with ionizing radiation via the photoelectric effect (Hainfeld et al 2004, Hainfeld et al 2008). Photothermal therapy (PTT) utilizes electromagnetic properties of nanoparticles that can cause them to heat up when exposed to certain frequencies of electromagnetic radiation, causing thermal damage to tumors (Baek et al 2011). The surfaces of nanoparticles can have various compounds attached to them and then be delivered to the tumor. This enables the compounds to infiltrate the tumor where they may interact directly as in the case of chemotherapeutic agents, or be used as a photosensitizer or photosensitizing agent that responds to certain frequencies of electromagnetic radiation depending on the nature of the compound and the type of interaction desired (Brown et al 2004, Trinidad et al 2014). These latter cases are examples of photodynamic therapy (PDT).

The nanoparticles used in the chemical and biological fields are typically concerned with structures that are on the order of less than 500 nm in size. There are solid metallic nanoparticles and mixed metal nanoshells. Metallic nanoparticles can have
differing shapes such as spheres, rods, or cages. The nanosphere is spheroidal in shape, the nanorod is cylindrical in shape with an axis that is longer than the radius, and the nanocage is a lattice type framework. Mixed metal nanoshells are composed of a central spheroidal core surrounded by a layer of material with a different dielectric constant than the core. The central core is typically a dielectric such as silica, e.g. silicon dioxide, (SiO$_2$) surrounded by a metallic shell, e.g. gold (Au) or silver (Ag) (Hirsch et al 2005).

Metallic nanoparticles are typically made of solid gold (Au), silver (Ag), or iron (III) oxide (Fe$_2$O$_3$). The latter is a paramagnetic material and has uses as an advanced contrast agent for MRI among other things and won’t be discussed further here (Babes et al 1999, Morales et al 2005).

The nanoparticles that are of particular interest in this paper are silica-gold nanoshells (NS) and solid gold nanoparticles (NP). When either of these particles is mentioned in a non-specific way they will be called nanoparticles.

1.1.1: Gold Nanoparticles

Gold nanoparticles are nanometer-sized particles that can come in a variety of shapes: spherical, rod, and polygonal among others. They are essentially solid particles of gold, which can be synthesized through a variety of chemical methods. This typically involves the reduction of a gold salt or the seeding of gold ions. The first monodisperse gold nanoparticles were synthesized in 1951 and enhancements and improvements on their manufacture improved over the years (Turkevich et al 1951, Frens et al 1972, Brown et al 1998). The nanoparticles typically exist as a colloid, a suspension of nanoparticles in a medium such as de-ionized water or citrate buffer. In order to reduce aggregation of the nanoparticles in the colloid, they typically have a stabilizing agent that is attached to the surface of the nanoparticle. In some cases the citrate buffer acts as the
stabilizer, in others polyethylene glycol (PEG) is coated on the surface of the nanoparticle to minimize aggregation.

1.1.2: Gold Nanoshells

The construction of mixed metal nanoshells is technologically more challenging than solid nanoparticles, and as such they were not synthesized until the 1990’s, although they were conceived of in 1951 (Aden et al 1951, Welch et al 1995, Averitt et al 1997, Oldenburg et al 1998). Having a gold outer shell gives the nanoshells the same chemical properties as gold nanoparticles, which implies that they should have similar toxicity and conjugation profiles as solid gold nanoparticles.

The first gold nanoshells were made of an Au₂S dielectric core surrounded by a shell of Au and were synthesized in 1994 by mixing two chemical compounds (Zhou, Welch et al 1995). This method of synthesis limits the control over the core to shell ratio as well as the size of the nanoshell. Advancement was made in 1998 through the construction of silica-gold core-shell nanoshells (Oldenburg et al 1998). Silica is the name for the molecule silicon dioxide, SiO₂. To create these nanoshells, a silicate compound is reduced which creates a spherical silica particle. Functionalization of these cores with amine groups allows gold colloid to be adsorbed onto their surface. The thickness of gold deposited on the nanoshell can then be controlled by reducing Au out of chloroauric acid.
The advantage to this method is that the core-shell ratios can be manipulated so that surface plasmon resonance effects can be maximized depending on the desired use. Surface plasmon resonance effects will be discussed in section 1.1.4.

1.1.3: Electromagnetic Interactions: Photoelectric Effect and others

The research that is being investigated in this thesis is to compare the effects of x-ray dose enhancement on the viability of cells that have been incubated with gold nanoparticles vs. silica-gold nanoparticles. In order to optimize photoelectric interactions and minimize Compton scattering, it is desirable that photons have energies less than 500 kVp (Mesbahi 2010). The details of the photoelectric effect and Compton scattering will be discussed below.

The proof of concept for using gold nanoparticles as a means to enhance dose to a tumor was done by injecting mice, which had subcutaneous EMT-6 mammary carcinomas, with 1.9 nm gold particles directly into the tumor site and then treating them with 250 kVp x-rays (Hainfeld et al 2004).

Dose enhancement effects due to materials with a high atomic number (Z) relative to the surrounding tissue, was first discussed concerning the interface between bone and
soft tissue (Spiers 1949). The phenomenon was also observed in patients receiving radiation treatments that had metal wires in their jaws following reconstructive surgery (Castillo et al 1988). Further historical investigation into the dose enhancing effects of high Z materials and ionizing radiation can be found in the literature (Matsudaira et al 1980, Santos Mello et al 1983, Nath et al 1990, Regulla et al 1998, Herold et al 2000).

There are five interactions that can occur which will attenuate the x-rays as they interact with the nanoparticles. The attenuation can be due to either scattering or absorption and the type of interaction that occurs is primarily dependent on the energy of the x-ray, and secondarily on characteristics of the nanoparticle, e.g. it’s atomic number, Z, or the electron density, \( \sigma_e \). The probability of a particular interaction occurring is given by the linear attenuation coefficient or the mass attenuation coefficient. The mass attenuation coefficient is the linear attenuation coefficient divided by the density of the material. The five interactions that can occur, in order of increasing probability with increasing energy are: Rayleigh (coherent) scattering, the photoelectric effect (absorption), Compton (incoherent) scattering, pair production (absorption) and photodisintegration (absorption).

Rayleigh scattering, also known as coherent scattering, is an interaction with an electromagnetic wave (photon) and an outer shell electron of the atom making up the material, which in this case would be gold, silicon, or oxygen, as these are the constituent elements of the nanoparticles or nanoshells used. This is an elastic event resulting in a change of direction of the photon while its energy remains unchanged. The scattered photon may go on to interact further in the region of interest or may escape. This effect
can be significant when the energy is low (<10 keV) and the atomic number is high, as the mass attenuation coefficient scales as approximately $Z^2$.

The interaction of electromagnetic waves and metals has been studied extensively for many years and details of the physics behind this can be found in books on electromagnetic theory and scattering (Jackson 1998, Newton 2002, van de Hulst 1981). The scattering of electromagnetic waves from small spherical particles, i.e. where the wavelength is much greater than the particle diameter is known as Rayleigh scattering. This is treated as an elastic scattering event and no energy from the photon is transferred to the material. The scattering of electromagnetic waves from particles that are a similar size to the electromagnetic wave is described by Mie scattering theory (Aden et al 1951, van de Hulst 1981). When the object has a size that is significantly greater than the wavelength, then geometrical scattering is used. However, when the wavelength of the incident radiation is small enough that its energy exceeds the binding energy of electrons in the atoms, then classical electromagnetic theory must be abandoned and quantum theory needs to be used.

