Manipulation of Gold Nanorod Physicochemical Properties to Enhance Biocompatibility, Uptake and Intracellular Preservation of Optical Properties for Bio-Imaging and Plasmonic Photo-Therapeutic Applications

Anthony B. Polito
Wright State University

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MANIPULATION OF GOLD NANOROD PHYSICOCHEMICAL PROPERTIES TO ENHANCE BIOCOMPATIBILITY, UPTAKE AND INTRACELLULAR PRESERVATION OF OPTICAL PROPERTIES FOR BIO-IMAGING AND PLASMONIC PHOTO THERAPEUTIC APPLICATIONS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

By

Anthony B. Polito III
B.S., Wright State University, 2002
M.S., Wright State University, 2004
M.S.H.S., George Washington University, 2009

2015
Wright State University
WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

August 10, 2015


__________________________
Saber Hussain, Ph.D.
Dissertation Co-Director

__________________________
David Cool, Ph.D.
Dissertation Co-Director

__________________________
Mill W. Miller Ph.D.
Director, Biomedical Sciences Ph.D. Program

__________________________
Robert E. W. Fyffe, Ph.D.
Vice President for Research and Dean of the Graduate School
ABSTRACT


Gold nanorods (GNRs) due to their tunable optical properties within the near infrared (NIR) region have been identified as strong candidates for new nano-based biomedical applications. Unfortunately, many have reported GNR cytotoxicity and it is still unclear how GNR aspect ratio (AR), surface charge and surface chemistry contribute to cellular association and cytotoxicity. GNR surface chemistry modifications have been reported to reduce cytotoxicity, however they can result in poor cellular uptake and loss of NIR optical properties preventing efficiency with bio-imaging and photo-thermal applications. The aim of this study was to determine if manipulation of GNR physicochemical properties could enhance biocompatibility while maintaining cellular uptake and preserving NIR optical properties for bio-imaging and plasmonic photo therapeutic applications. This study showed that surface chemistry is primarily responsible for cytotoxicity and cellular association of GNRs. In addition, tannic acid (TA) coated 11-mercaptoundecyl trimethylammonium bromide (MTAB) GNRs (MTAB-TA) displayed enhanced biocompatibility while maintaining high cellular uptake with preserved NIR optical properties, in vitro. Finally, MTAB-TA GNRs demonstrated significantly greater two photon luminescence microscopy image intensity and photo-
thermal cellular ablation compared to bare MTAB GNRs. These findings identify MTAB-TA GNRs as prime candidates for use in nano-based bio-imaging and photothermal applications.
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DEDICATION

To my wife and children, without your love and support I would never have been able to accomplish so much.
DISCLAIMER

The views expressed in this dissertation are those of the author and do not reflect the official policy or position of the United States Air Force, Department of Defense, or the U.S. Government.
I. INTRODUCTION

Properties of gold nanorods (GNRs)

Nanomaterials (NMs) are increasingly being developed for use in industrial, military and consumer products as well as in a vast array of biomedical applications (Keiper, 2003). The NMs have the potential to transform the medical diagnostics and treatment of diseases (Adlakha-Hutchison et al., 2009; Barreto et al., 2011). Gold NMs due to their unique optical properties have been identified as a prime candidate for nano-based biomedical applications. Another key property of gold NMs is that they can easily be joined with other materials because of their affinity to sulfhydryl (-SH) groups (Boca & Astilean, 2010; Hakkinen, 2012). This allows for enhancement by the functionalization of gold NMs with metal, organic or biomolecular functional groups, and in turn can significantly increase their efficiency in a range of applications (Vigderman et al., 2012).

Recent advances in nanotechnology made it possible to manipulate gold NMs into different sizes, shapes and surface structures (Ratto et al., 2010; Tan et al., 2012). Gold nanorods (GNRs), due to their anisotropic shape, possess a number of shape-specific advantages for biosystem use, including: enhanced surface area, unique spectral signature, and tunable optical properties via alteration of their aspect ratio (Agarwal et al., 2011; Alkilany & Murphy, 2010; Bouhelier et al., 2005; Kelly et al., 2003). The aspect ratio describes the proportional relationship between the nanomaterial’s width and height. Depending on the GNR’s aspect ratio, a narrow range of light frequencies induce
conduction band electron oscillation, a resonance called surface plasmon resonance (SPR) (Liang et al., 2012). This property of GNRs has been used in sensing and imaging applications resulting in greater sensitivity and resolution than traditional technologies (Huang et al., 2011; T. Wang et al., 2013). In addition, the spectral signature of GNRs extends into the near-infrared (NIR) region, where aqueous biological tissue absorbs minimal amounts of light (Huang et al., 2006; Weissleder, 2001). GNRs with a SPR between 650 and 900 nm fall within the “water window”, allowing for deep-tissue penetration making them ideal for minimally invasive nano-based bio-sensing and biomedical applications. Based on these unique characteristics, GNRs have shown great potential in a vast array of bio-applications including: imaging, sensing, photo-thermal therapy and drug or gene delivery (Agarwal et al., 2011; Alkilany & Murphy, 2010; Bouhelier et al., 2005; Kelly et al., 2003).

**Current barriers to bio-applications of GNRs**

While GNRs have shown great potential, several factors currently hinder further development for use in nano-based bio-applications. One major limitation is that it is still unclear how the physicochemical properties of aspect ratio, charge and surface chemistry contribute to GNR cellular association and uptake, and ultimately the material’s biocompatibility (Alkilany & Murphy, 2010; Alkilany et al., 2009; Alkilany et al., 2012; Sohaebuddin et al., 2010).

Bulk gold is considered to be “safe” and chemically inert. Gold has been used in bio-applications and medicine for decades in devices such as dental restorations, pacemakers and gold plated stents and for treatment of a variety of pathologies including: syphilis, tuberculosis and more recently rheumatoid arthritis (Panyala et al., 2009).
However, as the use of GNR for bio-applications is comparatively new a consensus on their overall safety has not been reached (Sohaebuddin et al., 2010). For example, several studies have reported toxicity both *in vitro* and *in vivo* after exposure to GNRs (Khlebtsov & Dykman, 2011; Kunzmann et al., 2011; Li & Chen, 2011; Tang et al., 2015).

In addition to GNR biocompatibility, the cellular association and uptake of GNRs is required for several biomedical applications (Webster et al., 2013). For example, cellular association and/or internalization of GNRs are critical for applications such as imaging, drug carriers and photo-thermal therapy (Hauck et al., 2008; Yang et al., 2012; Zhang et al., 2013). While still unclear, past studies suggest that GNR properties of size, charge, aspect ratio and surface chemistry can control their cellular uptake (Debroesse et al., 2013; Huff et al., 2007; Qiu et al., 2010).

**Functionalization of GNRs to reduce cytotoxicity**

One of the known causes of the toxicity of GNRs is believed to be due to cetyl trimethylammonium bromide (CTAB), a cationic surfactant used in the aqueous synthesis of GNRs (L. Wang et al., 2013; Wang et al., 2011). CTAB however, is essential during GNR synthesis as it forms a micelle around the gold, stabilizing it in a rod formation. Washing can be used to reduce the amount of free CTAB in the GNR suspension however, if the level of CTAB falls below the CTAB’s critical micelle concentration (1 mM in water and at 25°C), the GNRs will destabilize and fall out of suspension (Fuguet et al., 2005; Quirion & Magid, 1986). Therefore, new strategies had to be developed to address CTAB’s toxicity.
Two main strategies have been reported to overcome the cytotoxicity of CTAB: over coating by layering through electrostatic attraction and replacement by ligand exchange (Vigderman et al., 2012; Zhang et al., 2013). While these surface modifications have had varying levels of success at mitigating the cytotoxicity of “bare” CTAB GNRs, these modifications have often had the unintended consequence of altering cellular uptake and increasing particle aggregation (Alkilany & Murphy, 2010). For example, polyethylene glycol (PEG) functionalization, can disrupt cellular uptake of the GNRs into cells, significantly reducing their beneficial value in biomedical applications (Grabinski et al., 2011). Other surface modifications, such as functionalization with targeting peptides, are prone to particle aggregation and fusion when they are taken up by cells, resulting in loss of optical properties (Untener et al., 2013). Finally, these over coating and replacement procedures can be complicated multi-step processes that are difficult to scale up for commercial bio-applications.

Two GNR surface modifications that hold significant promise are the phenolic compound tannic acid (TA) and MTAB (11-mercaptohexadecyl trimethylammonium Br), a thiol analogue of CTAB. It has been previously reported that TA coated GNRs show reduced toxicity, demonstrate a distinctive form of endosomal uptake and display a unique intracellular distribution pattern that reduces particle aggregation and fusion (Alkilany et al., 2009; Untener et al., 2013).

Recently the Zubarev lab, using proton nuclear magnetic resonance spectroscopy, demonstrated that complete replacement of CTAB with MTAB was possible, unlike many other thiolated compounds (Vigderman et al., 2012). In MTAB replacement surface chemistry modification, the CTAB micelle bilayer around the GNR is exchanged with a
monolayer of MTAB that strongly bonds to the GNR. The MTAB GNRs showed no toxicity in human breast adenocarcinoma cell line MCF-7 even at high concentrations. In addition they reported 40% of MTAB GNRs to be taken up by the cells, exceeding previously reported uptake values. Unfortunately, this high uptake also resulted in extensive aggregation and fusion of intracellular particles, suggesting a loss of their key optical properties. In particular, they show side by side assembly of GNRs which results in a blue shift in plasmon resonance that would move the GNR spectra out of the target NIR “water window” needed for biomedical applications.

**Nano-based bio-imaging and plasmonic photo therapy applications**

Nano-based bio-imaging and plasmonic photo therapy hold great promise as tools to fight cancer. The use of GNRs has been described in a variety of bio-imaging modalities including photoacoustic tomography, surface enhanced raman scattering, x-ray computed tomography and two-photon luminescence (Boca & Astilean, 2010; Eghtedari et al., 2007; von Maltzahn et al., 2009; Wang et al., 2005; T. Wang et al., 2013). Of all these nano-based bio-imaging techniques, two-photon luminescence imaging has been shown to provide the highest contrast and spatial resolution (Tong et al., 2009).

In addition, GNRs have been successfully used in plasmonic photo therapeutic applications including photo-thermal therapy and photo-dynamic therapy (Huang et al., 2008; Vankayala et al., 2014). GNRs with ARs of approximately three and greater have spectral profiles that demonstrate longitudinal absorbance in the NIR region. When these GNRs are exposed to corresponding NIR laser irradiation, the GNRs absorb photons and this energy is converted to heat and can be used to kill targeted cells (Chatterjee et al.,
2011). Photo-thermal therapy requires no additional drugs/chemicals to kill cells, unlike photo-dynamic therapy.
II. HYPOTHESIS AND STRATEGIC AIMS

Hypothesis:

The manipulation of GNR aspect ratio, surface charge and chemistry can enhance their biocompatibility and cellular uptake while preserving key optical properties \textit{in vitro} for nano-based bio-imaging and plasmonic photo-thermal applications.

Strategic Aim 1: To determine the effect of GNR AR, surface charge and chemistry on cellular association/uptake and cytotoxicity, \textit{in vitro}.

