Development of Plasmonic Nanoplatforms for Diagnostics, Therapy, and Sensing

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biomedical Engineering in the Graduate School of Duke University

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ABSTRACT

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Abstract

Recent advances in nanotechnology have led to the application of nanoparticles in a wide variety of fields. In the field of nanomedicine, there is great emphasis on combining diagnostic and therapeutic modalities into a single nanoparticle construct (theranostics). In particular, anisotropic nanoparticles have shown great potential for surface-enhanced Raman scattering (SERS) detection due to their unique optical properties. Gold nanostars are a type of anisotropic nanoparticle with one of the highest SERS enhancement factors in a non-aggregated state. By utilizing the distinct characteristics of gold nanostars, new plasmonic materials for diagnostics, therapy, and sensing can be synthesized. The work described herein is divided into two main themes. The first half presents a novel, theranostic nanoplatform that can be used for both SERS detection and photodynamic therapy (PDT). The second half involves the rational design of silver-coated gold nanostars for increasing SERS signal intensity and improving reproducibility and quantification in SERS measurements.

The theranostic nanoplatforms consist of Raman-labeled gold nanostars coated with a silica shell. Photosensitizer molecules for PDT can be loaded into the silica matrix, while retaining the SERS signal of the gold nanostar core. SERS detection and PDT are performed at different wavelengths, so there is no interference between the diagnostic and therapeutic modalities. Singlet oxygen generation (a measure of PDT effectiveness)
was demonstrated from the drug-loaded nanocomposites. In vitro testing with breast cancer cells showed that the nanoplatform could be successfully used for PDT. When further conjugating the nanoplatform with a cell-penetrating peptide (CPP), efficacy of both SERS detection and PDT is enhanced.

The rational design of plasmonic nanoparticles for SERS sensing involved the synthesis of silver-coated gold nanostars. Investigation of the silver coating process revealed that preservation of the gold nanostar tips was necessary to achieve the increased SERS intensity. At the optimal amount of silver coating, the SERS intensity is increased by over an order of magnitude. It was determined that a majority of the increased SERS signal can be attributed to reducing the inner filter effect, as the silver coating process moves the extinction of the particles far away from the laser excitation line. To improve reproducibility and quantitative SERS detection, an internal standard was incorporated into the particles. By embedding a small-molecule dye between the gold and silver surfaces, SERS signal was obtained both from the internal dye and external analyte on the particle surface. By normalizing the external analyte signal to the internal reference signal, reproducibility and quantitative analysis are improved in a variety of experimental conditions.
Dedication

I dedicate this work to my family, friends, and colleagues who have supported me throughout this process.
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Chapter 1. Introduction

1.1 Hypotheses

1. SERS-labeled nanoparticles can be loaded with a photosensitizer to create a composite nanoparticle platform capable of both SERS detection and photodynamic therapy.

2. Functionalization of theranostic nanoprobes with a cell-penetrating peptide (CPP) can improve their efficacy by enhancing intracellular accumulation.

3. Coating gold nanostars with a thin shell of silver can preserve the tips of the gold branches as hot spots while increasing SERS due to the superior plasmonic properties of silver in the visible region.

4. Gold nanostars labeled with a small molecule Raman dye can be embedded in silver to provide both an internal SERS signal from the trapped dye, and external SERS signal from analytes on the particle surface.

1.2 Specific Aims

Specific Aim 1: Development of silica-coated gold nanostars for combined surface-enhanced Raman scattering (SERS) detection and singlet-oxygen generation

1.1 SERS-tagged nanostar synthesis and demonstration of SERS signal

1.2 Encapsulation of PDT drug

1.3 Demonstration of \(^{1}\text{O}_2\) generation

1.4 In vitro PDT study
Specific Aim 2: Demonstration of cell-penetrating peptide enhanced intracellular Raman imaging and photodynamic therapy

2.1 Synthesis of CPP functionalized SERS/PDT theranostic nanoprobes
2.2 Demonstration of CPP enhanced Raman imaging
2.3 Demonstration of CPP enhanced PDT

Specific Aim 3: Development of hybrid silver-coated gold nanostars for nonaggregated surface-enhanced Raman scattering

3.1 Synthesis and SERS characterization of silver-coated gold nanostars
3.2 Core nanostar comparison and SERRS evaluation
3.3 SERS nanoprobe for bio-applications

Specific Aim 4: Development of silver embedded nanostars for SERS with internal reference (SENSIR)

4.1 Particle synthesis and characterization
4.2 Evaluation of internal and external SERS
4.3 Comparison to silver embedded gold spheres
4.4 Internal reference improves SERS reproducibility

1.3 Motivation

This study aims to develop plasmonic nanoplatforms for use in imaging, therapy, and sensing applications. By combining imaging (diagnostic) and therapeutic components into a single platform by utilizing the unique properties of nanoparticles, a
theranostic agent can be created. This allows for the detection and treatment of disease in one construct, which is advantageous for the rapidly developing fields of personalized and precision medicine. The use of plasmonic nanoparticles for sensing applications is continually plagued by the need for higher sensitivity and reproducibility. Rational design of novel plasmonic nanoparticles can be used to optimize their signal enhancement and improve reproducibility.

1.4 Background

1.4.1 SERS Labels

Our group has been involved in the development and application of a variety of plasmonic nanostructures for SERS, ranging from colloidal nanostars, to nanopost arrays, nanowires, and nanochips,\textsuperscript{1-6} as well as their theoretical characterization using numerical and analytical techniques.\textsuperscript{7} In recent years, Raman-labeled nanoparticle probes have been gaining increasing interest in bio-labeling applications due to their advantages over conventional fluorescence methods.\textsuperscript{8-24} Fluorophores are highly susceptible to photobleaching, and solvent effects heavily influence fluorescence emission. Multiplex detection with fluorescence is also difficult because of the broad, featureless emission peaks, and the need for multiple specific excitation wavelengths. SERS spectra are generally unaffected by photobleaching and solvent or environmental effects. The potential for multiplexing is greater with Raman spectra, owing to the narrow fingerprint-like peaks and the need for only one excitation source. The use of a
Raman label whose absorption band overlaps with the laser excitation line can provide surface-enhanced resonance Raman scattering (SERRS), further increasing the signal by a few orders of magnitude.\textsuperscript{25}

**1.4.2 Photodynamic Therapy**

Photodynamic therapy (PDT) is a promising technique for the treatment of cancer and other diseases.\textsuperscript{26} PDT requires three components: light, a photosensitizer (PS), and oxygen. Upon irradiation at the appropriate wavelength, the PS becomes excited and can transfer its energy to oxygen in the surrounding media, producing reactive oxygen species (ROS) such as singlet oxygen.\textsuperscript{27} It is commonly accepted that singlet oxygen is the predominant cytotoxic agent produced during PDT.\textsuperscript{26-28} The generated ROS can react with nearby biomolecules, leading to cell death by apoptosis or necrosis.\textsuperscript{26} Therefore, PDT efficiency is largely determined by the production efficiency of singlet oxygen, which is a product of photosensitizer efficacy, light characteristics (intensity and wavelength), and oxygen concentration.

It has recently been shown that a mesoporous silica shell can be used to encapsulate various dye molecules onto a metallic core.\textsuperscript{29} This can be advantageous for PDT since many PSs are hydrophobic and cannot be adequately administered in a biological environment.\textsuperscript{30} Methylene blue (MB) is one common photosensitizer that has been used for a variety of applications, including PDT.\textsuperscript{31} Due to its high quantum yield of singlet oxygen generation ($\Phi_\Delta \sim 0.5$) in the therapeutic window (600–900 nm) and its
relatively low toxicity, MB has been considered as a promising candidate for PDT of cancer.\textsuperscript{28} However, clinical use of MB has been limited because of the lack of activity when applied systemically. The weak pharmaceutical effect results in part from poor penetration of MB into the cellular compartment of the tumor.\textsuperscript{28} Furthermore, the photodynamic activity of MB is usually eliminated in biological environments by reduction to leukomethylene blue.\textsuperscript{31} Embedding of MB in silica has been shown to protect it from this reduction in vitro.\textsuperscript{32} The encapsulated PS can be effectively delivered to the target site either by local injection or antibody targeting.\textsuperscript{33, 34} Due to the porous nature of the silica, it is not necessary for release of the PS at the target; singlet oxygen that is generated within the silica matrix can diffuse out into the surrounding tissue.\textsuperscript{28}

\subsection*{1.4.3 Theranostics}

The combination of therapeutic and diagnostic components into a single construct, \textit{i.e.} theranostics, is an emerging field of medical research that aims at further improving personalized medicine.\textsuperscript{35-39} Such composite materials allow for the imaging and detection of a specific target, monitoring biological and therapeutic processes, followed by localized release of therapeutic agents. In this way, theranostics can greatly improve the specificity and selectivity of various treatments, increasing efficacy while reducing unwanted side effects. Recently, we have developed plasmonic nanoprobes for various photon-triggered therapeutics, including photothermal and photodynamic therapies.\textsuperscript{40-43}
1.4.4 Cell-Penetrating Peptides

To further improve the efficiency of nanoparticle theranostic systems, sufficient delivery to the target cells is critical. One method for increasing intracellular accumulation of nanoparticles involves the use of a cell-penetrating peptide (CPP). One of the first CPPs discovered was the transactivator of transcription (TAT) peptide of the human immunodeficiency virus type 1 (HIV-1) viral genome. Conjugation of the TAT peptide to nanoparticles results in enhanced intracellular delivery, primarily through actin-driven lipid raft-mediated macropinocytosis. TAT has also been used in the development of nuclear-penetrating SERS nanoprobes and X-ray activated drugs.

1.4.5 Rational Design of Plasmonic Nanoparticles

Much effort has been devoted to the development of nanoparticles with the brightest SERS possible. While spherical gold and silver colloids have long been used in SERS studies, aggregation is typically required to generate the “hot-spots” of electromagnetic field for high SERS enhancement. Although this can give extremely low limits of detection, reproducibility becomes an issue when aggregation is relied upon. To overcome this problem, nanoparticles with intrinsic hotspots, such as nanorods and AuNSs, can be employed. AuNSs exhibit superior SERS properties owing to their tunable plasmon, for matching the excitation wavelength, and multiple sharp branches, each with a strongly enhanced electromagnetic (EM) field localized at its tip.
Our laboratory has extensively characterized the electromagnetic properties of AuNSs and their use in SERS.\textsuperscript{1, 43, 55} We have previously developed AuNS-based SERS nanoprobe for in vitro applications,\textsuperscript{40, 42, 56} and are now interested in developing ex vivo\textsuperscript{25} and in vivo applications, which present several challenges. The first issue we observed was the extremely high attenuation of SERS signal when attempting to detect the particles through tissue. The second issue found was the efficient photothermal transduction of AuNS solution, causing unwanted localized thermal trauma. It was interesting to note that when using a commercially available SERS nanoprobe based on aggregated AuNPs, the signal attenuation due to self-absorption was lower and heating of the solution after laser excitation was minimal. Such phenomena can be explained by the mismatch between the extinction maximum of these nanoparticles and the wavelength of the incident laser, hence limiting photothermal transduction and self-absorption of the Raman scattered light. Other reports have recently shown that plasmon matching is not as desirable as once thought when performing SERS measurements in solution.\textsuperscript{25, 57} It was therefore of interest for us to develop highly SERS active (\textit{i.e.}, highest brightness factor) nanoparticles without the aforementioned disadvantages.

There are a variety of strategies for enhancing SERS signal. One is to use resonant dyes to generate resonant SERS (SERRS). Another is to modify the composition and plasmon band of the nanoparticles to enhance their optical properties. Silver coating is a
well-known method to blue-shift the surface plasmon resonance and increase the SERS activity of gold nanoparticles. This process allows the monodispersity of gold nanoparticles to be preserved while taking advantage of the superior optical properties of silver.

**1.4.6 SERS with an Internal Reference**

Surface-enhanced Raman scattering (SERS) has emerged as a powerful spectroscopic tool for biological, chemical, and environmental analyses. Raman scattering is highly specific, providing a fingerprint-like spectrum of the vibrational modes in a molecule. The inherently weak Raman scattering of molecules can be enhanced by multiple orders of magnitude when placed within a few nanometers of a plasmonic nanostructure to generate SERS. Our laboratory has been investigating a wide variety of SERS-active platforms,\textsuperscript{3, 6, 58, 59} ranging from metal film on nanoparticle-coated microplates, nanorod arrays, nanodots, nanowires, and nanostars.\textsuperscript{1, 40, 43} While SERS has been shown to offer single-molecule detection sensitivity, quantification becomes an issue if the SERS platform is not reproducible. This is very evident when using colloidal solutions of nanospheres, as they rely on aggregation to provide SERS enhancement.\textsuperscript{60}

Recent developments in the synthesis of anisotropic nanoparticles have led to shapes that no longer require aggregation to generate SERS, such as nanocubes, nanorods, and nanostars.\textsuperscript{1, 43, 61, 62} These types of particles contain sharp points that concentrate the electromagnetic field, acting as intrinsic hot spots for SERS.\textsuperscript{59} Of these
different shapes, gold nanostars have been shown to provide the highest SERS enhancement.\textsuperscript{54} Although quantitative SERS analysis has been greatly improved by the use of anisotropic nanoparticles, there are still cases where the use of an internal standard would be necessary to obtain reliable data from a sample.

There have been reports concerning the use of an internal reference for SERS analysis, but none have involved nanostars or other anisotropic nanoparticles. The isotope edited internal standard method is perhaps the most accurate, though it requires an analyte that can be isotope-edited to produce shifted SERS peaks.\textsuperscript{63} This manuscript is focused on SERS nanoparticles that contain their own internal reference signal, requiring no additional modification of the analyte or sample preparation. The synthesis of SERS nanoparticles with embedded dye labels has been accomplished in a variety of ways. One method involved filling an Au-Ag nanoshell with the reference dye, then overcoating with silver.\textsuperscript{64} Another used dye-labeled polyelectrolytes to coat gold nanoparticles, which were then embedded into a silver shell.\textsuperscript{65} DNA has been used to create gold nanoparticles with a 1-nm, dye-containing interior gap.\textsuperscript{66} The strategy to embed thiolated molecules between the core and shell of a metal nanoparticle is what we have employed in this manuscript.\textsuperscript{57, 68}

Herein, we report the design of internal reference containing, silver-embedded gold nanostars and demonstrate their ability to reduce SERS signal variation due to matrix effects. The synthesis is a modified version of our previous report on silver-
coated gold nanostars. Unlike spherical nanoparticles, these nanostar-based platforms do not require aggregation to induce SERS, resulting in better reproducibility for quantitative analysis. Internal reference particles were made using spherical gold nanoparticles as a control, to establish that the nanostar core is necessary to achieve SERS without aggregation. This is, to the best of our knowledge, the first report of a plasmonic internal reference nanoparticle for non-aggregated SERS.

