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Polarized Angular Dependent Light Scattering from Plasmonic Nanoparticles: Modeling, Measurements, and Biomedical Applications

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ABSTRACT

Polarized Angular Dependent Light Scattering from Plasmonic Nanoparticles: Modeling, Measurements, and Biomedical Applications

by

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Several significant applications have been realized for light scattering in biomedical imaging. In order to improve imaging results with light scattering-based techniques, a variety of nanoparticles have been investigated as contrast agents, including gold nanoshells. As a method for studying the optical properties of plasmonic gold nanoparticles used as contrast agents for molecular imaging, we developed an automated goniometer instrumentation system. This system, which allows us to specifically study polarized angular-dependent light scattering of plasmonic nanoparticles, allowed us to perform a series of theoretical and experimental step-wise studies. The basic optical properties of the following gold nanoparticles were progressively investigated: (1) bare nanoshells at multipolar plasmonic resonances, (2) nanoshells with PEG modifications, (3) surface-textured nanoshells and (4) immunotargeted nanoshells (nanoshell-antibody bioconjugates) for cancer imaging. Based on the results from these studies, a new technique was developed to quantitatively measure the number of immunotargeted nanoparticles that bind to HER2-positive SKBR3 human breast cancer cells. Preliminary studies of determining the minimal incubation time of immunotargeted nanoshells with SKBR3 cells were also carried out to evaluate the potential clinical application of using
gold nanoshells intraoperatively. We, therefore, anticipate that our findings will provide the theoretical groundwork required for further studies aimed at optimizing the application of plasmonic nanoparticles in scattering-based optical imaging techniques.
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Dedication

To My Parents, Grand Parents and Husband
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Chapter 1: Thesis Overview and General Introduction

1.1 Introduction

In spite of the declining mortality rate caused by cancer since 1990, cancer has remained the second leading cause of death in the United States for the past several years [1-3]. Recent advances in optical technologies have resulted in a large variety of different applications in early cancer detection as well as cancer therapy, specifically, imaging techniques based on light scattering have been found to hold substantial promise in diagnostic applications [4-9].

Adapted from:


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In this Chapter, current light scattering-based optical imaging and spectroscopy techniques used in cancer diagnosis will be introduced. We will also discuss various nanoparticles which are being investigated for improving these optical diagnostic techniques by targeting specific disease biomarkers and providing enhanced contrast.

In order to develop optical technologies for diagnostic and therapeutic application, basic understanding of the interaction of light with biological tissue is essential. The interaction of light with biological tissue results in two different phenomena: the absorption of light by tissue and the scattering of light by tissue. That is, tissue can absorb the energy of photons which is then converted into heat and eventually causes tissue damage. However, this is precisely the process that permits photo-thermal therapeutic uses. In addition, light scattered by biological tissue may contain information, such as the size of nuclei or the scattering coefficient and hemoglobin content of the tissue, which can be collected and analyzed to facilitate diagnosis.

Non-invasive light scattering methods have the potential to provide information on tissue and cell morphology for clinical diagnostic purposes, and such methods have been investigated by several research groups [4, 5, 7-9]. Light scattering-based optical imaging and spectroscopy techniques, such as darkfield microscopy [8, 10, 11], optical coherent tomography (OCT) [9, 12, 13], reflectance confocal microscopy (RCM) [7, 14-16], diffuse reflectance spectroscopy (DRS) [4-6, 17], and intensity-based light scattering spectroscopy (LSS) [18, 19] have all developed very quickly over the past decade because of their high sensitivity, low cost, and strong potential for medical diagnostic
applications. Nevertheless, current practices in photonics technologies used for biomedical imaging and diagnostics also have their limitations which result from the limited variety of endogenous chromophores. In addition, these technologies sometimes provide insufficient image contrast and lack specificity to disease signatures. Therefore, there is an urgent demand for new materials and technologies that will improve image contrast, which will, in turn, lead to the improvement of diagnostic accuracy.

1.2 HER2 Biomarker

Current cancer diagnostic procedures require that tissue biopsies taken from patients be examined microscopically. A given diagnosis is then based on the morphology of individual cells and the patterns they form; e.g., some cancer cells feature enlarged nuclei and higher nuclei to cytoplasm ratios compared to normal cells. Since this method is highly dependent on the skill and experience of the individual carrying out the examination, false diagnoses can occur. On the other hand, scientific advances that merge nano- and biotechnologies can result in diagnostic techniques that eliminate human error and thus potentially improve cancer screening and early detection, as well as provide the basis for novel cancer therapies. As more cancer markers are identified, nanoparticle-assisted cancer imaging techniques and therapies are, correspondingly, drawing more interest.

Although some biomarkers are not unique to cancer, they are still suggestive of cancer when detected in much larger numbers. This is observed in the HER (erb) family
of proteins where abnormal overexpression of receptors may indicate the presence of cancer. The HER (erb) family consists of four closely related receptors, including the epidermal growth factor receptor (EGFR or erbB1), HER2/neu (erbB2), HER3 (erbB3) and HER4 (erbB4), as shown in Figure 1.1 [20]. Of these four receptors, HER2 has been found to be overexpressed in 10%-34% of invasive breast cancers [21], and HER2 overexpression is usually associated with the recurrence of disease and increased morbidity [22]. The commercially available targeted antibody therapy trastuzumab (Herceptin®, Genentech Corp., South San Francisco, CA) has become an important therapeutic option for HER2/neu positive breast cancer patients [23-25]. This breakthrough therapy is important because nanoparticles conjugated with anti-HER2 antibodies have also shown promising applications in breast cancer imaging and targeted cancer therapy [8, 10, 26].

![The HER (erbB) family](image)

**Figure 1.1** The HER (erb) gene family. This image was adapted from reference [20].
1.3 Scattering-Based Optical Molecular Imaging and Spectroscopy

In addition to those technologies based on fluorescence spectra or imaging, most current optical diagnostic technologies are based on the signals of light scattered or reflected (back scattered) by biological tissue. To obtain the best image from biological tissue, the working wavelength in which the images are acquired must be carefully chosen. Since the major absorbers in biological tissue are water, hemoglobin and oxy-hemoglobin, avoiding the high absorption of these substances can potentially lead to higher scattered signals for \textit{in vivo} diagnostics. Figure 1.2 shows the absorption spectra of tissue absorbers from the visible to the near infrared (NIR) region [27]. Hemoglobin mainly absorbs in the wavelength ranges lower than 650nm, while water mainly absorbs in the wavelength ranges higher than 900nm. Thus, we can see from Figure 1.2 that the NIR window of 650nm–900nm is ideal for \textit{in vivo} imaging applications.
Figure 1.2 Spectra of major tissue absorbers showing that the NIR region (650<λ<900) is ideal for in vivo diagnostic application. This figure was adapted from reference [27].

1.3.1 Darkfield Microscopy

Darkfield microscopy is ideal for imaging biological cells, as well as nanoparticles, because images in darkfield are formed only by the scattered light from objects, not from light transmitted by the light source. As shown in Figure 1.3, darkfield microscopy differs from traditional transmission microscopy in that there is one opaque disk between the light source and the object. As such, no directly transmitted light can pass through the objective and be visible to the observer, while, on the other side, only the light scattered by the objects can be detected. Therefore, the images of nanoparticles taken by darkfield microscopy appear to be bright spots against a dark background. Figure 1.4, for example, shows a sample darkfield image of gold nanoshells suspended in
de-ionized (DI) water. However, it should be noted that the conventional setup of the darkfield microscope strictly limits darkfield microscopy to *in vitro* imaging applications currently.

![Schematic of darkfield microscopy](image)

**Figure 1.3** Schematic of darkfield microscopy
Figure 1.4 Sample darkfield microscopy image of gold nanoshells in DI water using 40X objective obtained by a black and white CCD camera.

1.3.2 Optical Coherent Tomography

Optical coherent tomography (OCT) is a new imaging modality that can be used to acquire optical images of samples at different depths, up to several millimeters. As shown in Figure 1.5, OCT employs the principles of the Michelson interferometer to form images by using a low-coherence-length light source (usually a diode laser or a solid-state laser). The mechanics of the process may be explained as follows. The incident light hits a beam splitter and separates into two beams; one goes to the sample arm and the other goes to the reference arm. The light beams that are reflected, or backscattered, by the sample and the movable reference mirror, then meet at the beam splitter, are either reflected or refracted by the beam splitter, and interfere with each other
when the difference in the distance of light traveled on each arm matches within the coherence length of the incident light [28]. The resulted interference fringes contain the structural information within the sample. The signal is then collected and demodulated. Finally, an image is formed on the computer controlling the device. The depth in the sample can be resolved by analyzing the interference patterns as a function of the position of the reference mirror.

![Diagram](image)

**Figure 1.5** Optical coherent tomography (OCT) setup

### 1.3.3 Reflectance Confocal Microscopy

Confocal microscopy has found applications in fluorescence imaging, as well as reflectance imaging. Accessibility issues have limited the *in vivo* use of conventional
confocal microscopy to such areas as the skin [29, 30], cornea [31], lip and tongue [32]. Recent advances in fiber optics, however, have made it possible to integrate the components of confocal microscopy into an optical fiber bundle with a miniaturized objective which enables in vivo access to other organs, such as the cervix [7, 15, 16]. Reflectance confocal microscopy (RCM) can collect the backscattered light from the tissue and selectively image the sample at different depths. A pinhole is used to reject signals from out-of-focus planes, as shown in Figure 1.6.

Figure 1.6 Principles of reflectance confocal microscopy (RCM).
1.3.4 Diffuse Reflectance Spectroscopy

Diffuse reflectance spectroscopy (DRS) is recognized as a very promising technique for distinguishing between diseased and normal tissue [5, 6, 12]. To obtain diffuse reflectance spectra, a fiber bundle is used to deliver the light from a white light source to the sample and then to collect the diffuse reflected signal from the sample. The optical fiber bundle with a fiber probe makes it easy for non-invasive in vivo signal acquisition using DRS. From the obtained reflectance spectra, tissue optical properties, such as the absorption coefficient, scattering coefficient and hemoglobin concentration, can be extracted and diagnosis can be made based on the information derived from these same tissue optical properties [6].