Strategic Aim 2: To determine if manipulation of key physicochemical properties of GNRs can preserve optical properties after cellular uptake and evaluate their bio-imaging and plasmonic photo-thermal properties \textit{in vitro} using two-photon luminescence microscopy and plasmonic photo-thermal cellular ablation.
III. CHAPTER 1: EVALUATION OF THE EFFECT OF GOLD NANOROD ASPECT RATIO, SURFACE CHARGE AND CHEMISTRY ON CELLULAR ASSOCIATION AND CYTOTOXICITY, *in vitro*
SUMMARY

Gold nanorods (GNRs), due to their unique optical & electronic properties are popular candidates for novel nano-based biomedical applications. Unfortunately, previous studies have reported the potential for GNR cytotoxicity related to the materials physicochemical properties. Even so, it is still unclear how the physicochemical properties of aspect ratio (AR), surface charge and surface chemistry contribute to GNR cellular association and cytotoxicity. Here I report that surface chemistry is primarily responsible for cytotoxicity and cellular association of GNRs. Results demonstrated that with the removal or sequestration of cetyl trimethylammonium bromide (CTAB) both negatively and positivity charged GNRs had significantly enhanced biocompatibility. In addition, when GNR deposition was accounted for, the materials AR and primary surface charge had minimum overall impacted on cytotoxicity and cellular association of GNRs. These findings identify surface chemistry as primarily responsible for cytotoxicity and cellular association of GNRs, enabling the development of GNRs with enhanced biocompatibility for new nano-based biomedical applications.
INTRODUCTION

Nanomaterials hold the potential to transform an unprecedented range of industries including the medical diagnostics and treatment of diseases (Adlakha-Hutcheon et al., 2009; Barreto et al., 2011). Gold nanorods (GNRs), due to their unique optical properties, have been identified as a strong candidate for many nano-base biomedical applications. The optical properties of GNRs are tunable by changing the aspect ratio (AR) or surface chemistry (Bouhelier et al., 2005). The AR describes the proportional relationship between the nanomaterial’s width and its height. Depending on the GNR AR, a narrow range of frequencies of light induce conduction band electron oscillation and this resonance is called the surface plasmon resonance (SPR). For GNRs it occurs in the visible and near-infrared of the spectrum. GNRs with ARs of approximately 3 and greater have SPRs in the near-infrared range where light has minimal impact on living cells (Huang et al., 2006). In addition, by combining GNRs of differing ARs and therefore differing near-infrared SPR wavelengths would enable multiplexing of a multitude of biomedical applications. (Sepúlveda et al., 2009; Wijaya et al., 2009; Yu & Irudayaraj, 2006). These include diagnostic imaging, photo-therapies, and drug/gene delivery (Agarwal et al., 2011; Nagesha et al., 2007; Pandey et al., 2013; Pissuwan et al., 2008). Unfortunately, many studies have reported GNR cytotoxicity preventing the implementation of new GNR-based biomedical applications.
Physicochemical properties (size, shape, aspect ratio, charge and surface chemistry) of GNRs have all been suggested to influence both cellular uptake and cytotoxicity of these materials (Goodman et al., 2004; Grabinski et al., 2011; Schaeublin et al., 2011). Even so, there exists considerable uncertainty as these and previous studies failed to comprehensively control for the physicochemical properties of GNRs. The aim of this study is to determine how GNR AR and surface charge affects GNR cellular association and cytotoxicity, \textit{in vitro}. This will be evaluated by comparing different ARs (approximately 1, 2, and 3) of a negatively charged sets of GNRs to a positively charged sets of GNRs, (Tannic acid (TA) coated cetyl trimethylammonium bromide (CTAB-TA)) GNRs and (11-mercaptoundecyl trimethylammonium bromide (MTAB)) GNRs. I hypothesize that by manipulating GNR aspect ratio, surface charge and chemistry we can enhance their biocompatibility while maintaining their cellular uptake.
MATERIALS AND METHODS

Synthesis of GNRs

MTAB GNRs (MTAB-1, MTAB-3 and MTAB-3) were purchased from Nanopartz (Loveland, CO, USA). All other GNRs (CTAB, CTAB-TA-1, CTAB-TA-1, CTAB-TA-3, CTAB-TA and PEG) were synthesized according to a modified seed mediated procedure reported by Park and Vaia (Kyoungweon Park & Vaia, 2008). Briefly, a seed solution of CTAB (0.1 M) and chlorauric acid (0.1 M) is combined at room temperature with a growth solution of CTAB (0.1 M), chlorauric acid (0.1 M) silver nitrate (0.1 M) ascorbic acid (0.1 M). The CTAB was purchased from GFS chemicals (Powell, OH, USA). The chloroauric acid, ascorbic acid, silver nitrate, sodium borohydride, sodium Chloride, MOPS buffer and tannic acid were obtained from Sigma Aldrich (St Louis, MO, USA).

PEG functionalization of GNRs

CTAB GNRs were functionalized with PEG as previously reported (Untener et al., 2013) with modifications. Briefly, the GNRs were functionalized overnight with 1 mM thiol PEG (Nanocs, Boston, MA) two times (MW 20000 followed by MW 5000) to displace the surface bound CTAB molecules. The GNR samples were centrifuged at 8,000g and the supernatant was removed and replaced with sterile water to remove residual free CTAB.
**TA functionalization of GNRs**

**Method 1:** was used for the functionalization of CTAB-TA-1, CTAB-TA-2 and CTAB-TA-3 GNRs. CTAB GNRs were functionalized with TA as previously reported (Untener et al., 2013) with modifications. Briefly, the GNRs were functionalized overnight with 1 mM TA three times to overcoat the surface bound CTAB molecules. The GNR samples were centrifuged at 8,000g and the supernatant was removed and replaced with sterile water to remove residual free CTAB and TA.

**Method 2:** was used for the functionalization of TA-3 GNRs. CTAB GNRs were functionalized with TA according to a modified procedure reported by Ejima et al (Ejima et al., 2013) stepwise with TA (24mM), and MOPS buffer (100 mM, pH 7.4) with vortexing after each addition. The GNR samples were then centrifuged at 3,000g and the supernatant was removed and replaced with sterile water to remove residual TA. The MOPS buffer and tannic acid were obtained from Sigma Aldrich (St Louis, MO, USA).

**Characterization of GNRs**

GNR characterization was performed to determine their key physicochemical properties and to verify particle uniformity prior to experiments. The purity and spectral signature of the GNRs were analyzed before use with UV–Vis spectrometry on a Bio TEK Synergy HT (Winooski, VT, USA) instrument. For evaluation of rod size and morphology, nanoparticles in solution were placed onto a formvar carbon coated copper TEM grid (Electron Microscopy Sciences, Hatfield, PA, USA) and dried. They were imaged with transmission electron microscopy (TEM) using a Hitachi H-7600 with an accelerating voltage of 120 kV. To assess the surface charge of the GNRs, zeta potential
measurements were taken using laser Doppler electrophoresis on a Malvern Zetasizer, Nano-ZS. Agglomerate sizes of the GNRs in media were determined through dynamic light scattering (DLS), also on a Malvern Zetasizer (Malvern Instruments, MA, USA).

**Cell culture conditions**

The human lung, A549, cell line (American Type Culture Collection (ATCC), Manassas, VA, USA) and human liver, HepG2 cell line (ATCC, Manassas, VA, USA) were maintained in RPMI 1640 cell culture media (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin streptomycin. Cells were maintained in a humidified incubator controlled at 37 °C and 5% CO2. The same media composition was used for all GNR exposure procedures.

**Cellular viability assessment**

A549 human lung cell viability was evaluated using the CellTiter 96 Aqueous One Solution (MTS) (Promega, Madison, WI, USA) which monitors mitochondrial function and MultiTox-Glo Assay (LCDC) (Promega, Madison, WI, USA), which sequentially measures two protease activities; one is a marker of cell viability, and the other is a marker of cytotoxicity. Cells were seeded into a 96-well plate at a concentration of $2 \times 10^3$ cells/well and the following day treated with the stated GNR conditions. After exposure period, the cells viability was determined in accordance with the manufacturer’s protocol. Result represents three independent trials with the average ± the standard error reported.
**ROS assay**

The intracellular generation of reactive oxygen species (ROS) after GNR exposure was evaluated using CM-H2DCFDA (Life Technologies, Grand Island, NY, USA) which is based on intracellular esterases and oxidation that yields a fluorescent product that is trapped inside the cell. Cells were seeded into a 96-well plate at a concentration of $2 \times 10^3$ cells per well and the following day treated with the stated GNR conditions. After 1h, 6 h, and 24h the intracellular ROS generation was determined in accordance with the manufacturer’s protocol. Result represents three independent trials with the average ± the standard error reported.

**Quantification of cellular uptake of GNRs by ICP-MS**

A total of $1 \times 10^5$ cells/well were seeded on 12mm diameter glass slides in a 24-well plate and dosed with 5 μg/mL GNRs for 24 h. The cell samples were then washed three times with warm PBS (with the exception of the deposition study samples) and digested with an aqueous solution containing 0.05% Triton X-100, 3% HCl, and 1% HNO3. The intracellular gold concentration was determined through inductively coupled plasma mass spectrometry (ICP-MS) on a Perkin-Elmer ICP-MS 300D instrument (Santa Clara, CA). ICP-MS was conducted in standard mode with 20 sweeps per reading, at one reading per replicate, and three replicates per sample with a dwell time of 100 ms. A calibration curve was obtained using four gold standard solutions and the addition of an internal standard was done to ensure that no interferences were occurring. Result represents three independent trials with the average ± the standard error reported.
**Brightfield and darkfield microscopy**

A549 human lung cells were seeded at $1.25 \times 10^5$ cells per chamber on a 2-well chambered slide and grown for 24 h. The following day the cells were dosed with 10 μg/mL GNRs for 24 h. After 24 h, the cells were fixed with 4% paraformaldehyde and incubated with Alexa Fluor 555-phalloidin for actin staining and DAPI for nuclear staining (Life Technologies, Grand Island, NY). The slides were then sealed and imaged using a CytoViva 150 ultra resolution attachment on an Olympus BX41 microscope (Aetos Technologies, Opelika, AL). All experiments were performed at minimum three times. Care was taken to ensure full evaluation of each slide for well represented images.

**Transmission electron microscopy**

A549 human lung cells were seeded in a 6-well plate at $6 \times 10^5$ cells/well for 24 h then exposed to the stated GNRs concentration (5 and 20 μg/mL) and washed three times with warm PBS. The cells then fixed overnight in 2% paraformaldehyde and 2% glutaraldehyde after indicated duration (24 h, 4 days or 8 days). The cells were then stained with 1% osmium tetroxide, washed, and subsequently dehydrated with ethanol dilutions ranging from 50 to 100%. The cells were then embedded in LR White resin and cured overnight at 60 °C under a vacuum, after which the samples were sectioned using a Leica EM UC7 Ultramicrotome. Cell sections of 70 nm in thickness were placed on a formvar carbon coated copper TEM grid (Electron Microscopy Sciences, Hatfield, PA) and were imaged. Transmission electron microscopy (TEM) was performed using a Hitachi H-7600 with an accelerating voltage of 120kV. All experiments were performed
at minimum three times. Care was taken to ensure full evaluation of each sectioned sample for well represented images.