The photoelectric effect is the type of interaction with which this thesis is primarily interested. In this interaction, a photon is absorbed by an inner shell electron which is then ejected from its orbital and is known as a photoelectron. The vacancy left by the photoelectron is then filled by an electron in a higher shell, releasing its potential energy in the form of a characteristic x-ray, which in turn may escape the medium or interact with a higher shell electron. If a higher shell electron absorbs the characteristic x-ray, that electron is also ejected from the atom and is known as an Auger electron.
The photoelectric effect is highly dependent upon the energy of the x-ray and the atomic number of the material. The mass attenuation coefficient for this interaction is proportional to \((Z/E)^3\) (Khan 2010). It is because of this relationship that research is being conducted into the use of metallic nanoparticles and nanoshells as a means to enhance dose to tumors with kVp x-rays. The increase in dose to the tumor is known as the dose enhancement factor (DEF) (Cai et al. 2013). This factor is the ratio of dose required for a certain effect in the presence of nanoparticles vs. the dose required to achieve an identical effect in the absence of nanoparticles.

There are increases in the mass attenuation coefficients, which occur at the binding energies of the electron shell in question. For the K-edge in gold, which corresponds to the increase in the mass attenuation coefficient of the inner most shell, this occurs at an energy of 80.7 keV. The L- and M-edge correspond to the next two highest shells at energies of ~13 keV and ~3 keV respectively (Hainfeld et al. 2008). Although the absorption cross-section occurs at the stated energies, higher energies are required in order to impart enough kinetic energy to the electron so that it can cross the length of several cells. One effect of this is that, in order for the electron to be ejected with enough kinetic energy to traverse a few cells, the photon must be of a higher energy than the optimal absorption edge thus reducing the absorption cross-section (Hainfeld et al. 2008).

When the energy of the photon becomes significantly greater than the binding energy of the electrons, the photoelectric effect gives way to Compton scattering. In this inelastic event, a photon transfers some of its energy to an outer shell electron, typically ejecting the electron from the atom. The loss in energy of the photon may be enough that it is able to go on to interact via the photoelectric effect. The photon may only excite the
electron, which can then spontaneously decay as fluorescent photons, or Auger electrons. The first ionization energy of Au is 9.2 eV (Haynes et al. 2014). Compton scattering is essentially independent of the atomic number and is dependent on the electron density of the material, as the interaction involves “free” electrons. For almost all materials, this is on the order of $10^{23}$ electrons per gram of material (Khan 2010). This effect also diminishes as photon energy increases.

Pair production is an effect that occurs in the presence of matter, whereby a high-energy photon (>1.022 MeV) is transformed into an electron and a positron. The positron will then interact with an electron, annihilating both in the process and producing two 511 keV photons.

The final effect, photodisintegration, occurs at even higher photon energies than pair production. This involves a photon interacting with a nucleus directly, ejecting a proton, neutron, or alpha particle by a de-excitation of the nucleus.

Since this research involves X-rays with energies of 110-130 kVp, the role of pair production and photodisintegration will not be covered further as they involve interactions requiring megavoltage and higher energies.

1.1.4: Dose Enhancement

The main advantage to using kilovoltage x-rays and gold nanoparticles/nanoshells as a potential treatment of malignancies is the high cross section for photoelectric events compared to higher energy beams and/or lower Z materials. The increase in dose to tissue due to these events is called radiosensitization and the increase in dose to tissue can be quantified by the dose enhancement factor (DEF) or dose enhancement ratio (DER).

The dose deposited to tissue surrounding the gold nanoparticle from low-energy x-rays is due to photoelectrons, characteristic x-rays and Auger electrons. The
characteristic x-rays can cause secondary photoelectric events, and the effect of all of the electrons is to deposit their kinetic energy in the tissue. Dose is defined as the energy transferred by ionizing radiation per unit mass of material, with units of gray (Gy) where 1 Gy = 1 J/kg (Khan 2010).

As discussed earlier, the effectiveness due to increase in absorption cross-section at the energy of an electron shell edge is offset by the lack of kinetic energy of the photoelectron at these same energies. In order to increase the kinetic energy of the photoelectron, so that it can impart significant relative dose to tissue, requires choosing a higher incident photon energy that unfortunately has the disadvantage of decreasing the probability of a photoelectric event occurring. There is a significant increase in the relative absorbance of gold vs. tissue at 20 keV, on the order of 95 times (Hainfeld et al 2008). This is below the 80.7 keV K edge of gold but about 7 keV above the L edge of gold. At an energy of ~ 35 keV, there is a factor of ~ 50 increase in the ratio of gold attenuation relative to soft tissue (Hainfeld et al 2008).

Monte Carlo simulations have shown that for a Pd-103 source (20.48 keV γ-ray) the probability of a photoelectric event from the L shell is 76.13 % relative to all photoelectric events. By comparison, an I-125 source (26.07 keV γ-ray) and a Yb-169 source (62.11 keV γ-ray) yield a 76.35 % and 66.61 % probability of an L shell photoelectric event relative to all photoelectric events, respectively (Lechtman et al 2011). There have been many investigations into modeling radiosensitivity enhancements with gold nanoparticles (Rose et al 1999, Lechtman et al 2011, Lechtman et al 2013, Douglass et al 2013, Cai et al 2013).
1.1.5: Effective $Z$ of Silica-Gold Nanoshells

Composite materials will obviously have a certain probability for the photoelectric effect to occur. An element is determined by the number of protons in its nucleus (the atomic number, $Z$), and for a neutral element this also determines the number of electrons in it. A composite material has an effective atomic number, $Z_{\text{eff}}$, that is determined by

$$Z_{\text{eff}} = \sum_{i=1}^{n} f_i Z_i^{2.94}$$

where $f_i$ is the fractional weighting of the number of electrons of the $i^{th}$ element and $Z_i$ is the atomic number of the $i^{th}$ element (Mayneord 1937, Khan 2010).

A silica-gold nanoshell has a core of silicon dioxide surrounded by a layer of elemental gold. Assuming the compounds and elements are pure and the densities are standard, then $M_{\text{SiO}_2} = 60.08$ g/mole, $M_{\text{Au}} = 196.97$ g/mole, $\rho_{\text{SiO}_2} = 2.648$ g/cm$^3$, $\rho_{\text{Au}} = 19.3$ g/cm$^3$, $\#e_{\text{SiO}_2}/\text{molecule} = 30$, $\#e_{\text{Au}}/\text{atom} = 79$ where $M$ is the molecular/atomic mass and $\rho$ is the molecular/atomic density (Haynes WM (Ed.) 2014).

The volume of a sphere is given by $V_1 = \frac{(4/3) \pi}{r_1^3}$ and that of a shell surrounding the sphere is given by $V_2 = \frac{(4/3) \pi}{(r_2^3 - r_1^3)}$ where $r_1$ is the radius of the inner core and $r_2$ is the radius of the entire nanoshell. For the nanoshells used in this experiment $r_1 = 60$ nm and $r_2 = 75$ nm.
Using the values given above, it can be shown that $V_1 = 9.048 \times 10^{-16} \text{ cm}^3$, $V_2 = 8.624 \times 10^{-16} \text{ cm}^3$, $n_{SiO_2} = 7.202 \times 10^8$ and $n_{Au} = 4.019 \times 10^9$ so that $f_1 = 0.152$ and $f_2 = 0.848$. Inserting these values into the formula given above for the effective atomic number gives $Z_{eff} = 74.96$. The calculated effective atomic number of the silica gold nanoshell is close to $Z = 75$, the element rhenium, and is $\sim 95$ % of the value of a solid gold nanoparticle. Thus silica-gold nanoshells should not show a significant decrease in dose due to the photoelectric effect as compared to gold nanoparticles.