![Diagram of DRS setup](image)

**Figure 1.7** Schematic of the diffuse reflectance spectroscopy (DRS) setup.
1.4 Nanoparticle-Based Scattering Contrast Agents for Optical Molecular Imaging

In the area of biomedical imaging, nanoparticles have gained increased attention because of their potential as contrast agents. This attention arises from the unique optical properties of nanoparticles, which can improve imaging results at specific areas of interest. Some nanoparticles can either scatter or emit light at certain wavelengths, which range from the visible to the near infrared (NIR) region. This spectral range makes nanoparticles favorable for in vivo studies since the NIR is the most transparent region in biological tissue. A leading advantage associated with the use of nanoparticles in biomedical imaging results from their conjugation to bio-molecules which can specifically target molecular signatures of disease and thus provide accurate diagnostic results. Two additional advantages of nanoparticles include (1) the tunability of their optical properties which allows for the scattering or emitting of light over a broad range of wavelengths with strong signals and (2) their biocompatibility which allows nanoparticles to be safely used for in vivo studies by surface modification using Polyethylene glycol (PEG) or other biocompatible materials. Currently, scientific interest centers on developing this technique for in vivo diagnostic applications in the NIR region where the absorption of water and hemoglobin is the lowest, which we previously referenced as that area between 650nm–900nm where biological tissue is highly transparent (Fig. 1.2). Therefore, finding nanoparticles that can scatter or emit light in the NIR region has become more critical for biomedical researchers.
1.4.1 Colloidal Gold Nanoparticles

As early as 1977, colloidal gold nanoparticles were used in electron microscopy as molecular-specific stains of cells and tissues [33, 34]. Gold colloid conjugated with anti-HCG antibodies have also been widely used in pregnancy tests commercially available for easy use at home. Colloidal gold nanoparticles are currently being evaluated as a contrast agent for molecular imaging because they can resonantly scatter light with high efficiencies. Moreover, the gold surface can be readily modified using PEG-SH to improve biocompatibility. Colloidal gold nanoparticles exhibit much higher scattering cross sections compared to polymeric spheres of the same sizes, which makes them favorable for enhancing contrast in scattering-based imaging techniques [35]. Although colloidal gold nanoparticles can be fabricated to resonantly scatter light at different wavelengths by varying the size of the particles [36], the peak location is usually around 520-530nm for particle sizes ranging from 12-41nm. Since the surface plasmon resonance in pure gold colloid occurs in accordance with the 5 d-band to conduction band transition which occurs at 2.4 eV (corresponding to 517nm in wavelength), it is very difficult to get the peak resonance of colloidal gold nanoparticles up to the NIR region [37]. Therefore, the low NIR absorption and scattering of colloidal gold nanoparticles does limit their in vivo application.
Figure 1.8 Measured spectra of colloidal gold nanoparticles of r=6.5nm

1.4.2 Core-Shell Gold Nanoparticles

Nanoshells are spherical nanoparticles with a core-shell structure. The nanoshells discussed in this dissertation consist of a nanometer-sized SiO$_2$ core and a thin layer of concentric gold shell [38], as shown in Figure 1.9. $R_1$ refers to the radius of the inner silica core, $R_2$ refers to the radius of the entire nanoshell particle, and the unit is nm. $R_2-R_1$ is the thickness of the gold shell. Hereinafter, nanoshell will be referred to as “NS R $R_1/R_2$ nm”.
**Figure 1.9** Schematic of gold nanoshell structure. $R_1$ refers to the radius of the inner silica core, $R_2$ refers to the radius of the entire nanoshell particle, and the unit is nm. $R_2 - R_1$ is the thickness of the gold shell. Hereinafter, nanoshell will be referred to as NS $R_{1/R_2}$ nm.

Among the various nanoparticles under investigation, gold nanoshells stand out due to their high biocompatibility and strong, tunable optical signals. The plasmon resonance of gold nanoshells can be tailored to the NIR region simply by varying the core-shell ratio. The scattering cross section of the gold nanoshells is also much larger than the geometric cross section of the actual particle, which makes them good scatterers for scattering contrast agent applications.
Figure 1.10 Extinction spectra of gold nanoshells showing the tunability of optical properties (Mie theory calculations). Nanoshells with core radius of 55nm, and shell thickness of 5, 7.5, 10 and 15nm respectively are presented in this figure. The peak plasmon resonance is redshifted systematically from 715nm into the NIR region at around 1025nm as R1:R2 increases from 0.79 to 0.92.
1.4.3 Gold Nanorods

Gold nanorods represent another popular type of gold nanoparticle under investigation. The surface plasmon of gold nanorods has two peaks due to the special shape of the particle: a strong longitudinal peak in the NIR region, corresponding to the longitudinal axis of the particle, and a weak transversal surface plasma peak at 520 nm, corresponding to the transversal axis of the particle [39, 40]. A nanorod's characteristics are described in terms of its aspect ratio. That is, the aspect ratio determines the location of the nanorod's longitudinal peak, which is defined as the ratio of the longitudinal axis vs. the transversal axis. This peak commonly shifts to longer wavelengths as the aspect ratio increases.

![Graph of spectra of gold nanorods showing the redshifts of the longitudinal peak as the aspect ratio increases.](image)

Figure 1.11 Spectra of gold nanorods with different aspect ratio showing the redshifts of the longitudinal peak as the aspect ration increases. This figure is adapted from reference [40].
1.5. Thesis Overview

A series of step-wise studies were carried out, both theoretically and experimentally, to evaluate the use of plasmonic gold nanoparticles as a contrast agent for molecular imaging. The basic optical properties of the following gold nanoparticles were progressively investigated: (1) bare nanoshells at multipolar plasmonic resonances, (2) nanoshells with PEG modifications, (3) surface-textured nanoshells and (4) immunotargeted nanoshells (nanoshell-antibody bioconjugates) for cancer imaging. Based on the results from these studies, a new technique was developed to quantitatively measure the number of immunotargeted nanoparticles that bind to HER2-positive SKBR3 human breast cancer cells. Preliminary studies to evaluate the clinical application of gold nanoshells for tumor margin detection were also carried out.

Mie scattering theory and the solutions for nanoshell modeling are briefly introduced in Chapter 2 [38, 41, 42]. The general optical properties of nanoshells, including absorption, scattering and extinction are presented according to Mie theory calculations. Chapter 2 also discusses the fabrication of gold nanoshells and their surface modification with PEG and antibody.

To facilitate the application of nanoshells in scattering-based imaging techniques, it is essential to characterize their light scattering properties. Therefore, Chapter 3 presents data obtained from studying the light scattering from nanoshells at the
quadrupolar and octupolar frequencies of the surface plasmon resonance. Our measurements were found to be in good agreement with Mie theory calculations for both wavelengths [43]. However, \textit{in vivo} use of nanoparticles in biomedical imaging and therapy requires surface modification to enhance the stability and biocompatibility of these particles. Although polyethylene glycol is commonly used for the surface modification of metal and semiconductor nanoparticles, the influence of surface modification on the optical properties of nanoparticles has yet to be systematically studied. Thus, Chapter 3 also presents our findings based on the polarized and angularly-resolved light scattering properties of gold nanoshells before and after polyethylene glycol modification. It was observed that polyethylene glycol does not influence the extinction profile of gold nanoshells and that there is no significant change in the scattering phase function of nanoshells after polyethylene glycol modification.

In addition to the influence of surface modification, surface morphology also changes the polarized angular-dependent light scattering [44]. In Chapter 4, we demonstrate the aspects of this phenomenon by first fabricating gold nanoshells with smooth and roughened surfaces with dipolar and quadrupolar plasmon resonances tuned to 830 nm and then comparing the resultant light scattering properties. In the case of dipole resonance, small but measurable variations in the angular-dependent light scattering (ADLS) due to the introduction of surface roughness are observed. In the quadrupole case, the distinctive side lobe scattering characteristic of quadrupolar emission is strongly quenched for roughened nanoshells. These observations may have important implications in the interpretation of light scattering characterization of complex
nanostructures, and may lead to new ways of modifying the emission properties of resonant nanostructures.

Quantitative information of the binding concentration of nanoparticles to cancer cells is also essential in order to achieve optimal imaging and therapeutic results from in vivo applications of nanoparticles. While there are many studies which report the number of antibodies that bind per nanoparticle [26], there are almost no reports of the key factor which influences diagnostic or therapeutic efficacy: the number of targeted nanoparticles that bind per cell. We know that gold nanoparticles can be conjugated to various biomolecules in order to target specific molecular signatures of diseases [14], and we know that this targeting provides enhanced contrast in scattering-based optical imaging techniques.

In Chapter 5, we combine this knowledge with the results of our own studies involving light scattering of nanoshells with different surface modification and surface morphology. The result is the development of a new technique, which uses polarized light scattering, to quantify the number of immunotargeted plasmonic nanoparticles which bind to live SK-BR-3 human breast carcinoma cells. This novel "negative" method measures the binding concentration of those antibody/nanoparticle bioconjugates which are specifically targeted to breast cancer cells. Unlike previously reported methods, we collected unbound nanoparticle bioconjugates and measured the light scattering from dilute solutions of them in order to obtain quantitative binding information. By following this process, the interaction effects of adjacent bound nanoparticles on the cell membrane
can be avoided simply by measuring the light scattering from the unbound nanoparticles. In other words, by using nanoshells of two different sizes, the binding concentrations of anti-HER2/nanoshell and anti-IgG/nanoshell bioconjugates targeted to HER2-positive SK-BR-3 breast cancer cells can be compared. Hence, for anti-HER2/nanoshell bioconjugates, approximately 800-1600 nanoshells are bound per cell, while for anti-IgG/nanoshell bioconjugates, the binding concentration is significantly lower, at nearly 100 nanoshells bound per cell. These results are also supported by darkfield microscopy images of the cells labeled with anti-HER2/nanoshell and anti-IgG/nanoshell bioconjugates. The images demonstrate that greater contrast can be achieved with anti-HER2/nanoshell-labeled cells than with anti-IgG/nanoshell-labeled cells.

The quantitative binding of gold nanoshells to cancer cells that we obtained in Chapter 5 provides important information in determining the amount of nanoparticles necessary for tissue imaging studies. Finally, in Chapter 6, we carried out preliminary studies in examining how gold nanoshells can be clinically applied to the detection of tumor margins. Traditional breast tumor margin detection requires preparing and examining frozen histological tissue specimens removed from the suspected tumor site while the patient is under anesthesia. Here we proposed the use of immunotargeted nanoshells in targeted tumor margin detection. In order to evaluate the application of nanoshells in tumor margin detection directly in the operation room while the patient is under anesthesia, an initial study on incubating immunonanoshells and cancer cells at different time points is carried out. Our study supports the feasibility of using nanoshells
to target human breast cancer cells with minimal processing time, as little as 5-10 minutes.

This dissertation is focused on the study of light scattering from plasmonic nanoparticles as well as its applications in biomedical imaging. The findings and results offer in-depth understanding of the basic optical properties of nanoparticles with various surface modification and surface morphology. It also provides important information in optimizing the application of plasmonic nanoparticles in scattering-based optical imaging techniques.
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Chapter 2: Mie Scattering Theory and Layered Structure

Plasmonic Nanoparticles

Chapter 1 presented a brief overview of current light scattering-based optical imaging and spectroscopy techniques used for cancer diagnosis. Various types of scattering nanoparticles now being investigated as contrast agents for these optical techniques were also described. Chapter 2 focuses on basic light scattering theory and the types of nanoparticles used for the studies undertaken in support of this thesis, to include, primarily, gold nanoshells. In addition, since quantification of light scattering optical properties is a core issue in this thesis, Rayleigh and Mie scattering theories are compared. Specifically, Mie theory simulations for the optical properties of gold nanoshells are discussed. Finally, since surface modification, as well as surface morphology, can change the optical properties of nanoparticles, the fabrication of gold nanoshells is introduced in the context of surface modification using PEG-SH and antibody conjugation.

2.1 Introduction: Light Scattering Theories

When a beam of light penetrates a medium consisting of a suspension of small particles, the light will be attenuated. This attenuation is called extinction, which is characterized by both the scattering and absorption of light from these particles. A simple equation applies:
Extinction = scattering + absorption \hspace{1cm} (Eq. 2.1)

The studies in this dissertation involve only light scattering principles. To quantitatively study the light scattering from small particles, we can use the light scattering theories of Rayleigh or Mie. Specifically, when the particles are much smaller than the wavelength ($\ll \lambda / 2\pi$) of the incident light, Rayleigh scattering predominates. This scattering is strongly wavelength-dependent ($I \sim 1/\lambda^4$) and less sensitive to the scattering angle [1, 2], where the scattering angle $\theta$ is defined as the angle between the direction of the scattered light and the direction of incident light. Thus, the quantification of light scattering by Rayleigh theory tends to produce results that are more isotropic and less angular-dependent. Under the assumptions of Rayleigh scattering, the scattered intensity of a particle incident by a beam of light with intensity $I_i$ can be written as

$$I_s = \frac{(1 + \cos^2 \theta) k^4 |\alpha|^2}{2r^2} I_i$$ \hspace{1cm} (Eq. 2.2)

where $k$ is the number defined by $k = 2\pi \lambda$ ($\lambda$ is the wavelength of incident light in the surrounding medium) and $\alpha$ is the polarizability of the particle, as defined by the electrostatic formula $p$ and the applied field of the incident light $E_i$

$$p = \alpha E_i$$ \hspace{1cm} (Eq. 2.3)

In contrast, Mie theory applies to homogeneous particles of any size. Since the intensity of scattered light is a strong function of the scattering angle, the scattering becomes more of a geometric phenomenon when the particle size is larger than or similar to the wavelength of incident light. As such, quantification by Mie theory produces
results which demonstrate a scattering pattern with a sharper and more intense forward lobe for larger particles.