**Statistical analysis**

All experimental results represent a minimum of three independent trials unless otherwise stated. Data are expressed as the mean ± the standard error of the mean (SEM). Statistical calculations were performed using SAS (Version 9.1) or GraphPad Prism (version 5.02, GraphPad Software Inc. La Jolla, CA, USA) to determine statistical significance at p values of <0.05 (*), <0.01 (**), or <0.001 (***).
RESULTS AND DISCUSSION

GNR characterization

GNR characterization was performed to determine their key physicochemical properties and to verify particle uniformity prior to experiments. ARs of 1, 2, and 3 were selected to determine the role of AR on cytotoxicity and cellular association/uptake in study. The GNRs had a diameter of 23 ± 0.76 nm on average, with the exception for the AR 1 CTAB TA coated GNRs (CTAB-TA-1) that had a diameter of 9 ± 0.9 nm. AR 2 GNRs had a length of 48 ± 1.0 nm on average and AR 3 GNRs had a length of 65 ± 4.0 nm on average. TEM images demonstrated that GNR sets were uniform in size and morphology (Figure 1 and Table 1). UV−Vis analysis confirmed predicted SPR peaks based on calculated AR (Figure 1J) (Jun et al., 2008). AR 3 GNRs had an average SPR peak of 700.3 ± 5.0, nm which falls in the NIR “water window” region, making the GNRs candidates for biomedical applications. To determine GNR surface charge, zeta potential analysis was performed on each particle (Table 1). Zeta potential analysis measures the velocity a charged GNRs move in a voltage field and can be quantified by tracking the GNRs as they migrate in the voltage field. From this analysis, it was shown that both CTAB and MTAB GNRs were positively charged as expected due to the quaternary ammonium cation. PEG GNRs exhibited a neutral charge, while CTAB-TA GNRs displayed a negative surface charge, confirming that functionalization with PEG or TA was successful. Dynamic light scattering was used to determine hydrodynamic size of GNR groupings after exposure to a protein rich environment (culture media).
Hydrodynamic size of GNRs in media showed that TA coated GNRs were on average larger than the MTAB GNRs of the same AR.
Figure 1. GNR characterization.

Representative TEM images of A. CTAB-TA-1, B. CTAB-TA-2 C. CTAB-TA-3 D. MTAB-1 E. MTAB-2 F. MTAB-3 G. CTAB H. CTAB-TA I. PEG; J. UV-Vis absorption spectra of MTAB-1 (dark red), MTAB-2 (dark green), MTAB-3 (purple), CTAB (red), TA-3 (blue) and PEG (light green) GNRs.
Table 1. Characterization of GNRs

<table>
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<tr>
<th>Name</th>
<th>Primary Size (nm)</th>
<th>Aspect Ratio</th>
<th>Surface Chemistry</th>
<th>Surface Charge (mV)</th>
<th>Hydrodynamic Diameter in Media (nm)</th>
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<tr>
<td>CTAB-TA-1</td>
<td>9x9 ±0.9</td>
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<td>CTAB-TA</td>
<td>-23.4</td>
<td>93.7 ±1.8</td>
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<tr>
<td>CTAB-TA-2</td>
<td>23x49 ±4.9</td>
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<tr>
<td>CTAB-TA-3</td>
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<td>-26.0</td>
<td>653.0 ±68.9</td>
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<tr>
<td>MTAB-1</td>
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<td>MTAB</td>
<td>25.1</td>
<td>117.4 ±1.1</td>
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<tr>
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<td>TA-3</td>
<td>22x60 ±2.1</td>
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<td>416.4 ±35.2</td>
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<td>2.5</td>
<td>PEG</td>
<td>2.1</td>
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</table>

Cellular association of negatively charged GNRs of differing ARs

Visualization of cellular morphology and association of CTAB-TA GNRs of differing ARs were examined using brightfield, darkfield and TEM microscopy (Figure 2, 3 and 4). After 24 h exposure to the GNRs (5 µg/mL), the cells were assisted by brightfield microscopy. CTAB-TA-1 and CTAB-TA-2 exposed cells showed no change in appearance when compared to control cells. However, CTAB-TA-3 exposed cells no longer appear confluent with possible changes in cellular morphology (Figure 2). To confirm any changes in cellular morphology and to examine GNR cellular association, darkfield microscopy was performed. Results demonstrated that CTAB-TA GNRs of all ARs interacted with A549 cells and had a high level of cellular association. In addition, darkfield images revealed both CTAB-TA-2 and CTAB-TA-3 exposed cells displayed
altered cellular morphology compared to control cells (Figure 3). Finally, TEM microscopy was performed to examine GNR internalization and further observe any changes in cellular morphology. TEM images showed the presence of internalized negatively charged GNRs of all ARs (Figure 4). However, the GNRs with an AR greater than 2 displayed altered cellular morphology. CTAB-TA-2 exposed cells show vacuoles with a ribbon like structure, possibly lamellar bodies (Figure 4B). Alterations in lamellar bodies in A549 human lung epithelial cells have been reported as an indication of pre-cytotoxicity (Davoren et al., 2007). In addition, CTAB-TA-3 exposed cells displayed extremely large vacuoles with a small number of GNRs seen in the cells and a majority of the GNRs around the cells (Figure 4C and black arrow). These changes in cellular morphology could be an indication of cytotoxicity. Therefore, an assessment of cellular viability was performed.

Figure 2. Negatively charged CTAB-TA GNRs AR3 alter A549 cells.

Representative brightfield images following CTAB-TA-3 GNR (5 µg/mL) exposure. A. Control, B. CTAB-3 GNRs. Results illustrate CTAB-TA-3 exposed cells no longer appear confluent with possible changes in cellular morphology.
Figure 3. Higher AR CTAB-TA GNRs alter A549 morphology.

Representative Darkfield Images. Fluorescent images following GNR exposure (5 µg/mL) of differing AR A. Control, B. CTAB-TA-1, C. CTAB-TA-2 and C. CTAB-TA-3. Fluorescent images results illustrate the cellular association of GNMAs and the morphology of A549 cells. CTAB-TA-1 exposure showed no apparent change in morphology of A549 cells. However, both CTAB-TA-2 and CTAB-TA-3 displayed altered cellular morphology. A549 cells underwent actin (red) and nuclear (blue) staining with GNRs (reflecting white).
Figure 4. Negatively charged CTAB-TA GNRs are internalized by A549 cells.

Representative TEM images of A549 cells after exposure to negatively charged GNRs (5µg/ml) for 24h. A. CTAB-TA-1 B. CTAB-TA-2 C-D. CTAB-TA-3 E. Control. Results demonstrate the presences of internalized negatively charged GNRs of all ARs (1-3). However, A549 cells display altered morphology after exposure to CTAB-TA-2 and CTAB-TA-3 GNRs compared to control.

Cytotoxicity of negatively charged GNRs of differing ARs

A549, human lung cells were exposed to GNRs (10 µg/mL) for 24 h. Membrane integrity and mitochondrial function was determined to assess the cytotoxicity of the negatively charged GNRs of differing ARs. Cells that were exposed to CTAB-TA-3 GNRs assays showed a significant decrease in cell viability (Figure 5) and concentration dependent cytotoxicity. As all GNRs tested have a negative surface charge, the demonstrated cytotoxicity of the CTAB-TA-3 GNRs may be due to their higher AR or surface chemistry. To test these possibilities, I examined the cytotoxicity of positively charged GNRs of differing ARs.
Figure 5. Cytotoxicity of negatively charged higher AR GNRs.

A549 human lung cells were exposed to negatively charged CTAB-TA GNRs of differing ARs (up to 10 µg/mL) for 24 h, cell viability was assessed using A. LCDC and B. MTS assays and represented relative to the control cells. C. Results showed CTAB-TA-3 GNRs significantly decrease in cell viability after exposure to concentrations as low as 5 µg/mL. Statistical significance was determined using a one way ANOVA with Dunnett’s post-hoc tests.
Biocompatibility of positively charged GNRs of differing ARs

A549 human lung cells were exposed to GNRs (10 µg/mL or 20 µg/mL) for 24 h. Next, membrane integrity and mitochondrial function was determined to assess the cytotoxicity of the positively charged GNRs of differing ARs. After exposure to MTAB GNRs (10 µg/mL), A549 cells showed no significant effects on their cellular viability (Figure 6A-B).

To confirm that MTAB GNRs enhanced biocompatibility they were evaluated with a second cell line, HepG2 human liver cells. After exposure to MTAB GNRs (10 µg/mL), HepG2 cells also showed no significant decrease of cellular viability (Figure 7A-B). Finally, A549 cells were exposed to MTAB GNRs at four times the cytotoxicity concentration of CTAB-TA-3 GNRs (20 µg/mL). This again resulted in no significant decrease of cellular viability (Figure 8).

Next I examined cellular stress by measuring changes in reactive oxygen species (ROS) after cellular exposure to the GNRs as this has been reported as an indicator of GNR biocompatibility (Wang et al., 2011). The 6 h time point was chosen based on maximum ROS response before cell death. A549 and HepG2 cells showed no significant increase in ROS levels after exposure to MTAB GNRs (Figure 6C and 7C).

One possible explanation of the differences in cytotoxicity may be due to differences in GNR cellular association and/or uptake. To check these possibilities I tested MTAB GNR cellular association by TEM and uptake by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).
Figure 6. Positively charged MTAB GNRs do not decrease A549 cell viability.

A549 human lung cells, were exposed to positively charged MTAB GNRs of differing ARs (10 µg/mL) for 24 h, cell viability was assessed using A. LCDC and B. MTS assays and represented relative to the control cells. Results showed MTAB GNRs do not significantly decrease in cell viability. C. ROS assay demonstrate no significant increase in ROS after exposure to MTAB GNRs. Statistical significance was determined using a one way ANOVA with Dunnett’s post-hoc tests.
Figure 7. Positively charged MTAB GNRs do not decrease HepG2 cell viability.

HepG2 human liver cells were exposed to positively charged MTAB GNRs of differing ARs (10 µg/mL) for 24 h, cell viability was assessed using A. LCDC and B. MTS assays and represented relative to the control cells. Results showed MTAB GNRs do not significantly decrease in cell viability. C. ROS assay demonstrate no significant increase in ROS after exposure to MTAB GNRs. Statistical significance was determined using a one way ANOVA with Dunnett’s post-hoc tests.
Figure 8. Biocompatibility of positively charged MTAB GNRs.

A549 human lung cell were exposed to MTAB GNRs at 4 times the cytotoxic concentration of CTAB-TA-3 GNRs (20 µg/mL) for 24 h, cell viability was assessed using A. LCDC and B. MTS assays and represented relative to the control cells. Results showed MTAB GNRs do not significantly decrease in cell viability regardless of AR. Statistical significance was determined using a one way ANOVA with Dunnett’s post-hoc tests.

**Cellular association of positively charged GNRs of differing ARs**

TEM was used to verify the presence of MTAB GNRs (5 µg/ml) within the A549 cells after 24 h exposure. Results demonstrate the presence of the positively charged GNRs in the cells regardless of their AR (Figure 9).

Quantification of cellular uptake of the MTAB GNRs (5 µg/ml) of differing AR in A549 and HepG2 cells was determined by ICP-MS. Results indicated that as GNR AR increased so did cellular uptake. In both A549 and HepG2 cells MTAB-1 had the lowest uptake (3% and 2% of treatment, respectively). MTAB-2 and MTAB-3 reported higher uptake in HepG2 cells (45% and 57% of treatment, respectively) compared to A549 cells.
(30% in A549 and 29% of treatment respectively). However, when the differences in GNR deposition was accounted for, the difference in % uptake was greatly reduced in both cell lines (Figure 10C-D). These findings indicated that differences in GNR deposition may have a greater impact on cellular uptake than their AR and is in agreement with the findings that sedimentation rates and diffusion effects cellular uptake of GNMs (Cho et al., 2011).

Results show that the MTAB GNRs are being taken up by A549 and HepG2 cells. This indicates that the lack of GNR uptake is not the reason for the absence of cytotoxicity of higher AR MTAB GNRs. This further suggests that the AR of the GNR is not the cause of the cytotoxicity. Another possible explanation could be due to differences in surface chemistry. To determine the cause of the cytotoxicity in the CTAB-TA-3 GNRs, I evaluated the toxicity of free CTAB and TA. Finally, I evaluated the biocompatibility of GNRs of approximately the same AR (~AR3) with differing surface chemistries.
Figure 9. Positively charged MTAB GNRs are internalized by A549 cells.
Representative TEM images of A549 cells after exposure positively charged GNRs (5µg/ml) for 24h. A. MTAB-1 B. MTAB-2 C. MTAB-3 D. Control. Results demonstrate the presence of internalized positively charged GNRs of all ARs (1-3), suggesting MTAB GNRs are taken up by A549 cells.