1.1.6: Electromagnetic Interactions: Surface Plasmon Resonance

There is a unique effect that can be exploited due to the interaction of optical wavelengths of electromagnetic radiation (approximately 400 – 1100 nm) and gold nanoparticles, specifically with silica-gold nanoshells. When an electromagnetic wave of
a certain frequency interacts with a medium of differing dielectric constants, of which the
surface one is a thin layer, a resonance effect occurs due to the collective oscillation of
the conduction electrons on the surface layer. This couples the incident electromagnetic
field to the conduction electrons and then propagates in a direction parallel to the
interface between differing dielectrics. This effect is known as surface plasmon
resonance (SPR) and is sensitive to changes in the boundary layer, i.e. its thickness or the
chemical structure of the layer. When this effect occurs where there is a nanoscale size
spherical object with differing dielectric constants, e.g. a silica-gold nanoshell, and the
wavelength of the incident electromagnetic field is on the size order of the nanoshell, the
plasmon is constrained to the surface of the nanoshell. In this case the effect is called
localized surface plasmon resonance (LSPR).

The location (wavelength) of the LSPR is effectively an extinction of the
electromagnetic field with a peak at that wavelength and contains an absorption
component and a scattering component. Silica-gold nanoshells can have the location of
the plasmon resonance peak “tuned” to a wavelength window of approximately 600 nm
to 1000 nm by varying the core to shell ratio. This dimensionless quantity is the ratio of
the radius of the core to the radius of the entire nanoshell. It will be denoted as \( R = r_1/r_2 \),
where \( r_1 \) is the radius of the inner core and \( r_2 \) is the radius of the entire nanoshell. As the
thickness of the shell decreases relative to the size of the core, i.e. as \( R \) increases, the
resonance peak shifts to longer wavelengths. As mentioned earlier, the total extinction
contains absorption and scattering components. The proportion of absorption to
scattering, normalized to total extinction can also be manipulated by adjusting \( R \).
The ability to specify the location of the resonance peak and the proportion of absorbance to scattering, gives silica-gold nanoshells a distinct advantage over gold nanoparticles. The plasmon resonance peak window of nanoshells contains the near infrared (NIR) tissue window, which ranges from 700 nm to 900 nm. Tissue is quite transparent in this range and this opens up the potential of using gold nanoshells for PTT and PDT (Baek et al 2011, Trinidad et al 2014). By comparison, the resonance peak of gold nanoparticles is located at ~ 520 nm (Hirsch et al 2006). This wavelength is not suited for most light-based therapies due to its strong absorption and hence limited penetration in biological tissues.
1.1.7: Toxicity

There are several reasons that gold is a desirable element to construct nanoparticles out of. One is that it is relatively inert and biocompatible. This is an important feature for use in biological applications. The low toxicity of gold is required if these structures are to be left in the target for an indefinite amount of time and not cause additional damage. Gold in its metallic form (non-ionized) is inert to chemical processes that occur in the body and so is not susceptible to being transformed into toxic gold compounds that could potentially be toxic (Merchant 1998). The incubation of macrophages in vitro with silica-gold nanoshells has been found to be non-toxic to the macrophages (Shukla et al 2005). Various in vivo studies have been performed to verify the non-toxicity of silica-gold nanoshells (Hirsch et al 2003, O’Neal et al 2004, Stern et al 2008, Gad et al 2012).

1.1.8: Conjugation

The gold surface of nanoparticles is capable of having a variety of different compounds conjugated to its surface. In one method there is a passive attachment of molecules or proteins to the gold surface which is known as adsorption. Adsorption may not lead to a permanent attachment of the compound to the surface and that could limit its usefulness. This method may also cause an active area of the attached compound to be on the gold face, which renders it inert for whatever biological effect it is intended.

Another method of attachment is using a chemical linker, such as PEG to permanently attach molecules to the surface of the nanoparticle. In this manner, the required compound can be attached to the nanoparticle so that the active area of interest is not in contact with the surface of the nanoparticle. The conjugation of certain
compounds to the gold surface has been shown to limit systemic clearance or to have increased affinity to malignant cells (Kong et al 2008, Mody et al 2009).

1.2: MONOCYTES and SPHEROIDS

1.2.1: Enhanced Permeability and Retention Effect

In the absence of a tumor, bare nanoparticles that are circulating in a host’s vascular system will continue to circulate until they are engulfed by macrophages (Madsen et al 2011, Maeda et al 2000.). This is due to normal blood vessels being essentially impermeable to nanoscale particles (Dvorak et al 1988, Jain 1999). Tumors have the ability to emit a vascular epithelial growth factor (VEGF), which enables the tumor to create new blood vessels (angiogenesis) (Goto et al 1993). This vasculature is “leaky” and it lacks the relative impenetrability of normal blood vessels. As such, nanoparticles with sizes on the order of hundreds of nanometers are able to extravasate into the tumor through the leaky vasculature (Yuan 1995). The enhanced permeability of the tumor vasculature, along with decreased lymphatic drainage associated with most tumors results in passive accumulation of nanoscale structures in tumors. This is known as the enhanced permeability and retention effect (EPR) (Maeda et al 1999).

1.2.2: Tumor Associated Macrophages

Solid tumors typically have a necrotic center consisting of dead or dying cells. Surrounding this is a zone of cells that are in stasis due being in a zone that is low in oxygen (hypoxic). Outside of this is the region of proliferating cells that have a well developed capillary network and high oxygen levels. The necrotic core occurs due to the rapid growth of malignant cells, which increasingly push out the proliferating cells which have the ability to create the vasculature required to nourish the tumor (Hall et al 2012).
The lack of vasculature in the hypoxic region essentially nullifies the effectiveness of exploiting the EPR effect for nanoparticle delivery to the tumor (Choi et al 2007).

Monocytes are the precursors to macrophages, the white blood cells that are derived from the myeloid progenitor cells in the bone marrow (Madsen et al 2011). Once monocytes leave the circulatory system and cross the endothelial basement membrane, they differentiate into macrophages (Owen et al 2004, Choi et al 2007).

Tumors are able to attract monocytes via a chemo-attractive gradient. Once differentiated into macrophages inside the tumor, there is evidence that the tumor is able to manipulate them into promoting tumor growth (Lewis et al 2006). Macrophages that have been recruited by the tumor are called tumor associated macrophages (TAMs). TAMs can exist in significant numbers in tumors and have been shown to make up ~ 65% - 70 % of the mass of a tumor (Kelly et al 1988, Fleige et al 2001). Exploiting the ability of monocytes/macrophages to phagocytize nanoparticles, has led to the idea of using them as a delivery vector to transport the nanoparticles to the tumor. This has been called a cellular “Trojan Horse” (Choi et al 2007)

1.2.3: Spheroids

Spheroids are an in vitro agglomeration of cells that have grouped together into a spheroidal shaped mass that roughly mimics a tumor. This model was first developed and used as a model to study in vitro responses to tumors in the early 1970’s (Inch et al 1970, Sutherland et al 1971). One of the main advantages of spheroids over monolayer cell cultures is the appearance of an oxygen gradient that decreases radially towards the center of the spheroid (Santini et al 1999). The spheroid contains a necrotic core that is located at 50 to 300 µm from the outer rim of the spheroid (Sutherland 1988). It is believed that hypoxia is a key factor in creating the necrotic core of the spheroid.
(Mueller-Klieser 1997). The proliferating cells of the spheroid are located in the outer 3 to 5 cell layers with a quiescent layer of cells surrounding the necrotic core. The quiescent cells can become viable and enable further growth under the appropriate conditions (Madsen *et al* 2006).