Figure 2.1 compares the calculations of Rayleigh theory to those of Mie theory based on the light scattering from small particles. The Rayleigh scattering particle is a colloidal gold particle with a radius of 30nm, and the Mie scattering particle is a gold nanoshell with \( R_1 = 134 \) and \( R_2 = 155 \)nm. The wavelength of the incident light is 660nm, and the surrounding medium is water.

(a) **Rayleigh Scattering**

Colloidal gold nanoparticle \((r=30\text{nm})\) in water; \( \lambda = 660\text{nm} \)
(b) Mie Scattering

Gold nanoshell (R 134/155nm) in water; \( \lambda = 660 \text{nm} \)

**Figure 2.1** Light scattering patterns: Rayleigh scattering vs. Mie scattering

### 2.2 Single Scattering vs. Multiple Scattering

In conducting laboratory experiments, we usually work with a suspension of many particles rather than a single particle. In this case, each particle in the suspension is excited by the applied external field (the incident light) as well as the scattered field of other particles. When the concentration of the particles is sufficiently low and the separation of particles in the suspension is sufficiently large, the scattered field of neighboring particles can be ignored, when compared to a strong external field. Under these conditions, we can assume that the criteria for single scattering have been satisfied since individual particles can be considered as having only been excited by the applied
external field. To illustrate this, we take a dilute suspension containing $N$ small particles; the scattered intensity by the suspension is then $N$ times the intensity scattered by a single particle when the criteria for single scattering is satisfied.

In contrast, when the concentration and proximity of particles in a suspension are such that the scattered field of neighboring particles cannot be ignored, even when compared to a strong external field, multiple scattering must be considered. Under these conditions, a diffusion event occurs whereby light scatters many times over. Although this phenomenon raises no new physical problems, the mathematics required to solve the challenges of multiple scattering can be much more complicated compared to those involved in single scattering. Therefore, both experimentation and findings that support this thesis exclude the mathematical complications of multiple scattering, and we assume that each individual particle is acted on by an external field in isolation from other particles, or single scattering.

In addition to simplifying mathematical solutions, single scattering is very sensitive to particle sizes and concentrations, making it easier to define a significant linear relationship between the intensity of scattered light and particle concentration [1]. Moreover, under laboratory conditions, a simple, yet effective, way to prove that a single scattering event predominates in a sample is to dilute the concentration of particles in the scattering media to half. If the scattered light intensity (at every single scattering angle) also drops by half, only single scattering is observed in this sample. We have utilized this
very important feature of light scattering to characterize the number of nanoparticles binding to cancer cells, and the issue is fully discussed in Chapter 5.

### 2.3 Mie Theory Solutions for Spherical Particles

When Gustav Mie first published the solution to light scattering from the dielectric particle in 1908, it was only applied to homogeneous spheres [3]. When a plane scalar wave of infinite extent from the negative z-direction is incident onto a particle at the origin of the coordinate, as shown in Figure 2.2, the direction of propagation of the incident wave, and the direction of the scattered wave being detected, form a "scattering plane" (shadowed plane in Figure 2.2). The angle between the direction of the scattered light and the incident light is defined as the scattering angle $\theta$, while $\varphi$ is an azimuthal angle.

The field of the incident light may be written as [1, 2]

$$u_i = e^{-ikz + i\alpha} \quad \text{(Eq. 2.2)}$$

The scattered wave in the far field (distance $r$) from the particle can then be written as

$$u_s = S(\theta, \varphi) \frac{e^{-ikr + i\alpha}}{ikr} \quad \text{(Eq. 2.3)}$$

In this equation, $k$ is the wave number defined by $k=2\pi/\lambda$ ($\lambda$ is the wavelength in the surrounding medium) and $S(\theta, \varphi)$ is defined as the amplitude function of the scattering particle, which is a matrix containing four amplitude functions describing the scattering...
in any direction; thus $S_1, S_2, S_3$ and $S_4$, are all functions of $\theta$ and $\varphi$, and the subscripts $i$ and $s$ refer to the incident and the scattered light, respectively.

Figure 2.2 Light scattering from a small particle: electric vectors of incident and scattered light

Combining Equation 2.2 and Equation 2.3, we have

$$u_s = S(\theta, \varphi) e^{-ikr + ikz} \frac{e^{-ikr}}{ikr} u_i$$

(Eq. 2.4)

When polarization is considered, the scattered field can be written as
\[
\begin{pmatrix}
E_{\parallel s} \\
E_{\perp s}
\end{pmatrix} =
\begin{pmatrix}
S_2 & S_3 \\
S_4 & S_1
\end{pmatrix}
\frac{e^{-ikr+ikz}}{ikr}
\begin{pmatrix}
E_{\parallel i} \\
E_{\perp i}
\end{pmatrix}
\quad \text{(Eq. 2.5)}
\]

where \(E_{\parallel}\) and \(E_{\perp}\) refer to the electric fields parallel with and perpendicular to the scattering plane.

For spherical particles, \(S_3 = S_4 = 0\). Therefore, only two amplitude functions need to be considered for scattering in any direction, \(S_1(\theta)\) and \(S_2(\theta)\), which only depend on the scattering angle \(\theta\). For light scattered in any direction, or \(\theta \neq 0\), the electric field can be written as

\[
E_{\perp s} = S_1(\theta) \frac{e^{-ikr+ikz}}{ikr} E_{\perp i}
\]

\[
E_{\parallel s} = S_2(\theta) \frac{e^{-ikr+ikz}}{ikr} E_{\parallel i}
\quad \text{(Eq. 2.6)}
\]

Since the intensity of a scalar wave is proportional to the square of the amplitude,

\[
I = E \cdot E^* = |E|^2
\quad \text{(Eq. 2.7)}
\]

the intensity of scattered light with perpendicular and parallel polarizations, as well as the intensity of unpolarized light, can be obtained by

\[
I_{\perp s} = \frac{i_1}{k^2 r^2} I_i
\]

\[
I_{\parallel s} = \frac{i_2}{k^2 r^2} I_i
\]

\[
I_s = \frac{I_{\perp s} + I_{\parallel s}}{2} = \frac{i_1 + i_2}{2k^2 r^2} I_i = \frac{S_{11}}{k^2 r^2} I_i
\quad \text{(Eq. 2.8)}
\]

where \(i_1 = |S_1(\theta)|^2\), \(i_2 = |S_2(\theta)|^2\) and \(S_{11} = (|S_1(\theta)|^2 + |S_2(\theta)|^2) / 2\).
The ratio

\[ P = \frac{I_\perp - I_\parallel}{I_\perp + I_\parallel} = \frac{i_1 - i_2}{i_1 + i_2} \]  

(Eq. 2.9)

is defined as the *degree of polarization* of the particle and \(|P| \leq 1\). When \(P > 0\), the scattered light is partially polarized perpendicular to the scattering plane, but when \(P < 0\), the scattered light is partially polarized parallel to the scattering plane. For spherical particles of any size and index of refraction, \(P(0^\circ) = P(180^\circ) = 0\), since \(I_\perp = I_\parallel\) at these two specific positions.

Figure 2.3 shows polarized light scattering from small particles for both Rayleigh and Mie scattering. The scattered light with polarizations parallel and perpendicular to the scattering plane is plotted, as well as the unpolarized scattering phase functions. The parameters are the same as those used in Figure 2.1.
(a) Polarization plot of Rayleigh scattering
Figure 2.3 Polarized light scattering from small particles: Rayleigh scattering vs. Mie scattering. Please note that here in these figures, the unpolarized light scattering is plotted as \((I_r+I_\perp)\) rather than \((I_r+I_\perp)/2\) for easy viewing purpose.

For the scattered light, the intensity at a large distance \(r\) from the particle can also be written as

\[
I_s = \frac{F(\theta, \varphi)}{k^2 r^2} I_i
\]  

(Eq. 2.10)
In this equation, \( F(\theta, \varphi) \) is a dimensionless function of the direction. When \( F(\theta, \varphi) \) is divided by \( k^2 C_{\text{sca}} \), the scattering phase function \( P(\theta, \varphi) \) can be obtained, where \( C_{\text{sca}} \) is the scattering cross section of the particle, i.e., when the energy of the scattered light in all directions is the same as the energy incident on an area of \( C_{\text{sca}} \), or

\[
C_{\text{sca}} = \frac{1}{k^2} \int_{4\pi} F(\theta, \varphi) d\Omega
\]  

(Eq. 2.11)

where \( d\Omega = \sin \theta d\theta d\varphi \) is the unit solid angle and the integral is taken over all directions.

Similarly, the absorption cross section can be obtained when the energy absorbed by the particle is equal to the energy of the incident light falling on the area \( C_{\text{abs}} \), and when the energy of the total attenuation by the particle is equal to the energy of the incident light falling on the area \( C_{\text{ext}} \), the extinction cross section can be obtained.

According to Equation 2.1, we then have

\[
C_{\text{ext}} = C_{\text{abs}} + C_{\text{sca}}
\]  

(Eq. 2.12)

The scattering phase function is defined as

\[
P(\theta, \varphi) = \frac{F(\theta, \varphi)}{k^2 C_{\text{sca}}}
\]  

(Eq. 2.13)

where the phase function has no physical dimension and is normalized so that the integration over all directions is 1

\[
\int_{4\pi} P(\theta, \varphi) d\Omega = 1
\]  

(Eq. 2.14)
The anisotropy factor (asymmetry parameter) $g$, which is defined as the average cosine of the scattering angle, is another important parameter related to the angular-dependent scattering properties of small particles:

$$g = \langle \cos \theta \rangle = \frac{\int_{4\pi} P(\theta, \varphi) \cos \theta d\Omega}{4\pi}$$  \hspace{1cm} (Eq. 2.15)

In the case of spherical particles, the following equation applies:

$$g = \frac{\int_{0}^{\pi} S_{11}(\theta) \cos \theta 2\pi \sin \theta d\theta}{\int_{0}^{\pi} S_{11}(\theta) 2\pi \sin \theta d\theta}$$  \hspace{1cm} (Eq. 2.16)

As defined in Eq. 2.15, $g$ is used to describe the amount of forward direction retained after a single scattering event: (1) $g=0$ when a particle scatters light isotropically or when the scattered light is symmetric about $\theta=90^\circ$; (2) $g>0$ when the scattering is directed more in the forward direction towards $\theta=0^\circ$ ($g=1$ when the light only scatters at $\theta=0^\circ$); and (3) $g<0$ when the particle scatters more light in the backward direction towards $\theta=180^\circ$ ($g=-1$ when the light only scatters at $\theta=180^\circ$).

The efficiency factors of extinction, absorption and scattering can be defined, respectively, as

$$Q_{ext} = \frac{C_{ext}}{G}$$

$$Q_{abs} = \frac{C_{abs}}{G}$$  \hspace{1cm} (Eq. 2.17)

$$Q_{sca} = \frac{C_{sca}}{G}$$
where $G$ is the geometrical cross sectional area of the particle, or the particle cross sectional area projected onto a plane perpendicular to the incident light. In the case of a spherical particle of radius $a$, $G = \pi a^2$.

Additionally, readers who have further interest in the derivation of Mie solutions to light scattering problems will find comprehensive discussions of this extensive and complicated process in the literature of van de Hulst [1] and Bohren and Huffman [2].

