Preparation of Gold Nanoparticle-Cisplatin Conjugates and Investigation of their Toxicity in Zebrafish

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Preparation of Gold Nanoparticle-Cisplatin Conjugates and Investigation of their Toxicity in Zebrafish

A Senior Project submitted to
The Division of Science, Mathematics, and Computing
of Bard College

by

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Annandale-on-Hudson, New York
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Dedication

I dedicate this project to Geoffrey “Woody” Wooding, who died on January 26th, 2010 at the age of 55 from pancreatic cancer. Woody was like a second father to me from the age of 5, and his intense, passionate approach to life played a major role in inspiring me to become the person I am today.
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Abstract

The chemotherapy drug cisplatin is used to treat a wide variety of cancers; however, it has a number of side effects, such as damage to hearing or balance functions of the ear defined as ototoxicity, that can limit its use. The goal of this study was to use larval zebrafish as an in vivo model to investigate whether conjugation to gold nanoparticles (AuNPs) reduces cisplatin’s ototoxicity. AuNPs-cisplatin conjugates were synthesized using a seeded-growth approach, and the loading of cisplatin on the nanoparticles was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES). The conjugates synthesized were found to contain too low of a concentration of cisplatin to perform ototoxicity testing with. The results of systemic toxicity assays showed that the high (> 5 nM NPs) concentrations of AuNPs in the conjugates were themselves acutely toxic to zebrafish larvae. The findings suggest that severe systemic toxicity reduces the feasibility of using larval zebrafish as a model to study the effects of AuNPs at high (> 5 nM NPs) concentrations, providing important information about the concentration-dependence of AuNPs toxicity. However, no conclusions could be made about whether nanoconjugation reduces cisplatin’s ototoxicity.
Introduction

Cancer and Chemotherapy

For almost everyone, the word cancer evokes painful memories of a dearly beloved and departed friend or family member. Cancer is a large group of diseases involving uncontrolled cell division that leads to the growth of malignant tumors. As the average human lifespan has increased steadily in recent history, an increasing number of people are being diagnosed with cancer. Rightly so, cancer has the reputation as a deadly disease, and as of 2008 cancer accounted for almost 14% of the global death rate (Jemal, 2011). While advances in modern medicine over the past few decades have drastically decreased death rates from many illnesses, such as the two leading causes of death worldwide heart disease and infectious disease, cancer death rates have not decreased significantly (Varmus, 2006). There is no cure for cancer, but clinicians use a wide variety of treatments to manage the disease. The primary options for cancer treatment are surgery, radiation therapy, and chemotherapy all of which have the goal of completely eradicating cancerous cells, but unfortunately that goal is rarely achieved.

Traditional chemotherapeutic drugs act by impairing cell division, and correspondingly they kill rapidly dividing cells (Gerber, 2008). Rapid cell division is one of the defining features of cancer cells, but there are also many cells in the body that divide rapidly under normal conditions. As a result, chemotherapy not only kills cancer cells but also causes numerous common side effects by killing cells in the bone marrow, digestive tract, nervous system, and other healthy tissues (Love, 1989). The severe toxicity often associated with chemotherapy limits the dose of drugs that can be given, and with the lower drug doses tumors often develop resistance to the treatment (Luqmani, 2005). While there are certainly limitations to chemotherapy, chemotherapeutic drugs often offer clinicians the best chance to treat cancer patients.
Cisplatin

History. Cisplatin, or cis-diamminedichloroplatinum(II) [PtCl$_2$(NH$_3$)$_2$], is a paradigmatic chemotherapeutic drug that is widely used to treat a variety of cancers [Figure 1]. The compound itself has a long and interesting history that began with its initial synthesis and description as Peyrone’s Salt in 1844 by the Italian chemist Michele Peyrone (Peyrone, 1844). After its synthesis, the structure of Peyrone’s Salt was hotly debated for almost 50 years (Alderden, 2006). The structure was finally elucidated in 1893 by the German chemist Alfred Werner who deduced its square planar configuration and distinguished between the cis and trans isomers (Werner, 1893). Werner’s work with cisplatin played a part in his becoming the first inorganic chemist to win Nobel Prize, doing so in 1913, and the last one to do so for sixty years. After its structural elucidation, cisplatin existed in obscurity for decades until the accidental discovery of biological activity by the American chemist Barnett Rosenberg in 1965 (Rosenberg, 1965). Rosenberg was trying to measure the effect of electrical currents on bacterial cell growth, but instead he found that a compound formed in a reaction between constituents of the bacterial solution and platinum electrodes inhibited cell division itself. Rosenberg isolated ten platinum compounds formed in the ammonium chloride solution and determined that the molecule with biological activity was in fact cisplatin (Rosenberg, 1967). A very short time later, Rosenberg published results showing that cisplatin displayed potent antitumor activity in cancer model rats with solid tumors (Rosenberg, 1969). Encouraging results in animal studies led to cisplatin entering clinical trials in 1971, and it was approved for cancer treatment by the U.S. Food and Drug Administration (FDA) in 1978 (Loehrer, 1984).
Cisplatin’s translation from basic research discovery to clinical medicine occurred unthinkably fast by today’s standards, which indicates how much of a game-changing factor its introduction was for cancer treatment.

**Clinical Use.** Almost forty years after its serendipitous discovery, cisplatin is still one of the most successful anticancer drugs available. Even though it was first approved for treatment over thirty-five years ago, cisplatin is still prescribed as a first line treatment for many cancer diagnoses. In fact, cisplatin and its few effective derivatives are used in the treatment regimens of more than 50% of cancer patients (Gasser, 2011). Cisplatin’s early development and wide use to treat a variety of cancers has led to it being dubbed the “penicillin of cancer drugs,” a moniker that reveals its essential importance to chemotherapy (Trzaska, 2005). When new cancer treatments are developed, cisplatin is often used at the reference standard to which the novel treatments are compared. As one of the most popular chemotherapeutics, sales of cisplatin in the United States alone are about $500 million annually (Abu-Surrah, 2006). Initially approved to treat testicular and ovarian cancer, it has since been found to be effective against a wide range of solid-tumor cancers, including malignancies of the lung, head, neck, and bladder (Hambley, 2007). Cisplatin is most effective against testicular cancer, with a cure rate of between 85-90%, which is particularly impressive considering the 10% cure rate before the drug’s introduction (Wang, 2005). The drug is perhaps most famous for its role in helping cyclist Lance Armstrong win his battle against testicular cancer. The clinical success of cisplatin spawned great interest in the use of metal compounds as chemotherapy agents, but even as researchers have learnt more about cisplatin’s mechanism of action they still have been unable to significantly improve upon its cytotoxicity with newer generations of drugs.
**Mechanism of Action.** Although cisplatin was swiftly approved for treatment after the discovery of its cytotoxic activity, the exact mechanism by which cisplatin kills cells is still somewhat under debate. After intravenous administration, the drug moves from venous blood into cells by both passive diffusion and active transport (Gately, 1993). It had been postulated that cisplatin enters cells exclusively through passive diffusion, but evidence gathered over the past twenty years has implicated active mechanisms. In cancer cells, the copper transporter Ctr1 (Ishida, 2002) and the organic cation transporter Oct2 have been shown to play important roles in cisplatin uptake (Filipski, 2008). Before the drug is able to react with its target, the neutral cisplatin molecule must be activated by sequential aquation reactions that replace the cis-chloro ligands with water molecules (Hincal, 1979) [Figure 2]. The significant drop in chloride

![Figure 2](image-url)  
*Figure 2.* Depiction of the path taken by cisplatin from i.v. injection to DNA-adduct formation, including both passive and active cellular uptake and the activating aquation process.
concentration in the cytoplasm relative to the blood facilitates the aquation of cisplatin (Davies, 2000). The aquated form of the drug is highly reactive and can be inactivated in the cytoplasm by reacting with many thiol-containing nucleophiles, such as glutathione (GSH) (Kelland, 1993). After aquation, cisplatin is believed to exert its cytotoxicity by binding to DNA bases and forming adducts that eventually lead to cell death via apoptosis (Pinto, 1985).

Cisplatin has been shown *in vitro* and *in vivo* to covalently bond with the nucleophilic N\(^7\) atom of purine bases of DNA to form interstrand and intrastrand crosslinks (Eastman, 1987; Jung, 2007) [Figure 3]. Cisplatin-nucleobase binding occurs preferentially with intrastrand

![Cisplatin binding to DNA](image)

*Cisplatin forms:*
- ~65% intrastrand GG adducts
- ~25% intrastrand AG adducts
- ~5% interstrand GG adducts
- ~5% other intrastrand adducts

*Figure 3.* After entry into the nucleus, cisplatin preferentially forms covalent bonds to the nitrogen at position seven of purine bases. There is a preference for GG adducts over AG adducts and intrastrand adducts over interstrand adducts.
guanines. In all cases of binding, the ammine groups remain bound to the platinum. It was found that there is a linear correlation between the amount of platinum bound to DNA and the level of cisplatin cytotoxicity (Fraval, 1979). This result led researchers to explore the mechanisms by which cisplatin-induced nucleic lesions promote cell death. The DNA adducts formed by cisplatin are known to affect DNA replication, but there is no correlation between cisplatin’s DNA synthesis inhibition and cytotoxicity (Sorenson, 1988). The adducts cause distortions in DNA structure, namely bending and unwinding (Takahara, 1996). Numerous signaling proteins recognize and bind to these structural distortions in the DNA, which initiates various signaling sequences that can lead to apoptosis (Chaney, 2004). Formation of the platinum-DNA adducts does not invariably lead to cell death, as the adducts can be removed by DNA-repair pathways to repair the damage and promote cell survival. Of the major DNA-repair pathways, nucleotide-excision repair (NER) plays the leading role in removing cisplatin-induced lesions, and its level of activity is inversely correlated with cytotoxicity (Ferry, 2000). Ultimately, the relative intensities of the pro-survival and pro-apoptotic signals initiated after cisplatin exposure are integrated to determine the fate of the cell.