![Figure 4: Cross Section of Spheroid with Oxygen Gradients (Mirams *et al* 2013)](image)

As mentioned previously, *in vitro* spheroids mimic a non-vascularized tumor. This makes their use as a model to investigate the treatment of tumors more clinically relevant than single cell monolayers. In particular, hybrid spheroids containing malignant cells and monocytes are a way of modeling a true tumor that contains TAMs.
1.3: SCOPE OF WORK

There are two main areas of research in this thesis. The first is to investigate the efficacy of using 130 kVp x-rays on multicellular hybrid spheroids using silica-gold nanoshell incubated monocytes. The multicellular hybrid spheroids consist of human ACBT glioblastoma multiforme as the malignant cell line and P388D-1 murine lymphocytic monocytes as the nanoparticle/nanoshell delivery vector. It has been shown that cells containing gold nanoparticles are susceptible to dose enhancement effects from kilovoltage x-rays due to the photoelectric effect. It is hypothesized that: (1) x-ray irradiated hybrid spheroids containing nanoparticle loaded monocytes will show decreased survival compared to irradiated hybrid spheroids with empty monocytes, and (2) no statistically significant difference in survival between hybrid spheroids containing gold nanoparticles and silica-gold nanoshells will be observed.

The second area of research is to establish parameters for the MTS assay which will be used to measure the effects of ionizing radiation on P388D-1 monocytes. This includes determining the number of cells to be plated in each well of a flat bottom 96 well plate, determining the number of days after radiation treatments for measurable cell death to occur, and determining the number of hours the cells are incubated with the MTS reagent. Two different radiation doses (8 and 20 Gy) will be tested on silica-gold nanoshell, and gold nanoparticle incubated monocytes as well as controls (empty monocytes). It is hypothesized that: (1) the 20 Gy dose will yield greater cell death than the 8 Gy dose, (2) a significant decrease in survival will be observed following irradiation of the nanoparticle loaded monocytes compared to empty monocytes at both doses, and (3) no statistically significant difference will be observed when comparing survival of irradiated monocytes containing either gold nanoparticles or silica gold nanoshells.
CHAPTER 2: MATERIALS AND METHODS

2.1: MATERIALS

2.1.1: Cell Lines and Culturing

All experiments were carried out in the Bigelow Health Sciences building (BHS) and the Chemistry building (CHE) at the University of Nevada, Las Vegas (UNLV). The malignant cell line used in the experiments, both as the basis for spheroid formation and to measure the efficacy of the treatment techniques was a human grade IV glioblastoma multiforme (GBM) (ACBT - G. Granger, University of California, Irvine, CA). The cell line used to represent the cellular vector for the delivery of gold nanoparticles/nanoshells to the tumor spheroids was the murine lymphocytic monocyte cell line P388D-1 (ATCC CCL-46, American Type Culture Collection, Manassas, VA). This cell line was also used as the basis for experiments measuring treatment efficacy and the establishment of measurement parameters for the MTS assay.

All cultured cells and spheroids were kept in a CO\(_2\) Water Jacketed Incubator (Sheldon Manufacturing Co., Cornelius, OR) which maintained a temperature of 37 °C, 80 % humidity and a CO\(_2\) level of 5%. Culturing was performed in a Labconco™ Purifier Class A2 Biological Safety Cabinet (Labconco, Kansas City, MO). The ACBT cultures were contained in T-25 BD Falcon tissue culture flasks and the P388D-1 cultures were contained in T-75 BD Falcon tissue culture flasks (BD Biosciences, Franklin Lakes, NJ).

Culturing was done once per week in gibco™ Dulbecco’s Modified Eagle Medium (1X DMEM) with 4.5 g/L D-Glucose, L-Glutamine, 110 mg/L Sodium Pyruvate, 25 mM HEPES, and no phenol red (Thermo Fisher Scientific - Life Technologies, Carlsbad, CA). The media was supplemented with 50 mL of 10% fetal bovine serum (FBS) and 5 mL Pen-Strep (10,000 U/mL Penicillin and 10,000 μg/mL Streptomycin).
2.1.2: ACBT

ACBT exists as an adherent monolayer in a flask with media. The culturing of ACBT cells was done by aseptically pipetting the old media out of the flask, which leaves the adherent monolayer on the bottom of the flask. The flask was then lightly rinsed with 5 mL of gibco phosphate buffered saline (PBS), pH 7.4. To detach the adherent cells, 1 mL of 0.25 % Trypsin-EDTA (1X) with phenol red (Thermo Fisher Scientific - Life Technologies, Carlsbad, CA) was added to the flask. In order to ensure proteolytic cleavage, it was left in the flask for 5 minutes and sporadically rocked back and forth. Once detached, 4 mL of PBS was added to the flask, aspirated by pipetting and then transferred to a 15 mL centrifuge tube. The tube was then centrifuged for 5 minutes at 200 g in a Heraeus™ Megafuge™ 16 (Thermo Scientific, Waltham, MA). The supernatant was pipetted off and 5 mL of media was added to the remaining cellular pellet. The purpose of this step was to remove any dead cells, trypsin and old media. The cells were then re-suspended using a VWR Digital Vortex Mixer (VWR International, Radnor, PA) at 3000 rpm and a 1 mL aliquot was transferred to a T-25 flask containing 4 mL media.

2.1.3: P388D-1

Murine monocytes are essentially non-adherent and exist as a suspension in media. The culturing of the P388D-1 murine monocytes was done by aseptically pipetting a flask containing the monocytes into a centrifuge tube, stirred using a VWR Digital Vortex Mixer (VWR International, Radnor, PA) at 3000 rpm, and then centrifuging for 5 minutes at 200g using a Heraeus™ Megafuge™ 16 (Thermo Scientific, Waltham, MA). The supernatant was then removed via pipetting and 10 mL of fresh media was added to the tube and the cells re-suspended using the mixer. The purpose of
this step was to remove dead cells and old media. A 3 mL aliquot of the suspension was transferred to a T-75 flask containing 15 mL of media.

2.1.4: Silica-Gold Nanoshells and Gold Nanoparticles

The experiments were carried out using two different types of nanoparticles:

1. Silica-Gold Nanoshells (NS): The AuroShell™ nanoparticle is a silica-gold core-shell coated with a functionalized poly(ethylene glycol) (PEG) hydrophilic outer layer (Nanospectra Biosciences Inc., Houston, TX). It is kept as a suspension in deionized water. The purpose of the PEGylated coating is to reduce aggregation of the nanoparticles. The silica core has a diameter of 150 nm and the gold coating is 15 nm thick, which gives the overall diameter of the nanoparticle, sans PEGylated coating, as 150 nm. The stock concentration is 2.82 x 10^{11} particles/mL.

2. Gold Nanoparticles (NP): Cytodiagnostics™ 150 nm Stabilized Gold Nanoparticle, supplied in a 0.1 mg/mL citrate buffer with a proprietary stabilizing surfactant (Cytodiagnostics, Burlington, ON). The purpose of the citrate buffer and stabilizing surfactant was to reduce aggregation of the nanoparticles. The stock concentration was 3.9 x 10^{9} particles/mL.
2.1.5: X-Ray Sources

There were two radiation sources used for the experiments. The first source was used for the experiments concerning the spheroids. This was the Faxitron RX-650 (Faxitron X-Ray Corp., Wheeling, IL). This machine is capable of delivering x-rays using an x-ray tube with peak energy of 135 kVp. The average voltage is approximately 1/3 of the peak voltage, which in this case delivers x-rays with an average energy of ~ 43 keV. It delivers an exposure rate of 170 R/min at a distance of 0.66 m when operated at 130 kVp. The exposure to air in Roentgens can be converted to dose in tissue by the
conversion factor of 1 R = 0.0096 Gy. Thus the dose rate to tissue is ~ 1.6 Gy/min at this setting.

Figure 6: Faxitron RX-650 X-Ray Machine
(http://www.uta.edu/physics/main/faculty/wchen/nbmain/facilities.html)

The Faxitron RX-650 malfunctioned on March 30, 2014 and was deemed unusable for subsequent experiments. A second machine was delivered from the Department of Anthropology to the Department of Health Physics on June 10, 2014. This was the Faxitron Model 4385A (National X-Ray Corp., Flowery Branch, GA). This machine is capable of delivering x-rays using an x-ray tube with peak energy of 115 kVp. It delivers an exposure rate of 105 R/min at a distance of 0.61 m when operated at 110 kVp. The dose rate to tissue is ~ 1.0 Gy/min at this setting.
Both machines undergo a warm-up procedure where they are operated for 5 minutes at 30, 60, and 90 kVp respectively. After this is done they are ready for operation at the desired voltage.