2.4 Gold Nanoshells

2.4.1 Gold Nanoshell Fabrication

Spherical gold-silica nanoshells are fabricated as described by Oldenberg et al. [4]. First, nano-sized spherical $\text{SiO}_2$ cores are fabricated using the Stöber method [5]. To do this, silica particle sizes are initially characterized using scanning electron microscope (SEM, Phillips XL30 ESEM-FEG). In this way, $\text{SiO}_2$ cores with diameters ranging from 100 nm to 500 nm can be easily synthesized, and only the particle polydispersity that is < 10% is considered acceptable. The $\text{SiO}_2$ cores are then functionalized with amine groups by immersing and boiling them with aminopropyl-triethoxysilane (APTES). In the meantime, gold colloid is grown as outlined by Duff et al. [6]. In order to grow gold shell on the functionalized silica cores, the cores need to be seeded with concentrated gold colloid, which adsorbs to the amine groups on the silica surface. Then nanoshells are grown by mixing a stock solution of $\text{HAuCl}_4$ with the seed particles under formaldehyde
catalysis. This process reduces gold onto the adsorbed gold colloid, causing the surface to grow and eventually form a continuous shell over the silica core. Nanoshell growth is monitored by comparing the measured extinction spectra to the calculations from Mie theory, which is accomplished by using a UV-Vis spectrophotometer (Varian Cary 300). The size of the particle is further confirmed by SEM.

2.4.2 Optical Properties of Gold Nanoshells - Mie Theory Simulations

Mie theory solutions to core-shell structured particles, such as gold nanoshells, have been presented in different publications [7-9]. Briefly, Mie theory applies to such concentric sphere core-shell structures when the boundary condition at one additional interface is specified in solving the Maxwell's Equations. Here we use a Mie code (Gshell) developed by the group of Dr. Naomi Halas at Rice University for the simulations of optical properties of gold nanoshells [10].

To calculate the spectra and light scattering of gold nanoshells using Mie theory, the following parameters are necessary: the size of the particle ($R_1$ and $R_2$), the dielectric function of the core ($\varepsilon_1$), the gold shell ($\varepsilon_2$) as well as the surrounding medium ($\varepsilon_3$), the wavelength of the incident light and the scattering angles we want to calculate, as shown in Figure 2.4.
According to the publication of Stöber and Fink [5], we use an index of refraction, $n_1 = 1.45$, for the silica cores. The indices of refraction for gold are taken from those published by Johnson and Christy in 1972, as listed below in Table 2.1. In most cases, our measurements were made with nanoshells suspended in deionized water (DI water) or phosphate buffered saline (PBS); hence, the index of refraction of the surrounding medium is that of water, $n_3 = 1.33$.

Table 2.1 Optical properties of gold as well as the approximate errors in $n$ and $k$ [11]

<table>
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<tr>
<th>$eV$</th>
<th>Wavelength (nm)</th>
<th>$n$</th>
<th>$k$</th>
<th>$\Delta n$</th>
<th>$\Delta k$</th>
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<td>6.35</td>
<td>195.35</td>
<td>1.34</td>
<td>1.226</td>
<td>±0.02</td>
<td>±0.007</td>
</tr>
<tr>
<td>6.47</td>
<td>191.73</td>
<td>1.32</td>
<td>1.203</td>
<td>±0.02</td>
<td>±0.007</td>
</tr>
<tr>
<td>6.60</td>
<td>187.95</td>
<td>1.28</td>
<td>1.188</td>
<td>±0.02</td>
<td>±0.007</td>
</tr>
</tbody>
</table>

A sample calculation of extinction spectra of gold nanoshells (R 134/155nm) by Mie theory is shown in Figure 2.5. The individual contributions of the dipole (e1), quadupole (e2) and octupole (e3) components are also included in this figure. This nanoshell has a strong quadupole resonance peak in the NIR region, at around 755nm,
and a strong dipole resonance peak farther in the NIR region, at around 1225nm. A small octupole resonance peak at around 625nm can also be observed from the spectra.

**Figure 2.5** Mie theory plots of the extinction spectra of gold nanoshell R 134/155nm. The individual contributions of the dipole (e1), quadupole (e2) and octupole (e3) components are also shown in this figure. From this plot, we observe that this nanoshell has a dipole resonance peak at 1225nm, a quadupole resonance peak at 755nm and an octupole resonance peak at 625nm.

Figure 2.6 shows the polarized angular-dependent light scattering of the same nanoshell (R 134/155nm) at the dipole, quadupole and octupole peaks, respectively, as well as the graph of degree of polarization at each resonance wavelength.
(a1) NS134/155nm dipolar scattering @ 1225nm

- - - - \( I_1 \) (Vertical @ 1225nm)
- - - - \( I_2 \) (Horizontal @ 1225nm)
- - - - Phase Function @ 1225nm

Phase Function

Angle (degree)

0 30 60 90 120 150 180

0.1

1

10

(a2) Polarization: NS 134/155nm @ 1225nm

- - - - Polarization @ 1225nm

Polarization

0 30 60 90 120 150 180

-1.0

-0.5

0.0

0.5

1.0

(a) dipole resonance at 1225nm
(b1) NS134/155nm quadrupolar scattering @ 755nm

Phase Function

- - - I1 (Vertical @ 755nm)
- - I2 (Horizontal @ 755nm)
- Phase Function @ 755nm

Angle (degree)

(b2) Polarization: NS 134/155nm @ 755nm

Polarization

- Polarization @ 755nm

Angle (degree)

(b) quadrupole resonance at 755nm
Figure 2.6 Polarized angular dependent light scattering and degree of polarization of gold nanoshells R 134/155nm at the dipole, quadrupole and octopole resonances.
2.5 Immunotargeted Gold Nanoshells

For the in vivo application of nanoparticles in targeted imaging and therapy, antibody conjugation and surface modification with polyethylene glycol (PEG) is critical. Therefore, studying the optical properties of surface-modified nanoparticles is equally important. Accordingly, we outline here the preparation of nanoshell-antibody bioconjugates, which involves three precise steps: (1) using OPSS-PEG-NHS for the PEGylation of the antibody; (2) tethering the OPSS-PEG-antibody to the nanoshell surface at the optimal antibody concentration and (3) determining the optimal concentration of PEG-SH necessary for the stabilization of nanoshells in a saline environment.

2.5.1 PEGylation of Antibody

PEG is a water soluble polymer that has the chemical formula of H-(O-CH\textsubscript{2}-CH\textsubscript{2})\textsubscript{n}-OH [12]. The viscosity and freezing point of liquid PEGs generally increase with greater molecular weights (MW). In the course of carrying out our studies, antibodies were coupled to the gold nanoshell surface through a bi-functional PEG linker, OPSS-PEG-NHS (Necktar®). The NHS group of this PEG linker can be attached to the antibody (protein), while the OPSS group on the other end of the PEG linker can be effectively attached to the gold surface of the nanoshell, thus completing the nanoshell-antibody bioconjugation.
According to the PEG manufacturer [Nektar® catalog], the OPSS-PEG-NHS can couple to the targeted antibody at physiological pH, but higher pH can help accelerate the PEGylation process. Usually, the PEG and antibody are reacted on a 1:1 molar ratio under normal PEGylation conditions (pH 7-9 under room temperature for 30 minutes). However, for some antibodies or proteins, it is necessary to add up to 10-fold molar amount of PEG relative to the antibody. The reaction rate is also increased with higher pH values. Lower temperature may also be used for some antibodies. While under low temperature, a longer reaction time may be necessary to allow sufficient PEGylation. For our studies, the optimal PEGylation condition is determined to be reacting the OPSS-PEG-NHS with antibody on ice for 2-6 hours at pH 8.5. Figure 2.7 shows a schematic of the PEGylation of antibody and the reaction during this process, as well as the structure of the final product [Nektar® catalog].

\[
\text{OPSS - mPEG - C - O - N} \quad + \quad \text{H}_2\text{N} - \text{Antibody}
\]

1 part

react for 2-6 hours or overnight on ice

pH 8.5

\[
\text{OPSS - mPEG - C - NH - Antibody}
\]

Fig.2.7 Schematic of the PEGylation of antibody using OPSS-PEG-NHS
2.5.2. Nanoshell-Antibody Conjugation

After the PEGylation of antibody, the antibody can be tethered to the gold surface by using the sulfa group (OPSS) on the other end of the bi-functional PEG. The OPSS-PEG-antibodies were reacted with gold nanoshells at 4°C for 1 hour to make the nanoshell-antibody bioconjugates. In order to obtain the best results, nanoshells were reacted with antibody at different concentrations. By monitoring and comparing the extinction spectra of the product, an optimal concentration of antibody was selected and used to produce the nanoshell-antibody bioconjugates for further experiments [13].

2.5.3. PEGylation of Gold Nanoshell Using PEG-SH

PEG-thiol (MW=5000, 0.25μM) is also used to modify the nanoshell surface. PEG-SH was synthesized by reacting PEG-amine (MW 5000, Shearwater Polymers, Nektar 2M2U0H21) with Traut’s Reagent 137.9 MW (2-iminothiolane, Sigma I-6256) for 1 hour. The product was then dialyzed in deionized water for about 2 hours using a dialysis membrane with a molecular weight cutoff of 1000 to remove excess reagent. Product was stored in working aliquots at -20 °C.

To PEGylate the nanoshells, first, PEG-SH with a series of different concentrations in 10% potassium carbonate is prepared and mixed with nanoshells, and the mixtures is left undisturbed for 1 hour. A positive control of nanoshells without PEG-SH is prepared at the same time. Then 10% NaCl is added to the above suspensions (the
same amount of DI water is added to the control to maintain the same concentration. After the lapse of 15 to 30 minutes, the UV-Vis spectrum of each nanoshell sample is recorded and compared with the control. Since NaCl aggregates nanoshells which possess insufficient quantities of the stabilizing PEG-SH, the unstable suspensions will flocculate, and consequently spectral broadening, red-shifting and reduction of peak absorbance can be observed in the extinction spectra [13]. On the contrary, the well PEGylated nanoshells remain stable in the salt solution and do not exhibit such spectral changes, showing enhanced stability and biocompatibility under saline environments.

Once the antibody is tethered to the nanoshell surface using the bifunctional PEG linker, additional unoccupied sites remaining on the nanoshell surface are blocked using PEG-SH at the optimal concentration, as determined above. Having followed these procedures, the nanoshell-antibody bioconjugates are now ready to use in a saline environment for future experiments. An illustration of the structure of immunotargeted nanoshells is shown in Figure 2.8.

\[\text{PEG-SH (MW=5000)}\]
\[\text{OPSS-PEG-antibody (PEG MW=2000)}\]

Figure 2.8 Structure of immunotargeted gold nanoshells.
References:


Chapter 3: Mie Theory Modeling and Polarized Angular Dependent Light Scattering Measurements of Bare and PEGylated Gold Nanoshells

As Mie theory solutions for gold nanoshells have been briefly introduced in Chapter 2, this chapter will focus on the experimental measurements of light scattering from gold nanoshells in comparison to Mie theory predictions, especially the effects of surface modification on the light scattering from nanoshells.

3.1. Introduction

Gold nanoshells have been studied for a wide variety of biomedical applications, such as whole blood immunoassay [1, 2], photo-thermal tumor ablation [3, 4], cancer imaging [5-7], and tissue welding [8], etc. These works have been based on the extinction spectra of nanoshells. However, the individual effects of extinction, absorption and scattering have not been investigated separately. To help facilitate the development of nanoshell-assisted imaging techniques and non-invasive optical diagnostics, it is essential to characterize the light scattering properties of gold nanoshells. We are interested in the

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*a Adapted from:

angular dependency of light scattering in nanoshells and the design and optimization of specific nanoshells to facilitate biomedical applications. For in vivo applications of nanomaterials, it is necessary to modify the surface of the nano-materials with PEG or other biomacromolecules to enhance their biocompatibility. This surface modification may influence the optical properties of the nano-materials [9, 10]. Therefore, the potential discrepancy in optical properties between PEGylated and bare nanoshells is also an important subject of this study.