Figure 10. Quantification of cellular uptake of positively charged GNRs.

A549 human lung cells were exposed to positively charged MTAB of differing ARs (5 µg/mL) for 24 h, GNR Uptake was Quantified using ICP-MS. A. Uptake in A549 cells were 3% (MTAB-1), 30% (MTAB-2) and 29% (MTAB-3) of treatment. B. Uptake in HepG2 cells were 2% (MTAB-1), 45% (MTAB-2) and 57% (MTAB-3) of treatment. Results indicated on average that the higher the AR, the more GNRs were present in the
cells. However, when the differences in GNR deposition was accounted for, the
difference in % uptake was greatly reduced in both cell lines C. A549 cells 74% (MTAB-
1), 95% (MTAB-2) and 90% (MTAB-3) D. HepG2 cells 98% (MTAB-1), 89% (MTAB-
2) and 89% (MTAB-3). Statistical significance was determined using a one-way ANOVA
with a tukey post-hoc tests.

**Evaluation of cytotoxicity related to GNR surface chemistry**

To determine the cause of the cytotoxicity seen in the CTAB-TA GNRs with
higher ARs, the cytotoxicity of free CTAB and TA was tested (Figure 11). Results
indicated that free TA only demonstrated a decrease in viability at concentrations above
0.1 mg/mL, which is greater than the amount of TA present with CTAB-TA GNRs. In
contrast, free CTAB significantly decreased A549 viability at concentrations as low as 1
µg/mL. MTAB GNRs do not have a CTAB present on their surface as the CTAB-TA
GNRs do. As the AR of the CTAB-TA GNR increases so does the amount of CTAB
present on the surface of the GNRs. Results indicated that the primary cause of the
cytotoxicity seen in the CTAB-TA GNRs of higher AR may have been caused by leaking
of CTAB from the GNRs. Therefore, it may be possible to reduce or eliminate the
cytotoxicity seen in the higher AR CTAB-TA GNRs with optimization of TA
overcoating to more completely sequester the CTAB. To test this supposition, CTAB
GNRs were functionalized with TA according to a modified procedure reported by Ejima
et al (Ejima et al., 2013). This modified TA functionalization procedure resulted in
greater stability of the TA overcoating by raising and stabilizing the pH of the
suspension. This is because at a pH of 5 or less the TA overcoating can disassemble
allowing the release of CTAB (Ejima et al., 2013).
To evaluate the biocompatibility of the new TA-3 GNRs, I evaluated membrane integrity and mitochondrial function using A549 human lung cells. In addition, these results were compared to other GNRs with an ARs of approximately 3 with differing surface chemistries (CTAB, PEG, CTAB-TA-3, and MTAB-3). Results demonstrate the new functionalization procedure enhanced the biocompatibility of the TA coated CTAB GNRs (TA-3) (Figure 12). In addition, results show the higher AR positively charged GNRs (MTAB-3), neutral GNRs (PEG) and negatively charged GNRs (TA-3) all show no decrease in cell viability. In contrast, both positively charged CTAB GNRs (CTAB) and negatively charged CTAB-TA-3 displayed significant cytotoxicity. Results demonstrate that cytotoxicity is not mediated by surface charge or AR. Instead, these findings further indicate that surface chemistry is the primary driver of cytotoxicity and that controlling and reducing or eliminating CTAB is critical for biocompatibility.
Figure 11. Cytotoxicity of free CTAB and TA.

A549 human lung cells were exposed to increasing concentrations of CTAB and TA (0.00001, 0.0001, 0.001, 0.01, 0.1, 1 μm/mL) in the complete absence of GNRs for 24 h, cell viability was assessed using MTS assay. Free TA only demonstrated a decrease in A549 viability at concentrations greater than 0.1 mg/mL. Free CTAB significantly decreased in A549 viability at concentrations as low as 1 μg/ml. Results indicated that primary cause of the cytotoxicity seen in the higher AR CTAB-TA GNRs may have been caused by leaking of CTAB from the GNRs.
Figure 12. Surface chemistry is responsible for cytotoxicity of GNRs.

A549 cells were exposed to GNRs (10 µg/mL) ~AR3 with differing surface chemistries for 24 h, cell viability was assessed using A. LCDC B. MTS assays results demonstrate the enhanced biocompatibility of the new TA coated GNRs (TA-3). In addition, results show the higher AR positively charged GNRs (MTAB-3), neutral GNRs (PEG) and negatively charged GNRs (TA-3) all show no decrease in cell viability. Statistical significance was determined using a one way ANOVA with Dunnett’s post-hoc tests.
CONCLUSIONS

In this study, negatively and positivity charged GNRs of differing ARs were used to determine if they contributed to the cellular association and cytotoxicity of GNRs, *in vitro*. Results demonstrated the removal or sequestration of CTAB significantly enhanced biocompatibility of the GNRs regardless of their surface charge. In addition, when GNR deposition was accounted for, the material’s AR and primary surface charge had minimum overall impact on cytotoxicity and cellular association of GNRs. These results are in agreement with the recently published work by Wan et al (Wan et al., 2015) that also conclude surface chemistry, not AR, mediates the biological toxicity of GNRs. In their work they evaluated four ARs (1-4) of bare CTAB GNRs and conclude that because the bare CTAB (AR 1-4) were all cytotoxic, AR does not mediate biological toxicity. However, in order for bare CTAB GNRs to be stable (not aggregate), the concentration of free CTAB has to be higher than the critical micelle concentration. The critical micelle concentration of CTAB is 1 mM (365.45 1 µg/mL) when in water and at 25°C (Fuguet et al., 2005; Quirion & Magid, 1986). In their study, they failed to use a supernatant control to evaluate the cytotoxicity of the free CTAB in the GNR suspension. The cytotoxic concentration of free CTAB was found to be as low as 1 µg/mL (Figure 11) and therefore the cytotoxicity seen in their study was most likely due to free CTAB. This work demonstrated AR is not the main cause of GNR induced cytotoxicity by showing the biocompatibility of three ARs (1-3) of MTAB GNRs in two cell lines.

In conclusion, this study has determined that a GNRs AR and primary surface charge had minimum overall impact on cytotoxicity and cellular association of GNRs and
finds that surface chemistry is the primary driver of cytotoxicity and that controlling and reducing or eliminating CTAB is critical for biocompatibility of GNRs. These findings pave the way for the development of GNRs with enhanced biocompatibility for nano-based bio-medical applications.
IV. CHAPTER 2: SURFACE CHEMISTRY MANIPULATION OF GOLD NANORODS DISPLAYS HIGH CELLULAR UPTAKE WHILE PRESERVING OPTICAL PROPERTIES FOR BIO-IMAGING AND PHOTO-thermal APPLICATIONS, in vitro
SUMMARY

Due to their anisotropic shape, gold nanorods (GNRs) possess a number of advantages for biosystem use, including enhanced surface area and tunable optical properties within the near infrared region (NIR). However, a combination of cetyl trimethylammonium bromide (CTAB) related cytotoxicity, overall poor cellular uptake following surface chemistry modifications and loss of NIR optical properties due to material intracellular aggregation remain as obstacles for nano-based biomedical GNR applications. Here I report that tannic acid (TA) coated 11-mercaptoundecyl trimethylammonium bromide (MTAB) GNRs (MTAB-TA) show no significant decrease in \textit{in vitro} cell viability or stress activation after exposures to A549 human alveolar epithelial cells. In addition, MTAB-TA GNRs demonstrate a substantial level of cellular uptake while displaying a unique intracellular clustering pattern. This clustering pattern significantly reduces intracellular aggregation, preserving the GNRs NIR optical properties, vital for biomedical applications. MTAB-TA GNRs demonstrate significantly greater two photon luminescence microscopy image intensity and photo-thermal cellular ablation compared to bare MTAB GNRs. These results demonstrate how TA surface chemistry modification enhances biocompatibility and allows for a high rate of internalization while preserving the GNRs NIR optical properties. These findings identify MTAB-TA GNRs as prime candidates for use in nano-based bio-imaging and photo-thermal applications.
INTRODUCTION

Nanomaterials are increasingly being developed for use in industrial, military and consumer products, including a vast array of biomedical applications (Adlakha-Hutcheon et al., 2009; Barreto et al., 2011). Recent advances in solution chemistries for synthesis of solid phase nanomaterial technology make it possible to manipulate gold nanomaterials into different sizes, shapes and surface structures (Sun & Xia, 2002). Gold nanorods (GNRs) are of particular interest due to their unique optical region absorbance, emission and electronic properties. GNR optical properties are tunable by preparing nanofeatured structure based upon their dimensional aspect ratio (AR) or through surface modification chemistry (Bouhelier et al., 2005). The AR describes the two dimensional proportional relationship between the nanomaterial’s width and height. Depending on the GNR’s AR, a narrow range of light frequencies induces conduction band electron oscillation, termed surface plasmon resonance (SPR) (Liang et al., 2012). The spectral signature of the GNRs longitudinal plasmon resonance extends into the near-infrared (NIR) region and GNRs with a longitudinal SPR between 650 and 950 nm fall within the non-absorbing region of water and carbon based substances, termed the “water window” (Weissleder, 2001). This feature allows for deep-tissue penetration and sensing with GNRs and makes them useful for nano-based biomedical applications. As such, GNRs have been used in a vast array of biomedical applications, including diagnostic imaging, photo-therapies, and drug/gene delivery (Agarwal et al., 2011; Nagesha et al., 2007; Pandey et al., 2013; Pissuwan et al., 2008). Further, GNRs also show great potential for “theragnostic” uses that combined diagnostic imaging and therapeutic applications (Choi et al, 2012; Jelveh
Chithrani, 2011; Wang et al., 2009; Yang et al., 2013). GNRs synthesized with AR 4 will have longitudinal SPR around 800nm, allowing for the interaction with readily available 800nm lasers and therefore making GNRs with an approximate AR of 4 an ideal theragnostic platform. Even so, the combination elicited toxicity, poor cellular uptake and loss of NIR optical properties due to intracellular aggregation remain as obstacles for using GNRs in many nano-based biomedical applications (Alkilany & Murphy, 2010; Panyala et al., 2009).

The toxicity of GNRs is largely a product of free and possibly surface associated cetyl trimethylammonium bromide (CTAB), which is a cationic surfactant used in the aqueous synthesis of GNRs (Takahashi et al., 2005; L. Wang et al., 2013; Wang et al., 2011). During synthesis, CTAB provides a growth micelle environment around the gold seedings stabilizing them to form a rod (Jana et al., 2001; Nikoobakht & El-Sayed, 2003) and is present, in both the supernatant and as a bi-layer around the GNRs, after synthesis. Two strategies have been reported to overcome this surfactant’s cytotoxicity: replacement by post-synthesis ligand exchange or non-covalent overcoating by chemical cover layering via electrostatic attraction (Vigderman et al., 2012). Unfortunately, some commonly used surface modifications (e.g. polyethylene glycol; PEG) can significantly lower cellular uptake of the GNRs into cells, thus reducing their utility in biomedical applications (Grabinski et al., 2011; Huff et al., 2007). In addition, other surface modifications (e.g. polymer coatings, peptide functionalization etc.) are prone to particle aggregation when they are taken up by cells (Untener et al., 2013; Zhang et al., 2013) and result in alteration and/or loss of key optical properties (Kelly et al., 2003; Sosa et al., 2003). Furthermore, over-coatings can break down in biological environments over time.
(Ejima et al., 2013). This can result in surface leaching of the CTAB and therefore does not guarantee that the CTAB toxicity is completely mitigated by over-coating. Finally, over-coating and surface replacement procedures often require complicated multi-step functionalization processes (e.g. silica over-coating) that are difficult to scale up for biomedical applications (Gui & Cui, 2012).