2.1.6: MTS Assay

The MTS assay (Promega Corp., Madison, WI) is a colorimetric method to determine cell viability. It does this by counting the number of viable cells with a well plate reader. Viable cells are metabolically active and they contain dehydrogenase enzymes which, in the presence of phenazine methosulfate (PMS; Sigma, St. Louis, MO), can convert MTS \[3-(4,5\text{-dimethylthiazol-2-yl})-5\text{-}(3\text{-carboxy-methoxyphenyl})-2\text{-}(4\text{-sulfophenyl})-2H\text{-tetrazolium}] into a purple formazan compound. This compound is soluble in media and has an absorption peak at a wavelength of 490 nm (Santos et al 2014).

To determine the net number of viable cells, a negative control was subtracted from the wells containing the cells of interest. For any particular experiment, 100 µL of media containing a concentration of cells that is required, was pipetted into a minimum of 2 wells in a Costar™ flat bottom 96-Well Cell Culture Cluster Plate (Corning Inc., Corning, NY). Most experiments performed in this research used 16 wells of interest and 16 wells of negative control for each trial. In the negative control group, 80 µL of the clear media was removed and replaced with 80 µL of 95 % ethanol to kill the cells. The 96 well plate was placed in the incubator for 10 min and then 80 µL of the media-ethanol mixture was removed and replaced with 80 µL of clear media. To this, 20 µL of the MTS assay was added to all wells containing cells and then allowed to incubate for 1 to 4 hours. Thus there was a 5:1 ratio of media to MTS assay with a total volume of 120 µL/well. Longer incubation times resulted in a darker color but cytotoxic effects due to
the MTS assay will occur eventually. The MTS Assay was performed on a Tecan Infinite M1000 PRO microplate reader with Magellan 6 data analysis software (Tecan Group Ltd., Männedorf, Switzerland).

Figure 7: Differing Incubation Times of MTS Assay
(http://en.wikipedia.org/wiki/MTT_assay#mediaviewer/File:MTT_Plate.jpg)

Figure 8: Tecan Well Plate Reader
(http://www.news-medical.net/Infinite-M1000-PRO-Microplate-Readers-from-Tecan)
2.2: METHODS

2.2.1: Incubation of Monocytes with Nanoshells and Nanoparticles

The monocytes need to be loaded (incubated) with the nanoparticles in order for the experiment comparing the efficacy of radiation on the incubated vs. bare cells. It has been shown that the optimum time for the uptake of 150 nm PEG silica-gold nanoshells is approximately 24 hours (Chhetri 2013). Previous experiments performed at UNLV have used a concentration of $4.285 \times 10^9$ NS/mL and $5 \times 10^6$ cells/mL for the PEG silica-gold nanoshells (Makkouk 2010, Chhetri 2013).

The cell concentration was determined using an iNCYTO™ C-Chip disposable hemocytometer (Neubauer Improved) (Incyto, Korea). Counting was performed under a VWR inverted microscope (VWR International, Radnor, PA). The monocyte culture (10 μL was pipetted into the C-Chip and the number of monocytes in the central large square was determined. This number was then multiplied by $10^4$ to give the concentration of monocytes/mL. The counting was done under a VWR microscope using a hand counter. To obtain the required concentration of monocytes, an existing flask of culture was centrifuged, the supernatant removed followed by the addition of the requisite amount of media and re-suspension of the pellet. This step was done whether loaded or empty monocytes were used.

2.2.1.1: Incubation of Silica-Gold Nanoshells

As mentioned above, the stock concentration of NS was $2.82 \times 10^{11}$ NS/mL in deionized water. To get the required concentration of $4.285 \times 10^9$ NS/mL the following formula was used: $V_1 C_1 = V_2 C_2$, where V is the volume and C is the concentration. This calculation yielded a required volume of 15 μL NS per mL of media-nanoshell mixture.
This was then incubated for 24 hours and centrifuged for 5 minutes at 200 g to remove un-endocytosed nanoshells. This step was repeated twice more using 10 mL of media and the supernatant was removed each time. Resuspension was accomplished by the addition of 10 mL of fresh media to the pellet. A cell count was performed and the suspension diluted to achieve $5 \times 10^5$ / mL of nanoshell incubated monocytes. It has been shown that there is an uptake of 3.9 % of the silica-gold nanoshells by the P388D-1 monocytes over a 24 hour incubation period (Chhetri 2013).

### 2.2.1.2: Incubation of Gold Nanoparticles

The stock concentration of the NP was $3.6 \times 10^9$ / mL in a citrate buffer solution. This concentration was lower than the concentration used for the nanoshell experiments. The experiment involving the nanoshells had 1.5 % of the total volume being attributable to the nanoshells. Since the stock concentration of NP was lower than needed, it was not possible to have the same ratio of nanoparticle to media volume. The experiment performed used a ratio of 1 mL of nanoparticles to 1 mL of monocytes at a concentration of $5 \times 10^5$ monocytes / mL. This resulted in a final concentration of $1.8 \times 10^9$ monocytes / mL of nanoparticles and a concentration of $2.5 \times 10^5$ monocytes / mL.

The uptake of the gold nanoparticles by the P388D-1 monocytes was not known as a blank citrate buffer could not be obtained for a background measurement.

### 2.2.2: Preparation of Incubated Monocytes for Spheroid Formation or MTS assay

As stated above, 24 hours prior to the creation of the spheroids, 4 mL of the monocytes at $5 \times 10^5$ / mL were placed into a 10 mL petri dish, and to this was added either 15 µL of nanoshells or 4 mL of nanoparticles. This mixture was gently aspirated with the pipette to mix the contents. The dish was then placed in the incubator until the next day. One hour prior to the formation of the spheroids, mitomycin-C was added to
the dish in order to halt the division of the monocytes in a concentration of 20 μL/mL of culture, i.e., 80 μL of mitomycin-C for the nanoshells and 160 μL of mitomycin C for the nanoparticles. In cases where the MTS assay was used, mitomycin C was NOT added and, as such, monocyte division was not halted.

2.2.3: Spheroid Creation

The spheroids were kept in well plates that had an agar mixture added to each well. This agar is a nutrient base that the spheroids rest on while they are being monitored for growth. The same day that incubation of the monocytes with nanoparticles was started, the well plates with the agar mixture were prepared. These are Costar™ Flat Bottom 48-Well Cell Culture Cluster Plates (Corning Inc., Corning, NY). First a 2% agarose gel was heated in boiling water on a hot plate. Once the agarose was liquefied, it was mixed in a 1:1:0.005 ratio of agarose: 2x DMEM : 1 M NaOH. 0.25 mL of this mixture was pipetted into each well of the plate. This was then placed in the incubator to cool. Once the agar had solidified in the well plate, 0.75 mL of media was added to each well.

Spheroid creation was based on the rapid generation technique using a centrifuge (Ivascu and Kubbies 2006). Two ratios of ACBT-to-P388D-1 cells were used (2:1 and 5:1). For the 5:1 ratio experiments, hybrid spheroids were created that initially contained 5000 ACBT cells and 1000 monocytes. For the 2:1 ratio experiment, 5000 ACBT cells and 2500 monocytes were used. The same amount of ACBT per spheroid was used since monocyte division was inhibited with the addition of mitomycin-C. Once the mitomycin-C was added to the monocytes, the ACBT cells were prepared for the experiment. This was done first by detaching them as described above in the culturing section. The
Spheroids were formed in Costar™ Ultra Low Cluster 96-Well Round Bottom Plates via centrifugation. 200 μL of combined ACBT/macrophages were pipetted into each well. For the 5:1 ratio experiments a concentration of 50,000 ACBT cells/mL was used and for the 2:1 ratio experiment, a concentration of 40,000 ACBT cells/mL was used.