Polarized light scattering has been proven to be sensitive to both the size and shape of the index variation and the refractive index discontinuities in scattering materials. This technique can therefore be used to determine the size of small particles as well as the concentration of dilute suspension of these particles. Polarized light scattering has also been developed as a possible non-invasive tissue diagnostic tool by several research groups [10-14]. Drezek et al., for example, have discovered that cellular scattering is sensitive to changes in the nuclear morphology that accompanies neoplastic progression in cervical cells [11]. Mourant et al. have analyzed the polarized angular dependent spectroscopy of epithelial cells and epithelial cell nuclei and have found that light scattering is likely sensitive to structures smaller than those commonly investigated by standard pathology methods [14].

To experimentally study the angular dependent light scattering, a goniometer can be constructed to accurately measure light scattering from dilute suspension of small particles such as polystyrene spheres and gold nanoshells. This device measures the
intensity of polarized light scattering as a function of scattering angle, which is known as
the scattering phase function $P(\theta)$. At the same time, Mie theory can also be utilized to
calculate the scattering phase function of gold nanoshells, even though this involves the
more complicated concentric sphere geometry of those structures [15, 16]. In our
simulations, a refractive index of $n=1.45$ for silica [17] and the refractive indices
published by Johnson and Christy for gold [18] are used. The measured phase function
from the goniometer can, in turn, be compared to Mie theory to verify this device’s
accuracy.

3.2. Angular Dependent Light Scattering Experiments: the Goniometer

As noted above, an automated goniometer has been constructed to perform the
angular dependent light scattering measurements, together with different combinations of
linear polarizers for light delivery and detection. A schematic of the measurement system
is shown in Figure 3.1.(a). The light from a 633nm He-Ne laser (or an 830nm GaAlAs
laser diode) is incident onto a cylindrical cuvette (1 inch pass length, quartz) containing
the samples through the first polarizer (Newport, 10LP-Vis) and a φ500µm Precision
Pinhole (Edmund Optics, U56-287), the size of incident beam on the cuvette is about
1mm. The scattered light is then collected by a silicon detector rotating around the
cuvette through the second polarizer; the signal is read by a Picowatt Optical Power
Meter (Newport® Model 1830-C), which is connected to a computer through a GPIB
interface and controlled by LabView. The direction of propagation of incident light and
the direction of scattered light define a scattering plane. The parallel polarized light
scattering is detected when both polarizers are oriented so that to pass light polarized parallel to the scattering plane. The detection of perpendicular polarized light scattering is thus achieved when both polarizers rest on the cross-position. Our measurements range from $20^\circ$ to $160^\circ$, with a step size of $1.8^\circ$.

The angular resolution of the goniometer is another key issue for the accuracy of the goniometric measurement. The angular resolution is a function of the visible cross section of the beam inside the cuvette and the length of the rotating arm, which is the distance between the detector and the center of the cuvette, as shown in Figure 3.1(b) [19]. The angular resolution is also dependent upon the viewing angle $\theta$. In our experiment, using a 25.4mm (1 inch) pass length cylindrical cuvette and a 20cm rotating arm, the angular resolution ranges between $0.286^\circ$ and $7.27^\circ$, showing that the best angular resolution can be achieved at $\theta = 0^\circ$ while the angular resolution between $\theta = 87.7^\circ$ and $\theta = 92.3^\circ$ is the poorest.
**Figure 3.1** Schematic of the goniometer system and angular resolution. The light from a 633nm He-Ne laser (or an 830nm GaAlAs laser diode) is incident onto a cylindrical cuvette containing the samples through the first polarizer; the scattered light is then collected by a silicon detector rotating around the cuvette through the second polarizer. The direction of propagation of incident light and the direction of scattered light being detected define a scattering plane. (a) Schematic of the goniometer system. (b) Angular resolution of the goniometer at different viewing angles.

The goniometer is first tested using a dilute aqueous suspension of monodisperse polystyrene spheres with a diameter of 0.17μm (Bangs laboratory) and a concentration of 9.44×10⁷/ml. The buffer used for diluting the samples in this study is deionized water if not otherwise specified, and cuvette filled with deionized water is used as reference. The results of polystyrene spheres are in good agreement with Mie theory calculations, as
shown in Figure 3.2, which demonstrates that the goniometer is functioning properly. Note that the increased measured values relative to Mie theory predictions when approaching 90°. Several reasons may cause this discrepancy: the finite angular resolution of the goniometer in this area, as shown in Figure 3.1.(b); the limitation of the detector when detecting very low power; and/or the size distribution of the particles (around 10%).

![Graph showing light scattering measurements and Mie theory calculations](image)

**Figure 3.2** Comparison of light scattering measurements (scattered symbols) and Mie theory calculations (line curves) for 0.17μm diameter polystyrene spheres; Concentration of polystyrene sphere is ~ 9.44×10⁷ particles/ml; top line: Perpendicular polarization; middle line: Unpolarized phase function; bottom line: Parallel polarization.
3.3. Results and Discussion

Nanoshells with \( R_1 = 157 \pm 5 \text{nm} \) and \( R_2 = 187 \pm 7 \text{nm} \) are designed and fabricated with a strong octupole resonance at 680nm and a quadrupole resonance located at 815nm. Nanoshells with \( R_1 = 148 \pm 10 \text{nm} \) and \( R_2 = 168 \pm 12 \text{nm} \) are also fabricated for the multi-wavelength study, and these nanoshells also have a strong quadrupole resonance at 830nm and an octupole resonance located at 660nm, as shown in Figure 3.3.(a)[20]. PEGylated nanoshells are prepared as described in Chapter 2. Figure 3.3.(b) shows the measured extinction for nanoshells (\( R_1 = 157 \pm 5 \text{nm}, \ R_2 = 187 \pm 7 \text{nm} \)) with and without PEG in comparison to Mie theory calculation for bare nanoshells with \( R_1 = 157 \text{nm} \) and \( R_2 = 192 \text{nm} \). There is little difference between the extinction spectra of the bare and PEGylated nanoshell suspensions, and both are in good agreement with the Mie theory, showing that PEG doesn’t influence the extinction spectral shape of the gold nanoshells. The broadened linewidth at the quadrupole and octupole resonances of the nanoshell is dominated by the electron-interface scattering within the shell layer although the size distribution of the core and shell may also contribute to some degree [21].
Figure 3.3 Comparison of measured extinction spectra to Mie theory calculation of gold nanoshells; SEM images: magnification=50000x; scale bar=500nm. (a) Comparison of measured extinction spectra (dashed line; \( R_1 = 148\pm10\)nm; \( R_2 = 168\pm12\)nm) to Mie theory calculation (solid line; R150/170nm). (b) Extinction spectra of bare (dashed line) and
PEGylated (dotted line) nanoshells \( (R_1=157\pm5\text{nm}; \ R_2=187\pm7\text{nm}) \) compared to Mie theory (solid line; R157/192nm)

### 3.3.1 Light Scattering of Bare and PEGylated Gold Nanoshells

The angular light scattering properties of bare and PEGylated nanoshells \( (R_1=157\pm5\text{nm}, \ R_2=187\pm7\text{nm}) \) are studied using the goniometer at the wavelength of 633nm, which is near the octupole resonance of the fabricated nanoshells. Nanoshell suspensions are dispersed with an ultrasound probe for 2 minutes before each experiment to ensure the consistency of nanoshell dispersion and homogeneity in suspensions. First, the angular light scattering of the bare nanoshell suspension with a concentration of around \( 5.2\times10^7/\text{ml} \) is measured. The measurement on another nanoshell suspension diluted by a factor of 4 \( (\sim1.3\times10^7/\text{ml}) \) yields no difference in light scattering patterns. This demonstrates that single scattering predominates, while, at the same time, multiple scattering does not occur [12, 13]. PEGylated nanoshell suspension at a concentration of \( \sim1.3\times10^7/\text{ml} \) is prepared for light scattering measurement and compared to the bare nanoshell suspension at an equal concentration. Figure 3.4 shows that the parallel polarized angular scattering patterns of the bare and PEGylated nanoshells are very similar to each other. Measurements for both the bare and PEGylated nanoshells compare favorably with Mie theory predictions. The Mie theory calculation shown here is based on modeling a particle without any surface modification. In this case, both the shape of the extinction and the scattering phase function could be accurately predicted using this simplified model. Using more sophisticated modeling, a PEG layer can be imported if
desired [9, 10]. All of the angular dependent experimental results are averaged over three or more separate measurements. The error bar in Figure 3.4 represents the standard deviation of the three measurements for each sample.

**Figure 3.4** Parallel polarized light scattering from bare (solid red diamonds) and PEGylated (hollow circles) nanoshells ($R_1=157\pm5\text{nm};$ $R_2=187\pm7\text{nm}$). Normalized parallel polarized light scattering from bare and PEGylated nanoshells compared to Mie theory (solid line; $R$ 157/192nm) at 633nm, showing that the scattering phase function of the bare and PEGylated nanoshells are analogous to each other; error bar represents the standard deviation of 3 separate measurements.
3.3.2 Multipolar Scattering of Gold Nanoshells

Since it is believed that the scattering of gold nanoshells may be enhanced at certain wavelengths by the surface plasmon resonances [22], the multi-wavelength aspect of our studies is conducted at the quadrupole and octupole resonance frequencies and differences in the scattering patterns at these resonance frequencies are measured. Nanoshells of \( R_1 = 148 \pm 10 \text{nm} \) and \( R_2 = 168 \pm 12 \text{nm} \) with a strong quadrupole resonance at 830nm and an octupole resonance located at 660nm are fabricated for this purpose, as shown in Figure 3.5. Figure 3.6 shows the comparison of normalized parallel polarized

![Graph showing Extinction Efficiency vs. Wavelength](image)

**Figure 3.5** Calculated extinction spectra of gold nanoshells R 150/170nm showing the contribution from the dipole, quadrupole and octupole components of the spectra.

light scattering results with Mie theory predictions for nanoshells (R 150/170nm) excited at 830nm where the quadrupole resonance is located, and at 633nm, which is close to the
octupole resonance of the nanoshells. The quadrupole and octupole scattering patterns are well shown in the experiments. The scattering patterns for these two wavelengths are very different from each other, but both are well fitted by Mie theory, which therefore shows the potential of gold nanoshells for multi-wavelength imaging applications.

![Graph showing scattered intensity vs angle](image)

**Figure 3.6** Parallel polarized light scattering from nanoshells ($R_1=148\pm10\text{nm}$; $R_2=168\pm12\text{nm}$) excited at the quadrupole ($830\text{nm}$) and octupole ($633\text{nm}$) plasmon resonances respectively; concentration $\sim 1\times10^7$/ml; the line curves represent Mie theory calculations for light scattering from nanoshells ($R_{150/170}\text{nm}$) at 830nm (solid line) and 633nm (dashed line); while the scattered symbols represent the normalized experimental data at 830nm (hollow circles) and 633nm (solid diamonds).
3.4. Conclusions

The objectives of this study are (1) to investigate the optical properties of gold nanoshells before and after surface modification using polyethylene glycol (PEG) and (2) to evaluate the results in terms of advancing biomedical imaging applications of gold nanoshells. In summary, we have achieved these goals by successfully studying the angular dependence of light scattering on both bare and PEGylated gold nanoshells ($R_1=157\pm5\text{nm}$ and $R_2=187\pm7\text{nm}$) near the octupole resonance of the nanoshells, as well as for nanoshells ($R_1=148\pm10\text{nm}$ and $R_2=168\pm12\text{nm}$) at the quadrupole and octupole resonances respectively. For the multi-wavelength study at the quadrupole and octupole resonances of the nanoshells, our measurements are in good agreement with Mie theory calculations at both wavelengths. Our results therefore show that the optical properties of gold nanoshells can be accurately modeled by Mie theory. This offers insight into the efficacy of further design and additional research of gold nanoshells for future application in multi-wavelength \textit{in vivo} imaging techniques. Our study on the PEGylated nanoshells also indicates that PEG doesn’t change the scattering phase function of gold nanoshells. Finally, the experiments also indicate that the goniometer could be a very promising instrument for multi-wavelength inspection and for future studies on other nanoparticles being developed as novel contrast agents for biomedical imaging techniques.
References


Chapter 4: Light Scattering from Spherical Plasmonic Nanoantennas: Effects of Nanoscale Roughness

Since the influence of surface modification on the light scattering from plasmonic nanoparticles have been discussed in Chapter 3, in this chapter we will present the study of the effects of nanoscale surface roughness on the light scattering properties of plasmon resonant gold nanoshells.