**Resistance.** While cisplatin has had a major clinical impact, the main limitation on the drug’s utility has been the high rate of chemoresistance. Despite the fact that patients consistently respond well to initial cisplatin treatment, resistance to the chemotherapeutic effects, i.e. induction of tumor cell apoptosis, often develops through several possible mechanisms (Galluzzi, 2012) [Figure 4]. Cisplatin resistance can either be acquired during chronic drug treatment or can be an intrinsic tumor characteristic (Rabik, 2007), and researching cases of intrinsic resistance has helped produce a better understanding of the mechanisms of resistance to cisplatin in general. Studies have shown that cisplatin resistance is mediated by two broad mechanisms:
first, a failure of enough cisplatin to reach the DNA; and, second, a failure of adduct formation to result in cell death. In the first case, insufficient platinum DNA binding can be the result of decreased drug uptake (Holzer, 2006), increased drug efflux (Samimi, 2004), and/or increased levels of cytoplasmic thiol-containing species, such as glutathione, that bind to and inactivate the platinum center (Mistry, 1991). In the second case, resistance mediated after DNA binding can be the result of increased removal of adducts from DNA or increased tolerance to the adducts. As mentioned earlier, the main repair pathway for removing platinum-DNA adducts is NER, and resistance can occur in tumors with hyperactive NER due to factors such as increased activity of the endonuclease protein ERCC1 (Dabholkar, 1992). Increased tolerance to the presence of the adducts can also occur through a decrease of DNA mismatch repair, bypassing of the adducts by polymerase β and η, or a downregulation of apoptotic pathways (Kelland, 2007). Cisplatin-resistant tumor cells have often been known to display multiple mechanisms of resistance. To combat resistance, clinicians are forced to administer very high doses of cisplatin. Unfortunately, however, the maximum dose is often not determined by the concentration of drug needed to eradicate the tumor, but instead is limited by severe systemic toxicity.

Figure 4. Mechanisms that can inhibit cisplatin-induced apoptotic signals and cause resistance in tumor cells (Siddik, 2003).
**Dose-limiting Side Effects.** Administering the high doses of cisplatin that are necessary to counteract resistance can lead to severe systemic toxicity, because cisplatin, like all chemotherapy drugs, kills both cancerous and healthy cells. Cisplatin’s side effects have been called “notorious,” and it has been questioned whether its use would have been continued to be approved if not for the development of several co-treatments, such as prehydration, to ameliorate the unwanted toxicities (Borch, 1989). The most clinically relevant and dose-limiting side effect is nephrotoxicity, defined as damage to the kidneys (Arany, 2003). The nephrotoxicity may in part be explained by the fact that the kidney accumulates and retains more cisplatin than all other organs and is the main excretory organ for the injected drug (Litterst, 1977). Cisplatin is mainly taken up by the proximal tubule and distal cells of the nephron, and the apoptosis and necrosis it causes there can produce severe and potentially irreversible renal failure (Miller, 2010). Cisplatin also frequently causes peripheral neuropathy and myelosuppression, as well as occasional gastrointestinal toxicity (Rabik, 2007).

Ototoxicity, defined as damage to the hearing or balance functions of the ear, is another serious dose-limiting side effect that occurs in up to 80% of patients treated with cisplatin (Knight, 2007). Research suggests that children are even more susceptible to cisplatin-induced ototoxicity than adults (Li, 2004). Cisplatin’s ototoxicity is caused by the death of mechanosensory hair cells in both the inner and outer ear (Cardinaal, 2000). Mechanosensory hair cells transduce physical vibrations into neurological impulses that allow organisms to hear, and their death is the cause of at least 75% of human deafness (Hawkins, 2004). Although various mechanisms have been proposed for how cisplatin damages hair cells, evidence suggests that the drug triggers the production of reactive oxygen species (ROS) that can trigger cell death (Rybak, 2007). While multiple protective treatments are being explored (Rybak, 2009), there is
currently no effective way to prevent cisplatin-induced ototoxicity. The severe systemic toxicity caused by cisplatin limits clinicians’ ability to administer large enough doses of the drug to successfully eradicate tumors before inducing resistance.

**Beyond Cisplatin**

*Next-generation Platinum Drugs.* Cisplatin’s spectacular cytotoxic action but high prevalence of resistance and undesirable suite of side effects spurred researchers to develop derivatives with decreased toxicity (Anderson, 2012). Since cisplatin came to the market in the late 1970s, countless similar compounds have been synthesized and tested for anti-cancer activity, but only carboplatin and oxaliplatin have been approved in the U.S. as additional platinum-based chemotherapy drugs. The next generation platinum drugs were designed based on the hypothesis that replacing the chlorides with a more stable leaving group might reduce the toxicity without affecting the chemotherapeutic effect (Kelland, 2007) [Figure 5]. While carboplatin and oxaliplatin have comparable cancer killing effects and somewhat reduced toxicity in comparison to cisplatin, the slight increases in drug efficiency came with vast increases in price (Lokich, 1998; Jung, 2007). Thousands of platinum compounds have been developed and tested for chemotherapeutic activity, but none have been found to confer significant advantages over cisplatin, carboplatin, and oxaliplatin (Eckardt, 2009; Kelland, 2002).

![Structural comparison of the three Pt chemotherapy drugs approved by the FDA.](image)
The lack of success in the cisplatin derivatives is truly incredible in light of the fact that the seminal compound was stumbled upon completely by chance.

The inability to make more efficient platinum-based chemotherapy drugs led researchers to explore a century-old concept that seemed like pure science fiction when first proposed. In 1911, Paul Ehrlich, a Noble Prize-winning German physician and researcher who has been called the “father of chemotherapy,” proposed the idea of a “magic bullet,” which is a theoretical compound that could be made to selectively target disease-causing cells (Strebhardt, 2008). If a “magic bullet” could be used to deliver chemotherapy drugs directly to tumors, then there would no longer be any need to worry about off-target side-effects, because the drug would only be available for action at the intended target. Much research has been done to develop a system that can specifically target drug delivery to cancer cells, and progress in this area is accelerating. 

**Molecularly-targeted Drug Delivery.** One area of research has focused on conjugating cisplatin and other chemotherapeutic drugs to species whose biological characteristics favor their accumulation in tumor cells. When attempting to target cytotoxic action, researchers looked inward and took a page out of the immune system’s playbook by using antibodies to target drug payloads to tumor cells (Scott, 2012). The goal of such work has been to combine the cell killing activity of cytotoxic agents, which often display little selectivity, with the antigen-specific affinity of monoclonal antibodies. While this approach sounds very promising in theory, in practice it is hampered by the fact cancer cells have evolved with a selection pressure to not express surface proteins that will mark them as foreign to the immune system (June, 2012). There are particular cases in which individual patients have tumor-specific antigens, but there is much debate as to whether the extreme cost of developing personalized antibody treatments, which still have concerns about poor efficacy, for these individuals is worth the short increase in
lifespan that the treatments might afford (Maeda, 2012). Efforts have also been made to conjugate cisplatin to antibodies that bind to proteins specifically expressed on tumor vasculature, such as vascular endothelial growth factor (VEGF) (Ko, 2008); however, results have indicated that such a treatment would only benefit an extremely small subset of patients. These results are especially discouraging for the future of antibody-targeted chemotherapy drug delivery, because tumor vascular cells are known to have far more distinct surface protein expression than tumor cells themselves do (Ruoslahti, 2010). There are examples of patients with rare variants of particular cancers responding better to cisplatin-antibody conjugates than to the free drug alone, but this does not appear to be the case for a large percentage of the patient population (Pegram, 1998). In summary, attempts to molecularly target cisplatin and other chemotherapy drugs to tumor cells have largely been disappointing (Cleeland, 2012), and researchers are still searching for alternate methods to target chemotherapeutics.

**Enhanced Permeability and Retention Effect.** ‘Passive’ targeting is an alternative approach to directly delivering drugs to tumor cells that takes advantage of the fact that most solid tumors have unique microenvironments that are not observed in normal tissues. In contrast to ‘active’ targeting techniques, such as antibody conjugation, passive targeting methods exploit tumor-specific characteristics that are far more universally valid than any particular molecular markers (Torchilin, 2011). The practice of passively targeting of drugs to tumors was first developed by Japanese researcher Hiroshi Maeda over 25-years-ago. The Maeda lab discovered that macromolecules and macromolecular drugs (i.e. nano-sized) delivered into the blood accumulate preferentially in tumor tissues, reaching significantly higher concentration than in the plasma or other organs (Maeda, 1984). After observing the tumoritropism of macromolecular drugs, the Maeda lab determined that this altered biodistribution is the result of abnormalities of tumor
vasculature that make the endothelial lining of the blood vessel wall more permeable relative to vasculature of other tissues (Matsumura, 1986). Tumor vasculature is comprised of disorganized, poorly aligned endothelial cells with wide fenestrations up to 4 µm, which is vastly wider than in normal vasculature (Farnsworth, 2013). Tumor vessels also have a wide lumen and lack a smooth muscle layer, both of which contribute to the hyperpermeability in tumor tissues (Greish, 2010). In addition, tumor cells hyperproduce vascular mediators, such as VEGF and bradykinin, that also enhance vascular permeability (Fang, 2003). The combination of these vasculature alterations allows macromolecules, including nanoparticles, to permeate far more efficiently from plasma into tumors than into other tissues and organs (Iyer, 2006). Additionally, the lack of a functional lymphatic drainage system in most solid tumors means that macromolecules are retained in the tumor for up to weeks (Dreher, 2006). The Maeda group dubbed this altered biodistribution seen in cancer patients, which results from the unique nature of tumor vasculature and lymphatics, the Enhanced Permeability and Retention (EPR) effect [Figure 6]. In addition to altering biodistribution, the EPR effect also increases the plasma

Figure 6. Schematic depiction of the vascular deformities in and around tumor tissue that cause the EPR effect and tumoritropism of nanoparticles (Dand, 2013).
half-life of nano-size drugs, because their size exceeds the limit of renal excretion threshold (Greish, 2010). Since its initial description, the EPR effect has become one of the few tumor-specific characteristics that is a “gold standard” for antitumor drug delivery with minimized off-target effects (Maeda, 2000).

Gold Nanoparticles (AuNPs) for Drug Delivery

Desirable Characteristics of AuNPs. Nanomaterials are frequently conjugated to anticancer drugs for the purpose of selectively targeting non-macromolecular drugs to tumor via the EPR effect. The nanomaterials most often used as delivery vehicles include polymers (Maeda, 1984), dendrimers (Svenson, 2009), liposomes (Maruyama, 2011), and nanoparticles (NPs) of various shapes and sizes (Ghosh, 2008). In the past decade, gold nanoparticles (AuNPs) have emerged as a promising candidate for the delivery of drug payloads to solid tumor targets (Paciotti, 2004). AuNPs have a variety of unique chemical and physical properties that make them very attractive for use as drug delivery systems (DDS) to exploit the EPR effect. First, AuNPs are easily synthesized in a wide range of shapes and sizes and with a high degree of size monodispersity (Bastus, 2011), the latter of which is especially important for developing a reproducible treatment. Once synthesized, AuNPs are readily functionalized through thiol linkages that use notoriously strong gold-sulfur interaction (Hakkinen, 2012). Thiol linkages are often used to create self-assembled monolayers (SAM) of organic molecules on gold surfaces (Love, 2005). The purpose of an SAM is to anchor a molecule with a functional end group to a substrate via a different functional head group that possesses a strong affinity for the substrate [Figure 7]. The functional end group at the end of the SAM tail can be used to bind other molecules that would otherwise be unable to bind to the substrate directly, and through this mechanism the surface of AuNPs can be functionalized with an incredible diversity of molecules (Woehrle, 2005). In
addition, AuNPs have distinctive optical properties due to their localized surface plasmon resonance (SPR) at approximately 520 nm that allow for optical characterization (Haiss, 2007).