Monocytes were harvested one hour after the addition of mitomycin-C was added. They were then centrifuged to remove nanoparticles that had not undergone phagocytosis. This was done at 200 rpm for 5 min followed by the removal of the supernatant. Monocytes were then re-suspended in media and the process repeated twice. Media was added to the final pellet to get a concentration of 10,000 monocytes/mL for the 5:1 experiments and a concentration of 25,000 monocytes/mL for the 2:1 experiment.

The ACBT cells that were previously prepared were mixed with the respective nanoparticle/nanoshell loaded monocytes for the requisite concentration in a 50 mL centrifuge tube. The tube was then placed on a hand centrifuge to ensure the ACBT and monocytes were uniformly distributed. The contents were then put in a multi-channel reagent container and a multi-pipetter was used to transfer 200 μL of the mixture to each of the round-bottomed well plates.

In cases where control groups of spheroids were required, the exact same procedure as described above was performed, *sans* incubation of the monocytes. For the ACBT only spheroids, 5,000 cells per spheroid were used.

To create the spheroids, they were centrifuged in their well plates at 800 g for 10 minutes. The plate was then transferred to the incubator to give the cells time to form the spheroid structure. It has been observed that spheroid creation has not always been consistent, and can range from 1-2 days and may require additional centrifugation.
2.2.4: Spheroid Treatment

The spheroids were irradiated with the Faxitron RX-650 at an energy of 130 kVp. Experiments were performed using doses of 8, 10, 12, 14, and 20 Gy at a dose rate of 1.6 Gy/min.

Prior to irradiation, spheroids were transferred individually from each well of the round bottom plate into a 10 mL petri dish containing 4 mL of medium. There were 16 spheroids per petri dish per experiment. This gave six-petri dishes per well plate so that the spheroids could be treated at the five doses as well as having a control. The petri dishes were kept in the incubator until they were used in a treatment. Over the course of an experiment, a few spheroids were typically lost or damaged, and as such they were no longer tracked for the remainder of the experiment.

On the day of an experiment, the Faxitron was run through its warm-up procedure, which required approximately 15 minutes. One petri dish at a time was then moved from the room containing the incubator to the treatment room. In order to deliver the doses of 8, 10, 12, 14, and 20 Gy, the Faxitron was operated at 130 kVp for 4:54, 6:08, 7:21, 8:35, and 12:15 minutes respectively. The treatments were done with the lid of the petri dish removed to reduce x-ray attenuation and electron contamination. After each treatment was performed, the spheroids were transferred by micro-pipetting each spheroid into the flat bottomed well plates containing the agar and media.

2.2.5: Maintenance and Monitoring

The success of the treatments was based on monitoring the relative size of the spheroids over a period of three weeks. Two orthogonal measurements were made of the diameter of a spheroid using a calibrated micrometer on the VWR microscope. Each measurement was rounded to the nearest 25 μm and then the two measurements
averaged. The volume of each spheroid was then calculated by the formula $V = \frac{1}{6\pi}d^3$ where $d$ is the average diameter of the spheroid. The day of treatment was counted as day zero for measurements, and then two measurements were taken twice per week for the following three weeks. This gave 7 measurements per spheroid from day zero to day 21. On the day of every measurement, the media was refreshed by removing 400 μL of old media and replacing it with 425 μL of new media. The increase of new media was to account for evaporation.

2.2.6: Determination of Monocyte Concentration for MTS Assay

In order to perform the experiments using the MTS assay to check for cell viability, the optimum number of cells per well in a Costar™ 96-Well Flat Bottom Well Plate had to be determined. The signal strength of the absorbance at 490 nm should increase linearly as the number of cells increases per well. At a certain concentration, the signal will reach saturation and the absorbance reading will plateau. The first experiment was performed to determine the optimal number of monocytes per well. Once the linear trend had been established, a concentration corresponding to the midpoint of the trend line was used in subsequent experiments.

This experiment was performed by pipetting 100 μL of varying concentrations of monocytes into the well plates. For each trial, 32 wells were used, 16 of which were used for the negative control. The concentrations used were: 500, 1000, 2000, 5000, 10000, 20000, 50000, 100000, 150000, 200000, and 250000 monocytes per well. An MTS assay incubation time of 1 hour was used for this initial experiment. It was found that 50,000 monocytes per well for 100 μL of media was on the midpoint of the linear trend. These results are discussed further in Chapter 3.
2.2.7: Survivability and MTS Assay Incubation Time

The second set of experiments was performed to determine the optimal time to apply the MTS assay following irradiation. In the first part of this experiment, a dose of 8 Gy was delivered to a 10 mL petri dish containing 4 mL of $5 \times 10^5$ monocytes/mL. A control group consisting of an identical preparation of monocytes was brought into the treatment room, but was not irradiated. After each treatment, 50,000 monocytes were pipetted into each well plate and left in the incubator for 24, 48 or 72 hours. After each of the time periods cells were incubated with the MTS assay for one hour. The results of this experiment were not conclusive so a second experiment with a higher dose and longer post treatment times was done as described below.

In the second part of this experiment, a dose of 20 Gy was delivered to a 10 mL petri dish containing 4 mL of $5 \times 10^5$ monocytes/mL. As before, an unirradiated control group consisting of an identical preparation of monocytes was included. After each treatment, 50,000 monocytes were pipetted into each well plate and left in the incubator for 24, 48, 72, 96 or 120 hours. In this experiment, not only were there five days of experiments performed, but the MTS assay duration was also varied: 1, 2, 3 or 4 hour incubation times were examined.

2.2.8: Toxicity

The third part of the experiments was performed to determine whether there was any toxicity to the monocytes due to their incubation with gold nanoparticles or nanoshells. In the experiments with the spheroids, this was not relevant as the monocytes were given mitomycin-C and therefore they did not contribute to spheroid growth. Monocytes were incubated with nanoparticles and nanoshells as described in Ch. 2.2.1.1 and Ch. 2.2.1.2 and were prepared as in Ch. 2.2.2. The MTS assay was performed at 72
hours after irradiation using an MTS incubation time of one hour. As will be shown in Ch. 3, 1 hour of MTS incubation yielded an optimum result.
CHAPTER 3: RESULTS

3.1: SPHEROIDS

3.1.1: ACBT Spheroid Growth Kinetics

The degree of growth impairment of ACBT spheroids is clearly shown to be dependent on the dose delivered to the spheroids (Figure 9). Dose was delivered at ~ 1.6 Gy/min at an energy of 130 kVp. Spheroid volumes compared to the control group (0 Gy) at 21 days post treatment for doses of 8 Gy, 10 Gy, 12 Gy, 14 Gy and 20 Gy were 86%, 76%, 68%, 51% and 22% respectively.

Figure 9: ACBT Spheroid Growth Kinetics. Growth kinetics of non-incubated ACBT spheroids subjected to varying doses of 130 kVp x-rays. Each data point consists of the average of three separate trials and the error bars denote standard errors. The effect of dose was statistically significant at 21 days (p < 0.05) for all groups.