1.5 Research Summary and Significance

In this work, we have developed novel plasmonic nanostructures for use in diagnostics, therapy, and sensing. The first demonstration of a single nanoparticle construct capable of both SERS detection and PDT is presented in Chapter 2. The SERS response and \(^{1}\text{O}_2\) generation of the nanocomposite were characterized prior to demonstrating successful PDT in vitro. In conjunction with targeting moieties, such nanocomposites have the potential for theranostic applications. To further improve the theranostic capabilities of the developed nanoplatform, functionalization with a cell-penetrating peptide was employed (Chapter 3). It was demonstrated that the efficacy of SERS imaging and PDT were both increased after functionalizing the nanocomposite with the CPP. Chapter 4 describes the synthesis and characterization of silver-coated gold nanostars for non-aggregated SERS. At the optimal amount of silver coating, SERS signal intensity is increased by over an order of magnitude. To further improve quantitative detection with SERS, a silver-embedded nanostar with internal reference
was synthesized (Chapter 5). By normalizing the analyte signal with the reference signal, measurement variability is greatly reduced. We then conclude with some details about ongoing work and a future perspective (Chapter 6).
Chapter 2. Development of silica-coated gold nanostars for combined surface-enhanced Raman scattering (SERS) detection and singlet-oxygen generation (Aim 1)¹

2.1 SERS-tagged nanostar synthesis and demonstration of SERS signal

Nanostars were SERS-labeled by mixing the PEGylated nanoparticles with DTTC overnight. However, before silica coating, the SERS signal of the nanoparticles is overwhelmed by DTTC fluorescence at 785-nm excitation (data not shown). After silica coating, non-surface bound DTTC molecules were removed, and the SERS signal of DTTC is readily detected. It is presumed that the sulfur groups in the DTTC molecule aid in adsorption to the gold surface, although some DTTC remains within the layer of PEG. Condensation of silica onto the PEG is believed to displace any remaining DTTC that is not bound to the gold surface. Upon laser excitation at 785-nm, the silica coated, DTTC-tagged nanoparticles show little to no fluorescence signal, indicating that the dye is located at or near the gold surface, resulting in quenching. Figure 1 shows the strong SERRS signal from silica encapsulated MB DTTC-tagged nanoparticles (AuNS-DTTC@SiO₂-MB), while the gold nanostars with silica encapsulated MB without DTTC (AuNS@SiO₂-MB) exhibit no appreciable signal. Raman spectra of AuNS-DTTC@SiO₂

nanoparticles without MB are much noisier when excited at 633-nm (Figure 2). This is likely due to the fact that the excitation source is no longer resonant with the absorption band of DTTC or the plasmon band of the nanostars.

Figure 1. Raman spectra collected at 785-nm (dotted, dashed) and 633-nm (solid) excitation, 10 sec exposure time. Each spectrum was normalized to the laser power and to the $\omega^4$ dependence of Raman scattering. Spectra are offset for clarity and have had their backgrounds removed manually. Samples were dispersed in water. Gold nanostars tagged with DTTC and coated with MB encapsulated silica (AuNS-DTTC@SiO$_2$-MB; dotted) show a strong SERS signal at 785-nm. Gold nanostars without DTTC, coated with MB encapsulated silica (AuNS@SiO$_2$-MB; dashed) show little to no SERS at 785-nm. At 633-nm excitation (AuNS-DTTC@SiO$_2$; solid), the nanostars tagged with DTTC and coated with silica without MB have weak SERS.
Figure 2. Normalized Vis-NIR spectra of DTTC-tagged AuNS in water before (dotted) and after (solid) silica coating.

2.2 Encapsulation of PDT drug

We have utilized the method of silica coating presented by Fernández-López et al. to encapsulate MB in a mesoporous shell around gold nanostars. Previous work has shown that MB is loaded into the silica matrix when present in solution during silica condensation using the Stöber method. Silica coating of the AuNSs gives an expected red-shift in the Vis-NIR absorption spectrum due to an increase in the local refractive index around the nanoparticles. As can be seen in Figure 2, there appears to be a small shoulder around 680 nm for the silica-coated nanoparticles that may be attributed to the encapsulated MB. It is believed that the cationic nature of MB drives its adsorption to the negatively charged matrix during silica condensation. The initial SERS-tagging and the embedding of MB do not affect the quality of the silica shell, as seen in Figure 3, showing a TEM image of the silica-coated nanoparticles. Upon laser excitation at 633-nm, strong fluorescence is observed from the silica-coated nanoparticles.
containing MB (Figure 4). This is in contrast to a previous report where only residual fluorescence was observed after encapsulating MB in silica on gold nanoparticles. We propose that the initial tagging of the nanoparticles with DTTC prevents any MB adsorption directly to the gold surface, preserving its fluorescence and singlet oxygen generation capability. The AuNS-DTTC@SiO₂-MB have a blue shift of ~3 nm in the fluorescence spectrum compared to the AuNS-DTTC@SiO₂ sample spiked with 0.5 µM MB. Previous studies have shown that caging and confinement effects of the silica matrix on an embedded dye can cause a blue shift in the fluorescence emission. It was also observed that adjustment of the amount of TEOS added or the reaction time allows for control of the silica shell thickness if so desired (data not shown).

Figure 3. TEM micrograph of the silica coated nanostars.
Figure 4. Fluorescence spectra of AuNS-DTTC@SiO₂-MB (solid), MB-spiked AuNS-DTTC@SiO₂ (dotted), and AuNS-DTTC@SiO₂ (dashed) in water. Excitation was at 633-nm, 10 sec exposure time.

It was found that the as-prepared nanoparticles (~0.1 nM) have a MB concentration of 0.138 ± 0.001 µM. Quantification of the amount of MB encapsulated was performed by dissolving the silica shell using HF and measuring the resultant fluorescence intensity from MB. First, MB-spiked samples of AuNS-DTTC@SiO₂ were dissolved in HF, pelleted, and the supernatant collected. The MB fluorescence intensity of each sample, diluted in an equal volume of water, was measured to create a standard curve (Figure 5). The same procedure was carried out on the AuNS-DTTC@SiO₂-MB nanoparticles and the standard curve was used to calculate the concentration of MB.
Figure 5. Standard curve of MB fluorescence intensity from the supernatant of MB-spiked AuNS-DTTC@SiO$_2$ samples after being dissolved in HF/EtOH/H$_2$O solution. A linear regression was performed on the data set, obtaining a line with $R^2 = 0.997$. Error bars are ±$\sigma$ of three independent measurements.

2.3 Demonstration of $^1$O$_2$ generation

The MB-encapsulated silica-coated particles were found to produce significantly more singlet oxygen than silica-coated particles without embedded MB. Singlet oxygen generation from the nanoparticles was detected indirectly with the fluorescent probe, SOSG. Upon reaction with singlet oxygen, SOSG becomes fluorescent with excitation at a $\lambda_{\text{max}}$ of 504-nm and emission at a $\lambda_{\text{max}}$ of 525-nm. Singlet oxygen generation from AuNS-DTTC@SiO$_2$-MB, 0.14 µM MB-spiked AuNS-DTTC@SiO$_2$, and AuNS-DTTC@SiO$_2$ were compared (Figure 6). The measured fluorescence intensities at each time point were normalized to the initial fluorescence of the sample. A significant increase in the amount of singlet oxygen generation is observed from the MB-embedded and MB-spiked nanoparticles. It is interesting to note that the MB-embedded nanoparticles show higher singlet oxygen generation than the MB-spiked nanoparticles. It is possible that
trapping MB within the silica matrix reduces the number of dimers and other aggregates, increasing the quantum yield of singlet oxygen.\textsuperscript{74, 75} The plasmonic effect of the gold nanostars may also be contributing to the observed enhancement of singlet oxygen generation.\textsuperscript{76-78} These results indicate that such nanocomposites can effectively be used to carry the photosensitizer MB and generate singlet oxygen when excited at the appropriate wavelength.

![Figure 6](image.png)

Figure 6. Fluorescence intensity ratio of SOSG as a function of irradiation time (633-nm, 8 mW) for AuNS-DTTC@SiO\textsubscript{2}-MB (solid), MB-spiked AuNS-DTTC@SiO\textsubscript{2} (dotted), and AuNS-DTTC@SiO\textsubscript{2} (dashed). Error bars are ±σ of three independent measurements.

### 2.4 In vitro PDT study

A preliminary in vitro PDT test was performed to verify the efficacy of our nanoconstruct on cancer cells. The test was performed with BT549 human breast carcinoma cells. The treatment group was exposed to S30-DTTC@SiO\textsubscript{2}-MB with laser irradiation while the control group was exposed to S30-DTTC@SiO\textsubscript{2} with laser irradiation. Cell
viability was assessed by dual staining. The first stain was FDA, a membrane permeable dye, which is actively converted to fluorescein by living cells through hydrolytic cleavage. The second stain, PI, is membrane impermeable and only has detectable fluorescence once it has intercalated with DNA. Thus, PI is only seen in cells with damaged membranes.

As can be seen in Figure 7, only the MB-embedded particles produced a cytotoxic effect after laser irradiation and furthermore, this effect is only seen within the laser irradiated area. These results confirm previous findings about the nontoxicity of silica coated particles and show that SERS-tagged, silica coated particles do not produce any cytotoxic effects upon laser irradiation unless they are embedded with MB. Due to the mechanism of the photosensitizer, particle uptake is not required as singlet oxygen can be delivered to the cells by particles in contact with the cell membrane. It should also be noted that no photothermal effects were observed by these constructs (Figure 7B) due to the relatively low power density (~900 mW cm$^2$) and use of a 633-nm excitation source that did not overlap with the surface plasmon resonance of the particles (~800 nm).
2.5 Summary

To our knowledge, this is the first report of SERS-tagged nanocomposites possessing a combined capability for SERS detection and singlet oxygen generation for PDT. This work has demonstrated the relatively strong SERRS signal from DTTC-tagged nanostars using 785-nm laser excitation. Encapsulation of MB photosensitizing drug into a silica shell around the nanostars shows increased singlet oxygen generation upon laser excitation at 633-nm compared to silica-coated nanostars without MB. It was demonstrated that the MB-loaded nanostars produce a cytotoxic effect on BT549 breast cancer cells upon laser irradiation that was not seen in the silica-coated nanostars without MB. These multimodal nanoprobes have potential applications in theranostics, integrating SERS imaging and PDT. Future work will further investigate the behavior of
these nanoparticles in vitro and in vivo to test their efficacy as a PDT drug carrier and SERS imaging label.
Chapter 3. Demonstration of cell-penetrating peptide enhanced Intracellular Raman imaging and photodynamic therapy (Aim 2)\(^1\)

3.1 Synthesis of CPP functionalized SERS/PDT theranostic nanoprobes

Figure 8. Schematic depiction of the nanocomposite synthesis.

Figure 8 presents a visual overview of the steps required to prepare the theranostic nanoplateform. The Raman-labeled gold nanostars (AuNS) were prepared using a similar procedure described in our previous report.\(^4\) PEGylated AuNS were allowed to stir overnight in a solution containing 0.2 \(\mu\)M of the dye DTDC. The sulfur groups of the thiacarbocyanine dye aid in adsorption to the gold surface.\(^7\) Figure 8 shows the SERRS spectrum of the unwashed AuNS-DTDC particle solution before silica coating (solid line), indicating binding of the dye at or near the particle surface. After washing there was little to no change in the intensity of the SERRS spectrum. The decrease in SERRS intensity after silica coating is likely due to displacement of any

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DTDC that was not bound directly to the particle surface by the condensation of silica onto the PEG layer. Figure 9A shows the absorption spectra of PpIX and DTDC, indicating that excitation at 633 nm does not activate PpIX, and that this excitation is resonant with DTDC, thus producing SERRS.

Figure 9. SERRS spectra of AuNS-DTDC solution (solid, top), AuNS-DTDC@SiO2-PpIX-TAT solution (dotted, middle), and a point collection from a cell that had been incubated with AuNS-DTDC@SiO2-PpIX-TAT (dashed, bottom). All spectra were acquired at 633 nm excitation (8 mW) with a 10 second integration time. The solution spectra were recorded using a 10x objective with the particles suspended in water, while the intracellular Raman spectrum was recorded with a 40x objective. Spectra are baseline-subtracted and offset for clarity.
Figure 10. (A) Absorption spectra of free PpIX (solid) and DTDC (dashed) in ethanol. (B) Absorption spectra of the AuNS-DTDC before (solid, left axis) and after (dotted, left axis) silica coating (particles dispersed in water) and fluorescence emission from the AuNS-DTDC@SiO2-PpIX-TAT (dispersed in ethanol) under 415 nm excitation (dashed, right axis).

We coated the PEGylated, labeled AuNS with silica using a method described previously by Fernández-López et al. Adding PEG to the AuNS enhances particle stability in ethanol so that a modified Stöber method can be used to form the silica shell. It is believed that PEG facilitates the condensation of silica through hydrogen binding of PEG ether oxygens and silanol groups. As seen in Figure 9B, there is a red shift in the extinction spectrum of the AuNS after silica coating, caused by an increase in the local refractive index around the particles. PpIX loading of the silica shell was achieved by adding 1 µM of the photosensitizer to the reaction mixture prior to initiation of silica condensation. The drug was sequestered in the pores of the silica matrix, and fluorescence emission of PpIX was observed from the synthesized particles after being washed (Figure 9B). A calibration curve (Figure 11) was established using the fluorescence emission of PpIX under 415-nm excitation and it was estimated that 0.37 ±
0.03 µM of the initial 1 µM PpIX was encapsulated on the AuNS. The fluorescence intensity of PpIX remaining in the supernatant after the silica coating was used to make this estimation. When using the fluorescence intensity from the particle solution itself, a loaded PpIX concentration of 0.18 ± 0.03 µM is determined. This discrepancy can largely be attributed to the inner filter effects of the nanostars, which have an optical density around 0.65 in the excitation band for PpIX and an average optical density of about 0.8 in the PpIX emission band. It is also possible that there are quenching and enhancing effects on the PpIX caused by the gold nanostars; however, a more in-depth discussion of this topic is beyond the scope of this study.

Figure 11. A calibration curve created from the integrated fluorescence intensity of PpIX upon excitation at 415 nm. Error bars are ± one standard deviation of three measurements.

The particle samples were also tested for any PpIX leaching due to plasmonic heating of the nanostars. A HeNe laser (633 nm) was chosen due to the close matching of
the excitation wavelength with the maximum absorption of the nanostars. Aliquots of 100 µL of AuNS-DTDC@SiO$_2$-PpIX or AuNS-DTDC@SiO$_2$-PpIX-TAT were placed into a 96-well plate and irradiated with an 8 mW 633 nm laser for various amounts of time. The samples were spun down at 5k rcf, and PpIX fluorescence was measured from the supernatant. It is seen that after 15 minutes of irradiation, less than 25% of the PpIX has been leached from the nanoparticles (Figure 12). The TAT-coated particles also show a slightly lower rate of PpIX release, possibly due to partial blocking of the silica pores on the outer surface. It is worthy to note that when the delivered light flux is equal to that which is used for PDT (at 1.5 min irradiation time), only ~10% of the loaded PpIX had escaped from the silica shell.

![Figure 12](image.png)

**Figure 12.** PpIX release profiles from AuNS-DTDC@SiO$_2$-PpIX (solid) and AuNS-DTDC@SiO$_2$-PpIX-TAT (dashed) after irradiation with an 8 mW 633 nm laser. Error bars are ± one standard deviation of three measurements.
TEM was used to characterize the particle size and morphology. Figure 12 shows that addition of PpIX did not impact formation of complete silica shells on the AuNS. The hydrodynamic size of the AuNS-DTDC@SiO$_2$-PpIX was measured to be 123 ± 34 nm by Nanoparticle Tracking Analysis (Figure 14). The final particle modification step was conjugation with the TAT peptide. Electrostatic interaction between the negatively charged silica-coated particles and the positively charged TAT peptide induce an effective attachment method. This attachment is confirmed by the dramatic increase in intracellular particle accumulation observed for the TAT functionalized particles by two-photon luminescence imaging (Figure 15).

Figure 13. TEM micrograph of the silica coated AuNS. Scale bar is 100 nm.
Figure 14. Hydrodynamic size distribution of the silica-coated particles as measured by NTA.

Figure 15. Two-photon luminescence images of cells incubated with AuNS-DTDC@SiO₂-PpIX (left) and AuNS-DTDC@SiO₂-PpIX-TAT (right). The blue color is from a nuclear stain and the white color is from the nanostars. Particle concentration was 0.1 nM with a 1 hour incubation time for both samples.

Although silica nanoparticles are generally considered to be non-toxic, the pronounced increase in particle uptake caused by the TAT peptide warranted the use of a cytotoxicity assay to measure the impact of this dense particle loading. Cells in a 96-
well plate were incubated with various particle samples at a concentration of 0.1 nM for 1 hour, washed in PBS, and then cultured for 24 hours. After this time period, a Resazurin assay was used to assess the cytotoxicity of each particle sample (Figure 15). Each data set is the average fluorescence intensity from a column on the 96-well plate (8 measurements). It can be seen from the figure that there is no statistically significant difference in cell viability for any of the particle-incubated samples compared to the control sample.

![Graph](image)

**Figure 16.** Cell viability after a 1-hour incubation with various particle samples at a concentration of 0.1 nM. The error bars are ± one standard deviation of 8 measurements.

### 3.2 Demonstration of CPP enhanced Raman imaging

Raman images were created by taking a 5-second spectral acquisition centered at 1100 cm\(^{-1}\) (~600 cm\(^{-1}\) bandwidth) at each point on a grid with 2-µm spacing over the 2D area of a cell. The integrated DTDC peak intensity between 1120 and 1150 cm\(^{-1}\) was
displayed over the area using a color map to depict intensity variation. This peak was chosen because it showed the highest signal intensity. Representative Raman images are displayed in Figure 16. The color scale was kept constant across all of the images to allow for a fair comparison between them. In contrast, little to no Raman signal was detected from cells incubated with AuNP-DTDC@SiO$_2$-PpIX without TAT (Figure 18), which is in good agreement with the TPL imaging results (Figure 15).

![Figure 17. Three representative Raman images of cells incubated with AuNS-DTDC@SiO$_2$-PpIX-TAT, collected with 633 nm excitation (8 mW). The top row shows the brightfield image and selected area while the bottom row displays the calculated Raman image. The calculated intensity values came from the integrated peak intensity of the baseline-subtracted DTDC peak between 1120 and 1150 cm$^{-1}$. The intensity scale is shown beneath the images and was kept constant across all acquisitions.](image-url)
3.3 Demonstration of CPP enhanced PDT

The efficacy of our theranostic construct was demonstrated using cell viability staining after exposing nanoparticle-incubated cells to UV light. The treatment group was incubated with AuNS-DTDC@SiO2-PpIX-TAT for 1 hour while the control group was incubated with AuNS-DTDC@SiO2-TAT for 1 hour (particle concentration of 0.1 nM). The cells were washed 3x in PBS and then exposed to light for 30 seconds from a mercury arc lamp after passing through a DAPI filter (377/50 nm). A 40x objective was used to focus the light onto the cell sample, with a measured power density of 4.4 W cm$^{-2}$. After treatment, cells were cultured for 4 hours in complete growth medium prior to viability staining. Results of the viability staining are shown in Figure 18. Cell death due to PDT is highly evident in Figure 18 (left). There appears to be some cell detachment in the control group (Figure 18, right) due to heating of the nanoparticles, but the result is not as dramatic as that seen with the PpIX-loaded particles. The mechanism of photodynamic cytotoxicity is ascribed to the $^1$O$_2$ generated by PpIX when excited by the broadband
light within its absorption band. This $^1$O$_2$ can diffuse out of the porous silica matrix and travel on the order of tens of nanometers to affect cellular components. While the excitation light did heat the particles enough to cause some cell detachment, very few of the cells were actually ablated (red-stained cells, Figure 18, right). The effect of using PpIX-loaded particles without TAT was also tested. Light exposure after a 1-hour incubation with 0.1 nM AuNS-DTDC@SiO$_2$-PpIX did not produce any observable effect (Figure 20).

Figure 19. Viability staining of cells incubated with AuNS-DTDC@SiO$_2$-PpIX-TAT (left) and AuNS-DTDC@SiO$_2$-TAT (right) after 30 seconds of light irradiation. Live cells are stained green and dead cells are stained red. Scale bars are 250 µm.

Figure 20. Viability staining of cells incubated with 0.1nM AuNS-DTDC@SiO$_2$-PpIX for 1 hour after 30 seconds of light irradiation. Live/dead cells are stained green/red. Scale bar is 250 µm.
3.4 Summary

In summary, we present the first application of a theranostic, combined Raman imaging and photosensitizer nanoconstruct. The use of the cell-penetrating peptide, TAT, greatly increases particle uptake by the cells, enhancing the efficacy of our construct. Raman imaging and photosensitization were demonstrated on BT-549 breast cancer cells. When the same conditions were used for particles that were not functionalized with TAT, little to no Raman signal could be detected from the cells and no photosensitization was observed after light exposure. The particles exhibited no cytotoxic effect under dark conditions. Future work will involve the use of various targeting ligands and multiple Raman probes to investigate the specificity and multiplexing capability of our theranostic construct. The use of other drugs, not only photosensitizers, is also under investigation.
Chapter 4. Development of hybrid silver-coated gold nanostars for surface-enhanced Raman scattering (Aim 3)\textsuperscript{1}

4.1 Synthesis and SERS characterization of silver-coated gold nanostars

The AuNSs used in this study were prepared according to our previous report.\textsuperscript{81} To better characterize the silver coating process, three types of NSs were prepared: S5, which have low branch numbers and length, an average particle size around 50 nm, and an extinction maximum at 650 nm; S10, which have low branch numbers with an increased branch length, an average particle size around 60 nm, and an extinction maximum at 750 nm; and S30, which have high branching, an average particle size around 70 nm, and an extinction maximum at 850 nm. The particles are designated by the final concentration of AgNO\textsubscript{3} (used to control the branching) in the reaction mixture (e.g. S30 is prepared with 30 µM final concentration of AgNO\textsubscript{3}). After synthesis, CTAB was added as a surfactant to stabilize the particles, which were then purified by centrifugation to remove any unreacted reagents.

Silver coating of the NSs was performed in a similar manner to previous reports on the coating of gold nanorods with silver, and modified from our previous method for gold seeded growth of monodisperse silver nanospheres.\textsuperscript{82, 83} In this study, the CTAB-

stabilized AuNSs are used as seeds for the growth of a silver shell. Ascorbic acid serves as the reducing agent, with silver nitrate used as the precursor to elemental silver. After adding AA and AgNO$_3$ to the AuNS seed solution, NH$_4$OH is introduced to increase the pH, initiating the reduction of Ag$^+$ to Ag$^0$ by AA. An immediate color change is observed after the pH adjustment. The extinction maximum of the solution begins to blue-shift from the NIR region. After about 5 minutes, the color of the solution stabilizes, indicating completion of the silver coating reaction. A range of silver coated samples were prepared, from zero to 15 µL of 0.1M AgNO$_3$ added per mL of NS solution. These samples are referred to by the NS type@Ag#, where the # is the volume of silver added in µL per mL of NS solution; thus, 1 mL of S10 coated using 5 µL 0.1M AgNO$_3$ solution would be designated S10@Ag5.

To understand the silver coating process, we used S10 as a model where the coating process was monitored by UV/Vis spectroscopy and TEM. Figure 20 shows the extinction spectra of S10 coated with various amounts of silver, along with TEM images of a few representative samples. The plasmon band of the S10 progressively blue-shifts from around 750 nm down towards 500 nm as the amount of silver coating is increased. We have previously shown that the NIR plasmon maximum position of AuNSs is mainly controlled by the aspect ratio of the branches protruding from the core.$^{43}$ Upon silver addition, it can be seen from the TEM images that the coating develops from NS core and expands outward. This process effectively decreases the aspect ratio of the
branches that protrude from the silver shell, resulting in the observed blue-shift in their plasmon maximum and decreased plasmon intensity in the NIR range.

Figure 21. Extinction spectra of S10 with increasing silver coating (left). TEM images of the corresponding S10@Ag samples (right). Scale bars are 25 nm.

To further investigate the observed plasmon shift, theoretical modeling using the finite element method in COMSOL Multiphysics was performed. A 2D AuNS model was designed with a 50 nm core and six equally spaced branches of 25 nm in length. To simulate the silver coating process, increasingly larger spheres of silver were overlaid upon the AuNS core. The radius of the silver spheres used ranged from 30 to 50 nm, in steps of 5 nm. A plane wave polarized in the x-direction, travelling along the y-axis, was used to excite the particle in the wavelength range of 400 to 800 nm, evaluated in 10 nm steps. As can be seen in Figure 21, the theoretical model shows the same trend as the experimental data, with the plasmon blue shifting as the amount of silver is increased. The two plasmon peaks seen in the simulation can be attributed to the core for the shorter wavelength peak, and the branches for the longer wavelength peak. This model
also shows a decrease in the intensity of the branch plasmon peak relative to the core plasmon peak as the size of the silver shell is increased, which is in agreement with the experimental spectra.

**Figure 22.** Simulated $|E|^4$ as a function of wavelength for the models shown on the right. An x-polarized plane wave travelling along the y-axis was used for excitation. The value of $|E|^4$ was calculated at the tip of the upper-right branch in the particle models. Scale bars are 25 nm.

For SERS intensity evaluation, we chose to compare the overall SERS brightness of the nanoparticle samples in lieu of calculating their enhancement factors, which tend to be inaccurate as a consequence of assumptions made in their determination.\textsuperscript{51} Factors that would interfere with an enhancement factor calculation for these particles include: the irregular shape of the nanoparticles making it difficult to calculate their surface area to determine the number of dye molecules that can bind per particle, the use of CTAB leading to more than a monolayer of dye coverage per particle, and self-absorption of the particles reducing the measured Raman signal.\textsuperscript{25,57}

The SERS properties of these S10@Ag were then examined and compared with silver-coated gold nanospheres (AuNP@Ag). The AuNP were prepared by a seeded
growth method in CTAB solution. UV/Vis extinction spectra and TEM images of the AuNP can be found in Figure 23. All samples were labeled with 1 µM of DTDC and allowed to sit for 15 minutes before SERS measurements. Figure 23 shows the collected spectra from the various S10@Ag (Figure 23A) and AuNP@Ag (Figure 23B). The AuNP@Ag exhibit much lower SERS than S10@Ag, which is to be expected if the particles are in a non-aggregated state.\textsuperscript{51, 55} Note that the signal intensity from the S10@Ag can be an order of magnitude higher than that of the AuNP@Ag. To more accurately compare the SERS intensities, the spectra were background subtracted and then the intensity of the peak around 1580 cm\(^{-1}\) was integrated. The integrated intensities for the S10@Ag and AuNP@Ag samples were shown in Figure 23C and Figure 23D, respectively. The maximum enhancement for the S10@Ag is found at S10@Ag4, with an 11 ± 2 times increase in signal intensity than S10 alone. The AuNP@Ag exhibit a maximum enhancement at AuNP@Ag1, with a 2.2 ± 0.3 times increase in signal.

![Figure 23](image)

**Figure 23.** Extinction spectra and representative TEM images of gold nanospheres (AuNP) coated with various amounts of silver. Scale bars are 25 nm.
Figure 24. Raw SERS signal intensity of S10@Ag (A) and AuNP@Ag (B) samples with different amounts of silver coating. Spectra were acquired with a 1 second exposure time at 785 nm (150 mW power at the sample). Due to the low SERS intensity, spectra from AuNP@Ag contain visible background from the plastic vials used during the measurements. The integrated intensity of the DTDC peak around 1580 cm\(^{-1}\) is shown below the spectra for the S10@Ag (C) and AuNP@Ag (D) samples. Error bars are ± one standard deviation (n=7).

To examine the contribution of attenuation due to extinction on the observed increase in SERS intensity, ethanol was used as an internal standard to normalize the measured spectra. Since the ethanol Raman peaks are not enhanced by the nanoparticles, any observed intensity variation can be attributed to changes in the extinction of the solution near the laser wavelength. Silver-coated S10 samples were
prepared and labeled with pMBA. The pMBA dye was chosen for this study due to its thiol functionality, which will keep the dye molecules tightly bound to the nanoparticle surface in the presence of ethanol. Each sample was spiked to contain 10% ethanol before SERS measurements were performed. Spectra were acquired with a 1 second exposure time at 785 nm with 250 mW power at the sample. Figure 24 shows the integrated signal intensity recorded for the pMBA peak at ~1580 cm\(^{-1}\) (Figure 24A), the ethanol peak at ~880 cm\(^{-1}\) (Figure 24B), and the normalized pMBA peak intensity (Figure 24C). The maximum SERS brightness is observed around Ag5, which is similar to what was shown above in Figure 23. The Raman signal from the ethanol internal standard behaves as expected, which increases as the AuNS plasmon is progressively blue-shifted away from the laser wavelength. When the intensity of the pMBA peak is normalized by that of the ethanol, the measured SERS brightness actually decreases as more silver is added. This suggests that reduced attenuation, achieved by blue-shifting the nanostar plasmon away from the laser excitation wavelength, plays a significant role in the SERS signal enhancement that is observed in Figure 23. The silver coating itself may also be contributing to the enhanced un-normalized SERS brightness; however, single-particle SERS measurements would need to be performed to investigate this effect, which is beyond the scope of this study.
Figure 25. The recorded signal intensity of the pMBA peak at ~1580 cm⁻¹ (A), the ethanol peak at ~880 cm⁻¹ (B), and the ethanol normalized pMBA signal (C). Spectra were recorded at 785 nm, 250 mW, 1 second exposure. Error bars are ± one standard deviation (n=7).

4.2 Core nanostar comparison and SERRS evaluation

To compare the SERRS variation among different types of AuNSs, S5 and S30 were also evaluated to validate the synthesis method and experimental observations as obtained with the S10. Figure 26 shows the progression of S5@Ag and S30@Ag of increasing amounts of silver. As previously observed, the silver deposition begins mainly on the core of the particles, spreading outward as the amount of silver is increased until the branches are completely covered, resulting in a quasi-spherical shape. The smaller S5 are shown to have their branches mostly covered at lower amounts of silver than the larger S30. Magnified views of uncoated S30 and S30@Ag7 are shown in Figure 27, clearly showing branches of the NSs protruding from the silver shell.
Figure 26. UV/Vis extinction spectra of S5@Ag (A) and S30@Ag (B) of varying amounts of silver coating, as indicated in the legends, accompanied by representative TEM micrographs for both types of NS. Scale bars are 50 nm.

Figure 27. Magnified TEM views of uncoated S30 (left) and S30@Ag7 (right). Scale bars are 50 nm.

Figure 26 shows the extinction spectra for both S5@Ag and S30@Ag as the amount of silver is varied. The S5 plasmon is around 650 nm while the S30 plasmon is around 850 nm; in both cases, the extinction maximum blue-shifts to ~500 nm and increases in intensity with increasing amounts of silver. Note that there is no peak observed at ~420 nm, where the plasmon peak of silver nanospheres occurs, suggesting
no nucleation of silver particles. The blue-shifting NS plasmon, along with the absence of a plasmon peak at ~420 nm are indicative of silver shell formation on the AuNS.

To fabricate the SERS nanoprobes with the highest brightness, several factors were taken into consideration. Resonant SERS was employed because it generates multiple orders of magnitude higher SERRS signal than non-resonant SERS on non-aggregated AuNSs. In addition, we have shown that when using resonant dyes, a plasmon that is blue-shifted from the excitation provides the highest signal, as self-absorption effects are minimized when the plasmon is off-resonance from the excitation.\(^\text{25}\) Previously, we used sodium dodecyl sulfate (SDS) as a surfactant on AuNS to aid in stabilization and dye adsorption. It is believed that the hydrophobic bilayer formed by the SDS helps to entrap dyes near the particle surface. We have found that CTAB can act in the same manner, and exhibits about two to three times higher signal intensity than particles stabilized with SDS. The longer hydrophobic chain of CTAB (16 carbons) versus SDS (12 carbons) likely provides a larger volume for trapping dye molecules.

To investigate the effect of the various silver coatings on resonant Raman enhancement, AuNS@Ag samples were labeled with a NIR resonant dye, IR-780, for surface-enhanced resonant Raman scattering (SERRS) measurements. Figure 28 shows the SERRS brightness of S5@Ag and S30@Ag with various amounts of silver. The raw SERRS spectra of S5@Ag (Figure 28A) and the integrated, background-subtracted signal
intensity at 730 cm$^{-1}$ (Figure 28B) are shown. At its highest brightness, with S5@Ag3, the signal is enhanced 16 ± 2 times over the S5. For S30@Ag (Figure 28C,D), the highest intensity is observed at S30@Ag7, which is 9 ± 1 times higher than the signal of S30. It is worth noting that the maximal SERRS brightness was found on AuNSs with sub-total silver coating. Correlated with the TEM images from Figure 26, it is apparent that the maximum Raman signal enhancement occurs right before the gold tips become completely embedded in the silver shell. More silver does not always lead to higher SERRS response. A near-total silver coverage retains the hot-spots from the AuNS tips while lowering self-absorption from the NS solution to yield the strongest SERRS. In contrast, spherical silver coating with a mismatched plasmon maximum but no sharp tips had a SERRS brightness that was only slightly greater than the initial NS sample.
Figure 28. SERRS measurement of S5@Ag (A and C) and S30@Ag (B and D) with various amounts of silver coating, labeled with IR-780. Raw SERRS spectra for S5@Ag (A) and S30@Ag (B) are shown. Spline chart of the background-subtracted integrated signal intensity at 730 cm\(^{-1}\) for S5@Ag (C) and S30@Ag (D) samples. Error bars are ± one standard deviation (n=3). All spectra were recorded with a 100 ms exposure time using a 785 nm laser (150 mW power at the sample).

To ensure these nanoprobes were not aggregated, which would cause anomalously high Raman signals, the size distributions of S30@Ag were evaluated by NTA, both before and after dye labeling (Figure 29). No significant increase in particle size was observed after dye labeling, adding confidence that the particles remained in a non-aggregated state. The observed drop-off in Raman signal intensity after a certain amount of silver coating further supports the claim that particles remain non-aggregated.
after dye labeling. Once the ideal amount of silver coating is surpassed, the mostly spherical-shaped particles can be prone to aggregation, which would cause a marked increase in Raman signal.

![Graph showing mean hydrodynamic particle size measurements](image)

Figure 29. Mean hydrodynamic particle size measurements (determined by NTA) of S30 when coated with varying amounts of silver. No significant increase in size is observed after dye labeling, indicating that the particles remain in an unaggregated state.

The amount of sliver coating required for optimal SERRS appears to depend on the AuNS used. Although the exact amount of silver is not available in this study, the progression in the extinction spectra and TEM images clearly characterize the silver amount semi-quantitatively. Among the 3 types of NSs, they vary in core size, branch length and number. Based on the TEM findings, the particle geometry (e.g. core size and branch length/number) may dictate the required silver amount to obtain sub-total coverage on the AuNS for optimal SERRS. With S5 having a smaller core and few broad branches, the amount of silver required for subtotal coverage is less than that from S30, having a larger core and more sharp branches. The actual amount of silver coating
required for optimal SERS/SERRS, however, needs to be determined experimentally based on particle size and shape.

4.3 SERS nanoprobe for bio-applications

In order to make SERS/SERRS nanoprobes suitable for bio-applications, it is necessary to encapsulate the particles in an inert material, e.g. silica. Coating the Raman nanoprobes with silica protects the dye on the particle surface for greater structural stability and prevents unwanted adsorption of other molecules that may generate their own Raman signal. We prepared SERS nanoprobes using three different carbocyanine dyes (DTDC, DTTC, and HITC) to demonstrate the potential for multiplex detection. Thiol-PEG was used to stabilize the particles when transferred into ethanol for silica coating by a modified Stöber method. The PEG layer also acts to facilitate silica condensation onto the surface of the particles, presumably through hydrogen bonding. This method was found to be equally effective at encapsulating both AuNS@Ag and bare AuNS. The measured particle size distribution (Figure 30) showed no obvious signs of aggregation after silica coating. Figure 31 compares the Raman signal intensity from silica-coated, DTTC-labeled S30 and S30@Ag7. It is shown that after silica coating, the order of magnitude in signal difference between the S30 and S30@Ag7 is maintained. The TEM micrographs show that both particle types were completely coated and in a non-aggregated state.
Figure 30. Particle size distribution for S30@Ag7-DTTC@SiO₂ as determined by NTA.

Figure 31. Comparison of Raman signal intensity from S30-DTTC@SiO₂ ((A), blue) and S30@Ag7-DTTC@SiO₂ ((A), red), collected with a 100 ms exposure time. The spectra have been background subtracted and offset for clarity. TEM micrographs of the Ag0 and Ag7 SERS nanoprobes are shown in (B) and (C), respectively. Scale bars are 100 nm.
To show the potential of our new AuNS@Ag in biological applications, the prepared SERS nanoprobes were injected into a rat pelt for ex vivo detection. To prepare the particles for injection, the solutions were concentrated ten times and dispersed in sterile PBS. The particle solutions were drawn up into 1 mL syringes with a 27G needle. About 25 µL of each SERS nanoprobe was injected into the skin at different locations. The injection volume produced a small welt, with the particle solutions clearly visible through the skin (Figure 32 inset). Note that the injection of the non-silver coated nanoprobe looks darker than the others because of the original solution color; the AuNS solution is a dark gray color while the AuNS@Ag solution is reddish orange. The injection area was then swabbed with an alcohol pad before optical interrogation with the RamanProbe.
Figure 32. Raman spectra (background subtracted) recorded from different SERS nanoprobes after intra-dermal injection into a rat pelt: (A) S30@Ag-DTTC@SiO$_2$, black; (B) S30-DTTC@SiO$_2$, blue; (C) S30@Ag-HITC@SiO$_2$, green; (D) S30@Ag-DTDC@SiO$_2$, orange. The inset at top right shows the injection sites, each outlined in the color that matches the corresponding spectrum above.

As seen in Figure 32, three AuNS@Ag@SiO$_2$ (DTTC, HITC, and DTDC) and one AuNS@SiO$_2$ (DTTC) were measured through the dermis. The S30@Ag7-DTTC@SiO$_2$ (Figure 32A) showed a higher signal-to-noise ratio than the S30-DTTC@SiO$_2$ (Figure 32B), demonstrating stronger SERRS from S30@Ag@SiO$_2$ than from S30@SiO$_2$. The difference in signal intensity from resonant (DTTC and HITC) and non-resonant (DTDC) dyes is shown in Figure 32, with the resonant dyes providing an order of magnitude higher signal than the non-resonant dye. Also, non-resonant S30@Ag-DTDC@SiO$_2$ has
comparable intensity as the resonant S30-DTTC@SiO₂. SERRS on AuNS@Ag@SiO₂ clearly has stronger signal intensity than its non-resonant SERS and non-silver-coated counterparts.

Another benefit of the AuNS@Ag SERRS nanoprobes is that the extinction maximum no longer occurs in the region of the laser excitation. When the plasmon peak matches the laser wavelength, a small area of burnt tissue can be seen in the center of the AuNS@SiO₂ injection site (blue circle, Figure 32 inset) after the measurement had been performed (150 mW power, 1 mm spot size, ~20 seconds exposure). The laser power at the sample had to be decreased below 40 mW in order to prevent any visible damage when measuring the AuNS based probes. In contrast, no adverse effects on the tissue were seen for the AuNS@Ag@SiO₂ after laser interrogation, even under 250 mW for 20 seconds. Importantly, the photothermal effect did not affect the SERS signal of the particles over this period of time, even when the laser power was increased to 250 mW (Figure 33). It should be noted that there are many variables that could affect the observed photothermal response, including depth of the particle injection, concentration of the particle solution, volume of solution injected, and the type and pigmentation of skin. Therefore, although matching the laser excitation to the surface plasmon resonance of nanoparticles will generate the highest electromagnetic field enhancement for SERS, we show here that this is not always desirable, as doing so can lead to unintended tissue damage, along with extinction-based attenuation of SERS signal.
4.4 Summary

In this report we describe in detail the synthesis, characterization, and application of a novel hybrid bimetallic platform, AuNS@Ag, for SERS/SERRS detection. The amount of silver coating was optimized to blue-shift the plasmon while retaining the hot-spots on sharp tips, hence giving the greatest SERS/SERRS brightness. The morphology of the particles was assessed by TEM, while the optical properties were characterized with UV/Vis spectroscopy and Raman spectroscopy. In the optimized configuration, AuNS@Ag provided over an order of magnitude of signal enhancement compared to uncoated AuNS. The enhancement is most likely due to the off-resonance plasmon that reduces background self-extinction. Silica-coated, dye-labeled AuNS@Ag and AuNS were prepared for entrapping the dyes and preserving the non-aggregated
state of the particles. To demonstrate the potential for these particles in bio-labeling applications, ex vivo detection was performed following intradermal injection of the SERS/SERRS nanoprobes into a rat pelt. Raman signal was detected from all three AuNS@Ag nanoprobes, and the measurements did not cause any noticeable photothermal damage, which could occur when the particles’ surface plasmon resonance coincide with the incident light. These new, ultra-bright SERS particles will be further developed for sensing and imaging applications.
Chapter 5. Development of silver embedded nanostars for SERS with internal reference (SENSIR) (Aim 4)\(^1\)

5.1 *Particle synthesis and characterization*

The procedure to prepare the nanostars with internal reference is similar to our previous report of silver-coated gold nanostars.\(^5\) In this case, the gold nanostars were labeled with 4-mercaptobenzoic acid (pMBA) prior to the silver coating process. The thiol functionality of pMBA allows it to bind strongly with the gold surface through the formation of Au-S bonds. Tween 20, a non-ionic surfactant, was used to stabilize the particles during the washing and silver coating steps. The pMBA-labeled nanostars then served as seed particles for the silver growth, with silver nitrate used as the silver precursor and ascorbic acid as the reducing agent. While in the presence of 0.02% Tween 20, this reduction process proceeds very slowly at room temperate and neutral pH. Ammonium hydroxide was used to increase the pH of the solution and speed up the reaction rate. The particles were left undisturbed for at least one hour prior to further experiments.

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Figure 34. TEM images of gold nanostars (A) and silver-coated gold nanostars (B). The extinction spectra of the gold nanostars (blue) and silver-coated gold nanostars (red) are shown in (C). Scale bars are 50 nm.

The silver coating was monitored via UV/Vis absorption spectroscopy and TEM. Figure 34A,B shows the TEM images of gold nanostars before and after being coated with silver. It can be seen that the silver growth occurs mainly on the core of the gold nanostar, allowing the ‘hot-spot’ branches to remain accessible to external analytes. The extinction spectra of gold nanostars and silver-coated gold nanostars are shown in Figure 34C, with the gold nanostar plasmon starting in the NIR at ~700 nm, and blue-shifting down to ~485 nm after the silver coating. These findings are consistent with our previous work and indicate that embedding of the pMBA between the gold nanostar
core and silver shell does not have a significant effect on the obtained particle morphology.

5.2 Evaluation of internal and external SERS

![Graphs showing SERS intensity vs. amount of Ag added](image)

Figure 35. (A) SERS spectra of pMBA functionalized gold nanostars with different amounts of silver; inset zoomed in on the 613 cm⁻¹ Rh6G peak, (B) Integrated SERS intensity from 10 µM Rh6G, (C) Integrated SERS intensity from embedded pMBA on gold nanostars with different amounts of silver coating, (D) Integrated Rh6G signal intensity, (E) integrated pMBA signal intensity, (F) pMBA normalized Rh6G signal intensity at different concentrations of Rh6G. The spectra in (A) have been offset for clarity. Error bars are ±1 standard deviation.

The SERS intensity of both the embedded pMBA and external Rh6G, selected as a model analyte, were investigated at different amounts of silver coating. Figure 35A contains representative spectra that demonstrate the greatly enhanced pMBA SERS...
signal that is obtained after embedding the nanostars in different amounts of silver. The integrated intensity of the Rh6G peak at 613 cm⁻¹ on nanostars with different amounts of silver coating is shown in Figure 35B. The 1583 cm⁻¹ peak of pMBA was also integrated and plotted against the amount of silver added, seen in Figure 35C. After a certain amount of silver coating is reached, both the Rh6G and pMBA signals begin to decrease. For the external Rh6G, once the nanostars become completely embedded in the silver, the nanostar branches can no longer act as hot spots of electromagnetic field enhancement, resulting in a decrease of the SERS signal. The embedded pMBA signal increases rapidly up to the point where most of the gold surface has been covered in silver; after this point, there is a gradual decrease in signal as the silver coating is increased, suggesting that the signal is resulting from pMBA molecules embedded between the gold surface and silver coating. This is further evidenced by functionalizing AuNS@Ag with pMBA on the outer surface instead of the gold surface before coating, resulting in about two orders of magnitude lower signal (Figure 36). Thus, pMBA can be used as an internal reference to normalize the signal from analytes on the surface of the particle, reducing variability due to environmental factors. The Ag5 sample (as defined in the experimental section) with the highest pMBA signal was selected for further measurements and is what was shown in Figure 34.
Figure 36. The Raman spectrum of 0.1 M pMBA in 1 M NaOH, along with the SERS spectrum from 1 μM pMBA on AuNS@Ag particles. Spectra have been offset for clarity.

The SERS enhancement factor (EF) for the internal standard and external analyte on the SENSIR particles was determined using pMBA. A 0.1 M solution of pMBA in 1 M NaOH was used as the Raman standard. For the external EF, 1 μM pMBA was added to AuNS@Ag that were prepared without embedded pMBA. These spectra can be found in Figure 36. The SERS enhancement factor calculation was performed as follows:
The 1583 cm$^{-1}$ peak of pMBA was integrated for the 0.1 M pMBA Raman spectrum, the 1 µM pMBA on AuNS@Ag5 SERS spectrum, and SERS signal from 1 µM pMBA embedded in AuNS-pMBA@Ag5, after background subtraction. The exterior enhancement factor was found to be $4.1 \pm 0.2 \times 10^4$, with an interior enhancement factor of $4.2 \pm 0.2 \times 10^6$. The calculated external EF matches our previous report on gold nanostars.$^{55}$ We found the internal EF to be much higher, as could be observed in Figure 35A, at $4.2 \times 10^6$.

While the internal EF provides a very high internal reference signal, this could interfere with the detection of external analytes with low Raman cross-sections. However, we have observed that the presence of the reference signal does not significantly impact the external analyte signal in regions where their peaks do not overlap (Figure 37). When comparing the signal from 10 µM Rh6G on SENSIR particles and AuNS@Ag without embedded reference, the 613 cm$^{-1}$ Rh6G peak intensities are within 10% of each other. It is also possible to tune the nanoparticle structure (size/branching of gold nanostar and amount of silver coating) depending on the wavelength of the laser excitation and/or resonance of the analyte to optimize the SERS enhancement.$^{5}$ If spectral overlap of the peaks is an issue, other reference dyes (i.e. small
thiol molecules) may be used. In our preliminary results, p-aminothiophenol is another dye suitable for embedding (data not shown).

![SERS spectra graph](image)

Figure 37. The SERS spectra of 10 µM Rh6G on SENSIR particles (blue), and silver-coated gold nanostars without the embedded internal reference (red). Spectra have been offset for clarity.

The use of these particles for quantitative detection was demonstrated by establishing a calibration curve for Rh6G concentrations ranging from 1 to 10 µM while the particle concentration used was 0.1 nM. The integrated intensity of Rh6G, pMBA, and the pMBA normalized Rh6G intensity are shown in Figure 35D, Figure 35E, and Figure 35F, respectively. A linear fit was performed on the Rh6G calibration curve, obtaining an R2 value of 0.986. The normalized Rh6G curve improved the R2 value to
0.996, demonstrating that even under ideal conditions the use of an internal standard can aid in quantitative measurements.

### 5.3 Comparison to silver embedded gold spheres

![TEM images](image1.png)

**Figure 38.** TEM images of gold spheres (A) and silver-coated gold spheres (B). (C) shows the SERS spectra, offset for clarity, of pMBA-embedded silver-coated gold nanospheres with 10 μM Rh6G, before (red) and after (blue) the addition of 5 mM MgCl₂. Note the appearance of the 613 cm⁻¹ Rh6G peak after MgCl₂ addition. (D) is the extinction spectrum of AuNP@Ag with (blue) and without (red) 5 mM MgCl₂, indicating rapid aggregation of the particles after salt addition. Scale bars are 50 nm.

To confirm that the nanostar geometry is what provides the unique capability to combine an internal reference signal with the ability to perform non-aggregated SERS analysis on external analytes, a control experiment with gold nanospheres was performed. The 50 nm gold nanospheres were prepared by citrate reduction, resulting in a particle concentration of ~0.1 nM. An image of the particles can be found in Figure 38A. Functionalization with pMBA was performed in the same manner as the nanostars,
and silver coating followed the Ag5 protocol from the experimental section. Figure 38B is a TEM image of the silver coated spheres (AuNP@Ag), where the more electron dense gold core is clearly visible through the silver shell. It was observed that no SERS from Rh6G on AuNP@Ag could be obtained unless salt was added to induce nanoparticle aggregation (Figure 38C). The 613 cm\(^{-1}\) peak from Rh6G appears after addition of 5 mM MgCl\(_2\) to the solution. In the presence of this salt, the plasmon band of the AuNP@Ag is greatly reduced (Figure 38D), indicative of extensive aggregation in the nanoparticle solution.

The dependence upon aggregation of nanoparticles to produce SERS signals from the AuNP@Ag introduces significant issues with reproducibility. This is very apparent in the calibration curve for Rh6G that was constructed in Figure 39, resulting in a linear fit with an \(R^2\) value of 0.578. The random nature of the aggregation process results in a distribution of monomers, dimers, trimers, and higher clusters, all of which have different SERS enhancements. As such, the internal reference signal can no longer be used to normalize the analyte signal due to the inconsistent SERS enhancement factors throughout the sample. This problem is avoided when using the nanostars in place of spherical nanoparticles; since nanostars have intrinsic hot spots that do not require aggregation, there is a more uniform distribution of SERS enhancement throughout the sample.
Figure 39. (A) Integrated Rh6G signal intensity, (B) Integrated pMBA signal intensity, (C) pMBA normalized Rh6G signal at different concentrations of Rh6G on AuNP@Ag.
5.4 Internal reference improves SERS reproducibility

![Image](image.png)

Figure 40. Integrated SERS intensity of 10 μM Rh6G (blue) and the normalized Rh6G intensity (red) when used for analysis in a highly scattering medium (A) and at various particle concentrations (B). Error bars are ±1 standard deviation.

The utility of the internal reference nanostars for reducing SERS signal variability was demonstrated in a highly scattering medium, and also when using different particle concentrations. Polystyrene beads were added at different concentrations to solutions containing 0.1 nM AuNS@Ag and 10 μM Rh6G. This simulates the optically dense environments that are often found in unpurified biological or ecological samples. Figure 40A demonstrates the exponential decay in SERS signal as the concentration of polystyrene beads is increased. The extinction spectra of the polystyrene beads in
solution can be found in Figure 41. There is over an order of magnitude change in Rh6G signal intensity going from no polystyrene beads up to 0.26% w/v. Normalizing the signal by the internal reference significantly reduces this variation, with the lowest normalized intensity falling within two standard deviations of the original sample.

![Extinction spectra of 1µm polystyrene beads in water at the designated % (w/v).](image)

In cases where the concentration of SERS nanoparticles may be variable, such as in vivo, where the particles and/or their solvent could diffuse from the site of delivery, the analyte signal will be affected. The effect of particle concentration on signal from 10 µM Rh6G was investigated by varying the AuNS@Ag from 20 pM to 100 pM (Figure
As shown, the Rh6G signal intensity is correlated with the concentration of particles. At the lowest particle concentration tested, Rh6G signal is less than half that at the highest concentration, whereas the normalized Rh6G signal is essentially a flat line; performing a linear fit on the data in Figure 40B gives a slope of -0.0007. In both of these examples, quantification using SERS would be nearly impossible without the use of an internal reference. The obtained results provide a proof of concept for the use of these novel nanoparticle structures for quantitative SERS detection in complex samples.

To further demonstrate this point, we have taken the raw Rh6G intensity and the normalized intensity from Figure 40A at 0.026% polystyrene, and used the calibration curves from Figure 35 to calculate the predicted concentration of Rh6G. These results have been plotted in Figure 42. As shown, the predicted concentration of 10 µM Rh6G is 3.7 ± 0.3 µM when only using the raw signal intensity. After normalization to the pMBA peak, the predicted concentration is now 8.6 ± 0.9 µM, falling within two standard deviations of the actual value. Thus, we have clearly shown how the internal standard signal can aid in quantitative SERS analysis.
Figure 42. The calibration curves from Figure 2 are plotted together. The Rh6G intensity linear fit is shown in blue, with data points as diamonds (left axis). The normalized linear fit is shown in red, with data points as triangles (right axis). The measured 10 µM Rh6G intensity in 0.026% polystyrene and its predicted concentration is shown as the blue square. The normalized 10 µM Rh6G intensity in 0.026% polystyrene and its predicted concentration is shown as the red square. Error bars are ±1 standard deviation.

5.5 Summary

We have developed a silver-embedded gold nanostar platform that entraps an internal reference dye between the gold and silver surfaces. The unique geometry of the nanostar provides SERS enhancement in a non-aggregated state for analytes on the exterior surface of the particle. When gold nanospheres were used to create silver-embedded particles, salt had to be added to aggregate the particles before any SERS could be detected from the external analyte. The internal reference was shown to significantly reduce SERS signal variation in a highly scattering sample and at different particle concentrations, conditions that simulate some of the matrix effects that may be encountered in real-world samples. This internal reference nanoparticle structure
provides improvement over current strategies for quantitative SERS analysis in complex samples.
Chapter 6. Conclusion

All four of the aims presented at the beginning of this dissertation were completed in full. The studies presented here represent significant advances in the fields of SERS-based theranostics and sensing. In this work, we have demonstrated that nanostars can be used for SERS imaging in combination with photodynamic therapy, as well as the basis of rationally designed nanoparticles for SERS sensing. For imaging applications, the nanostar plasmon was tuned to match the laser excitation wavelength to maximize the SERS enhancement. A silica shell on the gold nanostars was used as a means to carry photosensitizer drug molecules for PDT. The drug-loaded silica coating was shown to have no detrimental effect on the SERS signal of Raman-labeled gold nanostars, and provided a high loading capacity (1000 drug molecules per particle). By functionalizing the theranostic nanocomposite with a CPP, intracellular accumulation of the nanoparticles was greatly enhanced, increasing the efficacy of both Raman imaging and PDT. For use in SERS sensing, the gold nanostars were coated with silver to enhance the SERS signal intensity up to an order of magnitude. Detailed investigation of the silver coating process revealed that the tips of the gold nanostar branches must be preserved, protruding out of the silver coating, in order to maintain the increased signal compared to uncoated gold nanostars. It was also found that a small-molecule Raman dye could be embedded between the gold and silver surfaces of the silver coated gold nanostars. This allowed for improved reproducibility and quantitative analysis of SERS
measurements by using the embedded dye as an internal standard. The dye-embedded particles exhibit SERS signal from the internal dye, as well as from external analytes on the particle surface. The embedded dye was shown to not interfere with the SERS enhancement obtained from analytes on the particle surface. These proof-of-concept studies show the potential of gold nanostars in SERS-based theranostic and sensing applications. We are currently in the process of setting up in vivo studies to test our theranostic construct in animals.

6.1 Ongoing work

6.1.1 Folate receptor targeted theranostic nanoconstruct for surface-enhanced Raman scattering (SERS) imaging and photodynamic therapy (PDT)

Theranostics, the seamless combination of therapy and diagnostics into a single construct, has become an active area of research in the field of nanomedicine.\textsuperscript{38, 84} This combination offers to provide more personalized treatment, specific to the molecular phenotype of the disease, in order to improve patient outcomes and reduce off-target effects.\textsuperscript{85} Recent advances in nanotechnology and the unique properties of nanoparticles make them ideal for use as theranostic agents, combining an imaging modality with a therapeutic function.\textsuperscript{38, 86} Plasmonic nanoparticles are one class of nanoparticles that have been used extensively for imaging applications.\textsuperscript{87}

The strong electromagnetic field enhancement produced by plasmonic particles under the appropriate excitation light can be used for surface-enhanced Raman
scattering (SERS), a very sensitive and specific spectroscopic technique. Recent advances in nanoparticle synthesis and modification have brought about the use of Raman dye labeled nanoparticles as contrast agents in vitro, ex vivo, and in vivo. It has been shown that SERS tags can be as bright as fluorescence tags, while not suffering from photobleaching effects. SERS-tags are also more suitable for multiplex detection, exhibiting sharp, fingerprint-like spectra, in contrast to the broad, featureless emission spectra of fluorescent tags.

Photodynamic therapy (PDT) is a promising treatment for a variety of diseases, including cancer. When a photosensitizer is activated with the appropriate wavelength of light, it can undergo intersystem crossing to an excited triplet state. In the presence of molecular oxygen ($^{1}\text{O}_2$), the photosensitizer in its excited triplet state can transfer its energy, producing singlet oxygen ($^{1}\text{O}_2$) and other cytotoxic reactive oxygen species (ROS), destroying cells in the immediate vicinity. One of the main obstacles to applying PDT clinically is the inactivation or poor solubility of the photosensitizer in biological environments. Loading of the photosensitizer onto a nanoparticle can overcome these problems by both protecting the molecule from degradation, and acting as a carrier for delivery to the intended target.

In order to create a true theranostic construct, the ability to detect and treat must be specific to a subtype of cells. Folic acid (FA) is one of the most common targeting ligands employed for nanoparticle delivery. Many cancer cells overexpress the folate
receptor, while normal cells typically have little to no folate receptor expression. By functionalizing the surface of our nanoparticles with FA, they can be used to specifically label FR-positive cells for detection by SERS, followed by PDT treatment.

In this report, we present the synthesis of a folate receptor targeted theranostic nanoparticle. The diagnostic aspect of the construct comes from a silver-embedded gold nanostar that acts as a SERS tag for Raman imaging. Photosensitizer molecules are loaded onto the SERS tag by encapsulating them in a silica shell for PDT treatment. Selective detection and treatment of folate receptor positive cells are demonstrated using HeLa cervical cancer and SK-BR-3 breast cancer cells.\textsuperscript{97,99} MDA-MB-468 breast cancer cells are used as a folate receptor negative control.\textsuperscript{97,98}
Figure 43. TEM images of gold nanostars (A), pMBA-embedded silver coated gold nanostars (B), and silica-coated, pMBA-embedded silver coated gold nanostars (C; scale bars are 100 nm). (D) SERS signal from the embedded pMBA; 10 s acquisition, 0.65 mW laser power. (E) UV/Vis extinction spectra of the silver-coated nanostars before (dotted) and after (solid) silica coating. (F) PpIX absorption (dotted) and emission (solid) at 415 nm excitation. (G) Particle size distribution of the silver-coated gold nanostars before (dotted) and after (solid) silica coating.

The nanotheranostic agent presented in this work was synthesized by combining different aspects from our previously developed protocols. The gold nanostars were prepared using a modified version of the surfactant-free nanostar procedure that was first reported by our laboratory.\textsuperscript{40} By adding the silver nitrate immediately before ascorbic acid, batch-to-batch variability is reduced compared to simultaneous addition. Figure 43(A) shows a transmission electron microscopy (TEM) micrograph of the gold nanostars that were prepared and subsequently functionalized with para-
mercaptobenzoic acid (pMBA). As we have shown in a recent report, pMBA-labeled gold nanostars can be coated with silver, embedding the pMBA between the gold and silver surfaces, and generating high SERS enhancement of the embedded dye.\(^\text{100}\) The silver-embedded gold nanostars are shown in Figure 43(B). A silica shell was then coated on the silver-embedded gold nanostars in order to load the photosensitizer, protoporphyrin IX (PpIX), onto the nanoparticles.\(^\text{42}\) The silica shell also allows for amine modification with (3-aminopropyl)triethoxysilane (APTES), which is easily conjugated to folic acid – polyethyleneglycol – NHS (FA-PEG-NHS) in order to provide both stability in biological environments and folate receptor targeting capability. The final theranostic construct is shown in Figure 43(C).

A representative SERS spectrum of the pMBA-embedded particles is presented in Figure 43(D), showing the characteristic 1076 cm\(^{-1}\) and 1580 cm\(^{-1}\) Raman peaks of pMBA ring breathing and stretching, respectively.\(^\text{56}\) The silver-embedded nanoparticles were also characterized by UV/Vis absorption spectroscopy (Figure 43(E)), before (dotted) and after (solid) silica coating and functionalization. The plasmon peak shifts from 506 nm to 514 nm after silica coating, as expected due to the increase in refractive index around the particles.\(^\text{69}\) The absorption and emission spectra of the photosensitizer PpIX are shown in Figure 43(F). The amount of PpIX encapsulated in the silica shell was estimated using the fluorescence intensity of PpIX remaining in the supernatant after silica coating (Figure 44). An estimated 0.33 ± 0.03 \(\mu\)M PpIX was loaded on 0.1 nM of
particles, which is in good agreement with our previous report.\textsuperscript{42} The particle size
distribution was measured by Nanoparticle Tracking Analysis (NTA) (Figure 43(G)),
and found no significant aggregation of particles either before (dotted) or after (solid)
silica coating and functionalization.

![Figure 44](image_url)

**Figure 44.** Calibration curve for the quantitation of PpIX encapsulated in the
theranostic nanoparticles. Of the initial 1 $\mu$M PpIX, there was found to be $0.67 \pm 0.03$
$\mu$M remaining in the supernatant after silica coating. This results in an estimated $0.33$
$\pm 0.03 \mu$M loaded on 0.1 nM of particles.

An in vitro demonstration of SERS-based diagnostics was performed. Two folate
receptor-positive cell lines (HeLa and SK-BR-3) and one folate receptor-negative cell line
(MDA-MB-468) were used. The cells were incubated with the folate conjugated
nanocomposite for 4 hours in fresh medium. Following the incubation period, cells were
washed 3 times in PBS prior to being fixed and dried for imaging. In this case, the cells
were fixed because our Raman microscope is not set up to perform live cell measurements. If equipped with a microscope incubator chamber, the SERS detection could be carried out on live samples. The cell sample was placed on the microscope stage and cells were located using a 10x objective. An area for Raman mapping was designated in the microscope software (white boxes in Figure 45). Raman spectra were acquired at each point in a grid across the sample at 20 μm step sizes. The intensity of the 1076 cm⁻¹ Raman peak of pMBA was then used to generate the false-color Raman maps by integrating the signal to baseline from 1050 to 1100 cm⁻¹ in the WiRE software. The color scale between all three samples was kept the same. As shown in Figure 45, both the HeLa and SK-BR-3 samples show high signal intensity from the FA-targeted nanoparticles, with low background signal outside of the cell-containing region. As expected, the MDA-MD-468 samples only show a background level of SERS signal following particle incubation. These results demonstrate the selective detection of folate receptor positive cells using the developed theranostic construct.
Figure 45. Raman mapping of the three different cell lines after 4 hr incubation with the FA-targeted theranostic nanoparticles. The sample was scanned in a grid pattern with 20 μm step size, taking a 1 s acquisition at each point. The Raman peak intensity at each point was then integrated to create the false-color map that is overlaid on the brightfield image. The folate receptor positive cell lines (HeLa and SK-BR-3) show high Raman intensity coming from the cluster of cells, while the folate receptor negative cell line (MDA-MB-468) shows little to no Raman signal.

The cells used for PDT were treated with particles in the same manner as for SERS detection. Instead of being fixed after PBS washing, fresh medium was replenished. An area of cells was focused on with a 40x objective, and then exposed to filtered red light (640/30 nm) from a mercury arc lamp for 15 seconds; the measured power at the sample was 11.5 mW. After irradiation, the cells were returned to the incubator for 4 hours prior to live/dead staining. The results of the PDT treatment can be seen in Figure 46. The complete theranostic construct (row 1) showed almost complete cell death for HeLa, a high percentage of cell death for SK-BR-3, and little to no cell death for MDA-MD-468. The amount of cell death in the SK-BR-3 sample is likely underestimated, as many of the cells in the irradiated area became detached, as observed in Figure 46. The SK-BR-3 cells may be more sensitive to environmental stresses than
HeLa, causing them to detach after PDT. Otherwise, these results are what was expected based on the folate receptor expression of the cell lines.

To verify that the cell death was due to PDT rather than photothermal or other light-induced effects, the targeted nanoparticle construct without PpIX was used as a control (row 2). As shown, there is no cell death after irradiation in the absence of PpIX. Likewise, without the FA for targeting (row 3), there are not enough particles uptaken by cells during the incubation period to cause any PDT effects after irradiation.

The biocompatibility of the theranostic construct was evaluated by a resazurin-based cell viability assay after the 4 hr incubation (Figure 47). There is a slight decrease in cell viability measured after particle incubation; however, none of observed differences were found to be statistically significant.
Figure 46. Cell viability staining after PDT treatment with the theranostic construct, a targeted without drug control, and an untargeted with drug control. The scale of each photo is ~1.86×1.24 mm. The number of live and dead cells in the irradiated area, compared to non-irradiated cells, were used to create the histogram displayed to the right of the cell photos. Error bars are one standard deviation (n=4).
Figure 47. Resazurin-based cell viability assay of the three cell lines after 4 hr incubation with the theranostic nanoparticles. There is a slight decrease in viability observed after particle incubation, though none are statistically significant.

In summary, we report the synthesis of a folate receptor targeted theranostic nanocomposite for SERS-based detection and PDT. The selective detection of folate receptor positive cells was demonstrated by Raman imaging, showing high SERS signal from folate receptor positive cells and little to no signal from folate receptor negative cells. The selective therapeutic effect was demonstrated by performing an in vitro PDT study. Again, only the folate receptor positive cells were affected by the treatment. The HeLa cells in the irradiated area were almost completely dead and the SK-BR-3 showed a high percentage of cell death, even though many of the cells detached from the treatment area. The folate receptor negative MDA-MB-468 cells showed no cell death in
the irradiated area. These data suggest that the silica coated, silver embedded gold nanostars are a promising platform for the development of future theranostic agents. Use of other small-molecule Raman dyes can provide the particles with unique SERS spectra for application in multiplex detection when combined with other specific targeting agents, such as antibodies.

6.1.2 Gold nanostars for adipose-derived stem cell tracking and photothermal ablation

Previous studies have shown that gold nanostars (GNS) are a suitable labeling agent for the tracking of mesenchymal stem cells. We are currently investigating the use of nanostars for the tracking and ablation of adipose-derived stem cells (ASCs). When compared the current standard for cell tracking, Qtracker quantum dots, the nanostars exhibited brighter intensity and longer signal retention within cells (Figure 48).
Figure 48. Undifferentiated ASCs are shown under phase-contrast (A) and stained with Hoechst 33342 following a 24h incubation with 0.14nM of TAT-GNS, and imaged with MPM (B). In (A), the TAT-GNS (yellow arrows) can be seen as black dots in the cytoplasm under phase contrast microscopy. In (B), the cytoplasmic localization of the GNS (white dots denoted by yellow arrows), in relation to the nucleus that is stained blue, is shown. Undifferentiated ASCs are shown following a 24h incubation with 0.14nM TAT-GNS and 0.14nM Qtracker (C). Cells were fixed on days 1, 2, and 4 and then imaged to determine the relative TPL emitted for each optical label. Each cell could be roughly distinguished by the predominant TPL. Qtracker (red dots) and TAT-GNS (white) could be seen in each cell. TPL was measured over a period of 4 days. There was a significantly greater degree of TPL emitted by those cells labeled with GNS vs. Qtracker throughout all 4 days of cellular proliferation. Images (B, C) were taken at 800nm using a laser power of 0.04W/cm². Scale bars represent 50 µm (A, C) and 20 µm (B).
It was also hypothesized that the nanostar-loaded stem cells could be ablated by photothermal therapy due to the high loading of particles and efficient light to heat conversion of the gold nanostars. By harnessing the ability of ASCs to migrate to tumors, it may be possible to ablate tumors in vivo after intravenous injection of GNS-loaded ASCs. Preliminary testing in vitro has shown that the GNS-loaded ASCs can be ablated by photothermal therapy (Figure 49). When the GNS-loaded ASCs are co-cultured with breast cancer cells, either SK-BR-3 or SUM-149 in this case, both cell types within the irradiated area are ablated.
Figure 49. Photothermal therapy effects on GNS-ASCs alone (A). ASCs were incubated with GNS and unlabeled media for 24 hours at 37 °C, and then exposed to an 800 nm laser for 3 minutes. The laser power used was 11 W/cm$^2$, 22 W/cm$^2$, and 55 W/cm$^2$. Representative images are shown following laser exposure. Cells were stained with fluorescein diacetate and propidium iodide which stains live cells green and dead cells red respectively. Following laser exposure, the control group displayed no cell death at all treatment powers. This is as compared to the GNS-labeled ASCs which resulted in a clear zone of cellular ablation as denoted by the empty space. A treatment power of 22 W/cm$^2$ was judged to be the most efficient and specific with respect to the size and area of ablation, and was used for co-culture photothermal treatments (B). SKBR3 cells and SUM-149 cells were co-cultured with either GNS-ASCs, or ASCs alone for 24 hours and then exposed to a 22 W/cm$^2$ laser for 3 minutes using the MPM. As compared to the unlabeled ASCs, those cells co-cultured with GNS-ASCs demonstrated a clear area of cellular death following treatment. Both samples were stained with fluorescein diacetate and propidium iodide which stains live cells green and dead cells red respectively.
6.2 Future work

6.2.1 Antibody targeting of theranostic constructs

A logical next step after the development of the folate receptor targeted theranostic agent is to use other targeting agents to provide multiplex capabilities. Antibodies offer high specificity and affinity for binding to their antigen. By conjugating antibodies to the theranostic nanoparticles, specific cell surface receptors can be targeted. Different SERS tags can be used with each antibody to achieve multiplexed detection based on the various SERS spectra.

6.2.2 In vivo SERS/PDT

A theranostic nanoprobe for in vivo SERS and PDT is currently under development. A 785 nm resonant Raman dye will be used for the SERS tag, taking advantage of the deeper penetration depth of light in tissue at NIR wavelengths. A recently commercialized photosensitizer, IRDye 700DX, will be used for photodynamic therapy. A 690 nm diode laser has been acquired for activation of the photosensitizer. Current testing in our lab has shown that IRDye 700DX can be covalently linked into a silica shell coated onto a gold nanostar. The NHS ester of the dye is readily conjugated to APTES, which is then incorporated into the silica matrix through hydrolysis and condensation reactions during the standard coating procedure. We have demonstrated
that these dye-loaded particles are capable of producing a photosensitizing effect in vitro after light irradiation.

In vivo studies will be initiated in a murine breast cancer model. The theranostic nanoparticles will be PEGylated to provide stability under physiological conditions and stealth behavior. Nanoparticles can be delivered by intratumoral injection or intravenously, relying on the EPR effect for particle accumulation in the tumor. The 690 nm laser light will be delivered to the tumor by a fiber optic either in contact with or held above the tumor.
Appendix A: Experimental Procedures

Chapter 2

Materials

Gold(III) chloride trihydrate (HAuCl₃·3H₂O), trisodium citrate dihydrate (C₆H₅O₇Na₃·2H₂O), 1N HCl, L(+)-ascorbic acid (AA), tetraethyl orthosilicate (TEOS), O-[2-(3-Mercaptopropionylamino)ethyl]-O′-methylpolyethylene glycol (mPEG-SH, MW 5k), methylene blue hydrate (MB), 3,3′-Diethylthiatricarbocyanine iodide (DTTC), fluorescein diacetate (FDA), propidium iodide (PI), and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA) at the highest purity grade available. Silver nitrate (AgNO₃, 99.995%) was supplied by Alfa Aesar (Ward Hill, MA, USA). Pure-grade ethanol and NH₄OH (29.5%) were obtained through VWR (Radnor, PA, USA). Ultrapure water (18 MΩ cm⁻¹) was used in all preparations. Singlet Oxygen Sensor Green (SOSG) reagent was procured from Invitrogen (Carlsbad, CA, USA). Hydrofluoric acid (HF, 52%) was bought from EMD Chemicals (Gibbstown, NJ, USA). Caution: HF is highly toxic and must be handled with the proper protective equipment.

SERS-tagged Nanostar Synthesis

Detailed synthesis and characterization of the nanostars will be presented elsewhere. A modified Turkevich method was used to prepare the seed solution.²³,²⁴ Nanostars were grown from the seed by simultaneous addition of 100 µL 2 mM AgNO₃ and 50 µL 0.1 M AA to a solution containing 10 mL of 0.25 mM HAuCl₄, 10 µL of 1N
HCl, and 100 µL of the gold seed solution under vigorous stirring. After 10 seconds the solution turned from light red to a dark grey. The stock concentration of nanoparticles is approximately 0.1 nM, as determined by Nanoparticle Tracking Analysis (NTA).

Freshly synthesized nanostars (10 mL) were capped with 5 µM mPEG-SH under gentle stirring for 15 minutes. The PEGylated particles were then centrifuged (10k rcf, 15 minutes) twice at 4 °C to remove excess PEG and redispersed in water. 5 µM DTTC in methanol was added to this solution and allowed to stir overnight. The DTTC-tagged particles were centrifuged (5k rcf, 15 minutes) twice at 4 °C to remove excess DTTC and resuspended in 2.3 mL of ethanol.

**Encapsulation of PDT Drug**

A modified Stöber method was used for formation of the silica shell.25 Under gentle stirring, 2.25 mL of the nanostars in ethanol was added to a solution containing 2.0 mL of water and 6.8 mL ethanol. Methylene blue (final concentration 5 µM) in ethanol and 160 µL of NH$_4$OH were added to the mixture. Silica coating was initiated by the addition of 30 µL 10% TEOS in ethanol, and the reaction was allowed to proceed for three hours. The nanoparticles were then centrifugally purified (3.5k rcf, 15 minutes) until no MB absorption could be detected from the supernatant (typically 2-3 times) and redispersed into 5 mL of water. The concentration of MB encapsulated in the sample was determined by dissolving the silica shell of the nanoparticles using a 26% HF, 50% EtOH, 24% H$_2$O solution. After reacting for 30 minutes, the sample was centrifuged at
18k rcf and the supernatant was collected. The supernatant was diluted with an equal volume of H₂O and fluorescence emission was recorded using 633-nm excitation.

**Characterization**

Raman spectra with 785-nm excitation (40 mW) were recorded on a HORIBA Jobin Yvon LabRAM ARAMIS system (Edison, NJ, USA) using a 1200 g mm⁻¹ grating. Fluorescence and Raman spectra with 633-nm (8 mW) excitation were recorded on a Renishaw inVia Raman microscope (Gloucestershire, UK) using an 1800 g mm⁻¹ grating. Transmission electron microscopy (TEM) was performed on a FEI Tecnai G² Twin transmission electron microscope (Hillsboro, OR, USA) with an accelerating voltage of 160 kV. Vis-NIR spectra were acquired on a Shimadzu UV-3600 (Columbia, MD). Particle concentrations and size distributions were measured by NTA with a NanoSight NS500 (Amesbury, UK).

**Singlet Oxygen Generation**

The fluorescent probe SOSG was used to indirectly measure singlet oxygen generation from the nanoparticles. In a 96-well plate, 90 µL of the coated nanoparticles were mixed with 10 µL of ~100 µM SOSG in methanol. The sample was excited using 633-nm laser light focused into the solution with a 10x objective. The laser power was 8 mW at the sample. Fluorescence intensity was measured at different time points with a BMG LABTECH FLUOstar Omega (Durham, NC, USA) using an excitation filter at 500 ± 10 nm and an emission filter at 530 ± 10 nm.
**In vitro PDT Study**

The BT549 cancer cells were a gift from Dr. Victoria Seewaldt. Cells were incubated in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% of fetal bovine serum, 25 mM HEPES and 0.023 U/mL of insulin, in an incubator with a humidified atmosphere (37 °C, 5% CO₂). Cells in exponential growth phase were used in the experiments. The nanoparticles were concentrated to ~0.2 nM and redispersed into the RPMI media. Cells were incubated with the particle-containing media during 1 hour of irradiation from a 633-nm HeNe laser (Model 1145, JDS Uniphase, Milpitas, CA) at 37 °C. The laser was coupled to a fiber that was routed into the incubator and then focused on the sample using a 10x microscope objective. Laser power at the sample was 16 mW with a spot size of ~1.5 mm in diameter, resulting in a laser power density of approximately 900 mW cm⁻². After treatment, the cells were washed twice with PBS, and incubated with fresh media overnight. Cell viability was assessed by incubating cells for 5 minutes in a solution of PBS containing 1 µg/mL FDA for live cells and 50 µg/mL PI for dead cells, and imaging on a fluorescence microscope. Images were recorded with a Canon EOS Rebel XTi (Tokyo, Japan) mounted to the front port of the microscope.
Chapter 3

Materials

Gold(III) chloride trihydrate (HAuCl₃·3H₂O), trisodium citrate dihydrate (CsH₃O₇Na·2H₂O), 1N HCl, L(+)-ascorbic acid (AA), tetraethyl orthosilicate (TEOS), O-[2-(3-Mercaptopropionylamino)ethyl]-O’-methylpolyethylene glycol (mPEG-SH, MW 5k), Protoporphyrin IX (PpIX), 3,3’-Diethylthiadicarbocyanine iodide (DTDC), fluorescein diacetate (FDA), propidium iodide (PI), and Resazurin based Toxicology Assay Kit (TOX8) were purchased from Sigma-Aldrich (St. Louis, MO, USA) at the highest purity grade available. Silver nitrate (AgNO₃, 99.995%) was supplied by Alfa Aesar (Ward Hill, MA, USA). Pure-grade ethanol and ammonium hydroxide (NH₄OH, 29.5%) were obtained through VWR (Radnor, PA, USA). Ultrapure water (>18 MΩ cm⁻¹) was used in all preparations. All glassware was cleaned with aqua regia, washed with copious amounts of water, and dried prior to use. Cell culture media and supplements, ProLong Gold Antifade Reagent, and Hoescht 33342 were purchased from Invitrogen (Carlsbad, CA). TAT-peptide (residues 49–57, sequence Arg-Lys-Lys-Arg-Arg-Arg-Gln-Arg-Cys-CONH₂) was ordered from SynBioSci (Livermore, CA).
**Instrumentation**

Raman spectra were recorded on a Renishaw inVia Raman microscope (Gloucestershire, UK), controlled by WiRE 2.0 software, using an 1800 g mm\(^{-1}\) grating with 633 nm (8 mW) excitation. Fluorescence emission spectra were collected using an Edinburgh Photonics FLS920 fluorescence spectrometer (Livingston, UK). Transmission electron microscopy (TEM) was performed on a FEI Tecnai G\(^2\) Twin transmission electron microscope (Hillsboro, OR, USA) with an accelerating voltage of 200 kV. Absorption spectra were acquired on a Shimadzu UV-3600 (Columbia, MD). Particle concentrations and size distributions were measured by Nanoparticle Tracking Analysis (NTA) with a NanoSight NS500 (Amesbury, UK). The fluorescence intensity of the Resazurin-based toxicology assay was measured by a FLUOstar Omega plate reader (BMG LABTECH GmbH, Germany). Photodynamic therapy treatment and cell viability imaging were performed on a Zeiss Axiovert 200M inverted microscope (Thornwood, NY) equipped with an X-Cite Series 120 mercury arc lamp (Lumen Dynamics, Mississauga, ON, Canada). Images were recorded with a Canon EOS Rebel XTi (Tokyo, Japan) mounted to the front port of the microscope. The TPL images were recorded using a commercial multiphoton microscope (Olympus FV1000, Olympus America, Center Valley, PA) with a femtosecond Ti:sapphire laser (Chameleon Vision II, Coherent, Santa Clara, CA) used for excitation.
Raman-Labeled Nanostar Synthesis

The nanostars were synthesized according to a previously published protocol. A gold seed solution was prepared by bringing 100 mL of 1 mM HAuCl₄ to a rolling boil and adding 15 mL of 1% trisodium citrate under vigorous stirring. The solution was kept boiling for 15 minutes, cooled, filtered with a 0.22 µm nitrocellulose membrane, and stored at 4 ºC. Nanostars were grown from the seed by adding 100 µL of the gold seed to a solution containing 10 mL of 0.25 mM HAuCl₄ and 10 µL of 1N HCl, followed quickly by simultaneous addition of 100 µL 1 mM AgNO₃ and 50 µL 0.1 M AA under moderate stirring. Within 10 seconds the solution turned from light red to a deep blue. The stock concentration of nanoparticles is approximately 0.1 nM, as determined by Nanoparticle Tracking Analysis (NTA).

Freshly synthesized nanostars (10 mL) were conjugated with mPEG-SH (1 µM final concentration) under gentle stirring for 15 minutes. The PEGylated particles were then centrifuged (3.5k rcf, 15 minutes) twice at 4 ºC to remove excess PEG and redispersed in water. DTDC (0.2 µM final concentration) in ethanol was added to this solution and allowed to stir overnight. The DTDC-tagged particles were centrifuged (3.5k rcf, 15 minutes) twice at 4 ºC to remove excess DTDC and resuspended in water (AuNS-DTDC).
Encapsulation of Protoporphyrin IX and TAT Conjugation

A modified Stöber method was used for formation of the silica shell. The labeled nanostar solution was centrifuged at 4 °C (3.5k rcf, 15 minutes) and resuspended in 2 mL of ethanol. Under gentle stirring, the solution of nanostars was added to a 20 mL glass vial containing 2.0 mL of water and 7.0 mL ethanol. Protoporphyrin IX (1 µM final concentration) in ethanol and 180 µL of NH$_4$OH were added to the mixture. Silica coating was initiated by the addition of 30 µL 10% TEOS in ethanol, and the reaction was allowed to proceed for three hours. The nanoparticles were then centrifugally purified (3.5k rcf, 15 minutes) two times and redispersed into 5 mL of ethanol. TAT conjugation was achieved by passive adsorption; a final concentration of 100 µm TAT was added to the ethanolic solution of particles and allowed to stir overnight.

Cell Culture and Nanoparticle Incubation

The BT-549 breast cancer cells were a gift from Dr. Victoria Seewaldt. Cells were cultured in modified RPMI 1640 medium (Gibco 22400-089) supplemented with 10% fetal bovine serum and 0.023 IU/mL insulin, and incubated at 37 °C in a humidified 5% CO$_2$ atmosphere. For PDT studies, cells were seeded into 6-well plates. Cells prepared for Raman mapping were grown on sterilized glass coverslips in 6-well plates. Cytotoxicity was assessed using cells grown in a 96-well plate. Cell samples for two-photon luminescence imaging were grown in 35 mm Petri dishes. All samples were grown to ~80% confluency before use.
The nanoparticle solution was prepared for cellular incubation by centrifugally washing once with water, then resuspending into complete growth medium to a particle concentration of 0.1 nM. Cells were incubated with the particle-containing medium for one hour. After incubation, the medium was aspirated and the cells were washed three times with PBS. For the cytotoxicity assay, growth medium was replaced and the cells were cultured for 24 hours. Resazurin (10% v/v) was added and the plate was kept in the incubator for 1 hour. Resazurin (blue, nonfluorescent) is reduced by live cells to resorufin (pink, fluorescent). The fluorescence intensity of resorufin was then measured by a plate reader. For two-photon luminescence imaging, cells were fixed in 4% paraformaldehyde and stained with Hoescht 33342 (2µg mL⁻¹ in PBS) 30 minutes prior to imaging.

**Raman Mapping**

After particle incubation the cells were fixed with a 4% paraformaldehyde solution and rinsed with water to remove any remaining salt. The coverslips were removed from the 6-well plate and mounted onto glass slides following the protocol for the ProLong Gold Antifade Reagent. After curing for 24 hours, the edges of the coverslip were sealed with clear nail polish to extend the sample life. Raman mapping was performed on the Renishaw inVia Raman microscope. Cells were located under brightfield illumination with a 40x objective. Spectra were collected with the grating centered at 1100 cm⁻¹ (~600 cm⁻¹ bandwidth) during a 5-second data acquisition. The
Raman image maps were created by collecting spectra at multiple points on a grid with 2-µm spacing over the 2D region of a cell. The baseline-subtracted intensity from the DTDC peak between 1120 and 1150 cm⁻¹ was integrated and then displayed over the grid using a color scale to depict the intensity variation across the area.

**Photodynamic Therapy**

After particle incubation, the cells were kept in PBS to prevent any optical interference from the phenol red in the cell culture medium. A region of cells was focused on using a 40x phase contrast objective, and then irradiated with light from the mercury arc lamp after passing through a DAPI filter (377/50 nm). The measured power density was 4.4 W/cm². After treatment, the PBS was replaced with growth medium and cells were cultured for 4 hours prior to viability staining. Cell viability was assessed by incubating cells for 5 minutes in a solution of PBS containing 1 µg mL⁻¹ FDA for live cells (green) and 50 µg mL⁻¹ PI for dead cells (red), and imaging on a fluorescence microscope.

**Data Analysis**

Smoothing and baseline subtraction of Raman spectra was performed in MATLAB R2012a. Spectra were smoothed using the ‘smooth’ function with parameters: span = 15, method = ‘sgolay’, degree = 2. The baseline was removed using a numerical algorithm developed in our laboratory, which uses a moving window to locally determine the background fluorescence. Unprocessed versions of the Raman spectra
presented in the text can be found in the electronic supplementary information.

Mathematica 8.0.4 was used to integrate the area under the curve for fluorescence spectra of PpIX. Scale bars were added to images using ImageJ 1.46j. All graphs were created in Microsoft Excel for Mac Version 14.2.3.
**Chapter 4**

**Materials**

Gold(III) chloride trihydrate (HAuCl₃·3H₂O), L(+)-ascorbic acid (AA), tetraethyl orthosilicate (TEOS), trisodium citrate dihydrate, sodium borohydride (NaBH₄), 1 N hydrochloric acid solution (HCl), hexadecyltrimethylammonium bromide (CTAB, product H9151), Dulbecco's phosphate buffered saline (PBS), O-[2-(3-Mercaptopropionylamino)ethyl]-O'-methylpolyethylene glycol (mPEG-SH, MW 5k), 4-Mercaptobenzoic acid (pMBA), IR-780 iodide, 3,3'-Diethylthiadiacarbocyanine iodide (DTDC), 3,3'-Diethylthiatricarbocyanine iodide (DTTC), and 1,1',3,3',3',3'-Hexamethylindotricarbocyanine iodide (HITC) were purchased from Sigma-Aldrich (St. Louis, MO, USA) at the highest purity grade available. Silver nitrate (AgNO₃, 99.995%) was supplied by Alfa Aesar (Ward Hill, MA, USA). Ammonium hydroxide (NH₄OH, 29.5%), carbon-coated copper TEM grids, 1 mL disposable syringes, 27 G x ½” needles, and 200 proof ethanol (EtOH) were obtained through VWR (Radnor, PA). All glassware and stir bars were thoroughly cleaned with *aqua regia* and dried prior to use. Ultrapure water (18 MΩ•cm) was used in all preparations.

**Instrumentation**

Raman spectra were recorded with a PIXIS:100BReX CCD mounted to a LS-785 spectrograph (1200 g mm⁻¹ grating), controlled by LightField software, from Princeton Instruments (Trenton, NJ). A 785 nm diode laser was fiber-coupled to an InPhotonics
RamanProbe (Norwood, MA) for excitation, with a power of 150 mW at the sample; the collection fiber of the RamanProbe was coupled to the entrance slit of the LS-785 spectrograph. Absorption spectra were collected with a FLUOstar Omega plate reader (BMG LABTECH GmbH, Germany). A FEI Tecnai G² Twin transmission electron microscope (Hillsboro, OR, USA) was used to acquire transmission electron microscopy (TEM) micrographs. Particle size distributions were measured by Nanoparticle Tracking Analysis (NTA) on a NanoSight NS500 (Amesbury, UK).

**Gold Nanostar Synthesis (AuNS)**

Three types of AuNS were synthesized as previously reported. A 12 nm gold seed solution was prepared by adding 15 mL of 1% trisodium citrate to 100 mL of a boiling solution of 1 mM HAuCl₄. This solution was kept boiling for an additional 15 minutes, cooled to room temperature in an ice bath, filtered through a 0.22 µm nitrocellulose membrane, and stored at 4 °C until use. To produce the AuNS, 100 µL of the gold seed was added to a 10 mL solution of 0.25 mM HAuCl₄ containing 10 µL of 1 N HCl, immediately followed by the simultaneous addition of 50 µL 0.1 M AA and 100 µL of AgNO₃ (0.5, 1, or 3 mM; samples designated S5, S10, and S30 based upon the final concentration of AgNO₃) under moderate stirring. The concentration of the silver nitrate solution controls the branch length and branch number of the resulting AuNSs. After synthesis, 100 µL of 0.1 M CTAB was added to the AuNS solution and left stirring for 5
minutes. The particles were then centrifuged at 2000 rcf for 20 minutes at 4 °C, the supernatant discarded, and the particles re-dispersed in 10 mL of 1 mM CTAB solution.

**Gold Nanosphere Synthesis (AuNP)**

CTAB stabilized AuNPs were obtained by the method of Jana *et al.* Gold seed was prepared by the addition of 0.6 mL freshly prepared, ice-cold NaBH₄ to a 20 mL aqueous solution containing 0.25 mM HAuCl₄ and 0.25 mM trisodium citrate. Growth solution (0.25 mM HAuCl₄ in 0.08 M aqueous CTAB) was prepared while the seed particles were aged for two hours. Seeded growth was performed by mixing 7.5 mL of growth solution with 50 µL 0.1 M AA, and then adding 2.5 mL of the gold seed solution while stirring; this is designated set A. After 10 minutes, 1 mL of set A was added to 9 mL of growth solution containing 50 µL 0.1 M AA, producing set B. This process was repeated two more times to obtain sets C and D. The particles from set D are ~30 nm in diameter and were used for subsequent silver coating. Set D was washed three times by centrifugation (3000 rcf for 30 minutes at 4 °C) and redispersion in 1 mM CTAB solution.

**Silver Coating of Gold Nanoparticles (AuNS@Ag and AuNP@Ag)**

A 1 mL aliquot of the washed AuNS/AuNP solution was transferred into a 1.5 mL centrifuge tube. The sample was briefly vortexed after each subsequent chemical addition. A small volume (varied between 0 and 15 µL) of 0.1 M AgNO₃ and an equivalent volume of 0.1 M AA were added to the solution. The reduction of silver by AA was initiated by the addition of NH₄OH (2 µL), at which point the color of the
solution began to darken. After about 5 minutes, the solution color had stabilized, indicating completion of the reaction. The various silver-coated AuNS samples were designated according to the volume of AgNO₃ added (e.g., S30@Ag5 for S30 AuNS coated using 5 µL of 0.1 M AgNO₃). The silver coated AuNS were then labeled with dye by adding 1 µM final concentration of the desired dye (dissolved in MeOH) to the solution, allowing it to sit for 15 minutes, centrifuging at 2000 rcf for 10 minutes, discarding the supernatant, and re-dispersing in water.

**Silica Coating (AuNS@Ag@SiO₂)**

Silica was coated onto the labeled AuNS@Ag using an established protocol. To the 1 mL sample of dye-labeled particles prepared above, 5 µL of 1 mM mPEG-SH was added and allowed to react for 1 hour. The solution was washed once by centrifugation (2500 rcf, 10 min) and then dispersed in 900 µL of EtOH with 200 µL of water. Silica coating was initiated by adding 18 µL of NH₄OH followed by 5 µL of 10% TEOS in EtOH to the solution. The reaction was allowed to proceed for 12 hours, at which point the sample was washed twice by centrifugation at 3000 rcf for 5 minutes and re-dispersed in water.

**Silver-Coated Nanostar 2D Modeling**

The 2D simulations were performed using COMSOL Multiphysics 4.3b with the RF module. The gold AuNS model consisted of a 50 nm core, with six equally spaced branches of ~25 nm in length. The silver coating process was modeled by overlaying
increasingly larger spheres of silver onto the AuNS, until it became completely embedded in the silver shell. The dielectric functions of gold and silver were calculated using the Lorentz-Drude model from Johnson and Christy. The nanoparticles were excited with an x-polarized plane wave propagating along the y-axis in the wavelength range from 400 to 800 nm.

**SERS Nanoprobe Injections**

A shaved rat pelt was provided by Dr. Bruce Klitzman. Prior to injection, 1 mL of AuNS@Ag@SiO$_2$ were centrifuged at 3000 rcf for 5 minutes and the supernatant discarded. The particles were then re-dispersed in 100 µL of PBS, giving a particle concentration of about 1 nM. A 1 mL disposable syringe with a 27G needle was used to draw up ~50 µL of the PBS particle solution. The needle was inserted tangentially to the skin (intradermal) with the bevel facing upward and ~25 µL of the solution was injected. The rat pelt was then placed under the focus of the RamanProbe to collect SERS spectra.
Chapter 5

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) at the highest purity grade available and used as received unless otherwise specified. Ammonium hydroxide (NH₄OH, 29%), and ethanol (200 proof) were acquired through VWR (Radnor, PA). Polystyrene beads (1.0 µm diameter) were obtained from Polysciences, Inc. (Warrington, PA). Glassware and stir bars were cleaned with aqua regia prior to use. Ultrapure water (18MΩ•cm) was used in all preparations.

Instrumentation

UV/Vis extinction spectra were acquired with a FLUOstar Omega plate reader (BMG LABTECH GmbH, Germany). Transmission electron microscopy (TEM) was performed with a FEI Tecnai G² twin transmission electron microscope (Hillsboro, OR). Raman spectra were recorded on a Renishaw inVia Raman microscope (Gloucestershire, U.K.) equipped with a 633 nm laser for excitation. Particle concentrations were measured with a Nanosight NS500 (Malvern Instruments Ltd, Worcestershire, U.K.).

Spherical gold nanoparticle synthesis (AuNP)

The AuNP were prepared using the citrate reduction method. An Erlenmeyer flask (250 mL) containing 100 mL of 0.25 mM HAuCl₄ was brought to a boil while stirring vigorously. To this solution, 0.8 mL of 1% w/v trisodium citrate dihydrate in water was quickly injected. The solution immediately became colorless, gradually
changed to dark grey, and then finally to a dark purple. After this point the solution was kept boiling for 30 minutes, then cooled to room temperature before use. The as-prepared particles were then labeled with *para*-mercaptobenzoic acid (pMBA, 10 µM final concentration), along with the addition of Tween 20 (0.02% final concentration) and left overnight. The particles were washed by centrifugation at 2000 rcf for 60 minutes and redispersed into 0.02% Tween 20 solution.

**Gold nanostar synthesis (AuNS)**

The AuNS were synthesized as previously reported. Briefly, a 10 mL solution of 0.25 mM HAuCl4 containing 10 µL of 1 N HCl was prepared in a 20 mL scintillation vial. With rapid stirring, 100 µL of 12 nm gold seed was quickly added to the above solution. After even dispersion of the gold seed (~5 s), 50 µL of 0.1 M ascorbic acid and 50 µL of 2 mM AgNO₃ were rapidly and simultaneously added, and the color of the solution changed from a pale red to deep blue. A final concentration of 10 µM pMBA and 0.02% Tween 20 were then added and allowed to incubate overnight. The sample was then washed by centrifugation at 2000 rcf for 20 minutes and resuspended in 0.02% Tween 20.

**Silver embedding of gold nanostars (AuNS@Ag) and spherical nanoparticles (AuNP@Ag)**

For 1 mL of the pMBA labeled nanostars, equivalent amounts (1, 3, 5, 7, or 10 µL) of 0.1M AgNO₃ (designated Ag1, Ag3, Ag5, Ag7, Ag10, respectively) and 0.1M ascorbic acid were added sequentially and vortexed to mix. The silver reduction process was
initiated by the addition of 2 µL NH₄OH (29%), causing the extinction spectrum of the solution to begin blue shifting. The reaction was allowed to proceed for at least one hour prior to using the particles in subsequent experiments. The AuNS@Ag5 were selected for further study as they provided the maximum internal standard signal observed; AuNP@Ag5 were prepared for control experiments.

**Model analyte SERS detection**

Rhodamine 6G (Rh6G) was used as a model analyte to demonstrate the SERS activity on the exterior surface of AuNS@Ag and AuNP@Ag. The prepared particles (~0.1nM concentration) were mixed with Rh6G dissolved in ethanol to achieve final concentrations in the range of 1 to 10 µM. The samples were transferred to glass vials for measurement on the Renishaw microscope. The laser (~6.8 mW at the sample) was focused into the solution with a 10x objective, and a 10 second exposure time was used to record the Raman spectrum. In the case of the AuNP@Ag, no SERS from Rh6G was observed when the particles were non-aggregated; to achieve a measureable Rh6G signal, 5 mM MgCl₂ was added to the solution immediately before collecting the spectrum to induce aggregation.

**Demonstration of reduced SERS variability by using an internal standard**

The variability of SERS signal in a scattering medium was investigated by adding 1.0 µm polystyrene beads to solutions of 0.1 nM AuNS@Ag containing 10 µM Rh6G.
After mixing, the solutions were transferred to glass vials for the Raman measurements as previously described.

We also studied the effect of particle concentration on the measured SERS signal. A range of AuNS@Ag concentrations from 20 to 100 pM were prepared and 10 µM final concentration of Rh6G was added. The samples were transferred to glass vials and Raman spectra were acquired as above.
Chapter 6

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) at the highest purity grade available unless otherwise specified. Ammonium hydroxide (NH₄OH; 50% v/v) and ethanol (EtOH; 200 proof) were obtained through VWR (Radnor, PA). Folic acid PEG NHS (FA-PEG-NHS) was acquired from Nanocs Inc. (New York, NY). All cell culture reagents, fluorescein diacetate (FDA), propidium iodide (PI), and phosphate buffered saline (PBS) were purchased from Invitrogen (Carlsbad, CA). Ultrapure water (> 18 MΩ · cm) was used in all preparations.

Instrumentation

Transmission electron microscopy (TEM) was performed on a FEI Tecnai G² Twin (Hillsboro, OR). Particle size distribution and concentration were measured by Nanoparticle Tracking Analysis (NTA) with a NanoSight NS500 (Malvern, Worcestershire, UK). Raman spectra were acquired with a Renishaw inVia Raman microscope (Gloucestershire, UK), equipped with a 633 nm HeNe laser. UV/Vis spectra and fluorescence intensity were recorded on a FLUOstar Omega plate reader (BMG LABTECH GmbH, Germany). Fluorescence emission spectra were collected on an Edinburgh Photonics FLS920 fluorescence spectrometer (Livingston, UK). Photodynamic therapy treatment and cell viability imaging were performed on an inverted microscope.
with a mercury arc lamp and appropriate filter cubes. Cell images were acquired with a Canon EOS Rebel XTi (Tokyo, Japan) mounted to the front port of the microscope.

**SERS-labeled nanoparticle synthesis**

Gold nanostars were synthesized as previously reported. In brief, 10 μL of 1N HCl was added to 10 mL of 0.25 mM HAuCl₄ under vigorous stirring. Then 100 μL of 12 nm gold seed, 50 μL of 2 mM AgNO₃, and 50 μL of 0.1 M ascorbic acid (AA) were added in rapid succession. The color of the solution changes from orange-red to blue-gray within seconds. A final concentration of 20 μM para-mercaptobenzoic acid (pMBA) and 0.02% Tween 20 were added to the as-prepared particles and allowed to gently stir for 24 hours. The solution was again brought to a rapid stir before adding 50 μL of 0.1 M AgNO₃, 50 μL of 0.1 M AA, and 10 μL of NH₄OH in sequence. Once thoroughly mixed, gentle stirring was continued overnight. The solution gradually changed to a highly scattering, deep red color. A 5 μM final concentration of thiol-PEG 5k was added and allowed to react for 1 hour. The particles were washed by centrifugation/resuspension (3k rcf, 15 min) in water three times and finally redispersed into 1 mL of water.
Silica coating and PpIX embedding

The SERS-labeled nanoparticles were coated with silica using a modified Stöber method. The 1 mL of concentrated nanoparticles was added to 8 mL of EtOH and 1 mL of DI in a 15 mL centrifuge tube and mixed. A final concentration of 1 μM Protoporphyrin IX (PpIX), 30 μL of 10% tetraethyl orthosilicate (TEOS), and 200 μL NH₄OH were then added, mixed, and allowed to sit undisturbed overnight to form PpIX-embedded silica shells on the particles. The silica-coated particles were washed in ethanol three times (3k rcf, 15 min) and redispersed in 8 mL EtOH and 2 mL of DI.

Folic acid functionalization

The particles were first modified with (3-aminopropyl)triethoxysilane (APTES) to provide free amine groups on the particle surface. A 1 μL aliquot of APTES was added to the EtOH/DI particle suspension and allowed to react for 6 hours. The APTES-modified particles were washed 4 times in EtOH and resuspended in 1 mL of PBS. To this solution, 2 mg of FA-PEG-NHS was added and mixed until dissolved. The reaction was allowed to proceed overnight at room temperature. The folic acid functionalized particles were washed three times (3k rcf, 10 min) in PBS with 0.02% Tween 20, with final resuspension in 1 mL of PBS (particle concentration ~1 nM).
Cell culture and nanoparticle incubation

HeLa, SK-BR-3, and MDA-MB-468 cells were obtained from ATCC (Manassas, VA). All three cell lines were cultured in high glucose DMEM (Gibco, 11995065) supplemented with 10% FBS, and grown at 37 °C in a humidified atmosphere containing 5% CO₂. For PDT experiments, cells were seeded into 6-well plates and grown to ~90% confluency before use. For Raman mapping experiments, cells were seeded into glass-bottomed 35 mm dishes and grown to ~50% confluency before use.

Immediately before particle incubation, the old medium was aspirated and replaced with fresh medium. To each well, 100 μL of 1 nM nanoparticle solution in PBS was added and the cells were returned to the incubator for 4 hours. The cells were then washed three times with PBS to remove any unbound particles, and fresh medium was replenished.

Toxicity of the theranostic nanoparticles was measured with a resazurin-base metabolic assay (TOX8, Sigma). Cells were incubated with particles for 4 hours, washed in PBS, and then had fresh medium added. The next day, 10% volume of resazurin solution was added to each well and allowed to incubate for 2 hours. The fluorescence intensity was then measured per the manufacturer’s protocol.
Photodynamic therapy

The cells were irradiated for 15 s on a microscope using a filtered (640/30 nm) mercury lamp through a 40x objective, with a power of 11.5 mW at the sample. After treatment, the cells were placed back in the incubator for 4 hours before staining. Live/dead staining was performed using fluorescein diacetate and propidium iodide in PBS at 50 µg/mL and 1 µg/mL, respectively. The live/dead cells in the treated area were counted to produce the cell viability histogram.

Raman mapping

After nanoparticle incubation, the cells were fixed in cold methanol for 15 minutes, rinsed with water, and allowed to dry. A Renishaw inVia Raman microscope equipped with a 633 nm HeNe laser, running WiRE 2.0 software, was used to acquire the Raman spectra. Cells were located under brightfield transillumination with a 10x objective. The motorized stage was then set to scan the sample in a grid pattern while acquiring a spectrum at each point. The grating was set to 1100 cm\(^{-1}\) and the exposure time was 1 second. The false-color Raman maps were created by integrating the signal to baseline of the 1076 cm\(^{-1}\) Raman peak of pMBA from 1050 to 1100 cm\(^{-1}\) in the WiRE 2.0 software.
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Biography

Andrew M. Fales was born in Towson, Maryland on January 24, 1988. He attended the University of Maryland, Baltimore County, where he graduated summa cum laude and was inducted into Phi Beta Kappa with a Bachelor of Science in Biochemistry and Molecular Biology in 2010. Andrew entered the Biomedical Engineering Ph.D. program at Duke University in the fall of 2010, where he was awarded the John T. Chambers Fellowship. He was also a fellow in the NIBIB sponsored Medical Imaging Training Program at Duke. His research has mainly been focused on the synthesis of novel plasmonic nanoparticles for surface-enhanced Raman scattering applications.

Book Chapters


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