Previous studies found that TA coated GNRs have reduced toxicity, demonstrate a distinctive form of endosomal uptake and display a unique intracellular distribution pattern that reduces particle aggregation (Debrosse et al., 2013; Untener et al., 2013). Unfortunately, as the CTAB-TA GNR’s AR increases so does its toxicity, possibly due to any remaining CTAB. Therefore procedures for exhaustive removal or exchange of the CTAB from the GNRs may be essential to help lower toxicity. GNRs coated with MTAB (11-mercaptopoundecyltrimethylammonium bromide), a thiol analogue of CTAB, represent a less toxic alternative though its biocompatibility and characterization within biological matrices has not yet been fully determined.

Vigderman et al recently used proton nuclear magnetic resonance spectroscopy to determine that complete replacement of CTAB with MTAB is possible due to its analogous chemical structure (Vigderman et al., 2012). MTAB replacement occurs as CTAB micelle bilayer around the GNR is exchanged with a monolayer of MTAB that strongly binds to the GNR. This study found that MTAB GNRs had no toxicity in the human breast adenocarcinoma cell line, MCF-7 even at high concentrations (Vigderman et al., 2012). In addition, 40% of MTAB GNR treatment was taken up by the cells, compared to less than 1% of their pegylated analogues, exceeding previously reported GNR uptake values (Vigderman et al., 2012). Even so, the Vigderman et al published
TEM images showed extensive aggregation of intracellular MTAB GNRs (Vigderman et al., 2012). This aggregation results in blue shift of plasmon resonance emissions, due to close proximity side by side assembly of GNRs, which moves the GNRs spectra MTAB GNRs out of the target NIR “water window” (Jain et al., 2006; Park, 2006). Therefore, new methods/techniques are needed to prevent the aggregation of MTAB GNRs in biological environment before they can be efficiently used in biomedical applications.

While the low toxicity and increased cellular uptake of MTAB GNRs are improvements over other GNR preparations, the loss of NIR optical properties described above limit their utility for biomedical applications. The aim of this study is to address this limitation by combining MTAB replacement with TA over-coating (i.e. MTAB-TA GNRs) and comparing their properties relative to MTAB and Silica GNRs, as well as CTAB GNRs with and without TA over-coating. TA coated GNRs taken up by cells display a unique intracellular distribution pattern that appears to reduce particle aggregation (Alkilany et al., 2009; Untener et al., 2013). Due to this, I hypothesized that MTAB-TA GNRs will exhibit enhanced biocompatibility and cellular uptake, while preventing particle aggregation to preserve key NIR optical properties within the A549 adenocarcinomic human alveolar basal epithelial cell line. The A549 cell line retains significant alveolar phenotype, and has been thoroughly characterized and used in numerous nano biocompatibility, bio-imaging and therapeutic studies (Foster et al., 1998; Kuo et al., 2012; Mason & Williams, 1980; Uboldi et al., 2009; Zhang et al., 2012).
MATERIALS AND METHODS

Synthesis of gold nanorods

GNRs with an approximate AR 4 (MTAB, MTAB-TA, and Silica) were purchased from Nanopartz (Loveland, CO, USA). GNRs of approximately AR 3 (CTAB, CTAB-TA, PEG) were synthesized according to a modified seed mediated procedure reported by Park and Vaia (Park & Vaia, 2008). Briefly, a seed solution of CTAB (0.1 M) and chlorauric acid (0.1 M) is combined at room temperature with a growth solution of CTAB (0.1 M), chlorauric acid (0.1 M) silver nitrate (0.1 M) ascorbic acid (0.1 M). The CTAB was purchased from GFS chemicals (Powell, OH, USA). The chloroauric acid, ascorbic acid, silver nitrate, sodium borohydride, sodium Chloride, MOPS buffer and tannic acid were obtained from Sigma Aldrich (St Louis, MO, USA).

PEG functionalization of GNRs

CTAB GNRs were functionalized with PEG as previously reported (Untener et al., 2013) with modifications. Briefly, the GNRs were functionalized overnight with 1 mM thiol PEG (Nanocs, Boston, MA) two times (MW 20000 followed by MW 5000) to displace the surface bound CTAB molecules. The GNR samples were centrifuged at 8,000g and the supernatant was removed and replaced with sterile water to remove residual free CTAB.
**TA functionalization of GNRs**

CTAB GNRs were functionalized with TA according to a modified procedure reported by Ejima et al. (Ejima et al., 2013) stepwise with TA (24mM), and MOPS buffer (100 mM, pH 7.4) with vortexing after each addition. The GNR samples were then centrifuged at 3,000g and the supernatant was removed and replaced with sterile water to remove residual TA. The MOPS buffer and tannic acid were obtained from Sigma Aldrich (St Louis, MO, USA).

**Characterization of GNRs**

The purity and spectral signature of the GNRs were analyzed before use with UV−Vis spectrometry on a Bio TEK Synergy HT (Winooski, VT, USA) instrument. For evaluation of rod size and morphology, nanoparticles in solution were placed onto a formvar carbon coated copper TEM grid (Electron Microscopy Sciences, Hatfield, PA, USA) and dried. They were imaged with transmission electron microscopy (TEM) using a Hitachi H-7600 with an accelerating voltage of 120 kV. To assess the surface charge of the GNRs, zeta potential measurements were taken using laser Doppler electrophoresis on a Malvern Zetasizer, Nano-ZS. Agglomerate sizes of the GNRs in media were determined through dynamic light scattering (DLS), also on a Malvern Zetasizer (Malvern Instruments, MA, USA).

**Cell culture conditions**

The human lung, A549, cell line (American Type Culture Collection (ATCC), Manassas, VA, USA) was maintained in RPMI 1640 cell culture media (Life Technologies, Grand
Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin streptomycin. Cells were maintained in a humidified incubator controlled at 37 °C and 5% CO₂. The same media composition was used for all GNR exposure procedures with the exception of the photo-thermal cellular ablation experiments where RPMI 1640 cell culture media without Phenol Red (Life Technologies, Grand Island, NY, USA) was used.

**Cellular viability assessment**

A549 human lung cell viability was evaluated using the CellTiter 96 Aqueous One Solution (MTS) (Promega, Madison, WI, USA) which monitors mitochondrial function and MultiTox-Glo Assay (LCDC) (Promega, Madison, WI, USA), which sequentially measures two protease activities; one is a marker of cell viability, and the other is a marker of cytotoxicity. Cells were seeded into a 96-well plate at a concentration of 2 × 10³ cells per well and the following day treated with the stated GNR conditions. After exposure period, the cells viability was determined in accordance with the manufacturer’s protocol. Result represents three independent trials with the average ± the standard error reported.

**ROS assay**

The intracellular generation of reactive oxygen species (ROS) after GNR exposure was evaluated using CM-H2DCFDA (Life Technologies, Grand Island, NY, USA) which is based on intracellular esterases and oxidation that yields a fluorescent product that is trapped inside the cell. Cells were seeded into a
96-well plate at a concentration of $2 \times 10^3$ cells per well and the following day treated with the stated GNR conditions. After 1h, 6 h, and 24h the intracellular ROS generation was determined in accordance with the manufacturer’s protocol. Result represents three independent trials with the average ± the standard error reported.

**RT-PCR for surfactant protein A and inflammatory gene expression**

The A549 human lung cells were seeded in 6 well plates at a cell density of $6 \times 10^5$ cells/well. Following seeding and overnight incubation, the cells were treated with 20 µg/mL of GNRs, while untreated cells served as a negative control. Following 8 and 24 h of exposure RNA was isolated using the RNeasy Mini Kit from Qiagen according to the manufacturer’s protocols. The RNA quantity and purity was assessed using a NanoDrop 1000 and 1 µg of RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY). The cDNA was then used in subsequent TaqMan® PCR assays to determine changes in gene expression for SPA1, Il-6, Il-6 and TNF -α and, while Hprt1 was used as a housekeeping gene to normalize expression changes. The gene expression is presented as a fold change determined using the $2^{\Delta\Delta C(T)}$ method (Livak & Schmittgen, 2001) and represents three independent trials with the average ± the standard error reported.

**Human cytokine immunoassay**

Secreted inflammatory proteins after GNR exposure were evaluated using the Bio-Plex Pro Human Cytokine 8-Plex Immunoassay (BIO-RAD, Hercules, CA). The multiplex assay detects the following cytokines: GM-CSF, IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-10 and
TNF-α. A549 cells were seeded in a 6-well plate at $6 \times 10^5$ cells/well for 24 h then exposed to GNRs (20 μg/mL) for 8 h. Next the cells were washed three times with warm PBS, media was replaced and cells were incubated for 16 h. The media was then collected and cytokine concentration was determined in accordance with the manufacturer’s protocol. Results represent three independent trials with the average ± the standard error reported.

**Quantification of cellular uptake of GNRs by ICP-MS**

A total of $1 \times 10^5$ cells/well were seeded on 12mm diameter glass slides in a 24-well plate in triplicate then dosed with 5 μg/mL GNRs for 24 h. The cell samples were then washed three times with warm PBS and digested with an aqueous solution containing 0.05% Triton X-100, 3% HCl, and 1% HNO3. For GNR retention study the cell samples were then washed three times after 24 h post exposure with warm PBS and media was replaced and repeated on day 4 and day 8. The intracellular gold concentration was determined through inductively coupled plasma mass spectrometry (ICP-MS) on a Perkin-Elmer ICP-MS 300D instrument (Santa Clara, CA). ICP-MS was conducted in standard mode with 20 sweeps per reading, at one reading per replicate, and three replicates per sample with a dwell time of 100 ms. A calibration curve was obtained using four gold standard solutions and the addition of an internal standard was done to ensure that no interferences were occurring. Result represents three independent trials with the average ± the standard error reported.
**Darkfield microscopy**

A549 human lung cells were seeded at $1.25 \times 10^5$ cells per chamber on a 2-well chambered slide and grown for 24 h. The following day the cells were dosed with 20 μg/mL GNRs for 24 h. After 24 h, the cells were fixed with 4% paraformaldehyde and incubated with Alexa Fluor 555-phalloidin for actin staining and DAPI for nuclear staining (Life Technologies, Grand Island, NY). The slides were then sealed and imaged using a CytoViva 150 ultra resolution attachment on an Olympus BX41 microscope (Aetos Technologies, Opelika, AL). All experiments were performed at minimum three times. Care was taken to ensure full evaluation of each slide for well represented images.

**Transmission electron microscopy**

A549 human lung cells were seeded in a 6-well plate at $6 \times 10^5$ cells/well for 24 h then exposed to the stated GNRs concentration (5 and 20 μg/mL) and washed three times with warm PBS. The cells then fixed overnight in 2% paraformaldehyde and 2% glutaraldehyde after indicated duration (24 h, 4 days or 8 days). The cells were then stained with 1% osmium tetroxide, washed, and subsequently dehydrated with ethanol dilutions ranging from 50 to 100%. The cells were then embedded in LR White resin and cured overnight at 60 °C under a vacuum, after which the samples were sectioned using a Leica EM UC7 Ultramicrotome. Cell sections of 70 nm in thickness were placed on a formvar carbon coated copper TEM grid (Electron Microscopy Sciences, Hatfield, PA) and were imaged. Transmission electron microscopy (TEM) was performed using a Hitachi H-7600 with an accelerating voltage of 120kV. All experiments were performed
at minimum three times. Care was taken to ensure full evaluation of each sectioned sample for well represented images.