3.1.2: ACBT/P388D-1 Hybrid Spheroid Growth Kinetics

Three groups of experiments containing nanoshell/nanoparticle incubated hybrid spheroids were conducted. Only one trial was completed since the Faxitron RX-650 malfunctioned after the first experiment. A control experiment of non-incubated hybrid spheroids was not performed due to this problem.
For the gold nanoparticle incubated spheroids with a 5:1 ACBT/P388D-1 ratio the trend in terms of response to dose was similar to the ACBT only spheroids (Figure 10). Dose was delivered at ~ 1.6 Gy/min at an energy of 130 kVp. Spheroid volumes compared to the control group (0 Gy) at 21 days post treatment for doses of 8 Gy, 10 Gy, 12 Gy, 14 Gy and 20 Gy were 90%, 85%, 82%, 79% and 51% respectively.

Figure 10: Gold Nanoparticle Incubated 5:1 ACBT/P388D-1 Spheroids. Growth kinetics of gold nanoparticle incubated hybrid spheroids subjected to varying doses of 130 kVp x-rays. Each data point consists of the average of one trial and the error bars denote standard errors.

For the silica-gold nanoshell incubated spheroids with a 5:1 ACBT/P388D-1 ratio there is an anomalous result with the 20 Gy dose (Figure 11). Dose was delivered at ~ 1.6 Gy/min at an energy of 130 kVp. Spheroid volumes compared to the control group (0 Gy) at 21 days post treatment for doses of 8 Gy, 10 Gy, 12 Gy, 14 Gy and 20 Gy were 92%, 83%, 72%, 61% and 79% respectively.
Figure 11: Silica-Gold Nanoshell Incubated 5:1 ACBT/P388D-1 Spheroids. Growth kinetics of silica-gold nanoshell incubated hybrid spheroids subjected to varying doses of 130 kVp x-rays. Each data point consists of the average of one trial and the error bars denote standard errors.

For the silica-gold nanoshell incubated spheroids with a 2:1 ACBT/P388D-1 ratio (Figure 12) the trend in terms of response to dose is similar to the 5:1 gold nanoparticle results (Figure 11). Dose was delivered at ~ 1.6 Gy/min at an energy of 130 kVp. Spheroid volumes compared to the control group (0 Gy) at 21 days post treatment for doses of 8 Gy, 10 Gy, 12 Gy, 14 Gy and 20 Gy were 90%, 86%, 85%, 81% and 56% respectively.
A comparison of normalized spheroid volume at 21 days post treatment of the different doses shows the anomalous result for the silica-gold nanoshell incubated spheroids with a 5:1 ACBT/P388D-1 ratio more clearly as well as the similar results of the 2:1 silica-gold nanoshell spheroids to the 5:1 gold nanoparticle spheroids (Figure 13). Compared to the non-irradiated control groups, the volumes of the 20 Gy irradiated ACBT cells-only, 5:1 NP, 5:1 NS and 2:1 NS spheroids at 21 days were 22%, 51%, 79% and 56% respectively. It should be noted that the 5:1 NP and 2:1 NS groups are within their standard errors but have $p > 0.06$.

Since only one trial could be completed, none of the results can be considered to be statistically significant.
3.2: Parameters for MTS Assay of P388D-1 Monocytes

3.2.1: Establishment of Concentration for MTS assay of P388D-1 monocytes.

As discussed in the materials section, the malfunction of the Faxitron RX-650 and its replacement 70 days later with the Faxitron 4385A led to a change in research focus using monocytes only. Since spheroids were no longer to be used, the MTS assay was the replacement to examine treatment efficacy. In order for measurements to be made, parameters needed to be established which included the optimum number of cells in each well. The cells exist in a monolayer in the well and the signal strength is linearly dependent on the number of viable cells per well.

Wells were seeded with 500, 1000, 2000, 5000, 10000, 20000, 50000, 100000, 150000, 200000 and 250000 cells. A 1 hour MTS incubation time was used for this experiment. The goal was to determine the concentration of cells resulting in a saturation
of the signal. A linear trend was observed with an increase of cell concentration with a saturation point reached at approximately 100000 cells per well (Figure 14).

![Graph of Determination of Number of Cells Required for MTS Assay Saturation](image)

Figure 14: Linear Trend and Saturation Concentration of P388D-1 Monocytes. Error bars denote standard errors.

Based on the results of this experiment it was decided that a concentration of 50000 cells per well would be used for future experiments since this was well below the saturation threshold.

3.2.2: 8 Gy Dose, 1 Hour MTS Incubation at 24, 48 and 72 Hours

The next experiment was to irradiate non-incubated monocytes with 110 kVp x-rays at a dose rate of ~ 1.0 Gy/min and a total dose of 8 Gy. Three trials were performed and MTS assays were performed at 24, 48 and 72 hours following irradiation. This experiment was a first step to test the response of monocytes to exposure and determine the length of time required to inhibit metabolic activity. No statistically observable effect
was shown with an 8 Gy dose after 24, 48 or 72 hours (Figure 15). Students t-test gave values of $p = 0.022$, 0.215 and 0.483 for those times respectively. The possibly statistically significant result at 24 hours is most likely attributable to a variation in initial concentration of cells per well.

Figure 15: 8 Gy Dose, 1 Hr MTS incubation at 24, 48 and 72 hours post-irradiation. Error bars denote standard errors.

3.2.3: 20 Gy Dose, 1,2,3,4 Hr MTS Incubation at 24,48,72,96 and 120 Hours

The next experiment was to monitor a 20 Gy dose delivered with 110 kVp x-rays at 1.0 Gy/min. The higher dose was used to ensure that there should be observable effects on cell viability and a longer period of tracking the cells to determine when cell death occurs. Varying the MTS assay incubation time was also investigated. Three trials were performed and all error bars in Figures 16–24 represent standard errors. The most statistically significant time after treatment (smallest p values) was observed at 120 hours post-irradiation. Of this group all of the MTS assay incubation times gave statistically
significant results, therefore to expedite the experiments, a 1 hour MTS incubation period was used in the NS and NP incubation experiments discussed in the next section. The data is displayed in two formats: Figures 16 – 20 show absorbance as a function of incubation time while Figures 21 – 24 illustrate absorbance vs. time post-treatment.

![MTS Incubation Times 24 Hours after Treatment](image)

Figure 16: 20 Gy Dose 24 Hours after Treatment
Figure 17: 20 Gy Dose 48 Hours after Treatment

Figure 18: 20 Gy Dose 72 Hours after Treatment
Figure 19: 20 Gy Dose 96 Hours after Treatment

Figure 20: 20 Gy Dose 120 Hours after Treatment
Figure 21: 1 Hour MTS Incubation

Figure 22: 2 Hour MTS Incubation
Figure 23: 3 Hour MTS Incubation

Figure 24: 4 Hour MTS Incubation
3.3: Treatment and Toxicity of NP and NS incubated P388D-1 Monocytes

3.3.1: Toxicity Test of NP and NS on P388D-1 Monocytes

An investigation was performed on the possible toxicity of gold nanoparticles and silica-gold nanoshells on P388D-1 monocytes. One of the factors that needed to be examined was whether toxic effects would be observed due to the high concentration of the NP citrate buffer relative to the media, 1:1. The de-ionized water used for the NS was at a much lower concentration during particle incubation, namely 1:72 and it has been shown in previous studies (Chettri 2013) that toxic effects weren’t observed after 24 hours of incubation. As shown in Figure 25, neither nanoparticles nor nanoshells were found to be toxic to murine monocytes.

![Toxicity Test 72 Hr after Incubation](image)

Figure 25: Toxicity Test of Incubated Monocytes
3.3.2: 20 Gy Dose to NP and NS Incubated P388D-1 Monocytes

The final experiment to be performed was a 20 Gy dose of 110 kVp x-rays delivered at a dose rate of ~1.0 Gy/min to NP and NS incubated monocytes. Three separate trials were performed. They showed an MTS absorbance at 490 nm of 43%, 87% and 64 % for the monocytes only, NS incubated monocytes and NP incubated monocytes respectively relative to a non-irradiated control group (Figures 26 and 27).