4.1. Introduction

The interaction of light with subwavelength structures is a topic of increasing interest and importance. Recent advances in clean room and chemical nanofabrication methods provide many opportunities to control both the geometry and composition of nanometer scale structures. These expanding capabilities allow us to broadly consider nanostructures as “nano-optical components” and to understand, in a more comprehensive manner, how optical frequency electromagnetic fields can be controlled and manipulated at nanoscale dimensions.

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* This study also appears in Hui Wang’s Ph.D. thesis.
Recently the influence on the optical properties of metallic nanoshells by the introduction of nanoscale “texturing” or the presence of defects on the nanoparticles has been reported [1]. There are several reasons for pursuing these studies. Surface roughness has long been known to relax the boundary conditions preventing the direct excitation of surface plasmon waves on macroscopic metal films [2], however, its role on a subwavelength structure where direct optical excitation is already possible has not yet been fully addressed. Experimentally realized nanoshells already have a small inherent degree of texturing or defects arising from the seeded growth method used to develop the spherical metallic shell layer on the surface of oxide nanoparticles. It is an interesting and important question whether or not the plasmon properties predicted by Mie scattering theory for analytically smooth nanoshells are significantly modified by surface roughness [3-7]. One would anticipate that nanoparticles in the quasistatic limit should behave as perfect dipoles. However, the introduction of phase retardation effects and the excitation of higher order modes that result when overall particle size is increased may introduce more significant deviations from the case of a perfectly spherical particle in the dipole limit. This more mesoscopic regime is one of general interest in light scattering studies, due to the importance of scattering properties in applications such as bioimaging [9]. In a more general sense, a wide variety of naturally occurring particles, such as biological structures [12-14] or atmospheric dust particles, are also known to have structured surfaces, yet the light scattering properties of such structures are not well studied.
The chemical method used to increase texturing on gold nanoshells has been recently reported, providing a systematic experimental approach with which to study the effects of nanoscale surface roughness on light scattering from nanoparticles [1]. The plasmon resonant extinction spectra of textured nanoshells were compared with theoretical simulations developed using a 3D finite-difference time domain method that accurately modeled the nonanalytical, highly textured nanoshell surfaces. Here it was seen that while the dipolar plasmon resonance extinction spectrum is robust with respect to surface roughness, the higher order plasmon resonances are significantly damped in the far field extinction spectrum with the onset of increasing nanoscale roughness, resulting in an overall redshifting of the nanoshell spectral envelope.

In the study presented in this chapter, we have examined the angular dependence of light scattering on resonance for both dipole and quadrupole resonant nanoparticles, and how this angular dependence varies with the introduction of surface roughness. In this case, the nanostructures must be fabricated such that the addition of texturing does not redshift the plasmon response of the nanostructures. This is accomplished by a chemical method which allows us to fabricate both smooth and textured plasmon resonant nanoparticles with the same far field spectral response. Using these tailored scatterers we were able to investigate the spatial distribution of resonant scattered light from nanoshells in two different geometries and sizes, selectively tuning the dipole and quadrupole resonances to the same laser wavelength. This approach allows one to suppress effects due to detuning of the plasmon resonance from the laser wavelength and
to the material dispersion of Au in the wavelength region of interest in our light scattering measurements.

4.2. Method

4.2.1 Surface Roughening of Gold Nanoshells

The fabrication of rough nanoshells is schematically illustrated in Figure 4.1(A). Gold nanoshells with relatively smooth surfaces were fabricated following a previously reported method [3]. Nanoscale roughness on the nanoshell surfaces was created by further deposition of metallic Au protrusions onto the surface of the smooth nanoshells. Ascorbic acid was used to reduce chloroauric acid (HAuCl₄) to metallic Au in the presence of cetyltrimethyl ammonium bromide (CTAB). It is known that CTAB and HAuCl₄ can form a Au(III)-CTAB complex, which can be reduced into Au(I)-CTAB complex by ascorbic acid. This Au(I)-CTAB complex can be further reduced to metallic Au upon introduction of gold nanoparticles as seed particles, an approach that has proven successful in the seed-mediated growth of gold nanorods [15-17]. In the present study, we use gold nanoshells as the seed particles, and nanoscale bumps are created on the surface of the nanoshells in the deposition processing. In the fabrication of Gold nanorods, single-crystalline nanoparticles are used as the seed, which results in the formation of single-crystalline nanorods. In contrast, nanoshells possess multicrystalline surfaces created by growth and coalescence of a large number of ultrasmall, randomly oriented single crystalline domains [1, 3]. These tiny domains grow into nanoscale
protrusions due to a slight variation in deposition with respect to domain orientation, thereby generating roughened surfaces on the nanoparticles. The as-fabricated rough nanoshells were washed twice by centrifugation and redispersion in water. The roughened nanoshells can be homogenously dispersed in water to form colloidal solutions.

Figure 4.1 (A) Schematic illustration of the fabrication of rough nanoshells. (B) Evolution of extinction spectra of the nanoshells during the bump growth on the surface. SEM images of the nanoshells (C) prior to bump growth and (D) after bump growth for 60 min.
The growth of bumps on the surface of nanoshells can be monitored by extinction spectroscopy over the time period of the chemical process. Figure 4.1(B) shows the evolution of extinction spectra of the nanoshells during the roughening process. In this set of experiments, we started from relatively smooth gold nanoshells with average core radius of 93 nm and shell thickness of 12 nm (Figure 4.1(C)). 10.0 μL of 1% HAuCl₄, 1.5 μL of 0.4 M ascorbic acid, and 90 μL of gold nanoshell solution (~ 6×10⁹ particles/mL) were introduced into 1 mL 25 mM CTAB aqueous solution in sequence. Both the quadrupole and dipole plasmon peaks blueshift (due to the increasing thickness of the shell layer) and the quadrupole resonance significantly intensifies during the growth of the bumps. This indicates an increase in the thickness of the nanoshells [3]. As the bumps gradually grow larger, they may coalesce on the surface, leading to an increase in overall shell thickness in addition to bumpiness. The growth of bumps terminates after 60 min due to reactant depletion. As illustrated in Figure 4.1(D), the surface roughness of the nanoshells significantly increases with a large number of nanoscale bumps (15 to 50 nm in size) randomly arranged over the surface of the nanoparticles. By adjusting the ratio between HAuCl₄ concentration, density of nanoshells in solution, and thickness of the initial nanoshell layer, the surface plasmon resonance of the resulting roughened nanoshells can be systematically tuned. This provides a unique approach to selectively place dipole, quadrupole or even higher-order multipole plasmon resonances of bumpy nanoshells at a wavelength of choice. In the case of this study, we tune the dipole and quadrupole plasmon resonances of the bumpy nanoshells to the same wavelength as that of two corresponding sets of smooth nanoshells.
4.2.2 Tuning the Plasmon Resonance of the Roughened Nanoshells

In the present study, smooth nanoshells with suitable core radii and shell thicknesses were fabricated with dipole and quadrupole resonances at 830 nm, respectively. Mie scattering theory appropriate for a concentric core-shell geometry [4, 5] was used to calculate the optimal core-shell dimensions for smooth nanoshells. The quadrupole or dipole resonance of rough nanoshells was also carefully tuned to 830 nm by controlling the growth of bumps on the surface of smooth nanoshells. In Figure 4.2(A), dipole resonance of both smooth and bumpy nanoshells are centered at ~ 830 nm, and a small shoulder at around 660 nm is also observed, corresponding to the quadrupole mode. In Figure 4.2(B), quadrupole resonances of smooth and bumpy nanoshells are both tuned to 830 nm and the small shoulder at 680 nm is assigned to the octopole resonance. The dipole resonance peak is shifted into the infrared beyond the scanning range of the spectral measurements. The peak positions and lineshapes in the extinction spectra of the roughened nanoshells are very similar to those of the corresponding smooth nanoshells, except that a slight broadening of the plasmon peaks is observed for the roughened nanoshells. The broadening of the plasmon peaks is probably arising from the inhomogeneity of shell thickness caused by increased surface roughness, as well as electron surface scattering within the gold shell [18].
Figure 4.2 Extinction spectra of smooth and bumpy nanoshells with dipole and quadrupole resonances at 830 nm. (A) Extinction spectra of smooth and bumpy nanoshells (homogeneously dispersed in water) with dipole resonance at 830 nm. The smooth nanoshells have core radius of 65±6 nm, and shell thickness of 14±1 nm according to SEM images. The blue curves are the measured spectra, and the red dash-dot
curve is the calculated curve according to Mie theory for a smooth nanoshell with 65 nm core radius and 14 nm shell thickness. The bumpy nanoshells were fabricated by adding bumps on the surface of smooth nanoshells with average core radius of 65 nm and shell thickness of 9 nm. (B) Extinction spectra of smooth and bumpy nanoshells (homogeneously dispersed in water) with quadrupole resonance at 830 nm. The smooth nanoshells have core radius of 148±10 nm, and shell thickness of 20±2 nm according to SEM images. The red dash-dot curve is the calculated curve according to Mie theory for a smooth nanoshell with 150 nm core radius and 20 nm shell thickness. The bumpy nanoshells were fabricated by adding bumps on the surface of smooth nanoshells with average core radius of 148 nm and shell thickness of 13 nm.

4.3 Results and Discussion: ADLS Measurements

An automated goniometer was used in the study of angular dependent light scattering (ADLS) measurements as shown in Figure3.1. The incident light source is a 30 mW GaAlAs diode laser with an output wavelength of 830 nm. The concentration of dipole resonant nanoshells is ~ 2×10^7 particles/mL and that of the quadrupole nanoshells is ~ 6×10^6 particles/mL. The light scattering data are collected in the range from 20° to 160°, with a step size of 1.8°.

The ADLS experiments were performed on dilute aqueous solutions of smooth and bumpy nanoshells with dipole or quadrupole resonance at 830 nm whose extinction
spectra are shown in Figure 4.2. Figure 4.3 shows a direct comparison of the ADLS patterns of the smooth and roughened nanoshells illuminated at their dipole scattering resonance, plotted in polar coordinates. Mie theory is used to theoretically predict the angular distribution of light scattering from perfectly smooth nanoshells. Because the angular scattering distributions are spatially symmetric, only one side (scattering angle ranging from 0° to 180°) is measured. For both the horizontal and vertical polarizations, the experimentally measured ADLS patterns of the smooth nanoshells match Mie theory very well. The bimodal dipole scattering distribution applies for the horizontally polarized incident light, while the scattering curve is almost isotropic for the vertical polarization. For both polarizations, the introduction of nanoscale roughness on the surface of nanoshells does not lead to significant differences in their ADLS patterns in the forward scattering direction; however, a marked decrease in the light scattering intensities in the backward direction is observed for the rough nanoshell case.
Figure 4.3 Angular dependent light scattering from smooth and bumpy nanoshells excited at their dipole resonance with horizontally (top) and vertically (bottom) polarized incident light. The light scattering patterns are plotted in polar coordinates. The solid curves are calculated curves for perfectly smooth nanoshells (65 nm core radius, 14 nm
shell thickness) according to Mie scattering theory. The blue circles and red squares are the experimentally measured data for smooth and bumpy nanoshells, respectively.