**Hyperspectral microscopy**

A549 human lung cells were seeded at $1.25 \times 10^5$ cells per chamber on a 2-well chambered slide and grown for 24 h and the following day was exposed to GNR (20µg/ml). After 24 h, the cells were fixed with 4% paraformaldehyde. The slides were then sealed and imaged using a CytoViva Hyperspectral Imaging System (Auburn, AL). Image capture times and setting remained constant for all samples. Finally, hyperspectral analysis was performed using CytoViva’s hyperspectral image analysis software. Result represents three independent trials with the average ± the standard error reported. Care was taken to ensure full evaluation of each slide for well represented images.

**Two-photon luminescence microscopy**

A549 cells were seeded at $1.25 \times 10^5$ cells per chamber on a 2-well chambered slide and grown for 24 h and the following day was exposed to GNR (20µg/ml). After 24 h, the cells were fixed with 4% paraformaldehyde. The slides were then sealed and imaged using an Olympus fv100 multi-photon confocal microscopy Imaging System with a 25x (NA 1.05) water immersion objective (Center Valley, PA). Ti-sapphire laser (Mia-Tai laser, Spectra-Physics, Mountain View, CA) at 810 nm set at 0.5% transmissivity was used as excitation light source and two-photon luminescence emission was detected by a photomultiplier tube with an ET660/40m-2p (640-680 nm) band pass filter (Chroma Technology, Bellows Falls, VT, USA). Cells were imaged at four times zoom and 0.15
µm/slices though the cells were captured. Image capture setting remained constant for all samples. Finally, image processing and intensity analysis was performed using Olympus’s Fluoview (Center Valley, PA) image analysis software. Extracellular MTAB GNRs were used as an image intensity control. Result represents two independent trials (minimum of 8 replicates per trial) with the average ± the standard error reported.

**Plasmonic photo-thermal cells ablation**

A549 cells were seeded in a 6-well plate at $6 \times 10^5$ cells/well for 24 h then exposed to GNRs (20 µg/mL) and washed three times with warm PBS. Cells were then exposed to the cell-permeant calcein AM (2 µM) (Life Technologies, Grand Island, NY) and the non-cell-permeant ethidium homodimer-1 (4 µM) (Life Technologies, Grand Island, NY) and after 15 min cells were irradiated with an 810nm 3W Ti-sapphire laser (Mia-Tai laser, Spectra-Physics, Mountain View, CA) for 60 sweeps at indicated power level. Cellular ablation was measured at the 0, 1, 5, 10 min time points. Result represents four independent trial with the average reported. Care was taken to ensure full evaluation of each slide for well represented images.

**Statistical analysis**

All experimental results represent a minimum of three independent trials unless otherwise stated. Data are expressed as the mean ± the standard error of the mean (SEM). Statistical calculations were performed using SAS (Version 9.1) or GraphPad Prism (version 5.02, GraphPad Software Inc. La Jolla, CA, USA) to determine statistical significance at p values of $<0.05$ (*), $<0.01$ (**), or $<0.001$ (***).
RESULTS AND DISCUSSION

GNR characterization

GNR characterization was performed to determine their key physicochemical properties and to verify particle uniformity prior to experiments. TEM images demonstrated that GNR sets were uniform in size and morphology (Figure 13 and Table 2). The AR 4 GNRs had a diameter 24.5 ± 1.1 nm and a length of 104 ± 1.2 nm on average. UV–Vis analysis confirmed predicted SPR peaks based on calculated AR (Figure 13D & H) (Jun et al., 2008). To determine GNR surface charge, zeta potential analysis was performed on each particle (Table 2). From this analysis, it was shown that MTAB GNRs were positively charged as expected due to MTABs quaternary ammonium cation. MTAB-TA GNRs displayed a negative surface charge, indicating that functionalization with TA was successful. When the GNRs were exposed to a protein rich environment (culture media) both MTAB and MTAB-TA GNRs displayed a negative surface charge, -15.5 and -18.1 mV respectively. Hydrodynamic size of GNRs in media showed that TA coated GNRs were on average larger than MTAB GNRs.
Figure 13. GNR characterization.

Representative TEM images of A. MTAB, B. MTAB-TA and C. Silica; D. UV-Vis absorption spectra of MTAB (gold), MTAB-TA (light blue) and Silica (pink) GNRs. Representative TEM images of E. CTAB F. CTAB-TA and G. PEG. H. UV-Vis absorption spectra of CTAB (red), CTAB-TA (dark blue) and PEG (green) GNRs.
Table 2. Characterization of GNRs

<table>
<thead>
<tr>
<th>Name</th>
<th>Primary Size (nm)</th>
<th>Aspect Ratio</th>
<th>Surface Chemistry</th>
<th>Surface Charge (mV)</th>
<th>Hydrodynamic Diameter in Media (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTAB</td>
<td>25x102 ±4.0</td>
<td>4.1</td>
<td>MTAB</td>
<td>36.0</td>
<td>288.2 ±9.5</td>
</tr>
<tr>
<td>MTAB-TA</td>
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<td>4.1</td>
<td>MTAB-TA</td>
<td>-15.7</td>
<td>509.6 ±66.9</td>
</tr>
<tr>
<td>Silica</td>
<td>25x106 ±3.0</td>
<td>4.2</td>
<td>Mesoporous Silica</td>
<td>11.6</td>
<td>455.8 ±11.3</td>
</tr>
<tr>
<td>CTAB</td>
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<td>2.9</td>
<td>CTAB</td>
<td>37.2</td>
<td>318.7 ±4.3</td>
</tr>
<tr>
<td>CTAB-TA</td>
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<td>CTAB-TA</td>
<td>-19.4</td>
<td>416.4 ±35.2</td>
</tr>
<tr>
<td>PEG</td>
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<td>2.5</td>
<td>PEG</td>
<td>2.1</td>
<td>75.2 ±0.9</td>
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</tbody>
</table>

MTAB-TA GNRs display enhanced biocompatibility

Since GNRs are known to exhibit toxicity linked to their physiochemical properties, we compared the biocompatibility of CTAB and MTAB GNRs with and without TA coating by evaluating membrane integrity and mitochondrial function (Figure 14). Overcoating CTAB and MTAB GNRs with TA resulted in enhanced biocompatibility, with no significant decrease in viability with exposure to MTAB-TA GNRs concentrations as high as 320 µg/mL (Figure 14B). After exposure to MTAB and MTAB-TA GNRs (20 µg/ml), A549 cells showed no significant effects on their cellular viability with viability remaining over 95% after 24 h and 48 h (Figure 14 A & C). The viability data demonstrates that the reported toxicity of CTAB-TA was not due to the TA over-coating, negative surface charge or the AR of these GNRs, suggesting that the residual CTAB bilayer was the cause of the toxicity (Alkilany et al., 2009).

Next, I examined cellular stress by measuring changes in reactive oxygen species (ROS) after cellular exposure to the GNRs. The 6 h time point was chosen based on maximum ROS response before cell death. A549 cells showed no significant increase in
ROS levels after exposure to MTAB and MTAB-TA GNRs, even at four times the treatment concentration of the CTAB GNRs that significantly increased ROS levels (Figure 14D).

Finally, as GNR surface chemistry has been shown to alter cellular response and gene and protein expression analysis has been used to detect subtle changes after exposure to GNRs and other NMs (Grabinski et al., 2011; Hauck, Ghazani, & Chan, 2008). Therefore, we evaluated the inflammatory response of exposure to MTAB and MTAB-TA GNRs in A549 human lung cells by measuring changes in surfactant protein A (SPA1) and inflammatory cytokine (SPA1, IL-6, IL-8 and TNF-α) mRNA expression and GM-CSF, IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-10 and TNF-α cytokine release.

A549 cells showed no significant change in the measured mRNA (SPA1, IL-6, IL-8 and TNF-α) levels after 8 h and 24 h (Figure 15) exposure to MTAB and MTAB-TA GNRs (20µg/mL). However, a lower concentration of CTAB GNRs (2.5µg/mL) resulted in a significant increase of IL-6, IL-8 and Tnf-α mRNA after 8 h and SPA1, IL-8 and Tnf-α mRNA after 24 h.

In addition there was no significant change in GM-CSF, IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-10 and TNF-α cytokine release after exposure to MTAB and MTAB-TA GNRs (20µg/mL). Overall cytokine release was low with only GM-CSF and IL-8 cytokines reaching concentrations greater than 1pg/mL (Figure 16). Together these results demonstrate the high in vitro biocompatibility of both MTAB and MTAB-TA GNRs.

In view of their biocompatibility based on these initial findings and their longitudinal SPR peaks in the NIR “water window”, we chose to further explore the
cellular association and \textit{in vitro} hyperspectral signature of MTAB GNRs with and without a TA coating.
Figure 14. MTAB-TA GNRs demonstrate enhanced biocompatibility.

A549 human lung cells were exposed to MTAB and MTAB-TA GNRs for 24 h or 48 h, cell viability was assessed using LCDC or MTS assays and represented relative to the control cells. A. LCDC assay showed after exposure to (20 µg/mL) for 24 h, there was no significant decrease in cell viability. However, exposure to CTAB and CTAB-TA GNRs (20 µg/mL) resulted in a significant decrease in cell viability. B. 24 h MTS assay results indicate the TA coating enhanced the biocompatibility of both CTAB and MTAB GNRs. MTAB-TA showed no significant decrease in viability concentrations as high as 320 µg/mL. C. 48 h MTS assay show that cytotoxicity of CTAB-TA GNRs (20 µg/mL) significantly increases over time whereas there was no significant decrease in viability after exposure to MTAB and MTAB-TA GNRs. D. ROS assay demonstrates no significant increase in ROS after exposure to CTAB-TA, MTAB and MTAB-TA GNRs. Taken together results demonstrate the high biocompatibility of both MTAB and MTAB-TA GNRs. Statistical significance was determined using a one way ANOVA with Dunnett’s post-hoc tests.
Figure 15. MTAB-TA GNRs do not alter inflammatory gene expression.

A549 human lung cells were exposed to GNRs for 8 h and inflammatory gene expression was assessed at 8h and 24h using RT-PCR. MTAB and MTAB-TA GNRs (20µg/mL) resulted in no significant change in A-B. SPA1, C-D. IL-6, E-F. IL-8 and G-H. TNF -α mRNA levels after 8 h or 24 h. In contrast, CTAB GNRs (2.5µg/mL) resulted in a significant increase of IL-6, IL-8 and TNF -α mRNA after 8 h and SPA1, IL-8 and TNF -α mRNA after 24 h in A549 human lung cells. Statistical significance was determined using a one-way ANOVA with a tukey post-hoc tests.
Figure 16. MTAB-TA GNRs do not alter cytokine release.

A549 human lung cells were exposed to GNRs for 8 h and inflammatory gene expression was assessed at 24h using a multiplex bead-based flow cytometric immunoassay. MTAB and MTAB-TA GNRs (20µg/mL) resulted in no significant change in released A. IL-8 and B. GM-CSF protein levels. Exposure to CTAB GNRs (2.5µg/mL) resulted in a significant increase of released IL-8 and GM-CSF protein in A549 human lung cells. Statistical significance was determined using a one-way ANOVA with a tukey post-hoc tests.

Cellular association and in vitro intracellular hyperspectral signature of MTAB-TA GNRs

Visualization of cellular association of MTAB and MTAB-TA GNRs was examined using darkfield microscopy (Figure 17A-C). Both GNRs interacted with A549 cells and had a high level of cellular association. MTAB and MTAB-TA GNRs appeared to be densely packed, suggesting the associated and/or internalized GNRs are in clusters.
In addition the morphology of A549 cells were retained, further confirming the biocompatibility of the GNRs.

Next we performed *in vitro* hyperspectral imaging (HSI) microscopy to investigate the GNR optical properties after cellular association. HSI combines the use of darkfield microscopy and spectroscopy for the measurement of the reflectance spectrum at individual pixels in a micrograph. HSI analysis has successfully been used for characterizing gold nanoparticle aggregation, protein adsorption and cell uptake in biological/cellular environments (Grabinski et al., 2013). This *in vitro* analysis of MTAB and MTAB-TA GNRs is critical, as many studies have shown that biological/cellular environments can alter the optical properties of GNRs and other nanomaterials. HSI analysis demonstrated that both GNRs had a strong association to A549 cells and appeared to indicate clustering of both MTAB and MTAB-TA GNRs (Figure 17D-E). However, the MTAB GNRs displayed a vast array of colors when compared to the primarily white appearance of the MTAB GNRs, indicating a shift out of the NIR to the visible spectra. Further, MTAB-TA GNRs have a more uniform clustering that allows for easier identification and delineation of the GNR clusters. Next, the reflectance spectrum of individual GNR clusters were measured and compiled to create a hyperspectral profile for both MTAB and MTAB-TA GNRs (Figure 17G). The hyperspectral analysis revealed that the spectral maxima were decreased in both hyperspectral profiles, as compared to their spectral profiles as synthesized (Figure 13). This blue shift in spectral maxima could have resulted from GNR intracellular aggregation with side-by-side assembly (Jain et al., 2006; Stacy et al., 2013). This would effectively lower the GNR AR resulting in the changes seen in hyperspectral profile. The hyperspectral profile of
MTAB-TA GNRs (n=385) revealed a sharper peak still within the NIR target “water window” (~732 nm). In contrast, the hyperspectral profiles of MTAB GNRs (n=194) and control (n=335) displayed broad peaks that were located primarily outside the target NIR window (~630-679 nm and ~550-634 nm respectively). In addition, MTAB-TA GNRs demonstrated a greater than 2.5 fold increase in scattering intensity after cellular association over the MTAB GNRs. Together these results suggest that the MTAB-TA GNR form uniform GNR clusters that are able to preserve their NIR optical properties after cellular uptake. This finding is significant for nano-based bio-imaging and therapeutic applications as a strong spectral profile in the NIR “water window” after exposure to biological/cellular environments is required for optimum efficacy in biomedical applications.

One possible explanation of the differences in hyperspectral signature may be due to differences in GNR uptake. Another possible explanation could be due to differences in aggregation states after cellular association and/or internalization (Aaron et al., 2009). To test these possibilities we set out to examine MTAB and MTAB-TA GNR uptake by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and intracellular aggregation states by TEM.
Figure 17. Intracellular MTAB-TA GNRs retain NIR optical properties.

Representative darkfield and hyperspectral images of A549 cells with analysis of intracellular GNR optical properties. Fluorescent images following 24 h GNR 20 µg/mL exposure A. Control, B. MTAB and C. MTAB-TA. Fluorescent images results illustrate clustering of GNRs with the morphology of A549 cells retained. A549 cells underwent actin (red) and nuclear (blue) staining with GNRs (reflecting white). Hyperspectral images following 24 h GNR 20 µg/mL exposure D. Control, E. MTAB and F. MTAB-TA. Results revealed that MTAB-TA presented a more uniform clustering and spectral profile based on their appearance in the hyperspectral images. G. Analysis of in vivo optical properties. MTAB hyperspectral profile (red) (n=194) displayed low intensity and loss of NIR optical properties of GNRs. In contrast, the MTAB-TA hyperspectral profile (green) (n=385) showed preserved NIR properties with intensity greater than 2.5 times that of the MTAB hyperspectral profile. Control (blue) (n=335).
High uptake with unique clustering pattern of MTAB-TA GNRs

Quantification of cellular uptake in the MTAB and MTAB-TA GNRs (5 µg/ml) was determined by ICP-MS. Both MTAB and MTAB-TA GNRs showed a high level (40% and 39% of treatment respectively) of cellular uptake compared to 1.5% with PEG GNRs or 15% with silica coated GNRs (Figure 18A). These results are similar to the 40% uptake of MTAB GNRs in MCF-7 cells reported by Vigderman et al (Vigderman et al., 2012). In addition, this finding suggests that the TA over-coating negative surface charge has minimal impact on cellular uptake of MTAB-TA GNRs. These findings appear to differ from other studies that report that GNRs with a positive surface charge have a greater cellular association and uptake than GNRs with a negative surface charge (Hauck et al., 2008; Qiu et al., 2010). It was originally proposed that the positive surface charge on the GNRs was attracted to the negatively charged membrane of the cell resulting in higher GNR cellular association (Hauck et al., 2008). However, when GNRs of differing charge are placed in a biological environment (or simulated biological environment such as culture media) the GNRs will take on the charge of the biological environment (Qiu et al., 2010). This is in agreement with our finding that both MTAB and MTAB-TA GNRs displayed a negative surface charge, -15.5 and -18.1 mV, respectively. It has been more recently proposed that the greater uptake levels seen with GNRs with a positive surface charge is because of their greater affinity to protein and formation of a protein corona that strongly influences the GNRs cellular uptake (Nel et al., 2009; Qiu et al., 2010; Walkey & Chan, 2012; Walkey et al., 2014). TA has a strong attraction for protein and cellular membranes that has been well documented (Van Buren & Robinson, 1969; Wagner, 1976). In addition, it has been reported that TA coated gold NMs have strong cellular
association and unique form of cellular uptake (Mukhopadhyay et al., 2012; Untener et al., 2013). Therefore the MTAB-TA GNRs may form a distinctive protein corona that may account for their uptake properties; thus, further research on the impact of the protein corona of TA GNRs and other NM is needed. The finding that there is no significant difference in uptake of the two GNRs further suggests that the difference seen in hyperspectral profiles is not significantly impacted by differences in cellular uptake of the two GNRs.

The concentration of GNR remained at that level for up to 8 days post exposure with 93% and 90% retention of the MTAB and MTAB-TA GNRs respectively (Figure 18B). This suggests that there is minimal exocytosis of the GNRs and/or high reuptake of exocytosed GNRs as supported by the previous finding on GNR trafficking (W. Zhang et al., 2013). Therefore, we next investigated the intracellular state of the GNRs, as previous studies have shown that the aggregation state of GNRs can affect their spectral profiles (Kelly et al., 2003; Sosa et al., 2003).

The distribution of MTAB and MTAB-TA GNRs (5 µg/ml) within the cell was observed using TEM (Figure 19). MTAB-TA GNRs demonstrated low aggregation of GNRs with a unique distribution pattern/clusters within the A549 cells. In contrast, MTAB GNRs appeared aggregated in dense clusters and/or tightly packed crest shape groupings. This suggests aggregation of the MTAB GNRs in the cell may account for the differences seen in the two GNRs spectral profiles after cellular association.
A549 human lung cells were exposed to GNRs (5 µg/mL) for 24 h, GNR uptake was quantified using ICP-MS. A. Results show high level of uptake of 40% for MTAB and 39% for MTAB-TA GNRs compared to PEG (1.5%) and Silica (15%) GNRs. B. After 8 days post exposure 93% and 90% of the MTAB and MTAB-TA GNRs were retained, respectively. Statistical significance was determined using a one-way ANOVA with a tukey post-hoc tests.

Figure 18. Uptake and retention of MTAB-TA GNRs
Figure 19. Visualization of MTAB-TA GNR uptake.

Representative TEM Images of A549 cells after GNR exposure 5µg/ml for 24h. A. MTAB B. MTAB-TA C. Control. Results demonstrate intracellular aggregation of MTAB GNRs and low aggregation of MTAB-TA GNRs.

Low intracellular aggregation of MTAB-TA GNRs.

Since concentration can influence nanoparticle aggregation, we studied this cellular patterning at 20 µg/ml. At this new concentration MTAB GNRs displayed small and large tightly packed clusters (Figure 20 A1,A4), crest (Figure 20 A3,5,6) and doughnut (Figure 20 A2) shaped groupings with aggregation of GNRs. MTAB-TA GNRs again displayed distinctive GNR cluster patterns with low aggregation of GNRs (Figure 20 B). Most GNRs are taken up through receptor-mediated endocytosis and further trafficked via an endo-lysosomal path (Chithrani, Stewart, Allen, & Jaffray, 2009).
Further, it appears that the MTAB-TA GNR clusters are less compressed/density packed than the MTAB GNR groupings. After 8 day post exposure the number of GNR clusters/groupings per cell in both the MTAB and MTAB-TA exposed cells decreased possibly due to consolidation of the GNR groupings and by cell division (Figure 21). However, there is only a slight decrease in the total amount of GNRs present in the sample (Figure 18B). This supports the finding of Zhang et al (Zhang et al., 2013) that demonstrated the long term retention of gold nanoparticles in NDA-MB-231 breast cancer cells with the concentration of GNRs in cells correlating with their rate of cellular division rather than exocytosis.

Figure 20. Visualization of intracellular clustering pattern of MTAB-TA GNRs.
Representative TEM Images of A549 cells after GNR exposure 20µg/ml for 24h. A. MTAB and B. MTAB-TA GNRs. Results demonstrate intracellular aggregation of MTAB GNRs and low aggregation and unique clustering of MTAB GNRs.

Figure 21. Visualization of MTAB-TA GNRs cellular retention.

Representative TEM Images of A549 cells 8 day post exposure after GNR exposure 20µg/ml for 24 h. Representative images of A. MTAB and B. MTAB-TA GNRs 8 days post exposure. Results show a decrease in the number of GNR clusters/groupings per cell in both the MTAB and MTAB-TA exposed cells.

Next, we analyzed the GNRs clusters/groupings with ImageJ software (Schneider et al., 2012) (Figure 22). The MTAB GNR groupings (n=28) were more densely packed
than the MTAB-TA GNR groupings (n=24) as reflected in the area fraction of GNRs over total area of clusters/groupings values of 64% vs 50% respectively (p<0.001). In addition, the MTAB GNR groupings had a smaller average diameter (calculated as the mean of smallest and largest diameter measurement for each grouping) than the average MTAB-TA GNR cluster at 343 nm vs 534 nm respectively (p<0.001). The total area of the MTAB-TA GNR clusters, as measured by ImageJ, appears to trend larger; however, the difference was determined not to be statistically significant (p=0.099). Taken together, these results indicate that the differences in the intracellular pattern may account for the preserved NIR spectral profiles of the MTAB-TA GNR clusters. Further, we examined the average diameter of extracellular GNR clusters in the TEM image with ImageJ. Results indicated that the diameter of extracellular MTAB-TA GNR clusters was larger than MTAB GNR clusters, 310 nm vs 165 nm, respectively (Figure 22 F). Based on these finding we next tested if these differences in the intracellular MTAB and MTAB-TA GNR clusters spectral profiles had any beneficial effect with nano-based biomedical applications, specifically two-photon luminescence microscopy and photothermal cellular ablation.
Figure 22. Analysis of intracellular MTAB-TA GNR clusters.

Representative images of intracellular clusters in A549 cells after GNR exposure 20µg/ml for 24 h and clusters analysis using ImageJ software. A. MTAB B. MTAB-TA C. Average intracellular cluster density D. Average intracellular cluster area E. Average intracellular cluster diameter F. Average extracellular clusters diameter. Results demonstrate that MTAB-TA GNR cluster (n=24) are on average less densely packed (50% vs 64%) and have a large average diameter (534 nm vs 343 nm) compared to MTAB GNR groupings (n=28) in A549 cells. However, there was no statistically significant difference in the average intracellular cluster area, 70 ±16 µm² (MTAB) vs 111±19 µm² (MTAB-TA). The average diameter of extracellular MTAB-TA GNR clusters were larger than the average diameter of extracellular MTAB GNR clusters, 310 nm vs 165 nm, respectively. Statistical significance was determined using t-tests.

MTAB-TA GNRs exhibit superior two-photon luminescence image intensity

The use of GNRs has been described in a variety of bio-imaging modalities (Boca & Astilean, 2010; Eghtedari et al., 2007; von Maltzahn et al., 2009; Wang et al., 2005; T. Wang et al., 2013). Of all these nano-based bio-imaging two-photon luminescence imaging has been shown to provide the highest contrast and spatial resolution (Tong et al., 2009). Therefore, two-photon luminescence imaging was the focus of our bio-imaging studies. A strong two-photon luminescence intensity essential for bio-imaging as it allows for deeper imaging in tissues with less power.

Two-photon luminescence microscopy was used to capture 3-D images of A549 human lung cells that were exposed to GNRs (20 µg/mL) for 24 h. Next, we analyzed the
intracellular MTAB and MTAB-TA GNR clusters with Fluoview software (Olympus, Pittsburgh, PA). The 3-D images were converted into 2-D images (Figure 23A-B) and the average intensity of MTAB and MTAB-TA GNR clusters was determined. Results demonstrate that intracellular MTAB-TA GNRs produce stronger two-photon luminescence intensity ($798 \pm 40.9$ AU (161% of control)) when compared to intracellular MTAB ($546 \pm 25.1$ AU (111% of control)). This indicates that the TA coating was able to reduce intracellular aggregation, protecting the GNRs key optical properties and NIR spectral signature, and in turn enhancing the GNRs bio-imaging capabilities.
Figure 23. MTAB-TA GNRs display greater two-photon luminescence intensity.

Representative images of A549 cells after GNR exposure 20µg/ml for 24 h A. MTAB B. MTAB-TA GNRs. C. Average image intensity D. Average image intensity (% of control). Results demonstrate on average that the image intensity for cells with MTAB-TA GNR (798 ± 40.9 AU and 161% of control) is greater than cells with MTAB (546 ± 25.1 AU and 111% of control). Statistical significance was determined using t-tests.

MTAB-TA GNRs demonstrate higher efficiency for photo-thermal cellular ablation.

The MTAB, MTAB-TA and silica GNRs all have spectral profiles that demonstrate longitudinal absorbance at approximately 810 nm. When these GNRs are exposed to corresponding 810 nm laser irradiation, the GNRs absorbs photons and this energy is converted to heat. This photothermal effect results in the GNRs becoming an extremely localized heat source that can be used to kill cancer cells via photo-thermal therapy (Cobley et al., 2011; Shanmugam et al., 2014; Zhang et al., 2012). The preserved spectral profile found in the intracellular MTAB-TA GNR suggests that they would be an excellent agent for photo-thermal therapy. To determine if the TA coating enhances the GNR photo-thermal properties in vitro, we used A549, adenocarcinomic human lung cells to evaluate the efficacy of MTAB and MTAB-TA GNR for photo-thermal cellular ablation. A549 human lung cells were exposed to the GNRs (20 µg/mL) for 24 h and washed three times. Next, calcein AM (2 µM) and ethidium homodimer-1 (4 µM) was added to RPMI 1640 cell culture media without Phenol Red and incubated for 15 min. The cells were then irradiated with 60 sweeps of an 810nm 3W Ti-sapphire laser (30-75mW). Cellular ablation was measured at the 0, 1, 5, 10 min post irradiation time points.
Results show that MTAB-TA is an effective photo-thermal therapeutic agent with cell viability decreasing as the laser power was increased (Figure 24). MTAB-TA GNR demonstrated the highest level of cell death and therefore the greatest efficacy for photo-thermal cellular ablation compared to MTAB and silica GNRs (Figure 25 & 26). Photo-thermal cellular ablation with silica GNRs resulted in non-uniform cell death in the irradiated field. This may be due to lower cellular uptake of silica GNRs (15%) compared to MTAB (40%) and MTAB-TA (39%) GNRs. Based on the cellular uptake results, the effective dose in the cell was approximately 3 µg/mL for the silica GNRs compared to approximately 8 µg/mL for MTAB and MTAB-TA GNRs. These photo-thermal cellular ablation results further confirms that the TA coating of GNRs preserves their optical properties and enhances their efficacy for photo-thermal applications.
Figure 24. MTAB-TA GNR shows efficiency as agent for photo-thermal therapy.

Representative images of A549 cells after exposure to MTAB-TA GNRs 20µg/ml for 24 h. Results show A549 cells before and after exposure to NIR laser irradiation. Results show viability of MTAB-TA GNR treated cell decreasing as the laser power was increased. Results demonstrate that MTAB-TA GNRs are an effective photo-thermal therapeutic agent.
Figure 25. Visual comparison of MTAB-TA GNRs efficiency for photo-thermal cellular ablation.

Representative images of A549 cells after exposure to GNRs 20µg/ml for 24 h. Results show A549 cells with MTAB, MTAB-TA, or silica GNRs before and after exposure to NIR laser irradiation. Results show that MTAB-TA has the greatest efficiency for photo-thermal cellular ablation as illustrated by the decrease in cell viability. Silica GNRs resulted in non-uniform killing of cells and GNR free control showed no decrease in cell viability.

Figure 26. MTAB-TA GNRs demonstrate greatest efficiency for photo-thermal cellular ablation.

A549 human lung cells were exposed to GNRs for 24 h and irradiated with 60 sweeps of an 810nm Ti-sapphire laser (75mW), cell viability was assessed using A. Mean calcein AM fluorescence B. Mean ethidium homodimer-1 fluorescence. Results shows that photo-thermal cellular ablation with MTAB-TA GNRs results in both a significant decrease in mean calcein AM fluorescence and a significant increase in mean ethidium homodimer-1 fluorescence.
fluorescence. This indicates a significant decrease in cell viability and significant increase in cell death and demonstrates MTAB-TA GNRS superior photo-thermal cellular ablation properties. Statistical significance was determined using t-tests at 10 min post exposure.
CONCLUSIONS

In this study, the GNR surface chemistry was modified by replacing CTAB with MTAB and over-coating with TA. This created a novel GNR (MTAB-TA) that formed unique clusters, showed no decrease in cellular viability, no indication of cellular stress and no alteration of cell morphology, confirming its enhanced biocompatibility. Further, in the A549 human lung cancer cell line, MTAB-TA GNRs demonstrated a cellular uptake rate 26 times greater than the commonly used PEG GNRs and 2.5 times greater than silica coated GNRs (Figure 17). This high uptake rate would enable a lower effective diagnostic and therapeutic working concentration. The MTAB-TA GNRs displayed unique intracellular distribution patterns that not only preserved their NIR optical properties within the water but also enhanced their spectral intensity greater than 2.5 times that of uncoated GNRs. This finding is critical for bio-applications because it allows for the use of minimally invasive NIR lasers, higher resolution imaging and more effective therapies. Finally we demonstrated that the MTAB-TA GNRs had the greatest efficacy for photo-thermal cellular ablation compared to MTAB and silica GNRs (Figure 25 & 26).

In conclusion, this study has identified the complete replacement of CTAB with MTAB and the use of TA to overcoat and create a soft shell around the GNR, reducing GNR aggregation, protecting and preserving the GNRs NIR optical properties intracellularly (Figure 27). Based on their biocompatible nature, high rate of in vitro cell internalization and low intracellular aggregation, MTAB-TA GNRs are prime candidates for use in vivo experimentation, nano-based bio-imaging and photo-thermal applications.
MTAB and MTAB-TA GNRs displayed high biocompatibility and cellular uptake, *in vitro*. However, after internalization by A549 human lung cancer cells, the MTAB GNRs displayed high intracellular aggregation. This resulted in a degradation of the GNRs optical properties. On the contrary, TA coated MTAB GNRs (MTAB-TA) displayed low intracellular aggregation and preserved NIR optical properties. This results in greater two-photon image intensity and photo-thermal cellular ablation making them ideal nano-based bio-imaging and photo-thermal applications.
V. CHAPTER III: CONCLUSION

An emerging field of nanotechnology is nano-based biomedical applications which have the potential to change how we diagnose and treat diseases including cancer. GNRs are of particular interest due to their unique optical properties that make them ideal for nano-based biomedical applications. To date, GNR toxicity, poor cellular uptake and loss of optical properties due to particle aggregation have been a barrier to new nano-based bio-imaging and plasmonic photo therapeutic applications. In addition, in the past it has also been unclear what physiochemical properties (AR, surface charge or chemistry) mediate these parameters. Therefore, in this study, negatively and positivity charged GNRs of differing ARs were used to determine if they contributed to the cellular association and cytotoxicity of GNRs, in vitro. The results of this work demonstrate that the removal or sequestration of CTAB from both negatively and positivity charged GNRs led to significantly enhanced biocompatibility. Finally, when GNR deposition was accounted for, the material’s AR and primary surface charge had minimum overall impact on cytotoxicity and cellular association of GNRs. These findings identified physiochemical properties that influence cellular association and cytotoxicity. In addition, MTAB GNRs were found to have low toxicity and increased cellular uptake compared to the other tested GNR preparations. However, the associated loss of NIR optical properties due to material intracellular aggregation limit their utility for biomedical applications.
Therefore, we next evaluated if combining MTAB GNRs with TA overcoating would result in creating a multifunctional “theranostic” GNR that would meet the following requirements: Demonstrate (1) enhanced biocompatibility, (2) high cellular uptake, (3) preserved optical properties in biological environments, and (4) efficacy with both bio-imaging and photo-thermal therapy. The MTAB-TA GNR showed no decrease in cellular viability, no indication of cellular stress and no alteration of cell morphology in vitro, confirming its enhanced biocompatibility. Further, in the A549 human lung cancer cell line, MTAB-TA GNRs demonstrated a significantly higher cellular uptake rate compared to PEG and silica coated GNRs enabling lower effective diagnostic and therapeutic working concentration of MTAB-TA GNRs. The MTAB-TA GNRs also displayed unique intracellular pattern that preserved their NIR optical properties. This resulted in MTAB-TA GNRs having greater two photon image intensity and superior efficacy for photo-thermal cellular ablation when compared to MTAB and Silica GNRs.

In conclusion, this study found that MTAB GNRs with TA overcoating led to enhanced biocompatibility, high cellular uptake, preserved optical properties in biological environments, and efficacy with both bio-imaging and photo-thermal therapy. This data strengthens the idea that complete CTAB removal is essential for enhanced biocompatibility of GNRs. Further it identifies TA overcoating as a simple, inexpensive surface chemistry manipulation that not only enhances biocompatibility, but also preserves GNRs optical properties in biological environments, with no significant impact on GNR uptake. Based on these results there is strong case and need for in vivo experimentation with MTAB-TA GNRs for nano-based bio-imaging and photo-thermal applications.
APPENDIX A

LIST OF ABBREVIATIONS

ATCC...............................................................American Type Culture Collection
AR...............................................................Aspect Ratio
CTAB.............................................................Cetyl trimethylammonium Bromide
DLS...............................................................Dynamic Light Scattering
FBS..............................................................Fetal Bovine Serum
GNMs.............................................................Gold Nanomaterials
GNRs.............................................................Gold Nanorods
HSI.................................................................Hyperspectral Imaging
ICP-MS.........................................................Inductively Coupled Plasma Mass Spectrometry
LCDC..............................................................Live Cell Dead Cell
MTAB...........................................................11-mercaptohexadecyl trimethylammonium Bromide
MTS...........................................................(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-
(4-sulfophenyl)-2H-tetrazolium)
NIR.................................................................Near-Infrared
NM...............................................................Nanomaterial
PBS..............................................................Phosphate Buffered Saline
PEG..............................................................Polyethylene glycol
ROS..............................................................Oxygen Species
SPR...............................................................Surface Plasmon Resonance
TA...............................................................Tannic Acid
TEM.............................................................Transmission Electron Microscopy
UV-Vis........................................................Ultra-Violet Visible Spectroscopy
VI. REFERENCES


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