![Comparison of Irradiation vs Control](image)

**Figure 26: Survival Curve after Irradiation**

The results of 20 Gy dose of radiation on the cells showed the most effect on the group that wasn’t incubated with either NS or NP. Relative to the NS incubated group which had the best survival, the NP incubated group had an MTS assay absorbance reading that was 68% of the NS group and the cells only group had a reading of 44% of the NS incubated group with $p < 1 \times 10^{-4}$ in both cases.
CHAPTER 4: DISCUSSION

The use of nanoparticles in cancer treatments, particularly gold nanoparticles and silica-gold nanoshells, is a novel and emergent technology that has been shown to enhance dose effects *in vitro*, *in silico* and *in vivo*. Additionally, nanoparticles show promise in other treatment modalities such as photothermal therapy, and photodynamic therapy. Silica-gold nanoshells have the advantage over gold nanoparticles of having tunable localized surface plasmon resonances (LSPR), which can take advantage of NIR wavelength light for photothermal or photodynamic therapy, while maintaining an effective atomic number that is within 10% of that of gold nanoparticles ($Z = 79$) allowing for their use in combined radiation and NIR treatments.

The proof of concept of using gold nanoparticles was established when mice with subcutaneous EMT-6 mammary carcinomas were injected with 1.9 nm gold nanoparticles directly into the tumor (Hainfeld *et al* 2004). Treatment proceeded with 250 kVp x-rays and a dose of 30 Gy. Tumor volume was significantly smaller 30 days post-treatment for these mice vs. irradiation only controls. The 1.9 nm NP were chosen to avoid high liver intake and to take advantage of the permeable vasculature of tumors (Dvorak *et al* 1988). An additional study has shown that 68 keV (42 Gy) x-rays are more effective than 157 keV (44 Gy) x-rays in terms of tumor growth suppression (Hainfeld *et al* 2010). Energies greater than 80.75 keV are required to take advantage of K-shell interactions in gold while energies greater than ~13 keV are required for L-shell interactions.

The concept of dose enhancement from x-ray radiation due to the presence of gold nanoparticles is based on the photoelectric effect. Not only have experiments proven the effectiveness of this method, but Monte Carlo simulations have been carried
out that show the validity of enhancing dose to cells via photoelectric interactions due to the presence of gold nanoparticles (Cho et al 2005, Cai et al 2013, Lechtman et al 2013). It was shown that a dose enhancement factor of at least 2 was realized for 140 kVp photon beams (Cho et al 2005). Furthermore, a peak in the DEF was shown at 30/40 keV in a separate simulation (Cai et al 2013). These studies show that the experiments carried out with the equipment used in this thesis project should be capable of producing observable dose enhancement effects (kVp 110-130).

Spheroids are a proven in vitro method to model tumors in laboratory experiments. Aside from lacking the vascularization of a true in situ tumor, spheroids mimic the oxygen gradients that are observed in tumors. Multi-cellular hybrid spheroids constitute a more accurate representation of tumors as they model the effects of tumor associated monocytes/macrophages. The recognition that monocytes/macrophages can compose a significant portion of a tumor (~ 60 % – ~ 70 %), has provided the rationale for using them as a transport mechanism of nanoparticles in a technique called the “Cellular Trojan Horse” (Choi et al 2007). The monitoring and measurement of spheroid growth post-treatment is a standard technique for determining treatment efficacy.

The results in Figure 9 demonstrate a clear dose response for pure ACBT spheroids. As expected, spheroid survival decreases with increasing x-ray dose. These results are in qualitative agreement with those of Madsen et al. (2002) who used a Cs-137 gamma source (0.66 MeV) to irradiate ACBT spheroids to doses up to 16 Gy at a dose rate of ~ 1.6 Gy/min.

The hybrid spheroid results presented in Figures 10-13 are somewhat difficult to interpret. With the exception of a few anomalies, the data do show a dose response,
although not as pronounced as the corresponding data for the pure ACBT spheroids. Unfortunately, as illustrated in Figure 13, the most pronounced dose effect was observed in the pure ACBT irradiated spheroids which showed greater volume reduction compared to the nanoparticle and nanoshell loaded hybrid spheroids at each dose investigated. This is the exact opposite result one would expect based on increased photoelectric interactions in the nanoparticle and nanoshell spheroids. These anomalous results are likely due to the fact that the experiment was performed only once (due to equipment malfunction) and, as such, the data cannot be considered statistically significant. Furthermore, as the experiment was conducted at the very end of the useful lifetime of the x-ray tube, one cannot rule out the possibility of significant output fluctuations resulting in substantial dose variations and, hence significant dosimetry errors.

In the interest of expediency, it was decided to forego further investigations with hybrid spheroids in favor of murine monocytes in which survival was monitored using a colorimetric (MTS) assay. MTS assays allows for rapid determination of cell viability (a few days) compared to the time consuming spheroid growth assay which requires at least 3 weeks.

The data presented in Figures 14-24 provide valuable information concerning the optimal parameters for the MTS assay, and collectively, they represent the most significant finding of this thesis. As illustrated in Figure 14, there is a relatively broad range over which the MTS signal is linear with cell concentration (approximately 100 – 100,000) and this provided the rationale for choosing 50,000 cells per well (the approximate midpoint). The data shows that the MTS signal saturates at a concentration of approximately 100,000 cells above which the assay is no longer accurate.
The results illustrated in Figures 16-24 show that the duration between monocyte irradiation and expression of damage was at least 96 hours (Figure 19) since this represents the first time point at which there was a statistically significant survival difference between irradiated and non-irradiated bare monocytes. Furthermore, the data show that, as long as this 96 hour time interval was satisfied, any of the investigated MTS incubation times (1 – 4 hours) could be used. For expediency, the shortest (1 hour) time was chosen.

At the concentrations used in these experiments, neither the nanoshells nor the nanoparticles were toxic to murine lymphocytic monocytes. This is in good agreement with the findings of other investigators (Chhetri 2013; Makkouk 2010).

As shown in Figure 26, radiation was most effective in bare monocytes. This is similar to the findings for the hybrid spheroids (Figure 13) and is clearly anomalous based on expectations of increased photoelectric absorption in nanoshell and nanoparticle loaded monocytes. The reasons for these anomalous results are not entirely clear, but are likely due to inaccuracies in dosimetry. The replacement x-ray source used in these experiments was very old and the reliability of the tube output was uncertain since detailed dose measurements were not performed.
CHAPTER 5: CONCLUSION

The overall aim of this work was to investigate the dose enhancing effects of ionizing radiation on multi-cellular hybrid tumor spheroids consisting of human glioma cells and nanoparticle-loaded murine monocytes. The failure of a key piece of equipment (Faxitron RX-650) after only one trial halted further experiments and, as a consequence, the results obtained were inconclusive.

A second set of experiments was conducted with a different x-ray source (Faxitron 4385 A) and utilizing the MTS assay to determine cell viability. It was determined that a concentration of 50,000 monocytes per well in a 96 well plate yielded a sufficiently accurate MTS signal. Furthermore, it was shown that the time interval from irradiation to application of the MTS assay must be at least 96 hours in this in vitro system. As long as the 96 hour interval is satisfied, any of the investigated MTS incubation times (1 – 4 hours) may be used. As was the case with the hybrid spheroid results, the monocyte studies were inconclusive. This was most likely due to fluctuations in the x-ray tube output.

Future studies should be focused on using more reliable radiation sources including commonly used x-ray irradiators based on radioisotopes such as $^{137}\text{Cs}$ and $^{60}\text{Co}$. With the opening of UNLV’s Accelerator Center, access to high energy (megavoltage) sources will become available. This will facilitate more clinically relevant studies since the energies are identical to those used in radiation therapy of cancer patients and, as such, the true clinical potential of gold-based nanoparticles can be evaluated.
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