In Figure 4.4 we compare the ADLS patterns of the smooth and bumpy nanoshells illuminated at their quadrupole resonances. The roughened nanoshells exhibit a significant deviation from Mie theory predictions for smooth nanoshells. Both the smooth and roughened nanoshells possess much stronger light scattering in the forward scattering direction than in the backward direction. For smooth nanoshells, a secondary maximum at 95° is observed, in agreement with Mie theory, when the incident light is horizontally polarized. However, this side lobe completely disappears when nanoscale roughness is introduced on the surface of nanoshells. In the case of vertically polarized incident light, the bumpy nanoshells present significantly weaker light scattering intensities in the range of 30°~100° than the corresponding smooth nanoshells.
Figure 4.4 Angular dependent light scattering from smooth and bumpy nanoshells excited at their quadrupole resonance with horizontally (top) and vertically (bottom) polarized incident light. The solid curves are calculated curves for perfectly smooth nanoshells (150 nm core radius, 20 nm shell thickness) according to Mie scattering theory.
The blue circles and red squares are the experimentally measured data for smooth and bumpy nanoshells, respectively.

4.4 Conclusions

In conclusion, roughened gold nanoshells have been fabricated by introducing nanoscale roughness on the surface of relatively smooth nanoshells. Both smooth and rough nanoshells were fabricated with dipole resonances and quadrupole resonances at the same laser wavelength of 830 nm. The effects of nanoscale surface roughness on the angular distribution of light scattering from these nanoshell antennas have been investigated both at the dipole and quadrupole resonances. Though the difference in far-field extinction spectra of the corresponding smooth and bumpy nanoshells is minimal, the introduction of nanoscale roughness on the surface of nanoshells does result in measurable differences in the angular light scattering distributions. These observations may have important implications in the interpretation of light scattering characterization of complex nanostructures, and may lead to new ways of modifying the emission properties of resonant nanostructures.
REFERENCES


Chapter 5: Quantifying Immunotargeted Plasmonic Nanoparticles

Binding to Cancer Cells

The basic optical properties of gold nanoshells with different surface modifications and surface morphologies have been presented, both theoretically and experimentally, in the previous chapters. Here in this chapter, we will focus on the biomedical applications of light scattering from immunotargeted plasmonic nanoparticles.

5.1 Background

Previous studies have shown that colloidal gold nanoparticles which have been conjugated with anti-EGFR monoclonal antibodies provide enhanced signals in labeled human cervical tumor cancer (SiHa) cells, as shown in confocal reflectance images [1, 2]. Similar results, when using darkfield microscopy, have also been seen in anti-EGFR/gold nanoparticles labeled HSC oral cancer cells [3]. Proof-of-concept studies show that gold nanoshells conjugated with anti-HER2 antibodies provide enhanced signals, specifically in labeled HER2-positive human breast cancer SK-BR-3 cells. These have been observed

Adapted from:

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in reflectance confocal and darkfield microscopy images [4, 5]. Given the diagnostic potential of various nanoparticles and their *in vivo* applications, the ability to quantify nanoparticle bioconjugates that can be targeted to cancer cells is significant for improved diagnostic and therapeutic results. Despite this potential, however, the key factor which influences diagnostic or therapeutic efficacy is not even reported, namely, the number of targeted nanoparticles that bind per cell. Thus far in the literature, only Sokolov *et al.* have reported \( \sim 5 \times 10^4 \) nanoparticle bioconjugates bound per cell for human cervical tumor (SiHa) cells labeled with anti-EGFR monoclonal antibodies-conjugated colloidal gold nanoparticles [2]. The method they use to get the binding number requires centrifugation of the mixture of labeled cells and nanoparticles, so that those nanoparticles which did not bind to the cell surfaces are separated out. This method may be limited by the size of the nanoparticles and material from which they are made, i.e., it may not be effective to separate cells and nanoparticles of all varieties by centrifugation. Other people attempted to study the binding efficiency by using the direct measurement of optical signals from nanoparticles which bound to cells. The problem with this method is that multiple scattering can happen when nanoparticles are in close proximity to each other on the cell membrane. Due to the nonlinear effect introduced by this multiple scattering, the nanoparticle binding information cannot be accurately assessed. It is also difficult to directly count the number of particles that bind to cells. Instead, we have developed a novel method to quantitatively characterize the binding concentration of gold nanoparticle bioconjugate-labeled HER2-positive SK-BR-3 breast cancer cells using polarized angular-dependent light scattering. We have called this method “negative” quantification because we first obtain concentration information and light scattering
spectra from nanoparticles that were originally added to the cells for incubation. We then collect unbound nanoparticles after the incubation period with the cells, and measure light scattering of these unbound nanoparticles. There is a significant linear relationship between polarized light scattering and particle concentration when the suspension is dilute enough. Therefore, by comparing the light scattering spectra of the original and the unbound nanoparticles, the concentration of the unbound nanoparticles can be effectively obtained. Combined with cell-counting data, the average number of nanoparticles bound per cell can be finally derived. By following this procedure, cells labeled with immunotargeted nanoparticles can be effectively separated from unbound nanoparticles, as will be discussed later in this chapter. Furthermore, multiple light scattering, which may influence the accuracy in deriving concentration of nanoparticles, can also be avoided simply by measuring light scattering from unbound nanoparticles.

5.2 Method

5.2.1 Preparation of Immunotargeted Gold Nanoshells

Gold-silica nanoshells are fabricated as described in Chapter 2. The growth of nanoshells is monitored using a UV-Vis spectrophotometer (Varian Cary 300) and also by comparing the measured extinction spectra to the calculations from Mie theory; the particle size is further confirmed by using a SEM. Mie theory is used to determine the absorption, as well as scattering and extinction coefficients, of nanoshells with a specific core radius and shell thickness. The concentration of the nanoshells can then be determined by relating the calculated extinction coefficient to the measured extinction from the spectrophotometer. Figure 5.1 shows the SEM images and the measured
extinction spectra of two different nanoshells used in this study. According to Mie theory calculations and SEM images, the smaller nanoshell is \( \text{R}_1 = 86 \pm 7 \text{nm} \) and \( \text{R}_2 = 111 \pm 10 \text{nm} \), which is interpreted as R 80/103nm; while the larger one is \( \text{R}_1 = 134 \pm 6 \text{nm} \) and \( \text{R}_2 = 157 \pm 7 \text{nm} \) which is interpreted as R 134/155nm.

![Graph and image of nanoshells](image)

(a) Nanoshells R 80/103nm

![Graph and image of nanoshells](image)

(b) Nanoshells R 134/155nm

**Figure 5.1** Measured extinction spectra of gold nanoshells. SEM images of gold nanoshell are taken at 50000X, and scale bar is 500nm. (a) Nanoshells R 80/103nm. (b) Nanoshells R 134/155nm
As discussed in Chapter 2, anti-HER2 (Labvision) or anti-IgG (Sigma) antibodies were tethered to nanoshell surfaces using bifunctional PEG linkers. The anti-HER2 (anti-IgG)/nanoshell conjugates were obtained by reacting the OPSS-PEG-antibodies with the nanoshells for one hour at 4°C. Additional nonspecific adsorption sites on the nanoshell surfaces were then blocked by reacting with PEG-SH (MW 5000) for an additional hour. The nanoshell bioconjugates were subsequently centrifuged so that excess reagent was removed. The nanoshell bioconjugates were then resuspended in phosphate buffered saline (PBS) to prepare for cell targeting. Concentration of nanoshell bioconjugates was determined to be \( \sim 1 \times 10^9 \) /ml, with peak optical density of the extinction spectra at around 2, depending on the size of the particle.

5.2.2 Cell Culture and Incubation with anti-HER2 (anti-IgG)/Gold Nanoshell Bioconjugates of SK-BR-3 Breast Cancer Cells

HER2-positive human breast epithelial carcinoma SK-BR-3 cells (ATCC) were cultured in McCoy 5A growth medium containing 10% fetal bovine serum (FBS) and 1% antibiotics at 37°C and 5% CO₂. The cells were grown in 25cm² cell culture flasks to a concentration of \( \sim 5 \times 10^4 \) cells/cm². Following three washes with PBS, anti-HER2 (anti-IgG)/nanoshell bioconjugates suspended in PBS were added to the cell culture flask together with 1% Bovine Serum Albumin (BSA) as a blocker to eliminate nonspecific interaction with nanoshell bioconjugates. The cells were then incubated with the
nanoshell bioconjugates for one hour. After incubation, additional unbound nanoshell conjugates were collected using a pipette. The cells were then rinsed with PBS three times to ensure that all the unbound nanoshells were removed. The rinsing PBS and unbound nanoshells were collected for light scattering measurements. Finally, the labeled cells were removed from the cell culture flask using trypsin-EDTA and resuspended in McCoy 5A growth medium for darkfield imaging and cell counting.

5.2.3 Polarized Angular-Dependent Light Scattering Measurement

The polarized angular-dependent light scattering spectra of gold nanoshell bioconjugates were studied using an automated goniometer. The experimental setup for the light scattering study is the same as shown in Figure 3.1, and details about it can be found in Chapter 3. Light scattering has been shown to be sensitive to both the size and concentration of small particles. Furthermore, it has been proven that light scattering from surface-modified nanoparticles can also be predicted by Mie theory [6-8], as discussed in Chapter 3. Therefore, the concentration of gold nanoshell bioconjugates can be reliably derived from the light scattering phase function that we measured. We additionally conclude, for the aforementioned reasons, that the goniometer has effectively facilitated our quantitative study and can thus be considered a promising tool with which to characterize the binding concentration of gold nanoshell bioconjugates to SK-BR-3 cells.
5.3 Results and Discussion

Figure 5.2 Flow chart explaining the method of quantifying immunotargeted nanoshell binding to cancer cells.

Figure 5.2 shows a flow chart explaining the method of quantifying immunotargeted nanoshell binding to cancer cells developed in this study. To accomplish our goal of characterizing the number of nanoshells bound on each cancer cell, nanoshell bioconjugates with different concentrations were first prepared, and light scattering from these initial samples was compared. The light scattering study showed a significant linear relationship with the concentration for these samples, as shown in Figure 5.3. This confirms the feasibility of using light scattering to characterize the concentration of unknown nanoshell bioconjugates. One of these diluted nanoshell samples, with known concentration, was then recorded as a standard reference, as shown in Figures 5.4 (a) and (b) (black solid curves). After cell targeting, the unbound nanoshell bioconjugates were collected and diluted for light scattering measurements. Once the polarized light scattering of both the unbound and reference nanoshell bioconjugates was obtained, the concentration of the unbound nanoshells could be derived by interpolating the scattered
Figure 5.3 Scattered light intensity at different scattering angles showing linear relationship between the light scattering signal and the concentration for nanoshell R80/103nm. Error bars represent the standard deviation of three separate measurements.

intensity between the lines in Figure 5.3. Specifically, the total number of nanoshells bound to the cells could then be calculated using the volume and concentration data of the unbound nanoshells and the nanoshells originally added to the SK-BR-3 cells. After counting the cells, the number of nanoshells bound per cell could be determined, thus confirming the validity of our "negative" approach, as referenced above. Figure 5.4 shows the parallel polarized light scattering from the reference and unbound gold nanoshells (anti-HER2 conjugated only) of different sizes. The number of anti-HER2
(anti-IgG)/nanoshells bound per cell is shown in Table 5.1. The errors in Table 5.1 represent the standard deviations of three separate measurements which come from the summation of the individual errors generated in the light scattering measurements and from cell counting. This data is also supported by the results from a previous study on the use of gold nanoshells for molecular imaging [4]. In that study, the authors used nanoshells of R120/155nm for cell labeling, and compared the intensity of darkfield microscopy images taken from different nanoshell-labeled configurations. Loo's histogram analysis of the resulting darkfield images shows that nanoshell targeting of the HER2 receptor resulted in significantly greater average contrast values in the anti-HER2 group (142±16) compared with the anti-IgG group (48±12) and with the group lacking nanoshells (26±4) [4]. These results show an average of 5 times greater efficiency in anti-HER2 targeting than in anti-IgG targeting. And this reinforces our own quantitative results of using the R134/155nm nanoshell (which is similar in size as reported in reference [4]) to increase the nanoshell binding efficiency with anti-HER2 targeting. We have found that the anti-HER2 targeting is on the order of 5-9 times more efficient than the anti-IgG targeting for this specific nanoshell, as shown in Table 5.1.
Figure 5.4 Parallel polarized angular dependent light scattering measurements of reference and unbound anti-HER2/nanoshell bioconjugates: the concentration of unbound nanoshells can be derived by comparing the light scattering to that of the reference
nanoshells. (a) NS 80/103nm. (b) NS 134/155nm. Error bars represent the standard deviation of three separate measurements for each sample.