While characteristics related to synthesis and characterization should be taken into account when designing a DDS to reduce the toxicity of a treatment it, it is of paramount importance that delivery vehicles do not have significant toxicity themselves.

**Toxicity of AuNPs.** The toxicity of AuNPs is an area of avid research, as fears about nanoparticles’ potentially toxic side effects are one of the major factors slowing their progression to mainstream clinical use. One thing that has been made clear by initial research on nanoparticle toxicity is that toxic side effects are dependent on both the concentration and chemical identity of the nanoparticles being tested (Braydich-Stolle, 2005). Bulk gold has long been used for medicinal purposes, including treating arthritis, but NPs are known to exhibit different properties from their bulk counterparts and much research is going into the cytotoxicity of AuNPs (Lim, 2011). So far, the majority of the research has shown that AuNPs have minimal toxicity, which has been found to depend on factors such as size, concentration, exposure time,
and surface functionality (Mironava, 2011; Bozich, 2014). Two seminal in vitro studies found that AuNPs are taken up by human cells, but that this uptake does not result in cellular toxicity (Connor, 2005; Chithrani, 2006). The results of other in vitro and in vivo studies have, on the other hand, produced decidedly mixed results. A study using human dermal fibroblasts found that incubation with AuNPs for six days caused an increase in apoptosis that was at least partially due to decreased expression of extracellular matrix proteins; however, their data also indicates that the cellular damage is not permanent and that cells can completely recover (Mironava, 2011). An in vivo mouse study found no evidence of toxicity after repeated intraperitoneal (i.p.) administration of 13 nm AuNPs (400 μg/kg/day) every day for eight days, even though all of the organs tested, including the brain, showed NP accumulation (Lasagna-Reeves, 2010). A different mouse study found that i.p administration of AuNPs of various sizes (8 mg/kg/week) for three weeks resulted in severe toxicity and lethality for mice exposed to AuNPs from 8 to 37 nm, but did not show harmful effects with AuNPs of 3, 5, 60, and 100 nm (Chen, 2009). The dearth of published studies on AuNPs toxicity and the lack of consistency of previous findings necessitates more research being done in this area. Still, there are a few specific examples of in vivo studies that suggest that AuNPs can effectively be used as drug delivery vehicles without inducing toxic side effects.

**AuNP-Cisplatin Conjugate Synthesis.** Recent research focusing on the vehiculation of cisplatin with AuNPs has provided very encouraging results. Cisplatin’s potent cytotoxic action but severe multiorgan toxicity profile makes it a perfect candidate for conjugation to nanoparticles. The use of AuNPs as a DDS for cisplatin was first proposed by Vazquez-Campos et al. as a means to increase the concentration of drug inside malignant tumor cells relative to healthy cells, and therefore reduce toxic side effects (Vazquez-Campos, 2008). Since the initial proposal of
AuNP-cisplatin conjugates, one research group non-covalently conjugated cisplatin onto hydrophilic assemblies of AuNPs-L-Aspartate nanostructures, and in *in vitro* tests found that the conjugates led to an increased susceptibility of cancer cells to treatment relative to free cisplatin alone (Tomuleasa, 2012). However, it is unlikely that Tomuleasa et al.’s results would translate to *in vivo* testing, as a comparison of the release of electrostatically-bound versus covalently-bound cisplatin from conjugates in the presence of serum showed that non-covalently-bound cisplatin conjugates are significantly less stable in physiological media (Comenge, 2010). It is critical that cisplatin remains bound to its delivery vector in physiological media, because release prior to entry into tumor cells would prevent the drug from exploiting the EPR effect as a conjugate. Comenge et al. developed a system in which AuNPs are first functionalized with mercaptoundecanoic acid (MUA) to create a SAM with carboxylic terminal groups, which is then reacted with cisplatin to form a coordination bond between the drug and the AuNPs (Vazquez-Campus, 2008) [Figure 8].

*Figure 8.* AuNP-cisplatin conjugate synthetic scheme, adapted from (Vazquez-Campus, 2008).
The key aspect of the covalent MUA-cisplatin bond is that the link is pH sensitive: at serum pH (~7.4) the coordination bond between the linker molecule and the drug is stable, but decreasing the pH (<5) leads to hydrolysis of the MUA-Pt bonds (Comenge, 2012). NPs are well known to be internalized via endocytic pathways (Nel, 2009) (Figure 9), and the decreasing pH within endosomes, especially late endosome and lysosome, promotes the release of cisplatin after internalization via protonation of the MUA carboxylic group. After release from AuNPs, cisplatin is able to escape the endosome and perform its cytotoxic action in the nucleus. By combining exploitation of the EPR effect to have AuNPs preferentially transport cisplatin to tumor cells and a pH sensitive coordination bond to promote drug release upon uptake by tumor cells, Comenge et al. were able to develop a “magic bullet” cisplatin delivery system.

*Figure 9.* Different endocytic pathways by which NPs are internalized (Research at Dordick J. S. Group). After uptake into endosomes, NP cargo must escape the endosome to reach the target of its cytotoxic action, which in the case of cisplatin is the nucleus.
**AuNP-Cisplatin Conjugate Testing.** After developing the AuNP-cisplatin conjugates, Comenge et al. showed in both *in vitro* and *in vivo* experiments that the conjugate design has significant, positive effects on cisplatin’s pharmacokinetics, biodistribution, and toxicity (Comenge, 2012). The researchers performed assays to quantify the Pt cell internalization and DNA accumulation in human lung carcinoma derived cell line A549 cells after treatment with free cisplatin or AuNP-cisplatin conjugates for up to 24 hours. Faster cellular uptake and higher cytoplasmic levels of Pt were found for the conjugate-delivered drug, which resulted in up to 125 times higher levels of Pt found in the DNA at 24 hours. The increased rate of uptake can be explained by the fact that AuNP-cisplatin enters cells via an active endocytic mechanism, as opposed to the passive diffusion and some active transport of free cisplatin into cells (Jung, 2007). The rapid accumulation of cisplatin in cells via endosomal uptake when delivered by AuNPs may help overcome resistance mechanisms that involved overexpression of cisplatin efflux proteins or underexpression of uptake proteins (Xue, 2013).

After proving that AuNPs-cisplatin conjugates have greater cellular uptake and better DNA targeting in *in vitro* experiments, the researchers tested the ability of the nanocarrier in comparison with free cisplatin to treat a mouse model of cancer. Tumor-bearing Severely Compromised ImmunoDeficient (SCID) mice were treated with two i.p. injections of saline, free cisplatin, or AuNP-cisplatin (1.5 mg/kg). It was found that nanoconjugation significantly reduced cisplatin-induced system toxicity and nephrotoxicity, as shown by tracking mouse body weight changes and histological studies of the kidney respectively, without affecting therapeutic benefit, as measured by tumor growth suppression. The finding of a clear reduction in nephrotoxicity is especially important, because nephrotoxicity is considered to be the dose-limiting factor in cisplatin treatment. The consistently positive results of the *in vitro* and *in vivo*
tests performed by Comenge et al. are very encouraging, and they show the need for future studies to further investigate whether AuNP conjugation is able to remove the toxic side effects of cisplatin while maintaining its therapeutic benefits. Currently, their mouse study is the only published *in vivo* study investigating the effects of AuNP-cisplatin conjugates.

**Zebrasfish Model**

Zebrasfish (*Danio rerio*) have emerged as useful and cost-effective model organism for *in vivo* testing that combines the scale and throughput of *in vitro* assays with the systems physiology of animal studies. Zebrasfish offer many advantages as a model organism including a fully-sequenced genome and relative genetic homology with humans, well-characterized mutant strains, large number of offspring, rapid embryonic development, embryonic transparency, low upkeep costs, and the ability to absorb drugs dissolved in liquid media (Zon, 2005). The feasibility of using zebrasfish to study the response of organisms to AuNPs and their derivatives has been demonstrated by various research groups. It was found that zebrasfish embryos raised from 5 hours post-fertilization (hpf) to 120 hpf in AuNP solution showed no toxic side effects at any of the sizes of AuNPs tested (Bar-Ilan, 2009). Another research group found that the diffusion of AuNPs in zebrasfish embryos led to slight, but not significantly different from the control, stochastic toxic effects, and concluded that AuNPs are biocompatible with zebrasfish (Browning, 2009). While the results of these studies are promising, a different study showed that zebrasfish embryos raised from 5 to 120 hpf in 1.5 nm AuNPs had significantly lower survivorship into adulthood and persistent behavioral deficits relative to controls (Truong, 2012). The lack of many research articles about AuNP zebrasfish toxicity is compounded by the fact that the existing articles vary greatly in factors such as nanoparticle size and treatment time, and there is a need for more comprehensive studies to address the toxicity of AuNPs to zebrasfish.
However, accessible evidence certainly does not rule out the possibility of performing tests involving AuNPs in zebrafish.

The use of zebrafish in pharmacological toxicology studies is becoming increasingly common, and is even being adopted by the pharmaceutical industry (Fleming, 2013). In fact, zebrafish are frequently used as a model to study cisplatin-induced ototoxicity (Ton, 2005; Ou, 2007; Vlastis 2012; Thomas, 2013). Zebrafish, along with all other fish and amphibians, express neuromasts- bundles of mechanosensory hair cells- along the sides of their bodies in a sensory system called the lateral line, which is closely related, and probably ancestral, to the human auditory system (Dambly-Chaudiere, 2003). The zebrafish lateral line is frequently used to study hair cells due to its easy access in comparison to the inner ear and its genetic homology relative to the human auditory system. Cisplatin is known to cause auditory impairment in cancer patients by killing mechanosensory hair cells in the inner ear, and it has also been shown to kill zebrafish lateral line hair cells in a dose-dependent manner (Ou, 2007).

Various mechanisms have been proposed for how cisplatin damages hair cells once inside the cell, including triggering oxidative stress (Ravi, 1995); however, until recently the mechanism of cisplatin uptake into hair cells was unknown. Efforts to prevent cisplatin-induced ototoxicity hinge on the development of a deeper understanding of the mechanisms through which this toxicity occurs. While transporter proteins Ctr1 and Oct2 have been demonstrated to play leading roles in cisplatin uptake into cancer cells, it was recently demonstrated that cisplatin-induced zebrafish lateral line hair cell death is dependent on the activity of mechanotransduction (MET) channels (Thomas, 2013). This finding strongly suggests that cisplatin enters hair cells through the MET channel, and that cisplatin-induced ototoxicity is dependent on that entry phenomenon. If this were the case, then it is likely that cisplatin-induced
ototoxicity could be reduced, if not prevented, by conjugation of the drug to a material, such as AuNPs, that is too large to fit through the MET channel, which has a diameter of approximately 1.2 nm (Fettiplace, 2009) [Figure 10].

![Figure 10. Hypothesized mechanism by which conjugation to AuNPs could reduce cisplatin’s ototoxicity. Once nanoconjugated, the effective size of drug is too large to fit through the MET channel and enter hair cells.](image)

**AuNP-Cisplatin Conjugate Testing in Zebrafish**

The goal of this study was to use larval zebrafish as an *in vivo* model to investigate whether conjugation to AuNPs reduces, if not eliminates, cisplatin’s ototoxicity. Comenge et al. have already demonstrated that AuNP-cisplatin conjugates retain the free drug’s cancer cell killing ability while significantly reducing its nephrotoxicity and systemic toxicity. The potential utility of AuNPs as cisplatin drug delivery systems would be greatly supported by evidence that nanoconjugation reduces the drug’s ototoxicity. The existing studies of AuNPs zebrafish toxicity, and AuNPs toxicity in general, have provided inconsistent results, so it is crucial that
more research is done on this subject. While AuNP-cisplatin conjugates were shown to be non-toxic to mice, it would be very significant to find that they are also non-toxic to a different model organism. The results of *in vivo* testing of AuNP-cisplatin conjugates with zebrafish larvae should provide information about the feasibility of using nanoconjugation to reduce ototoxicity, as well as about the general toxicity of high concentrations of AuNPs to zebrafish.
Experimental Methods

Synthesis of MUA-Capped Gold Nanoparticles.

Synthesis of Gold Seeds. AuNPs were synthesized according to a seeded growth strategy developed by Bastus et al. that is based on the reduction of gold salt by citrate (Bastus, 2011). Prior to synthesis, all glassware was thoroughly cleaned with acetone, rinsed in DI H$_2$O, and dried with pressurized air to help prevent unwanted nucleation and aggregation of AuNPs during the synthesis. A spatula fashioned from a plastic straw was used to transfer gold(III) chloride (Sigma Aldrich) due to the compound’s tendency to plate to other metals, and, before use in synthesis, the gold(III) chloride solution was syringe filtered using a 0.2 µm PTFE filter. Gold seeds were synthesized by using a micropipette to add 1 mL of 25 mM gold(III) chloride (AuCl$_3$) solution to a boiling solution of 150 mL of 2.2 mM sodium citrate dibasic sesquihydrate (Sigma Aldrich) in a 250 mL Erlenmeyer flask. The liquid was heated on a Corning hot stir plate and strongly stirred (stir setting = 5.5) by a 1” Teflon-coated magnetic bar. No reflux condenser was used with the goal of avoiding the presence of temperature gradients in the liquid that would result in uneven nanoparticle growth (Kimling, 2006). Upon addition of the gold, the color of the solution changed from colorless to yellow to bluish purple to pinkish red within three minutes. The reaction was allowed to run at 100 ºC for 10 minutes. Then, the temperature was decreased to 90 ºC and 2 mL of the solution were extracted via a Pasteur pipette for characterization by scanning electron microscopy (SEM) and UV-vis spectroscopy.

Seeded Growth of AuNPs. When the gold seed solution reached 90 ºC, 1 mL of 60 mM sodium citrate and 1 mL of 25 mM gold(III) chloride solutions were added by micropipette with a time delay of 2 minutes. After 30 minutes, another aliquot of 2 mL of the nanoparticle solution were
extracted for further characterization. By repeating this process of the sequential addition of sodium citrate and gold(III) chloride followed by a 30 minute reaction period, AuNPs of progressively larger sizes were grown. Six iterations of the sequential addition process were performed, and aliquots of the seven different AuNP solutions (including the seeds) were characterized by SEM and/or UV-Vis spectroscopy. After characterization of the AuNPs grown by the different number of seeded-growth steps, nanoparticles produced by 2 and 3 growth steps were selected for use in the rest of the study.

**Conjugation of MUA and Concentration of Functionalized AuNPs.** The conjugation of 11-Mercaptoundecanoic acid (MUA) (Sigma Aldrich) was performed based on the procedure developed by Comenge et al. (Comenge; 2010, 2012). 2.2 mL of 10 mM MUA basic solution were added to 50 mL of AuNP solution in a 125 mL Erlenmeyer flask and the solution was stirred gently (stir setting = 2, 1” Teflon-coated stir bar) overnight. The MUA solution required heat with stirring for 10 minutes and heated sonication for 15 minutes to fully dissolve the solid, and was basified with 7.5 µL of 2M NaOH per mL solution. Before addition to the AuNPs, the MUA solution was syringe filtered using a 0.2 µm PTFE filter. The MUA-solution was made fresh before each conjugation, because the thiol compound begins to aggregate and precipitate if left in solution for longer than a week. 50 mL of the MUA-AuNP conjugate solution formed overnight were decanted into a 50 mL centrifuge tube and centrifuged for 2 minutes at 2500 rcf. The quick centrifugation caused floating chunks of excess MUA to form a pellet, and the supernatant was decanted into another 50 mL centrifuge tube. 2.5 mL of a 200 mM, pH = 2.6 glycine (Sigma Aldrich)/ HCl buffer were then added to the 50 mL of MUA-capped AuNPs to destabilize them by protonation of the carboxylic acid functionality of the MUA self-assembled monolayer. The destabilized solution was then centrifuged for 30 minutes at 2500 rcf, the
supernatant was removed, and the nanoparticle pellet, which was actually more of a gel, was resuspended in 1 mL of 50 mM, pH = 8 tricine (Sigma Aldrich) buffer. It was critical that the centrifuge used was of swinging bucket style rather than fixed-angle style because the latter led to the accumulation of nanoparticles on one wall of the centrifuge tube, which prevented removal of the supernatant without disturbing the pellet and therefore decreasing the magnitude of concentration. The excess MUA that had not been removed by differential centrifugation was removed from the concentrated MUA-AuNP solution by a dialysis step (×500, overnight) using 50 mM, pH = 8 tricine buffer as the solvent and membrane tubing with a 3.5 kDa pore size, which is larger MUA but smaller than the AuNPs.

**Gold Nanoparticle Characterization Techniques.**

**UV-Vis Spectroscopy.** UV-visible spectra were acquired with an Agilent 8453 UV-Vis spectrophotometer. AuNP solutions (160 µL) were placed in a quartz Precision Cells Type 703M sub-micro cuvette with a 10 mm path length, and the spectral analysis was performed in the 200 to 1000 nm range at room temperature with a resolution of 1 nm. If the maximum absorbance ($\text{abs}_{\text{max}}$) of the solution was greater than 2 AU, then the sample was diluted so that the $\text{abs}_{\text{max}}$ was less than 2 AU. Citrate-capped AuNPs were diluted with DI H$_2$O (Narayanan, 2008), while concentrated MUA-capped AuNPs and AuNPs-cisplatin conjugates were diluted with 50 mM, pH = 8 tricine buffer. At the beginning of every analysis session, a background spectrum was collected with the cuvette containing 160 µL of DI H$_2$O. In between each individual sample analysis the cuvette was rinsed with DI H$_2$O, dried with pressurized air, and the outside was wiped with KimWipes. To facilitate comparison, all spectra were normalized to 400 nm (Rodriguez-Fernandez, 2006).
Scanning Electron Microscopy. AuNPs synthesized using the seeded-growth strategy were visualized using a TESCAN MIRA3 SEM operating at an accelerating voltage of 20.0 kV. Samples were prepared by pipetting 1μL droplets of the AuNP solutions onto copper tape and leaving them to dry in the air for at least two hours. The SEM images of the AuNPs were used for determining the size distribution and shape of the particles. For each sample analysis, a representative image was selected and the size of 100 particles was measured using ImageJ, which allowed for the calculation of the average size and standard distribution of the AuNPs.

Synthesis of Aquated Cisplatin \([\text{Pt}(\text{H}_2\text{O})_2(\text{NH}_3)_2]\)(\text{NO}_3)_2\).

The synthesis of aquated cisplatin was performed based on the procedure used by Comenge et al. (Comenge, 2012) [Figure 11]. An aqueous silver nitrate solution was prepared by dissolving 169 mg (1 mmol) \(\text{AgNO}_3\) (Sigma Aldrich) in 2.5 mL of DI \(\text{H}_2\text{O}\). The \(\text{AgNO}_3\) solution was added dropwise using a Pasteur pipette to a suspension of 150 mg (0.5 mmol) cisplatin (Sigma Aldrich) in 2.5 mL DI \(\text{H}_2\text{O}\) in a 25 mL Erlenmeyer flask. Upon the addition, \(\text{AgCl}\) precipitated and the yellow color of the initial mixture began to disappear. The resulting suspension was heated to 50 °C for 1 hour on a hot stirring plate. During this time, the reaction was shielded from light under a cardboard box, because light can initiate the conversion from \(cis-\left[\text{Pt}(\text{H}_2\text{O})_2(\text{NH}_3)\right](\text{NO}_3)_2\) to \(trans-\left[\text{Pt}(\text{H}_2\text{O})_2(\text{NH}_3)\right](\text{NO}_3)_2\) in solution (Wagstaff, 2012). When the reaction was complete the solution was a milky white, and the precipitated \(\text{AgCl}\) was removed by centrifugation at 3000 rcf for 15 minutes. The collected supernatant solution was syringe filtered using a 0.2 μm PTFE filter and evaporated to dryness on the rotovap in a 50 mL round bottom flask. The resulting light yellow, grayish residue (76±4% (mean±SD)) was further dried under high vacuum to afford a powder of the same color for further use and
characterization: Melting range (Recorded on Optimelt MPA100 with 1 °C/min heating) = 177.8-181.3 °C; FTIR (neat) 3309, 3237, 1481, 1339, 1275, 1185, 1140, 999 cm$^{-1}$ [Figure S3].

*Figure 11.* Depiction of the reaction performed to synthesize aquated cisplatin. Below the structure of the starting material and the product are photographs of their appearance.

**Conjugation of Aquated Cisplatin to MUA-Capped AuNPs**

The conjugation of aquated Cisplatin to MUA-Capped AuNPs was performed based on the procedure developed by Comenge et al. (Comenge, 2012). 5 μL of 16 mg/mL [Pt(H$_2$O)$_2$(NH$_3$)$_2$](NO$_3$)$_2$ aqueous solution were added to 1 mL of concentrated MUA-capped AuNPs suspended in 50 mM tricine buffer in a 1 Dram glass vial. The glass vials were used as reaction vessels because their small diameter allowed the minute volume of solution to reach a sufficient depth for a stir bar to be used. The solution was stirred minimally (stir setting = 1, 0.25” Teflon-coated stir bar) for 25 minutes, and then the reaction was stopped by removing the excess aquated cisplatin by dialysis (×500, overnight) using 50 mM, pH = 8 tricine buffer as the solvent and membrane tubing with a 3.5 kDa pore size. Quantification of cisplatin loading onto the AuNPs was performed using inductively coupled plasma optical emission spectrometry (ICP-OES).
ICP-OES Analysis of AuNPs-cisplatin Samples

ICP-OES data were obtained using a Teledyne-Leeman Labs Prodigy PS-1000 ICP spectrometer (Tufts University Department of Chemistry). Prior to analysis, both nanoparticle samples and standards were digested with 0.2 M nitric acid to avoid potential precipitation of metal salts, and a solution of 0.2 M nitric acid was used to collect a baseline reading. Samples of 50 mL of each standard and 5 mL of each nanoparticle solution were prepared. Nanoparticle solutions were diluted 1:50 to afford a large enough volume for analysis. Both platinum ($^{195}$Pt) and gold ($^{197}$Au) concentrations were measured.

Calibration standards were prepared containing both platinum and gold by serial dilutions of 1000 ppm platinum stock ICP solution and 1000 ppm gold stock ICP solution (Fluka) in 0.2 M nitric acid. The calibration standards contained: 0.025 ppm Pt and 0.25 ppm Au; 0.05 ppm Pt and 0.5 ppm Au; 0.2 ppm Pt and 2.0 ppm Au; 1.0 ppm Pt and 10.0 ppm Au; 2.0 ppm Pt and 20.0 ppm Au; 10.0 ppm Pt and 100.0 ppm Au. A 10.0 ppm solution of manganese was used as an internal standard to align the detector to the most intense portion of the plasma. Also, a 10.0 ppm solution of each element of interest was used to align the spectrometer to the most intense portion of the emission line for each element. The emission lines selected for each element were as follows: Pt = 214.423 nm; Mn = 257.610 nm; Au = 242.795 nm. Whenever a sample was not actively being run, DI H$_2$O was aspirated through the plasma.

After alignment of the detector, the ICP was calibrated using the standard solutions. Then, ICP-OES analysis of elemental concentrations in each of the nanoparticle sample solutions was performed. Instrument operating conditions used were RF power of 1.2 kW, 18 L/min coolant flow, 0.0 L/min auxiliary flow, and 32 PSI on the nebulizer. The spectrometer was kept at 34 °C, on low purge, and with an axial view of the plasma. The solution uptake rate was 1.4
mL/min, and a 40 second delay was programmed between beginning of sample uptake and start of emission recoding. The integration time was 10 seconds, and three integrations were performed per sample.

**Zebrailfish Strains and Husbandry**

Zebrailfish (*Danio rerio*) embryos of either sex were produced by matings of adult fish maintained on a 14:10 light-cycle in system water at 28.5 °C with pH ranging from 7.0-7.4 in the Bard College Biology Department zebrafish facility. All of these conditions are standard in zebrafish research (Lawrence, 2007). The adults used for mating were either Et4xTU/TL or Et20xTU/TL transgenic strains, so the offspring produced were either pure TU/TL or carriers of one of the two transgenes. Et4 transgenic zebrafish express green fluorescent protein (GFP) in hair cells of the lateral line (Lopez-Schier, 2006), and Et20 transgenic zebrafish express GFP in the neuromast supporting cells (Moon, 2011).

For matings, between 1-2 male and 2-3 female adult fish were placed together in a mating tank with fake vegetation during the day, and the next morning after 10 AM embryos were collected and sterilized in 0.5% bleach solution (Bates, 2006). Embryos were maintained in the zebrafish facility at densities of 20-50 animals per 100 mm² Petri dish in 28 °C 0.5× E2 media (7.5 mM NaCl, 0.25 mM KCl, 0.5 mM MgSO₄, 75 µM KH₂PO₄, 25 µM Na₂HPO₄, 0.5 mM CaCl₂, 0.35 mM NaHCO₃, 0.5 mg/L methylene blue). E2 media was changed daily, and larvae older than 4 days post fertilization (dpf) were fed once daily with micro powder food. At 3 dpf, larvae were imaged using fluorescence microscopy to observe GFP expression so that they could be sorted according to genetic strain. Et4 larvae were desired for use in the *in vivo* assays due to the easy imaging of their hair cells, but larvae of all strains had to be used as a result of the dearth Et4 embryos produced.
**Ototoxicity Assay**

To assess ototoxicity, 5 dpf zebrafish larvae were treated for 24 hours with test compounds that were dissolved in 50 mM, pH = 8 tricine buffer. The protocol was based on an established hair cell ablation procedure that involves treating zebrafish larvae in 50 μM cisplatin for 24 hours (Ton, 2005). Test compounds were dissolved in the tricine buffer instead of E2 media because the AuNP-cisplatin conjugates were suspended in tricine buffer, and keeping the solvent constant between treatment groups helped to control variance between the treatment conditions. Treatment conditions included 50 μM cisplatin, 20 μM cisplatin, 7 μM cisplatin, AuNP-cisplatin solution with 20 μM cisplatin, and 50 mM, pH = 8 tricine buffer as a control. For each treatment, between 3 and 6 larvae were used, and the small sample sizes were due to difficulty producing embryos in large numbers. For each treatment group, all of the larvae were placed into a well in a 24-well plate containing 1 mL of the particular treatment solution. At the end of drug treatment, the 6 dpf larvae were washed in E2 media (3×, 2 min), and their surviving lateral line hair cells were counted by fluorescence microscopy. The percentage of surviving hair cells following treatment was calculated relative to the tricine buffer controls.

**Fluorescence Microscopy Imaging**

The purpose of fluorescence imaging was to visually assess larval zebrafish hair cell integrity after exposure to cisplatin in the free form and conjugated to AuNPs. Due to the fact that all in vivo experiments were performed with a mixture of Et4, Et20, and TU/TL zebrafish larvae (not all of which expressed GFP in hair cells), the hair cells of all larvae were selectively labeled prior to imaging. All of the fish in each treatment group were labeled simultaneously and subsequently imaged individually. For vital labeling of hair cells, zebrafish larvae were immersed in a 50 μM solution of 4-(4-diethylaminostyryl)-1-methylpyridinium
iodide (DiASP, Sigma Aldrich) for three minutes at room temperature in the dark (Faucherre, 2009). The working DiASP solution was freshly prepared before each use from a 1 mg/mL stock stored in the freezer. Stained larvae were washed briefly in E2 media (3×, 1 min) to remove the excess fluorophore, anaesthetized in E2 media containing a few drops of Ethyl 3-aminobenzoate methanesulfonate (tricaine, Sigma Aldrich) solution for 30 seconds, and mounted in fresh E2 media on a glass slide. Once on the slide, larvae were aligned using a hair loop so that they were lying on their side [Figure 12]. Imaging consisted of counting the number of hair cells in each of the 3 most posterior tail neuromasts and acquiring representative images with one of two fluorescence microscope setups: Nikon Eclipse E600 microscope with a 100 W mercury lamp, a FITC filter, a ProgRes MF camera, and 4×, 10×, or 40× air objectives; Olympus IX71 microscope with a 100 W mercury lamp, 525/30 nm emission filter and 460/40 excitation filter, and a 20× air objective. After imaging, larvae were returned to Petri dishes marked with their treatment group.

Systemic Toxicity Assay

To assess the acute systemic toxicity of a single exposure to AuNP-cisplatin solutions, 5 dpf zebrafish larvae were treated in AuNP-cisplatin samples containing between 6 and 8 μM cisplatin for varying lengths of time. All AuNP-cisplatin solutions were suspended in 50 mM, pH = 8 tricine buffer. There were 5 fish per treatment group, and the groups were: AuNP-
cisplatin for 24 hours, AuNP-cisplatin for 12 hours, AuNP cisplatin for 4 hours, MUA-capped AuNPs for 24 hours, and 50 mM, pH = 8 tricine buffer for 24 hours. For each treatment group, all of the larvae were placed into a well in a 24-well plate containing 1 mL of the particular treatment solution. After the treatment time was complete, the number of surviving fish in each group was counted. The survival count was taken immediately after completion of the treatment, and did not take into account mortality occurring after this time that is still due to AuNPs exposure.

Data Analysis

Statistical significance values were obtained by independent-means t-test or a one-way analysis of variance (ANOVA) with a 95% confidence level using SPSS statistics program. Tukey post-hoc comparisons were used to analyze the significance of differences found by the one-way ANOVA. Statistical significance was set at p < 0.05.
Results and Discussion

Gold Nanoparticle Synthesis

Gold nanoparticles of different sizes were synthesized using a seeded-growth strategy that was based on reversing the order of addition of reagents in the Turkevich method (Kimling, 2006). Gold seeds were made by adding an aqueous AuCl$_3$ solution to a boiling solution of sodium citrate and allowing the reaction to run for ten minutes. Then, the temperature of the solution was decreased to 90 °C for the purpose of inhibiting the nucleation of new AuNPs, and therefore narrowing the size distribution of NPs grown from the seeds (Bastus, 2011). The seeded growth steps were carried out by sequentially adding more sodium citrate and AuCl$_3$ and allowing the reaction to run for 30 minutes between steps. The addition of Au$^{3+}$ precursor with each growth step acidifies the solution, and the sodium citrate is added to act as a pH buffer in the reaction (Ji, 2007). If the reaction solution becomes too acidic, then the carboxylic groups on the citrate molecules become protonated and can no longer provide electrostatic repulsion between NPs. So, the citrate is really fulfilling three roles by additionally acting as a reducing agent and capping agent throughout the synthesis of AuNPs. After synthesis, both Au seeds and AuNPs produced by subsequent growth tests were characterized. UV-vis spectroscopy was used to measure the optical properties of AuNPs solutions produced by the seeded growth synthesis [Figure 13]. For all solutions, the spectra show a symmetric SPR band that red shifts as a function of the number of growth steps. It is well known as the diameter of particles in AuNPs solutions increases the SPR band of those solutions undergoes a red shift in correlation with size increase (Amendola, 2009). The fact that the intensity of the SPR band increases with increasing number of growth steps also suggests that the seeded-growth strategy produces AuNPs of increasing size by performing additional growth steps on the seed solution.
Figure 13. Spectral characterization of AuNPs produced by seeded-growth synthesis. (a) UV-vis absorption spectra of AuNPs obtained after different number of growth steps. The increased red shift of the surface plasmon resonance (SPR) band after additional growth steps is due to an increase in diameter of the AuNPs. All spectra are normalized at 400 nm to facilitate comparison. Zoom of peaks shown in the inset. (b) Evolution of the SPR peak wavelength as a function of the growth step. (c) A photograph of AuNPs solutions produced by different number of growth steps.
Analysis of SEM images of Au seeds and AuNPs produced after two and six growth steps confirmed the growth of the nanoparticles [Figure 14]. The size of the AuNPs increased from $11.3 \pm 1.2$ nm as seeds to $14.9 \pm 1.7$ nm after 2 additions, and then to $22.1 \pm 2.2$ nm after 6 additions. In comparison, the group whose methods were being followed obtained AuNPs that increased from $13.5 \pm 2.1$ nm as seeds to $19.2 \pm 2.6$ nm after 2 additions, and then to $24.8.1 \pm 3.4$ nm after 6 additions (Bastus, 2011). While the standard deviations in diameter of the AuNPs produced in this experiment were lower than the standard deviations of the AuNPs produced by Bastus et al., the size distributions for the AuNPs in this experiment (Figure 14D-F) were not as narrow as the size distributions found by Bastus et al. Another discrepancy between the studies is that Bastus et al. found that growth decreases as the particle size increases, but the AuNPs synthesized in this experiment showed a linear relationship between number of growth steps and particle size [Figure S1]. It is expected that AuNPs growth should significantly decrease as the particle size increases, because the rate of growth is determined by the ratio of gold atoms added and those present at the seed surface (Bastus, 2011). Therefore, it is quite surprising that addition of a constant amount of gold to particles of increasing surface area led to linear AuNPs growth. One possible explanation for the discrepancy between the growth-rate findings in this experiment and established knowledge about the rate of this reaction is that the SEM used in this experiment for imaging has far lower resolution than the TEM used by Bastus et al. The relatively low resolution of the SEM could have resulted in imprecise measurements of AuNPs diameter. Still, the data from SEM imaging was very valuable, as it allowed AuNPs grown from 2 and 3 growth steps to be selected for further functionalization and use in the rest of the study. Also, attaining at least a rough measurement of AuNPs diameter was necessary for later calculations, such as the number of cisplatin molecules loaded per nanoparticle.
Figure 14. Scanning Electron Microscope (SEM) images of AuNPs synthesized by different number of growths steps in the seeded-growth synthesis. (a-c) SEM images of AuNPs obtained after 0 (Seeds), 2, and 6 growth steps, respectively. White scale bar in each image represents 200 nm. (d-f) Size distribution histograms of AuNPs obtained after 0 (11.3±1.2 nm), 2 (14.9±1.7 nm), and 6 (22.1±2.2 nm) growth steps, respectively. 100 NPs were measured for each group. The histogram insets show the appearance of AuNPs produced using that number of growth steps.
Gold Nanoparticle Functionalization

The process of attaching cisplatin to MUA-capped AuNPs to create an efficient antitumor drug was first proposed by Vazquez-Campos et al. (Vazquez-Campos, 2008) [Figure 15A]. The pH-sensitive bond between cisplatin and the linker molecule, MUA, promotes the release of cisplatin from AuNPs after endosomal uptake by tumor cells, which the conjugates preferentially accumulate near to due to the EPR effect. After the seeded-growth synthesis, AuNPs were modified with a self-assembled monolayer (SAM) of MUA. The gold-thiol linkages formed between MUA and the AuNPs displace the citrate molecules that had been capping the NPs. The MUA SAM increases the electrostatic stability of the AuNPs relative to their citrate-capped form, as measured by ζ-Potential (Comenge, 2012), and also provides carboxylic groups for further functionalization. UV-vis spectroscopy analysis showed that the SPR band of 2 growth step AuNPs shifted from 521 nm to 526 nm after MUA conjugation [Figure 15B]. Comenge et al. also reported a 5 nm red shift of the SPR band after conjugation of MUA to AuNPs (Comenge, 2012).

To enable increased cisplatin loading into AuNPs solution, the MUA-capped AuNPs were concentrated by a destabilization-resuspension process. pH 2.6 glycine/HCl buffer was added to the AuNPs solution to destabilize the NPs by protonating the MUA carboxylic acids. The destabilized solution was centrifuged, the supernatant was removed, and the pellet of MUA-capped AuNPs was resuspended in pH 8 tricine buffer. Dialysis was then performed on the colloidal solution to remove the excess, unbound MUA. UV-vis spectra of the MUA-capped AuNPs taken after concentration showed that the concentration process resulted in a 1 nm red shift of the solution’s SPR band [Figure 15B]. This finding suggests that slight aggregation could have occurred during the concentration process; although, the red shift could also just
reflect optical differences of the DI H\textsubscript{2}O and tricine buffer in which the preconcentration and concentrated MUA-capped AuNPs were diluted, respectively. Comenge et al. reported concentrating the MUA-AuNPs by up to a factor of 50 (Comenge, 2012), but in this experiment the MUA-AuNPs could only be concentrated by a little more than a factor of 3, as measured by UV-vis absorbance [Figure 15B]. Noticeable concentration could only be achieved after increasing the centrifugation time from 2 minutes, as suggested by Comenge et al., to 30 minutes, and even then the factor of concentration was far less than what was reported by Comenge et al. To gain a better measure of the exact factor of concentration that was achieved, future studies should measure [Au] before and after concentration using ICP-OES, instead of UV-vis. Ultimately, the concentration of cisplatin in the final drug-nanoparticle conjugate solution is limited by the concentration of NPs in the solution.

In the final synthetic step, MUA-capped AuNPs were functionalized with cisplatin through a coordinate covalent bond. As mentioned earlier, it has been shown that AuNPs with covalently-bound cisplatin are far more stable in physiological media than those electrostatically bound with cisplatin. Commercial cisplatin does not bind covalently with deprotonated MUA carboxylic groups on the NP, and instead forms an electrostatic attraction (Comenge, 2010). To enable the formation of a covalent bond, aquated cisplatin, [Pt(H\textsubscript{2}O)\textsubscript{2}(NH\textsubscript{3})\textsubscript{2}][NO\textsubscript{3}\textsubscript{2}], must be used in the conjugation reaction instead of commercial cisplatin. H\textsubscript{2}O is a better leaving group than Cl\textsuperscript{-}, which allows the aquated form of cisplatin to form covalent bonds with MUA carboxylic groups. Excess aquated cisplatin is initially added so as to achieve homogenous surface coverage by the partial conjugation. The conjugation reaction between aquated cisplatin and MUA-capped AuNPs must be carried out in solution with a pH between 8 (pK\textsubscript{a} value of MUA SAM) and 9 (pH at which aquated cisplatin is deprotonated to give less-reactive hydroxo
Figure 15. AuNP Functionalization. (a) Scheme showing the functionalization steps to produce the AuNPs-cisplatin conjugate drug delivery system, adapted from (Comenge, 2012). Photographs show the appearance of AuNPs at steps in functionalization process. (b) UV-vis spectra of AuNPs at the working conditions. Red-shift of the SPR peak occurs at the different stages of functionalization: the initial peak of 2 growth step, citrate-capped AuNPs shifted from 521 nm to 526 nm after MUA conjugation, from 526 nm to 527 nm after concentration, and from 527 nm to 529 nm after cisplatin conjugation. Zoom of peaks shown in inset.
complexes) to ensure formation of the conjugate (Comenge, 2012). After attachment of aquated cisplatin, the SPR band of the MUA-capped AuNPs solution red shifts from 527 nm to 529 nm [Figure 15B]. In comparison, Comenge et al. observed a 1.5 nm red shift upon conjugation of aquated cisplatin to MUA-capped AuNPs (Comenge, 2012). If too much aquated cisplatin is bound, then the negative charge of the MUA SAM is quenched and the AuNPs aggregate. So, the aquated cisplatin conjugation reaction was stopped after 25 minutes by removing the excess, unbound aquated cisplatin by dialysis. After completion of the synthesis of the AuNPs-cisplatin conjugates, the amount of drug loaded into the solutions was measured.

The concentration of both platinum and gold in the AuNPs-cisplatin conjugate solutions was measured using ICP-OES. The goal of the project was to investigate whether conjugation to AuNPs reduces cisplatin’s ototoxicity, and to do so the same concentration of the free drug and the NP-drug conjugate had to be given to different treatment groups for comparison. Therefore, it was essential to determine the concentration of cisplatin loaded onto the nanoparticles before the AuNPs-cisplatin conjugates were used in any in vivo assays. The fact that cisplatin is a simple inorganic molecule with one platinum atom per molecule means that the molarity of cisplatin bound to AuNPs can be measured by the molarity of bound platinum. To quantitatively determine the concentration of platinum and gold in the AuNPs-cisplatin solutions, the conjugates were first digested using 0.2 M nitric acid and then analyzed by ICP-OES. ICP-OES Calibration curves are shown in Supplemental Information [Figure S2]. Ten AuNPs-cisplatin samples were analyzed, including five samples from 2 growth steps AuNPs (14.9 ± 1.7 nm) and five from 3 growth steps AuNPs (16.7 ± 1.9 nm). One of the samples, Sample IX, was a AuNPs-cisplatin solution that had not had the excess cisplatin removed by dialysis. Sample IX was found to have vastly more platinum than any of the other dialyzed AuNPs-cisplatin solutions,
which revealed the effectiveness of the dialysis process. For eight of the nine AuNP-cisplatin conjugate solutions (excluding Sample X) the drug loading was approximately the same as had previously been demonstrated Comenge et al., which was about 500 molecules of cisplatin per AuNP (Comenge, 2012) [Table 1]. This result was expected, because the AuNPs synthesis and functionalization process performed in this experiment was almost identical to the one employed by Comenge et al. However, the total concentrations of platinum (up to 20.5 μM) and gold (up to 2680 μM) in all of the solutions were far lower than had been demonstrated by Comenge et al. (up to 380 μM Pt and 70 mM Au), because the AuNP concentration step was less successful.

Table 1. Analysis of AuNPs-cisplatin samples. NPs with a radius of 7.465 nm are from a two growth step synthesis, and NPs with a radius of 8.366 nm are from a three growth step synthesis. #Cisplatin/NP is given with calculated uncertainty (See Figure S4). See Table S1 also percent uncertainty values associated with these calculations. Even within the same synthetic methodology, samples differed greatly in both [Au] and [Pt] measurements. Samples I-VIII contained the expected number, based on past analysis of conjugates made using this synthetic scheme (Comenge, 2012) of cisplatin molecules loaded per AuNP. Sample IX contained drastically higher [Pt] because dialysis was not performed on this sample after addition of aquated cisplatin. Sample X contained higher [Pt] and a larger number of cisplatin/NP than all other dialyzed AuNPs-cisplatin samples. * determined from SEM data; # determined from ICP-OES data; $ calculated from SEM and ICP-OES data.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Radius/NP (nm)*</th>
<th>Au [uM]#</th>
<th>Pt [uM]$</th>
<th>#NP/mL$ x 10^{12}</th>
<th>NP [nM]§</th>
<th>#Cisplatin/NP$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample I</td>
<td>7.465</td>
<td>1510</td>
<td>6.38</td>
<td>8.85</td>
<td>14.7</td>
<td>435 ± 210</td>
</tr>
<tr>
<td>Sample II</td>
<td>7.465</td>
<td>696</td>
<td>4.17</td>
<td>5.07</td>
<td>6.76</td>
<td>617 ± 200</td>
</tr>
<tr>
<td>Sample III</td>
<td>7.465</td>
<td>1550</td>
<td>6.07</td>
<td>9.07</td>
<td>15.1</td>
<td>403 ± 450</td>
</tr>
<tr>
<td>Sample IV</td>
<td>7.465</td>
<td>1310</td>
<td>6.36</td>
<td>7.67</td>
<td>12.7</td>
<td>500 ± 820</td>
</tr>
<tr>
<td>Sample V</td>
<td>8.366</td>
<td>2680</td>
<td>6.95</td>
<td>11.1</td>
<td>18.5</td>
<td>376 ± 3800</td>
</tr>
<tr>
<td>Sample VI</td>
<td>8.366</td>
<td>2480</td>
<td>7.14</td>
<td>10.3</td>
<td>17.1</td>
<td>418 ± 1100</td>
</tr>
<tr>
<td>Sample VII</td>
<td>8.366</td>
<td>1440</td>
<td>4.60</td>
<td>6.00</td>
<td>9.96</td>
<td>462 ± 130</td>
</tr>
<tr>
<td>Sample VIII</td>
<td>8.366</td>
<td>2070</td>
<td>7.25</td>
<td>8.60</td>
<td>14.3</td>
<td>507 ± 1900</td>
</tr>
<tr>
<td>Sample IX</td>
<td>8.366</td>
<td>745</td>
<td>195</td>
<td>3.10</td>
<td>5.14</td>
<td>38100 ± 8400</td>
</tr>
<tr>
<td>Sample X</td>
<td>7.465</td>
<td>745</td>
<td>20.5</td>
<td>4.36</td>
<td>7.24</td>
<td>2840 ± 580</td>
</tr>
</tbody>
</table>

The greatly decreased gold molarity and number of NP/mL relative to the findings of Comenge et al. is likely a result of two factors. First, the fact that the conjugates synthesized in this experiment had between 3.10×10^{12} and 1.11×10^{13} NP/mL after concentration while
Comenge’s conjugates reportedly had $5.5 \times 10^{12}$ NP/mL before concentration suggests that AuNPs were synthesized in lower than expected initial concentrations. Second, UV-vis spectral data showed that the concentration process performed in this experiment only increased the NP concentration by slightly more than a factor of three, which is a far lower factor of concentration than was achieved by Comenge et al (up to 50 times). As expected there was more gold in the solutions of AuNPs from 3 growth steps than from 2 growth steps. An independent-samples t-test was conducted to compare the gold molarity of AuNPs-cisplatin solutions that had been made from AuNPs from 2 growth steps and 3 growth steps. There was not a significant different in gold molarity for 2 growth step AuNPs-cisplatin solutions ($1162 \pm 414 \mu M$) and 3 growth step AuNPs-cisplatin solutions ($1883 \pm 792 \mu M$); $t(8)=1.801$, $p=0.109$; however, the difference was approaching significance. However, none of the AuNPs-cisplatin solutions contained anywhere near the amount of AuNPs as the samples produced by Comenge et al. Therefore, even though the conjugates synthesized in this experiment had the same per NP drug loading as the conjugates synthesized by Comenge et al., there was simply too low a concentration of AuNPs in the solutions to achieve the previously published cisplatin concentrations.

AuNPs-cisplatin Sample X stands out as appearing to have significantly more cisplatin (~20$\mu M$) bound to the NPs than any other the other solutions. Yet, the very high number of calculated cisplatin molecules per NP for Sample X ($2840 \pm 580$ cisplatin/NP) indicates that something went wrong with this solution, because loading of more than about 500 cisplatin/ 15 nm AuNP has never been achieved (Comenge, 2012). It is possible that dialysis somehow failed for Sample X, which would explain the presence of excess platinum in the solution. Or the AuNPs could have aggregated sometime before cisplatin conjugation, as it is known that as NPs grow the number of cisplatin that can be attached to their surface increases exponentially (Craig,
 EITHER WAY, ICP-OES ANALYSIS REVEALED THAT AuNPs-CISPLATIN SAMPLES CONTAINED FAR LESS PLATINUM THAN HAD BEEN EXPECTED BASED ON PRIOR STUDIES, AND THIS FINDING MEANT THAT TESTING THE OTOTOXICITY OF AuNPs-CISPLATIN CONJUGATES AT DOES OF CISPLATIN THAT CAUSE HAIR CELL LOSE WAS NOT FEASIBLE WITHIN THE SCOPE OF THIS PROJECT.

ZEBAFISH TOXICITY TESTING

The original goal of this study was to use larval zebrafish as an in vivo model to investigate whether conjugation to AuNPs reduces cisplatin’s ototoxicity. Towards this end, it was first tested whether cisplatin kills larval zebrafish lateral line hair cells in a dose-dependent manner. Small groups of zebrafish larvae were treated in 50 mM, pH = 8 tricine buffer containing different concentrations of cisplatin for 24 hours, according to an established procedure (Ton, 2005). Many other groups performing cisplatin ototoxicity assays with zebrafish employ a 4 hour treatment period that requires up to 1 mM cisplatin to induce the same level of hair cell loss caused by treatment with 50 μM cisplatin for 24 hours (Ou, 2007; Uribe, 2013). The longer treatment time was employed in this study so that a lower concentration of cisplatin could be used to induce hair cell loss, and it is has been shown that the time of onset of hair cell death in the larval zebrafish lateral line is correlated with the dose of cisplatin delivered (Ou, 2007). Ultimately, this meant that with a longer treatment time a lower concentration of the drug would need to be incorporated into the nanoparticle conjugates, because the purpose of the study was to test to the ototoxicity of AuNPs-cisplatin solutions in comparison with the ototoxicity of the same dose of free cisplatin.

Results of the 24 hour ototoxicity assay showed that cisplatin does induce the death of larval zebrafish lateral line hair cells in a dose dependent manner [Figure 16]. While treatment with 7 μM cisplatin solution did not cause noticeable hair cell death relative to the control tricine
buffer treatment, the two higher cisplatin concentration solutions (20 and 50 μM) both caused significant cell death [Figure 17A-C]. During the same assay, one group of 6 larvae was treated with AuNPs-cisplatin Sample X, and, although 2 of the larvae died during treatment, the survivors did not have significant hair cell loss [Figure 17E]. One-way ANOVA testing revealed a significant difference in number of hair cells between the treatment groups in the ototoxicity assay (F(4,61) = 146.47, p < 0.001). A Tukey post-hoc test showed that there were no

![Figure 16. Cisplatin-induced ototoxicity. Dose response curve showing hair cell counts as a function of cisplatin concentration. Zebrafish larvae (N=3-5 fish per group) were treated in 50 mM, pH = 8 tricine buffer containing the indicated concentration of cisplatin for 24 hours. Then, larvae were stained with DiASP, a fluorophore that specifically labels hair cells, and imaged using fluorescence microscopy. Control larvae treated with 0 μM cisplatin were found to have 10.7 ± 0.7 hair cells per neuromast, and hair cell counts for other groups is given as a percentage of the control. The blue circle indicates the concentration of Pt in the AuNPs-cisplatin sample used to treat larvae, which did not induce any hair cell loss (See Fig 17).](image-url)
significant differences in hair cells counts for larvae in the control group, the 7 μM cisplatin group, and AuNPs-cisplatin Sample X group. However, larvae in the group treated with 50 μM cisplatin had significantly fewer hair cells than all other groups (p < 0.001). Larvae in the group treated with 20 μM cisplatin had significantly more hair cells than the 50 μM cisplatin group (p < 0.001), but significantly fewer hair cells than the other three groups (p < 0.001).

The ototoxicity assay provided important information, but was not able to produce definitive results about the ability of vehiculation to AuNPs to reduce cisplatin’s ototoxicity. The finding that a 20 μM dose of cisplatin was able to cause a significant reduction in hair cell count relative to the control suggests that AuNPs-cisplatin samples containing as low as 20 μM can be used for in vivo zebrafish ototoxicity assays. Initial analysis of ICP-OES data for elemental concentration showed that AuNPs-cisplatin Sample X contained 20.5 ±0.5 μM Pt [Table 1], and this sample was used for the ototoxicity assay on the assumption that it contained 20.5 μM cisplatin bound to

![Figure 17](image-url). Cisplatin-induced ototoxicity hair cell images. (a-e) DiASP-labelled bundles of hair cells of larvae treated in the 24 hour ototoxicity assay referenced in Figure 16. One representative image is shown per group. For scale, average diameter of a hair cell is 5 μm. Images show increasing hair cell loss with increasing cisplatin dose, but no hair cell loss for control larvae or AuNP-cisplatin Sample X treated larvae. It was determined that this sample likely did not have as much as 20 μM cisplatin, as was calculated (Table 1), so it probably was not a positive result. All images taken at 20× with an Olympus IX71 scope.
the AuNPs. The very low uncertainty (2\%) associated with the platinum concentration measurement shows the precision of this measurement [Table S1]. As mentioned previously, the high number of cisplatin molecules per NP in Sample X indicates it likely that either dialysis of excess cisplatin failed or aggregation of the AuNPs occurred prior to cisplatin conjugation. If dialysis of excess cisplatin failed, then unbound cisplatin in the AuNPs-cisplatin solution would lose cytotoxicity within a few days, because cisplatin in solution isomerizes into its trans form transplatin, which has greatly reduced bioactivity (Wagstaff, 2012). Therefore, failure of dialysis could explain why larvae treated with AuNPs-cisplatin Sample X showed no hair cell loss even though the solution had a concentration of platinum (20.5 \( \mu \text{M} \)) at which free cisplatin is known to cause hair cell death [Figure 17B,E]. If, instead of dialysis failure, aggregation of AuNPs before cisplatin addition explains the high drug loading per NP in Sample X, then the finding that treatment with this solution did not cause hair cell loss would support the hypothesis that conjugation to AuNPs can reduce cisplatin’s ototoxicity. However, without SEM images from which to measure the exact diameter of AuNPs in Sample X, it is impossible to know whether aggregation of this sample did occur and allow the actual loading of over 2800 cisplatin molecules per NP. Ultimately, results from the ototoxicity assay showed that cisplatin kills larval zebrafish hair cells at an over ten-fold lower concentration than achieved in AuNPs-cisplatin conjugates by Comenge et al., which suggests that it should be possible to perform AuNPs-cisplatin ototoxicity testing in zebrafish. Unfortunately, other than Sample X, none of the AuNPs-cisplatin samples produced in this experiment contained a high enough concentration of bound cisplatin that the same dose of the free drug is ototoxic.

The observation of 2 deaths out of the 6 larva treated for 24 hours with AuNPs-cisplatin Sample X prompted further testing of the systemic toxicity of the AuNPs-cisplatin conjugates to
zebrafish larvae. ICP-OES analysis revealed that all of the AuNPs-cisplatin samples contained a greater concentration of gold than was used in any existing study of AuNPs with zebrafish [Table 1]. The AuNPs-cisplatin samples prepared in this experiment were all measured to contain greater than 5 nM NPs, while the AuNPs zebrafish toxicity studies referenced in this paper used no more than 2 nM NPs for treatment (Truong, 2012). Seeing as how the toxicity of AuNPs has been shown to be dose dependent (Browning, 2012), the relatively high concentration of the AuNP-cisplatin conjugates suggested that they might cause greater toxicity than was observed in previous studies. Smalls groups (n=4-6) of larvae were treated in the AuNPs-cisplatin solutions for either 4, 12, or 24 hours, and Table 2 gives the exact assignments

<table>
<thead>
<tr>
<th>Treatment Solution</th>
<th>Treatment time (hr)</th>
<th>N</th>
<th>Fish Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample I</td>
<td>4</td>
<td>4</td>
<td>75</td>
</tr>
<tr>
<td>Sample IV</td>
<td>4</td>
<td>5</td>
<td>80</td>
</tr>
<tr>
<td>Sample VI</td>
<td>12</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>Sample VIII</td>
<td>12</td>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td>Sample II</td>
<td>24</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>Sample III</td>
<td>24</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Sample V</td>
<td>24</td>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td>Sample X</td>
<td>24</td>
<td>6</td>
<td>67</td>
</tr>
</tbody>
</table>

*Table 2.* Systemic toxicity testing of different AuNPs-cisplatin samples. The sample names refer to data given in Table 1. The average and standard deviation of percent survival were 78 ± 4% at 4 hours, 33 ± 11% at 12 hours, and 38 ± 24% at 24 hours.

of particular conjugate samples to the different treatment conditions, as well as the percent of larvae that survived in each sample [Table 2]. Larvae survival in the AuNPs-cisplatin solutions was found to generally decrease with time, but increasing the treatment time from 12 to 24 hours did not appear to significantly effect the mortality rate [Figure 18]. Although the very
Figure 18. Larval zebrafish systemic toxicity caused by treatment with AuNPs-cisplatin conjugates. The percent of larvae surviving AuNPs-cisplatin treatment for varying lengths of time is shown. Percentage of fish surviving the treatment was counted immediately after completion of the treatment. The 4 hour data point comprises two separate experiments with a total of 9 larvae; the 12 hour data point comprises two separate experiments with a total of 9 larvae; the 24 hour data point comprises four separate experiments with a total of 21 larvae. Only 25% (two experiments, n=8) of larvae treated in concentrated solutions of AuNPs-MUA for 24 hours survived (not shown), which indicates that it is likely that the systemic toxicity is due to the high concentration of AuNPs, instead of a specific trait of the AuNPs-cisplatin conjugates.

small samples sizes weaken any conclusions that can be drawn, the finding that only ~35% of larvae treated for 12 or more hours survive indicates the AuNPs-cisplatin solutions used in this experiment were acutely toxic. While the large standard deviation for the 24 hour data points shows the need for more data collection with larger sample sizes, it appears as if the systemic toxicity is not significantly increased by increasing the treatment time past 12 hours. To test whether the observed acute toxicity was due to the high concentrations of AuNPs themselves,
two groups with a total of eight larvae were treated in concentrated MUA-capped AuNPs solution for 24 hours. While ICP-OES data was not available for the concentrated AuNPs-MUA solutions, UV-vis absorbance data suggests that these solutions contained very similar concentrations of NPs to the AuNPs-cisplatin solutions [Figure 15]. Only 25% of larvae survived the 24 hour treatment in AuNPs-MUA solution, which strongly suggests that the acute systemic toxicity observed in the larvae treated with the AuNPs-cisplatin solutions was an effect of the high concentrations of AuNPs.

The link between AuNPs solution concentration and zebrafish larvae toxicity was supported by the finding of a modest correlation between the concentration of gold in AuNPs-cisplatin solutions and the acute systemic toxicity of those samples to zebrafish larvae [Figure 19]. When the survival percentages of the four different groups treated with AuNPs-cisplatin solution for 24 hours were compared, it was found that concentration of gold in the solutions moderately predicted their acute toxicity to the larvae. Given the prior similar finding of concentration-dependent AuNP toxicity in zebrafish, it is likely if this experiment had employed a greater number of groups in each treatment condition then the strength of the correlation between [Au] and acute zebrafish toxicity would have increased. The systemic toxicity results suggest that AuNPs acute toxicity to zebrafish is dose dependent in the concentration range of approximately 5 to 20 nM AuNPs, which has been shown to be the case in the concentration range of 0.025 to 1.2 nM AuNPs (Browning, 2009). The precision of the concentration data upon which this conclusion is based is demonstrated by the relatively low percent uncertainties associated with the ICP-OES gold concentration measurements [Table S1]. Considering the lack of published information available about the toxicity of 5 to 20 nM AuNPs to zebrafish and the
Figure 19. Relationship between gold concentration and systemic toxicity of AuNPs. The four treatment groups whose data is shown here are the four groups that underwent 24 hour treatment with AuNPs-cisplatin conjugates referenced in Figure 18. Percentage of fish surviving the treatment was counted immediately after completion of the treatment. Although the sample size is small, it appears that there is at least a moderate correlation between the concentration of gold and the acute systemic toxicity of that sample to zebrafish larvae. A line of best fit is shown and its $R^2$ valued is given.

small sample sizes used in this experiment, data from additional experiments is necessary before firm conclusions can be made about the toxicity of AuNPs in this concentration range to zebrafish. That being said, it appears as if concentrations of AuNPs above 5 nM are acutely toxic to zebrafish larvae, and the findings from this experiment provide evidence that this toxicity is concentration-dependent.
Conclusions

The intended goal of this project was to use larval zebrafish as an in vivo model to investigate whether conjugation to AuNPs reduces cisplatin’s ototoxicity. AuNPs-cisplatin conjugates were prepared, but two factors prevented their use in larval zebrafish ototoxicity assays. First, it was determined that the concentration of cisplatin bound to the conjugates was low enough that the same concentration of the free drug did not cause hair cell loss. The low cisplatin concentration prevented ototoxicity testing because the assay required that the same dose of drug given in conjugate form causes hair cell loss in free form. Second, it was found that the high concentration (>5 nM NPs) of AuNPs in the conjugate solutions was acutely toxic to zebrafish larvae over the 24 hour period required for ototoxicity testing. The small samples sizes and high deviation of the results of the systemic toxicity testing means that more data needs to be gathered before conclusions can really be made about the toxicity of >5 nM AuNPs to zebrafish larvae. Still, these initials results suggest that AuNPs at the concentrations used in this project are acutely toxic to zebrafish larvae. The systemic toxicity of AuNPs-cisplatin conjugates found in this study is intriguing, because Comenge et al. found that conjugates synthesized using the same protocol caused no systemic toxicity or nephrotoxicity in an in vivo mouse experiment (Comenge, 2012). The conjugates synthesized by Comenge et al. had up to 27 times higher AuNPs molarity than the conjugates prepared in this study, and yet they still did not induce systemic toxicity in mice upon i.p administration.

The different toxicities caused by AuNPs-cisplatin treatment in different model organisms both highlights the need for more studies to gather information about NP toxicity and also indicates that experiments using AuNPs at high concentrations should be performed using mice rather than zebrafish. As was mentioned earlier, the inconclusive findings of existing
studies on the toxicity of AuNPs necessitates the execution of a greater quantity of more detailed toxicity studies in a wider range of model organisms (Khlebtsov, 2011). Until more conclusive toxicity information is available, researchers will have to base experimental designs on the existing findings. In this vein, a logical future project would utilize a mouse model to investigate whether conjugation to AuNPs reduces cisplatin’s ototoxicity, because mice appear to tolerate significantly higher concentrations of AuNPs treatments than do zebrafish. It is possible that a contributing factor for why AuNPs-cisplatin solutions killed zebrafish larvae but tumor-bearing mice is that the biodistribution of the conjugates was altered by the EPR effect in the mice but not in the zebrafish. The use of zebrafish as a model for malignant tumors is well established (Amatruda, 2002), and future studies testing the efficacy of AuNPs-drug conjugates in zebrafish should utilize tumor-bearing fish so that the EPR effect can occur. However, before more AuNPs-drug conjugate testing is performed, thorough studies focusing on the acute systemic toxicity, and its concentration dependence, of AuNPs to zebrafish must be completed

Moving forward, the use of nanoparticles as delivery carriers to selectively target chemotherapeutic drugs to tumors is still very promising. The EPR effect remains a well documented mechanism by which drugs can be targeted to tumors, and researchers have even found that this tumoritropism can be enhanced by pre-treatment with drugs such as vasoconstrictors (Iyer, 2006). The passive targeting of NPs to tumors via the EPR effect has also been supplemented by the addition of tumor-targeting ligands, such as antibodies, to the NP surface that will also drive active targeting (Qian, 2008). Some research groups have added an imaging component to the antibody-functionalized NPs that allows for tracking of cellular uptake of the nanoparticles, and these groups have found that the combination of both passive and active tumor targeting leads to increased tumor cell drug uptake when compared to passive targeting
alone (Yang, 2009). The incredible precision of delivery achieved by the antibody-functionalized NPs-drug conjugates indicates that these systems have great potential for reducing the toxicity of chemotherapeutic drugs. Ultimately, the safety of these conjugates could be maximized by making the nanoparticle used for delivery out of a substance that is biodegradable with a lifespan that is limited to the therapeutic window of the drug being used (De Jong, 2008). Exciting work is being carried out to develop such biodegradable nanocarriers using genetically engineered drug material such as elastin-like polypeptides, silk-like polypeptides, and silk-elastin-like protein polymers (Shi, 2014). In the future, it is possible that biodegradable nanocarriers such as these will be used in the clinic to target the delivery of chemotherapeutic drugs like cisplatin to tumors, and therefore reduce chemotherapy’s toxic side effects.
References


Supplementary Information

Supplementary Figure 1. Linear relationship between number of growth steps in seeded-growth synthesis and diameter of AuNPs produced. Diameter measurements made from SEM images (Figure 14) using image analysis software ImageJ. It was expected that growth in diameter per growth step would decrease with increased number of growth steps, but this was not observed.

Supplementary Table 1. Percent uncertainties associated with the calculations given in Table 1 of the text.
Supplementary Figure 2. Calibration curves for ICP-OES analysis of [Au] and [Pt]. Standard solutions made by serial dilutions of 1000 ppm stocks in 0.2 M HNO₃.
Supplementary Figure 3. Comparison between IR spectra of aquated cisplatin (top) and commercial cisplatin (bottom). Differences between the two spectra help to confirm the synthesis of aquated cisplatin.