<table>
<thead>
<tr>
<th># of nanoshell per cell</th>
<th>NS R80/103nm</th>
<th>NS R134/155nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-HER2/ NS</td>
<td>1503±204</td>
<td>883±101</td>
</tr>
<tr>
<td>anti-IgG/ NS</td>
<td>90±11</td>
<td>113±16</td>
</tr>
</tbody>
</table>

Images of anti-HER2 (anti-IgG)/gold nanoshell bioconjugate-targeted SK-BR-3 breast cancer cells were taken under darkfield microscopy, a type of imaging modality that is only sensitive to the scattered light of visualized objects. This makes darkfield microscopy very suitable for the imaging of biological cells and small particles, as well as for nanoparticle-labeled cells. Images were taken with a Zeiss Axioskop 2 Plus microscope equipped with a color CCD camera using 40X objective. All images were taken using the same lighting conditions, more specifically, all same-sized nanoshell labeling had images taken under the same conditions. Figures 5.5 and 5.6 show the darkfield microscopy images of SK-BR-3 breast cancer cells labeled with anti-HER2 (anti-IgG)/gold nanoshell bioconjugates, as well as SK-BR-3 cells without any labeling. Based on the darkfield images, we can clearly see the difference in both specifically-(anti-HER2/gold nanoshell) and non-specifically- (anti-IgG/gold nanoshell) labeled cells, compared to those cells without nanoshell labeling. The cells with specific labeling demonstrate much better contrast under darkfield microscopy, due to the increase in
targeted nanoshells, as was determined in our light scattering study [4]. This also supports our results on the number of nanoshell bioconjugates bound per cell as shown in Table 5.1.

![Figure 5.5 Darkfield microscopy images of SK-BR-3 cells with (a) anti-HER2/nanoshell targeting (R80/103nm, specific), (b) anti-IgG/nanoshell targeting (R80/103nm, nonspecific) and (c) without targeting. All images were taken under same lighting conditions.](image_url)
Figure 5.6 Darkfield microscopy images of SK-BR-3 cells with (a) anti-HER2/nanoshell targeting (R134/155nm, specific), (b) anti-IgG/nanoshell targeting (R134/155nm, nonspecific) and (c) without targeting. All images were taken under same lighting conditions.

5.4 Conclusions

In conclusion, we have developed a new technique to quantitatively characterize the binding concentration of nanoparticles to living cancer cells. We have labeled HER2-positive SK-BR-3 human breast cancer cells with gold nanoshells of different sizes, and quantitatively compared the differences in anti-HER2 and anti-IgG conjugated nanoshell labeling. We have provided an easy and reliable method for quantitatively determining the amount of immunotargeted nanoparticles that can be targeted to SK-BR-3 breast cancer cells. Unlike the previous methods which measure cells labeled with nanoparticles, this method only requires the measurement of light scattering from nanoshell bioconjugates and provides accurate information on the binding concentration.
Following this method, multiple scattering effects can be avoided simply by measuring light scattering from the dilute suspension of unbound nanoshells. As a generalized methodology, it can also be easily applied to studies of other scattering nanoparticles targeted to cells that overexpress other biomarkers.
References:


Chapter 6: Conclusion and Future Work - Preliminary Data

for Clinical Application of Plasmonic Nanoparticles

6.1 Introduction

The unique optical properties of gold nanoshells, primarily their tunability in the NIR region, make them ideal candidates for a variety of applications in biomedical imaging and therapy. A better understanding of the optical properties of gold nanoshells can help facilitate the development of nanoshell-assisted imaging techniques and non-invasive optical diagnostics. In this dissertation, therefore, I have presented a theoretical, as well as experimental, step-wise study of the light scattering properties of gold nanoshells in the context of variable surface modifications and surface morphologies. This series of studies was designed to provide information useful for future clinical application of gold nanoshells. Specifically, by studying the light scattering of immunotargeted gold nanoshells, we have developed a novel technique by which to quantitatively determine the binding concentration of nanoshells to SK-BR-3 cancer cells in vitro. This study reveals a key factor that influences both the diagnostic and therapeutic efficacy of immunotargeted gold nanoshells, namely the number of targeted nanoparticles that bind per cell.
6.2 Preliminary Studies in Evaluating Clinical Applications of Plasmonic Nanoparticles: Incubation Time Trial

One important clinical application of gold nanoshells has yet to be investigated: targeted tumor margin detection. To determine tumor margins, current practices require histological study of frozen tissue specimens removed from the suspected tumor site while the patient is under anesthesia. This procedure is expensive, time consuming and sometimes inaccurate.

However, since nanoshells conjugated with cancer-specific antibodies can be targeted specifically to the cancer cells with much higher binding concentration, as suggested above, we have also proposed the application of gold nanoshells in targeted tumor margin detection. In order to evaluate the efficacy of immunotargeted gold nanoshells for this purpose, we compared the imaging results of nanoshell-labeled cancer cells at different incubation time points. Many studies have reported on the application of various nanoparticles in different imaging modalities. These studies specifically demonstrate that nanoparticles can effectively increase imaging contrast for the detection of specific types of cancer cells. However, the effect of incubation time on binding concentrations, in the context of tumor margin detection, has, to our knowledge, not yet been reported. Therefore, since tumor margins must now be determined within a brief clinical window, while the patient is under anesthesia, we have carried out experimentation with the aim of minimizing the incubation time of immunonanoshells
binding to cancer cells. Correspondingly, the imaging results were evaluated at different time points, as elaborated below.

6.2.1 Method

Although the materials required for this study were identical to those used in the study reported in Chapter 5, our methods were as follows. First, HER2 positive SK-BR-3 breast cancer cells were cultured in 2-well chamber slides for direct viewing under darkfield microscopy. Anti-HER2 antibodies were conjugated to gold nanoshells for specific targeting and cell labeling. Next, when the cells grew to nearly confluent in the chamber slides, nanoshell-antibody bioconjugates suspended in PBS with 1% BSA were applied to the cells. After the desired incubation time elapsed, additional unbound nanoshells were removed. Finally, following three washes using PBS, the chamber slides with labeled cells were cover slipped and imaged under darkfield microscopy.

6.2.2 Preliminary Results

To compare incubation time, our first set of experiments involved incubation increments of 10 min, 20 min, 40 min and 60 min. Figure 6.1 shows the resulting darkfield images of cells incubated with nanoshells at these different time points. Images of SK-BR-3 cells lacking nanoshells were also included as a standard baseline for cell scattering images.
<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Dark-field images 20X</th>
<th>Dark-field images 10X</th>
</tr>
</thead>
<tbody>
<tr>
<td>No NS</td>
<td>(a1)</td>
<td>(a2)</td>
</tr>
<tr>
<td>10 min</td>
<td>(b1)</td>
<td>(b2)</td>
</tr>
<tr>
<td>20 min</td>
<td>(c1)</td>
<td>(c2)</td>
</tr>
<tr>
<td>40 min</td>
<td>(d1)</td>
<td>(d2)</td>
</tr>
<tr>
<td>60 min</td>
<td>(e1)</td>
<td>(e2)</td>
</tr>
</tbody>
</table>

**Figure 6.1** Darkfield microscopy images of HER2 positive SK-BR-3 human breast cancer cells incubated with anti-HER2 conjugated gold nanoshells at different time points: (a) no nanoshells; (b) 10 min; (c) 20 min; (d) 40 min and (e) 60 min taken with (1)
20X objective and (2) 10X objective. All the darkfield images (obtained using the same objective) are taken with the same lightening conditions.

A numerical analysis of the histogram intensities of cells in the darkfield images is presented in Figure 6.2 and Table 6.1. The color images were converted into gray scale, and intensity values were extracted using a MATLAB code. Intensity values range from 0 to 255 in gray scale images, with 0 corresponding to pure black and 255 corresponding to pure white. The higher the intensity value is, the greater the contrast. Error bars in Figure 6.2 represent the standard deviation in intensities of 10 or more cells.

Figure 6.2 Histogram intensity analyses of individual cells in darkfield images at different incubation time points. The images were converted into gray scale, and intensity values were extracted using a MATLAB code. Intensity values range from 0 to 255 in
gray scale images, with 0 corresponding to pure black and 255 corresponding to pure white. The higher the intensity value is, the greater the contrast. Error bars represent the standard deviation in intensities of 10 or more cells.

**Table 6.1** Average intensities of cells in darkfield images at different incubation time points

<table>
<thead>
<tr>
<th>Cell-NS incubation time</th>
<th>No NS</th>
<th>10min</th>
<th>20min</th>
<th>40min</th>
<th>60min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intensity (a.u.)</td>
<td>8.8</td>
<td>17.3</td>
<td>19.7</td>
<td>20.1</td>
<td>23.3</td>
</tr>
<tr>
<td>Errors (a.u.)</td>
<td>1.3</td>
<td>5.4</td>
<td>3.3</td>
<td>5.6</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Our preliminary data show that we can, indeed, effectively minimize the incubation time of immunotargeted nanoshells with cancer cells and that we can do so with signal and contrast sufficient to differentiate the specific targeting. Thus, these results support the feasibility of using immunotargeted nanoshells in clinical tumor margin detection applications within the time constraints noted above.

**6.3 Future Directions**

Based on our cell studies to determine minimal incubation time, tissue experiments can now be carried out to further evaluate the application of immunotargeted nanoshells in tumor margin detection. Tissue images can be taken by either reflectance confocal microscopy or by darkfield microscopy, and these images can then be compared
to histological images to differentiate the presence of any correlations between the tumor margins as produced by these different diagnostic methods.

In addition to current applications of gold nanoshells in optical imaging and photo-thermal therapy in the NIR region, an extensive variety of promising clinical applications remain to be explored in order to exploit the clinical potentials of nanoparticles. For example, the use of gold nanoshells with conventional non-invasive diagnostic modalities, such as magnetic resonance imaging (MRI) and computed tomography (CT), has not yet been explored. Colloidal gold nanoparticles have been studied as contrast agent for MRI [1] and CT [2, 3]; therefore, gold nanoshells may also have the potential to be used for these applications. Although the optical resonance of gold nanoshells differs from that of gold nanoparticles, these two nanomaterials are similar in their chemical composition. Finally, in addition to diagnostic applications, the use of gold nanoshells in radiation therapy could open up still another potential field of study [4].
References:


