Surface-Enhanced Raman Scattering Nanoparticles as Optical Labels for Imaging Cell Surface Proteins

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Chemistry
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Abstract

Assaying the expression of cell surface proteins has widespread application for characterizing cell type, developmental stage, and monitoring disease transformation. Immunophenotyping is conducted by treating cells with labelled targeting moieties that have high affinity for relevant surface protein(s). The sensitivity and specificity of immunophenotyping is defined by the choice of contrast agent and therefore, the number of resolvable signals that can be used to simultaneously label cells. Narrow band width surface-enhanced Raman scattering (SERS) nanoparticles are proposed as optical labels for multiplexed immunophenotyping.

Two types of surface coatings were investigated to passivate the gold nanoparticles, incorporate SERS functionality, and to facilitate attachment of targeting antibodies. Thiolated poly(ethylene glycol) forms dative bonds with the gold surface and is compatible with multiple physisorbed Raman-active reporter molecules. Ternary lipid bilayers are used to encapsulate the gold nanoparticles particles, and incorporate three different classes of Raman reporters. TEM, UV-Visible absorbance spectroscopy, DLS, and electrophoretic light scattering were used to characterize the particle coating. Colourimetric protein assay, and secondary antibody labelling were used to quantify the antibody conjugation.
Three different *in vitro* models were used to investigate the binding efficacy and specificity of SERS labels for their biomarker targets. Primary human CLL cells, LY10 B lymphoma, and A549 adenocarcinoma lines were targeted. Dark field imaging was used to visualize the colocalization of SERS labels with cells, and evidence of receptor clustering was obtained based on colour shifts of the particles’ Rayleigh scattering. Widefield, and spatially-resolved Raman spectra were used to detect labels singly, and in combination from labelled cells. Fluorescence flow cytometry was used to test the particles’ binding specificity, and SERS from labelled cells was also detected using a modified flow cytometer. The work reported herein is a significant demonstration of SERS immunophenotyping using multiple cancer models, and adaptability to clinical instrumentation.
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<tr>
<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge Coupled Device</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>CLL</td>
<td>Chronic Lymphocytic Leukemia</td>
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<td>DCLS</td>
<td>Direct Classical Least Squares</td>
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<td>DF</td>
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<td>Dynamic Light Scattering</td>
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<tr>
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<td>LSPR</td>
<td>Localized Surface Plasmon Resonance</td>
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<td>B Lymphoma Cell Line</td>
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<td>MG</td>
<td>Malachite Green Isothiocyanate</td>
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<td>NP</td>
<td>Nanoparticle</td>
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<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
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<tr>
<td>SERS</td>
<td>Surface-Enhanced Raman Scattering</td>
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1 Introduction and Summary of Thesis

1.1 Introduction

The cell is the basic functional unit of eukaryotic organisms; understanding the normal development and function of different cell types can provide insight into the origins of disease through recognition of irregularities thereto. An important step on the path to accomplishing this is the advancement of tools to study cell structure. Myriad methods have been leveraged to image whole cells and/or their structural elements: electron microscopy [1], confocal and super-resolution fluorescence microscopies (ex. PALM/STORM, STED) [2,3], X-ray crystallography [4], near-field scanning optical microscopy (NSOM) [5], and medical imaging modalities such as computed tomography (CT), positron emission tomography (PET) or magnetic resonance imaging (MRI). These methods require complex instrumentation that may not be readily available. Additionally, complex sample preparation is required in some cases that precludes the investigation of live cell dynamics and/or native conformation of proteins. In this thesis, I explore experimental development of nanoscale probes with the aim of detecting disease at the cellular level through the identification of relevant biomarkers.

One of the approaches adopted for studying disease biomarkers is to assay the expression of proteins on the cell surface via immunophenotyping, and nanoscale probes are promising for use with this application. Because surface proteins are essential for the interaction of cells with their external environment, the landscape of protein expression is reflective of function, and irregularities thereto can be indicative of disease onset. The development of straightforward methods to characterize and study cell structure is important to fundamental research however such methods are of particular significance for clinical use whereby the distinction between normal and diseased cells, or the sensitive and specific detection of a disease state, must be reliably achieved. A number of different contrast agents have been adopted for immunophenotypic assays, and this thesis aims to describe the advancement of nanoscale probes for this purpose with the expectation that they may improve the limits of detection sensitivity, and accuracy with which multiple signals are resolved.
The nanoscale probes developed herein add to the repertoire of physical methods used to interrogate cells at the molecular level. Contrast agents are required to identify surface proteins using imaging modalities such as those listed above, and nanoprobes bring benefits to this application that will be discussed in the subsequent sections of this chapter, as well as throughout the remainder of the thesis. Both polymer coated-, and phospholipid bilayer encapsulated- gold nanoparticles with embedded reporters for surface-enhanced Raman scattering were prepared, characterized, and evaluated as immunolabels with three different in vitro tumour models. The nanoprobes were found to be detectable using a number of methods that are available to fundamental and clinical researchers including optical microscopy, Raman spectroscopy, and flow cytometry. The resolution of multiple signals can be a challenge in phenotyping, and the nanoprobe labels were found to be resolvable from mixtures of up to three label types. In all cases, the nanoprobes were targeted to cell surface receptor proteins that have integral function to each of the cell types evaluated, respectively. A number of different nanoscale labels have been forwarded for in vitro and in vivo biological applications and it is of interest to discuss how this work fits in to the broader context of nanomaterial research.

1.2 Cell Surface Proteins

In this thesis, nanoprobes are targeted to surface proteins that are implicated in signal transduction however ion channels, vesicular transporters and adhesion molecules are other common surface protein types that can be highly expressed. Cell surface proteins are embedded in the lipid bilayer structure of the cell membrane, and those involved in signal transduction transmit information from extracellular to the intracellular environments thus enabling the cell to generate appropriate physiological responses to stimuli. The signal could take the form of longer distance transmissions by endocrine factors that travel through the blood circulation however, signaling over shorter distances also occurs such as for paracrine signaling between adjacent cells, or via direct cell-cell or cell-matrix contacts such as between cadherins, integrins and components of the extracellular matrix. Upon binding its ligand, a cell surface receptor protein undergoes some conformational change that is detected on the intracellular surface of the cell membrane. These mechanical signals are transduced into chemical signals as intracellular proteins involved in the secondary messenger cascade are sequentially modified through
enzyme-mediated phosphorylation and dephosphorylation. Different classes of cell surface protein receptors that vary in the molecules involved in their respective cascades, effect the recruitment of transcription factors upon extracellular ligand binding. Examples of these different classes include: G protein-coupled-, tyrosine kinase-, TGFβ-, notch-, Hedgehog-, Wnt-, cytokine, and tyrosine phosphatase receptors of which CD45, a surface protein targeted in this thesis by nanoprobes, belongs to the later category. Once the signal reaches transcription factors that translocate to the nucleus, gene transcription and translation, and subsequent protein synthesis, are modulated [6].

The physiology of cellular responses to stimuli dictates the physiology of complex organisms; it follows that characterizing cellular phenotype, or cell protein expression profile, is key to understanding both normal biological function as well as for isolating the basis of disease transformation. Because the surface proteins expressed by individual cells are indicative of their function and stage of development, immunophenotyping is advantageous for discriminating between distinct cell types, as well as between normal and malignant cells in a mixed population. The nanoprobes developed in this thesis are promising as contrast agents for immunophenotyping, and proof-of-concept demonstration of their efficacy is made herein.

1.3 Nanotechnologies for Studying Biological Systems

Nanotechnologies, such as the SERS probes investigated in this thesis, are on a size scale that is advantageous for studying biological structures, in vitro and in vivo. Nanoparticles can enter, or bind to eukaryotic cells with micron scale dimensions, and can be used to study protein structure with dimensions on the order of nanometers, such as for an IgG antibody having a hydrodynamic diameter of 10 nm. They can be synthesized to be sufficiently small for crossing the blood-brain barrier, or entering the microcirculation. Not only do nanomaterials carry a size advantage, but their unique optical properties impart the potential for obtaining new kinds of information about biological structures.

Myriad nanomaterials in suspension can be delivered to cells and tissues, in vitro and in vivo. Examples include semiconductor nanocrystals (quantum dots) [7], carbon nanotubes [8], magnetic nanoparticles [9], fluorescent upconversion nanoparticles [10] mesoporous silica
nanoparticles [11], polymer nanoparticles [12], lipid micelles and vesicles [13], and noble metal nanoparticles [14]. All have been leveraged to extract information from, or modulate biological systems in some way. They act as contrast agents for imaging, as substrates for spectral enhancement, as delivery vehicles for other contrast agents.

Plasmonic noble metal nanoparticles are no exception. With resonances in the visible region of the electromagnetic spectrum, the resultant enhanced electromagnetic fields in close proximity to their surfaces amplify the intensity of their light scattering [15–17]. This can be exploited for imaging purposes using both the particles’ Rayleigh scattering, or of Raman scattering for molecular species proximal to the plasmonic surface, whereby exchange of energy between the incident light and vibrational energy levels of scattering species result in shifts in the wavelength of the scattered radiation that can be analyzed to determine the identity, conformation, and surrounding environment of the molecular scatterer(s). These applications are investigated in detail in the subsequent chapters.

This amplification is surface-enhanced Raman scattering (SERS) and has been used to study biological structures in many different ways. The narrow bandwidths of SERS spectra (approximately 1 nm FWHM) mean that multiple spectra with peaks in the same wavelength region can be deconvolved to obtain information about the scattering species to an extent that would be difficult for broadband fluorescence spectra. Additionally, the structures of plasmonic noble metal nanoparticles can be engineered through synthesis of nanorods [18], nanoshells [19], nanocages [20], nanostars [21], spiky structures [22], nanorice [23], and nanotriangles [24] among myriad other structures [25,26] in order to tune the optical resonances through the visible region to near-infrared wavelengths. Also notably, resonance with NIR wavelengths is significant for biological detection enabling probe lasers to excite contrast agents more deeply embedded in tissue, as well as mitigating the potential for heating and damage of the surrounding cells and tissues [27].

It can be seen how the application of SERS nanoparticles to studying cells and biological molecules could make feasible the collection of vital information in a way that was not possible before their advent. While plasmonic nanoparticles can be used to enhance endogenous Raman spectra of biological molecules, structures, and organelles in their immediate vicinity, they can also be used to enhance the Raman spectra of reporter molecules embedded in their coating
structure to construct optical probes that can be used to detect biomarkers of interest. A system where SERS labelling offers great promise is immunophenotyping of B lymphocyte cancers which is the predominant application I investigate herein.

1.4 Summary of Thesis

The first section provides a review of background concepts that are relevant to the techniques and approaches used to undertake the work in this thesis. The second section presents experimental work arranged to highlight the progression from SERS nanoparticle development, to increasingly complex imaging applications. The third and final section consists of a literature review pertaining to the use of metal nanoparticles for cell and tissue labelling applications other than those described herein. The experimental sections are largely comprised of material from published manuscripts that have been adapted to emphasize the intellectual contributions of the author.

1.4.1 Thesis Organization

Chapter 2: This chapter reviews the mechanisms of normal development and disease transformation of B lymphocytes, the in vitro model predominantly used in this thesis to develop targeted SERS-active gold nanoparticles. Current optical technologies used clinically for the diagnosis of leukemias and lymphomas will also be briefly discussed.

Chapter 3: The fundamental physical concepts that determine the enhanced optical properties of noble metal nanoparticles are discussed, with an emphasis on spheres. Localized surface plasmons are differentiated from surface plasmon polaritons that exist on planar surfaces. Extinction of light by metal nanoparticles is discussed, as well as Raman scattering and the electromagnetic and chemical enhancement mechanisms responsible for surface-enhanced Raman scattering.

Chapter 4: This chapter reviews concepts and rationale relevant to the instrumentation and methods used to prepare, and characterize antibody-targeting SERS gold nanoparticles, as well as to evaluate their efficacy and specificity as cell surface protein labels.
Chapter 5: The development and characterization of lipid bilayer (dioleoylphosphatidylcholine/sphingomyelin/cholesterol) encapsulated SERS-active gold nanoparticles are described in this chapter. Three different classes of Raman dyes were incorporated into the coating structure, and the encapsulating layer was characterized using Raman spectroscopy, UV-Vis absorbance spectroscopy, dynamic light scattering, and transmission electron microscopy. The lipid-encapsulated SERS gold nanoparticles were found to be stable both over longer-term storage, and suspension in acidic and high ionic strength conditions. The lipid coating is inherently biocompatible, and nanoparticles with zwitterionic surface functionalities are advantageous for biological applications.

Chapter 6: This chapter contains an account of CD20 targeting on primary human chronic lymphocytic leukemia cells using poly(ethylene glycol)-coated SERS-active gold nanoparticles conjugated to rituximab (anti-CD20) antibodies. The anti-CD20 SERS probes bound specifically to their CD20 targets, as evaluated using non-specific control particles and competitive binding experiments. Evidence of CD20 clustering upon ligand binding was observed using light scattering dark field microscopy images. This is a particularly interesting test system for SERS detection because rituximab is a therapeutic antibody used in the treatment of B lymphocyte neoplasms, and the target cells were derived from CLL patient samples.

Chapter 7: Triplexed SERS labelling of primary human chronic lymphocytic leukemia cells and the LY10 B lymphoma cell line is described in this chapter. Three different PEG-coated SERS-active gold nanoparticles, each functionalized with a different Raman reporter and corresponding monoclonal antibody, are demonstrated as effective optical labels for the specific detection of three different surface molecules on B cells, individually and simultaneously. The SERS nanoparticle binding is observed by collecting Raman spectra of cell suspensions, dark field microscopy images, and fluorescence flow cytometry. Spatially resolved triplex SERS spectra are deconvolved using least-squares fitting to identify the presence of three SERS probe types on a single cell. The SERS spectra of the nanoparticle labels were sufficiently intense to be detected using a modified flow cytometer. This work demonstrates a significant advance toward the application of SERS-active nanoparticles to immunophenotyping of multiple biomarkers, and towards use with clinical instrumentation.
Chapter 8: This chapter details the investigation of antibody-targeted SERS liposomes for specific in vitro labelling of CD19 and CD20 expressed by both primary chronic lymphocytic leukemia cells, and the LY10 B lymphoma cell line. SERS liposome bioconjugation was characterized, and dark field and confocal Raman microscopies were used to demonstrate the colocalization of SERS nanoparticles with cells. The in vitro and in vivo functioning of liposomal contrast agents are well understood, therefore demonstrating that SERS and Rayleigh light scattering functionalities could be successfully imparted to targeted liposomes is an important advance toward the wider adoption of SERS immunolabels.

Chapter 9: The targeting of PEG-coated SERS active gold nanoparticles to epidermal growth factor receptor in vitro is described in this chapter. The anti-EGFR SERS nanoparticles were used to label a different tumour model: lung adenocarcinoma, using the A549 cell line. Nanoparticle-treated cell cultures were interrogated using wide-field and spatially resolved Raman microscopy, in addition to dark field imaging. This study is significant because it demonstrated that the SERS-active gold nanoparticles investigated herein are suitable for use as immunolabels with multiple in vitro cancer models.

Chapter 10: In order to provide context to the work described herein, this chapter contains a brief literature review of multiple applications of noble metal nanoparticles to cell and tissue labelling.

Chapter 11: This chapter will summarize the material covered in the thesis, and detail suggestions for future work.

1.5 References


2 The Imaging Application: B Lymphocyte Development, and Disease Transformation

2.1 Overview

The following section provides a brief review of the developmental biology of blood cancers that will be the imaging target for SERS-active Au nanoparticles. Normal B cell development will be discussed with an emphasis on stages that have been implicated in the transformation of healthy cells to malignant diseases followed by a description of some of the mechanisms of transformation that have been proposed in the literature. Lastly, examples of currently used diagnostic technologies that rely on optical detection are provided along with factors determining some aspect of their detection limits.

Two B cell cancer models were studied in this thesis: primary human chronic lymphocytic leukemia (CLL) cells, and a human B cell lymphoma line (LY10). SERS Au NPs were conjugated to antibodies in order to target them to surface proteins of interest for immunophenotyping B cells. The significance of the development and application of contrast agents for immunophenotyping \textit{in vitro} will be made apparent in the following discussion.

2.2 Normal Development of B Lymphocytes

B (bursal) lymphocytes are key mediators of the adaptive immune response [1]. They play a role in humoral immunity by secreting soluble antibodies for identifying and cross-linking foreign antigens. Additionally, B cells phagocytose antigens and present partially degraded portions thereof on their surfaces bound to their major histocompatibility complex proteins, in order to activate T cells that have specificity for that antigen.

Each stage of the complex B lymphocyte development is error-prone and has the potential to transform cells, resulting in disease [2-5]. Antigen-independent development occurs in the bone marrow in adults (in the spleen in the fetus) and is dictated by expression of multiple transcription factors [6,7]. Cells undergo additional differentiation and proliferation in
secondary lymph organs and peripheral tissues upon response to antigen binding. The occurrence of three sequential but temporily overlapping processes demarcate the antigen-independent B lymphocyte development: V(D)J recombination which is the rearrangement of the immunoglobulin loci, expression of immunoglobulin, and expression of various B lymphocyte markers. Developmental stages can be identified by assaying cellular phenotype, and immunoglobulin (Ig) expression.

All blood cells develop from self-renewing pluripotent hematopoietic stem cells (HSC) in the bone marrow[1,7]. The HSCs maintain contact with bone marrow stroma ensuring their self-renewal, while daughter cells, upon loss of stromal contact, differentiate through increasingly specific stages of commitment to common myeloid, and common lymphoid progenitors; B lymphocytes, T lymphocytes, natural-killer (NK) cells and some dendritic cells originate from the later. Stage specific changes in protein expression during differentiation occur during development in the bone marrow. Many of the surface molecules expressed by leukocytes are given a cluster of differentiation (CD) designation. The CD nomenclature was developed by the Human Leukocyte Differentiation Antigens workshop and is a standardization method to characterize each cell surface antigen that has distinct binding characteristics to a set of monoclonal antibodies.

BCR repertoire diversity, and the ability to recognize antigens with which an individual has not yet been challenged, is created by the random assembly of gene segments on immunoglobulin loci to generate exons that will code for the Ig variable regions [1,6,8]. This is significant because from one gene, millions of combinations of segments are possible, thereby enabling the production of millions of different B cell clones that each have different antigen specificity. Three pools of gene segments (variable, diversity and joining) located on chromosome 22 are used to assemble the heavy chain variable region (V_{H}). Two gene segments (variable and joining) are used to generate light chain variable region loci, however these may be assembled from either V and J pools of kappa chain segments on chromosome 2, or V and J pools of lambda chain segments on chromosome 14. Kappa and lambda chains are functionally identical however a single B cell clone can produce Ig with only one of either kappa or lambda chains, in a 2:1 ratio in healthy individuals.
Pro-B cells express recombination activation genes 1 and 2 (Rag1/2) to initiate V(D)J recombination[6,10-12]. The enzymatic products of Rag1/2 introduce double strand DNA breaks in two places at recombination signal sequences (RSS) that flank each pool of V, D and J segments and the process is completed by random assembly via nonhomologous end joining pathways, and removal of introns and unused V, D and J gene segments. In the first stage of BCR synthesis and expression, the VH exons are joined with CH exons coding for a μ heavy chain. The nascent heavy chain associates with surrogate light chains (VpreB and λ5 proteins) as well as intracellular Igα and Igβ signal transduction proteins to form the pre-BCR at the cell surface. At this developmental checkpoint, lymphocytes that fail to express functional heavy chains for complexation to the surrogate light chain are removed via B cell tolerance. To ensure that each B lymphocyte produces monospecific BCR, the productive assembly and expression of pre-BCR coded by one allele prompts allelic exclusion to occur through one of the following processes occurring at the excluded Ig allele: production of an out-of-frame (non-productive) transcript, a germline (unrearranged) transcript, or a transcript that codes for non-pairing chains. Rag1/2 is expressed again, and V-J recombination occurs to produce the VL. An immature B cell expresses surface IgM and enters the blood stream. The structure of mature IgM is provided in Figure 2.1.
Circulating immature B cells enter the splenic marginal zone or follicles and each of these destinations resign the cells to a different fate [7]. In following the progress of follicular B lymphocytes, once in the spleen these cells move through three transitional stages (T1-T3) that can be isolated as different fractions based on changes in phenotype. When follicular B cells are challenged by antigen, they either differentiate and proliferate to become short-lived terminally differentiated plasma cells that secrete soluble Ig, or antigen binding may begin a germinal centre reaction that produces either B memory cells, or long-lived plasma cells.

A germinal centre (GC) is a lymph tissue microenvironment where B and T lymphocytes continuously interact, both with each other and with antigen, in a Darwinian process that yields the expansion of clones with high affinity for the particular antigen [13,14]. There are two processes that occur during the GC reaction that increase BCR antigen affinity and change the class of Ig expressed and secreted (and therefore its effector function): somatic hypermutation and class-switch recombination [11,15-17]. Both processes are mediated by activation-induced cytidine deaminase (AID) enzyme that introduces genetic lesions on to Ig single stranded DNA transcripts by transforming random cytidines to uracils resulting in a base-pair mismatch of U-G. These lesions are introduced at a high rate in to variable region exons for SHM, or in to large, repetitive switch region sequences upstream from C\textsubscript{H} exons for CSR. The lesions introduced during both processes are repaired by non-homologous end joining as for V(D)J recombination, as well as by base excision repair and mismatch repair mechanisms.

Dysfunctional clones that are autoreactive or fail to express a functional BCR are removed from the B cell pool by apoptosis, anergy or receptor editing (by repeated expression of Rag1/2) as part of B cell tolerance. Proliferation of, and failure to eliminate dysfunctional clones are both implicated in disease onset. Expression of a BCR that is chronically activated in response to self-antigen or long-term infection can result in clonal lymphocytoses [18].
2.3 B Lymphocytic Leukemias and Lymphomas

B cell leukemias and lymphomas are characterized by proliferation of a malignant B lymphocyte clone [8]. B cell cancers vary in age of onset, disease progression, cell count, histological and morphological appearance, phenotype, and probable cell of origin. General concepts in disease development will be presented however the focus of this chapter will be chronic lymphocytic leukemia and diffuse large B cell lymphoma because these were the cancer models used to study the brightness/performance and specificity of targeted SERS Au NPs.

Each stage of B lymphocyte development that contributes to a diverse, specific repertoire of antigen responses and the ability of immune cells to rapidly proliferate provides opportunity for the introduction of genetic lesions and malfunction of control processes. Failure of B cell tolerance mechanisms to neutralize this threat via apoptosis, anergy and receptor editing could ultimately result in expansion of malignant lymphocyte clones. Additionally, complex interactions with other immune cells such as T lymphocytes introduce further opportunities for inappropriate B cell activation and proliferation. Cancer types are distinguished by evaluating cell morphology and count, identifying an aberrant or distinct phenotype, and whether characteristic genetic lesions are present [2-4]. Diagnostic technologies will be discussed in Chapter 2.4.

CLL is the most common form of leukemia affecting 3.5 per 100,000 per year in North America with a median age of onset of 70 for men and 74 years for women [19]. CLL may be treated with monoclonal antibodies and/or cytotoxic chemotherapy but currently has no cure. This disease is characterized by absolute lymphocytosis of at least 5x10^9/L mature looking, terminally differentiated circulating B lymphocytes that express low levels of surface Ig, in addition to a characteristic phenotype that includes CD5 which is normally expressed by T cells and only 1-2% of B cells [19-21]. Morphologically, CLL cells are small and circular with a high ratio of nucleus to cytoplasm. Additionally, after slides are treated with histology stains for microscopy, “smudge cells” are present which are crushed during slide preparation due to the decreased mechanical stability of the CLL cells.

Though the term CLL describes a single disease at the genetic level, it has two different clinical courses: an indolent course that may not require treatment and a more aggressive course with poorer prognosis requiring early treatment. The two disease courses differ in their apparent cell
of origin. Tumours of the latter course express unarranged Ig variable regions genes that are similar to the germ line, while the indolent course expresses genes that have undergone somatic mutation. This points toward a role for antigen binding and the specificity of the BCR in disease progression either by an immature B cell for the aggressive course, or increased proliferation and inhibited differentiation by germinal centre lymphocytes.

There are a number of common genetic lesions associated with CLL cells. V(D)J recombination in CLL has been shown to use a $V_H$ repertoire biased toward non-random gene segment combinations that do not appear in healthy cells. Translocations involving Ig loci may result in the juxtaposition of potent Ig gene promoters with oncogenes resulting in overexpression of cancer causing proteins. Additionally, genetic translocations in CLL can involve genes important to cell cycle regulation and DNA repair. A deletion on the short arm of chromosome 17 (del17p13) encodes protein 53 ($TP53$) which is a tumour-suppressing transcription factor whose function is to arrest cell-cycle or trigger apoptosis upon activation by DNA strand breaks. Del11q23, a deletion on the long arm of chromosome 11, is the location of ataxia telangiectasia mutated gene (ATM) that codes for a kinase controlling $TP53$ expression. 13q14 deletions, the site of tumour suppressor genes $RB1$ and $D13S25$, are the most common lesion in CLL having multiple clinical outcomes depending on the length of the deletion and whether it is monoallelic or biallelic. Though the presence of particular lesions results in a diagnosis of CLL, all of the effected genes and therefore associated mechanisms of disease development have not yet been identified. An example of this is trisomy 12, despite the fact that it is one of the most commonly present lesions in CLL.

Diffuse large B cell lymphoma (DLBCL) is the most prevalent type of non-Hodgkin’s lymphoma. The designation of DLBCL includes B cell lymphomas with large, diffuse cells evident upon examination of tumor morphology and histology, respectively [21]. In contrast to CLL, DLBCL is a heterogenous disease that encompasses multiple disease entities, the definitions of which remain an area of active research and the following discussion is not exhaustive. Some classifications are made based on the purported cell of origin from which disease transformation occurred such as for germinal centre B (GCB) DLBCL and activated B cell (ABC) DLBCL, though each of these classifications include different mechanisms of disease transformation. Epstein-Barr virus positive- and anaplastic lymphoma kinase-positive DLBCL are distinctions made based specifically upon mechanisms of disease transformation by viral
infection and the presence of a characteristic genetic lesion, respectively. It is also common to distinguish DLBCL type based on distinct sites that are affected such as the central nervous system, as well as primary cutaneous, intravascular, and leg-type DLBCLs [2, 22-26].

2.4 Optical Technologies for B Lymphocyte Disease Diagnosis

Leukemias and lymphomas comprise a diverse group of diseases, therefore a multifaceted approach is advantageous for accurately identifying and diagnosing any specific cancer subtype. Current clinical approaches examine cell count and morphology, phenotype, and gene expression profile to differentiate healthy from malignant B cells. Additionally, these technologies enable the identification of cell of origin and corresponding mechanism of disease development, therefore providing insight into what may be the most specific and effective treatment strategy. Examples of diagnostic technologies that rely on optical detection are provided along with factors determining some aspect of their detection limits.

In order to examine cell morphology, histology stains are applied to sections of tissue biopsy samples or extracted blood cells. Histology stains contain a mixture of dyes that not only introduce contrast into optical microscopy images, but are also differentially absorbed by cellular organelles and tissue structures and therefore provide insight into morphology and development. Giemsa is an example of one such stain made up of methylene blue and eosin, and the effect observed upon staining is a pink appearing cytoplasm with blue or purple nuclei. Spatial and colour resolution are limited by the fundamental resolution limit of light microscopy, and either the viewer’s eyes or fidelity of colour representation by a camera connected to the microscope, respectively.

Monitoring phenotype and immunoglobulin expression of an individual’s leukocytes provides additional insight as to whether there has been an expansion of one or more malignant lymphocyte clones, as well as enabling identification of lineage and developmental stage of the clonal population [8]. The predominant method used in the clinic for this purpose is flow cytometry. Flow cytometry is a multiparametric technique used to acquire and correlate multiple types of information about individual cells in a large population [27,28]. A detailed description of flow cytometry and instrumentation are provided in Chapter 4. The expression of surface
proteins by a large B lymphocyte clone that are not appropriate for its lineage or maturation state may be indicative of the onset of disease. Disease subtype can be identified by characteristic expression signatures. Additionally, lambda and kappa Ig light chains can be fluorescently labelled to determine whether there is skewed distribution in the 2:1 ratio of κ:λ in healthy individuals. Because a lymphocyte clone can only express Ig with a single type of light chain, changes in the κ:λ can be used to identify expansion of a neoplastic clone.

The sensitivity, detection limits and dynamic range of flow cytometers are influenced by instrumental contributions, molecular contributions, and how closely the absorbance and emission bands of the fluorophores used for experimentation match the excitation and collection optics of the cytometer. Instrumental contributions include the quantum efficiency of the detectors for each channel over the wavelength range(s) being interrogated and the transmission efficiencies of all optical components. Molecular contributions that must be considered are how closely the excitation and emission profiles of the chosen fluorophore(s) overlap with the cytometer laser and optics, as well as each fluorophore’s extinction coefficient and fluorescence quantum yield at the probe wavelength. To normalize measurements between instruments and quote the sensitivity of different cytometers, NIST has defined units of Molecules of Equivalent Soluble Fluorochrome (MESF); usually stated with respect to fluorescein [29,30]. The degree of overlap between the absorbance and emission spectra of fluorophores used in multicolour flow cytometry protocols also determine whether multichannel compensation needs to be applied in order to deconvolve the intensities recorded at each detection channel. For fluorophores that emit wavelengths that can be collected by the optics in more than one detection channel, a percentage of the intensity for that fluorophore in its intended detection channel is subtracted from each non-specific channel to compensate for its contribution to the fluorescence intensity incorrectly recorded in each channel. Though applying compensation algorithms is necessary to correct the relative intensities recorded in each channel, it results in the loss of some intensity in each channel and therefore decreases the sensitivity of the fluorescence measurements.

Cytogenetics approaches are used to investigate the presence of genetic lesions that are characteristic to different subtypes of leukemia and lymphoma. Not only are such irregularities indicative of the presence of a tumour, they also provide insight as to the molecular processes involved in cellular transformation. The term cytogenetics encompasses multiple techniques, though karyotyping and in-situ hybridization are two of the most frequently used [31,32].
To construct a karyotype, cell division is arrested in metaphase when chromosomes are in their most compact form and isolated from cells for staining. Chromosomes may then be analyzed using G-banding. Metaphase chromosomes are stained with Giemsa histology stain and chromosomal regions differentially take up the dye which results in characteristic banding patterns along the length of each chromosome. Adenine-thymine rich regions (that are gene poor) absorb more dye and appear darker than gene-rich cytosine-guanine rich regions. The chromosomes are then arranged by size with the sex chromosomes last and observed for irregularities in banding pattern. G-banding enables visualization of large lesions with a resolution in the range of megabases for metaphase labelling. More precise information can be obtained using fluorescence labelling techniques such as spectral karyotyping (SKY) and fluorescence in-situ hybridization (FISH) to label particular genes involved in suspected genetic lesions. SKY involves arranging the metaphase chromosomes in a karyotype as for G-banding, while the genes being probed using FISH can be imaged directly from cells. Fluorescently labelled strands of DNA or RNA with sequence complementary to the strand of interest are incubated with thermally or chemically denatured DNA. Upon cooling, hydrogen bonds reform between complementary strands and the fluorescently labelled probe sequences anneal to the DNA. Ultimately, the resolution of both G-banding, SKY and FISH are limited by the resolution of a standard light microscope which is approximately 300 nm (depending on the wavelength of light being imaged) and how condensed the chromosomes are that are being studied. Interphase chromosomes can be resolved within thousands of kilobases, while the resolution limit for imaging metaphase chromosomes is usually on the order of megabases.

In this thesis, B cell malignancies are used as the predominant tumour model with which to investigate the use of targeted SERS-active Au NPs as optical probes for sensing cell surface molecules of interest in immunophenotyping. B cell leukemias and lymphomas are a useful example system for in vitro targeting because diagnosis of these diseases is typically accomplished using circulating cells which are extracted from patients prior to analysis. By reviewing the diversity of mechanisms through which B cell cancers develop, it becomes clear that maximizing the number of biomarkers that can be simultaneously detected will aid in sensitive, specific diagnosis of blood cell cancers thus indicating a role for SERS-active Au NPs in the diagnostic toolkit.
2.5 References


3 Physical Principles Dictating the Use of SERS Labels for Optical Detection

3.1 Overview

The following section reviews the fundamental physical properties that enable gold nanoparticles to be used as optical labels for the experiments described in this thesis. Excitation of surface plasmons by light in both the situation of planar surfaces and spherical nanoparticles will be described and contrasted. The quasistatic approximation to solving Mie’s equations will be defined as well as how it is applied to calculate the extinction of an electromagnetic field by spherical nanoparticles that are significantly smaller than the wavelength of the incident light. The relationship between the combination of metal and dielectric materials with the wavelength at which resonance with an external EM field occurs will be discussed. A basic explanation of the Raman scattering process will be provided and finally a description of how Raman intensity of molecules located at or near the nanoparticle surface are enhanced via the electromagnetic and chemical enhancement mechanisms of surface-enhanced Raman.

3.2 Surface Plasmons on Planar Surfaces

Surface plasmon polaritons (SPPs) are oscillations of conduction electron density relative to the metal nuclei along the interface between a metal and dielectric material [1,2]. SPPs propagate along the x-y plane and decay exponentially perpendicular to this plane into both the metal and dielectric layers (Figure 3.1).
Figure 3.1: Surface plasmon polariton wave on a planar surface at the metal-dielectric interface.

The relationship between the frequency of the SPP wave and its momentum (proportional to the absolute value of its wave vector, |k|) at the metal-dielectric interface is described by the dispersion relation for the component of the plasmon travelling in the x-y plane, parallel to the interface:

\[
k = \frac{\omega}{c} \sqrt{\frac{\varepsilon_1'(\omega)\varepsilon_2}{\varepsilon_1'(\omega) + \varepsilon_2}}
\]  

Equation 3.1

Where k is the wave vector (|k| is proportional to momentum), \(\omega\) is the SPP angular frequency, \(\varepsilon_1(\omega)\) is the real part of the complex dielectric constant of the metal, and \(\varepsilon_2\) is the dielectric constant of the dielectric medium. The complex dielectric constant for the metal (where refractive index \(n^2 = \varepsilon\)) is frequency dependent, and is of the form \(\varepsilon_1(\omega) + i\varepsilon_1'(\omega)\).

The resonant condition for SPPs occurs at the frequency where:

\[
\varepsilon_1'(\omega) = -\varepsilon_2
\]  

Equation 3.2

When this condition is met, the denominator in equation 3.1 approaches zero and k reaches a maximum value at the asymptote of the plotted dispersion relation (Figure 3.2).

Photons cannot directly couple to SPPs in a smooth metal surface, nor are these plasmons radiative. When light impinges on a smooth metal surface, the component of the light wave that travels parallel to the metal-dielectric interface has less momentum than an SPP with the same frequency. The dispersion relation for photons in the dielectric medium is the following:

\[
k = \frac{\omega}{c} \sqrt{\varepsilon_2}
\]  

Equation 3.3

Furthermore, this inability of photons to directly couple to plasmons along a smooth metal surface can be visualized in the plot in Figure 3.1. The SPP dispersion relation does not cross the
light line without some means through which to match the momenta of the two waves. This can be accomplished by using either a grating or prism coupler.

Figure 3.2: Dispersion relation for a surface plasmon at a smooth metal/air interface ($\omega_{SP}$); dispersion relation for photons travelling through air ($\omega/c$); dispersion relation for photons travelling through a prism ($n(\omega/c)$). SPP dispersion relation calculated using the dielectric function of gold from ref [1].

When perturbations are introduced in to the metal surface, such as for a grating, surface plasmons can reduce their wave vector/momentum and become radiative. Conversely, light impinging on a metal grating with grating constant (a), at a given angle ($\theta$), undergoes a change in the momentum of its component that travels parallel to the metal surface ($k_x$). If sufficient momentum is added to $k_x$, ($k_x + \Delta k_x \geq k_{sp}$) the dispersion relation for photons and plasmons ($k_{sp}$) will be equal for a particular metal-dielectric combination and light frequency, for some incident angle of light on the grating surface [1]:

$$k_x = \frac{\omega}{c} \sin \theta \pm \frac{\nu^2 \pi}{a} = \frac{\omega}{c} \sin \theta \pm \Delta k_x = k_{sp}$$

Equation 3.4

where $\nu$ is an integer. Metal surfaces of irregular roughness can also be used to couple photons and surface plasmons in which case the $\Delta k_x$ is better represented by a continuum rather than by discrete values associated with the grating constant.
Alternatively, an attenuated total reflection (ATR) or prism coupler may be used to increase the momentum of the incident photons by changing the refractive index of the medium through which they travel to approach the metal-dielectric interface. There are two principle configurations for the ATR coupler that vary in their placement of the prism relative to the dielectric medium. In the Kretschmann configuration, light passes through the prism which is in contact with a metal layer and is totally internally reflected at the prism-metal interface. Plasmons are excited at the opposing metal-dielectric interface by the evanescent wave from the totally internally reflected light. This configuration works so long as the metal film is sufficiently thin (10’s of Å). The Otto configuration places the prism adjacent to the dielectric medium. The wavelength at which plasmon coupling occurs can be observed by recording the spectrum of the specularly reflected light in order to observe a minimum in intensity where energy has been absorbed by the SP. Mathematically, the component of the photon wave vector travelling parallel to the metal–dielectric interface, $k_x$, increases upon travelling through the prism, with refractive index, $n = \sqrt{\varepsilon_2}$ [1,2]:

$$k_x = \sqrt{\varepsilon_2} \frac{\omega}{c} \sin \theta$$  

Equation 3.5

Therefore, as for the grating coupler, there is an angular condition whereby the dispersion relations for light and for SPs can be satisfied for a particular combination of metal and dielectric materials and wavelength of light. This can be visualized in Figure 3.2 whereby the slope of the light line decreases when the Kretschmann prism coupler is used, and crosses the SP dispersion relation.

### 3.3 Localized Surface Plasmons

When light impinges on a metal nanoparticle surrounded by a dielectric medium, its response differs from the situation of a planar metal surface at the metal-dielectric interface. Isolated nanoparticles are also capable of supporting plasmon oscillations however this plasmon does not propagate and can directly couple to light because there is no momentum mismatch between the photons and localized surface plasmons (LSPs) [1-3]. The metal nanoparticle is sufficiently thin
such that the incident electric field can drive a coherent oscillation of the conduction band electrons inducing polarized dipole and higher order multipole modes in the particle. The particle’s induced dipole experiences a restoring force to return the conduction band electrons to their original positions with respect to the metal nuclei and can be described as a driven damped harmonic oscillator. Photons with the same wavelength as the LSPs are absorbed and excite one of the plasmon modes. While the focus of the following discussion will be LSPs supported by spherical nanoparticles, a discussion of shape-dependent tunability of the plasmon resonance of anisotropic nanoparticles is located in Chapter 10.4. Resonance of the first-order, dipole, mode of spherical nanoparticles exists when the following condition is met:

\[ \varepsilon_1'(\omega) = -2\varepsilon_2 \]  

Equation 3.6

Extinction of light by the metal nanoparticle occurs through both absorbance and scattering processes [2-5]. Mie provided a full solution to Maxwell’s equations for the extinction of a plane wave by a sphere of arbitrary size that takes in to account the contributions of dipole and higher order multipole modes in the sphere, however Mie’s solution can be simplified for the case of nanoparticles having a diameter much smaller than the wavelength of the incident light (radius, \( a/\lambda < 0.1 \)) [6,7]. In the quasistatic approximation, the incident electric field can be approximated as being spatially static relative to the particle, oscillating only temporally (Figure 3.3).

Figure 3.3: Schematic representation of localized surface plasmon resonance of isolated metal nanoparticles in response to a temporally oscillated electric field (solid line). Electron clouds are labelled with e\(^-\). The direction of the field is indicated using arrows.
Additionally, the quasistatic approximation does not take into account possible excitations in the sphere by the magnetic field. The contribution to the nanoparticle’s extinction by its dipole mode may then be used to approximate the full solution for extinction by the nanoparticle. The Mie theory reduces to the following expression for the particle’s extinction spectrum is the following accounting only for the particle’s dipole mode [3]:

\[
E(\omega) = \frac{2\pi N a^3 \varepsilon_2^{3/2}}{\lambda \ln(10)} \left[ \frac{\varepsilon_1'/(\omega)}{(\varepsilon_1'/(\omega) + \chi \varepsilon_2)^2 + \varepsilon_1''/(\omega)^2} \right]
\]

Equation 3.7

Where \(\omega\) is the frequency of the incident field, \(N\) is the number of particles and \(\chi\) is the electric susceptibility of the metal which accounts for the shape of the nanostructure. This value is equal to 2 for a nanosphere.

By imposing the quasistatic approximation on extinction by the nanosphere, the amount of energy removed from the incident electric field, given by the extinction \((\sigma_{ext})\), scattering \((\sigma_{sca})\) and absorption \((\sigma_{abs})\) cross-sections for the nanoparticle (of volume, \(V\)), are calculated using equations 3.8 through 3.10 [2]:

\[
\sigma_{ext} = 9 \frac{\omega}{c} (\varepsilon_2)^{3/2} V \frac{\varepsilon_1'}{(\varepsilon_1' + 2\varepsilon_2)^2 + (\varepsilon_1'')^2}
\]

Equation 3.8

\[
\sigma_{sca} = \frac{3}{2\pi} \left(\frac{\omega}{c}\right)^4 (\varepsilon_2)^2 V^2 \frac{(\varepsilon_1' - \varepsilon_2)^2 + (\varepsilon_1'')^2}{(\varepsilon_1' + 2\varepsilon_2)^2 + (\varepsilon_1'')^2}
\]

Equation 3.9

\[
\sigma_{abs} = \sigma_{ext} - \sigma_{sca}
\]

Equation 3.10

For the frequency at which equation 3.6 is satisfied, it can be seen that equation 3.7 reaches a maximum value for extinction by the nanospheres. The resonance condition is impacted by the choice of metal and dielectric materials, and the wavelength of the external electric field (and therefore optical response of the metal at the chosen wavelength). Additionally, as the volume of the spherical NP increases, the ratio of its scattering to absorption cross-section increases indicating that larger particles are advantageous for use as optical contrast agents [5].
In addition to enhanced extinction by the metal nanoparticle at its plasmon resonant wavelength, it is important to consider the presence of an enhanced electric field at the surface of the particle with respect to the amplitude of the incident field [3,8]. The enhanced field is the superposition of the incident electric field, and the induced dipole field from the metal nanoparticle. First, the magnitude of the induced dipole field \( E_{\text{in}} \) inside the spherical particle (having radius \( a \)) is dependent upon the polarizability \( \alpha \) of the particle’s conduction electrons in response to the applied external field \( E_o \):

\[
E_{\text{in}} = \alpha E_o \quad \text{Equation 3.11}
\]

\[
\alpha = ga^3 \quad \text{Equation 3.12}
\]

\[
g = \frac{\varepsilon'(\omega) - \varepsilon_2}{(\varepsilon'(\omega) + 2\varepsilon_2)} \quad \text{Equation 3.13}
\]

Again, as follows from equation 3.6, the amplitude of the induced dipole field inside the sphere will be greatest when \( \varepsilon'_1(\omega) = -2\varepsilon_2 \). This relationship relates the choice of metal and surrounding dielectric material to the wavelength of incident light at which LSPR occurs.

The amplitude of the enhanced electric field at the nanoparticle surface can be calculated using LaPlace’s fundamental equation of electrostatics, in place of Mie’s electrodynamic calculations, for the case where the particle is sufficiently small and the quasistatic approximation applies [8]:

\[
E_{\text{out}}(x, y, z) = E_o \hat{z} - \alpha E_o \left[ \frac{\hat{z}}{a^3} + \frac{3z}{a^5} + \left( x\hat{x} + y\hat{y} + z\hat{z} \right) \right] \quad \text{Equation 3.14}
\]

where \( E_{\text{out}} \) is the magnitude of the enhanced near-field at the surface of the sphere, \( a \) is the radius of the sphere, \( x, y \) and \( z \) are Cartesian coordinates of the sphere, and \( \hat{x}, \hat{y} \) and \( \hat{z} \) are unit vectors along each Cartesian coordinate. The first term describes the contribution from the incident electric field and the second term originates from the induced dipole inside the sphere.
It is this enhanced near-field that increases the intensity of Raman scattering from molecules located at, or near the nanoparticle surface.

### 3.4 Raman Scattering

Raman scattering is the process of inelastic light scattering by a molecule due to exchange of energy between the molecule and incident light. Energy from the incident light can be lost to the molecule and therefore the scattering is of lower energy than the incident radiation (Stokes Raman scattering) or energy can be acquired by the incident radiation from molecules in an excited vibrational state (anti-Stokes Raman scattering) [9,10]. For Stokes scattering, absorption of energy from an incident electric field results in a transition from the vibrational ground state to an excited vibrational virtual state (which is a superposition of vibrations). The energetic transitions involved in Raman scattering are depicted in Figure 3.4:

**Figure 3.4: Jablonski diagram showing A) Stokes Raman scattering; B) anti-Stokes Raman scattering; and C) Rayleigh scattering processes.**

Because the ground vibrational state of molecule will be the most highly populated, Stokes shifted Raman scattering will be the more frequent and therefore of higher intensity than anti-Stokes Raman. The difference in energy between the incident and Raman-shifted frequencies is equal to the energy difference between vibrational states in the molecule. This relationship is given by the Bohr frequency rule (equation 3.15) where $h$ is Planck’s constant ($6.62606957 \times 10^{-34}$ kg m$^2$/s).
\(34 \text{ m}^2 \text{kg/s})\), \(\nu\) is the frequency of light absorbed or emitted by the molecule, and \(W_n\) and \(W_{n'}\) are the energies of vibrational states \(n\) and \(n'\), respectively[10]:

\[
\nu_{n'\rightarrow n} = \frac{W_{n'} - W_n}{h}
\]

Equation 3.15

For a molecule in an applied electric field, the positive and negative charges in the molecule (or the nuclei and electrons) will be attracted towards the opposite poles of the field, inducing a dipole moment in the molecule. The magnitude of the induced dipole is dependent upon the strength of the external field, and the response of the molecule to the field by distortion of its electron cloud. This is the polarizability \((\alpha)\) of the molecule [9,10].

\[
\mu_{\text{ind}} = \alpha E_o
\]

Equation 3.16

Where \(\mu_{\text{ind}}\) is the induced dipole moment, and \(E_o\) is the external electric field. The optical response of the molecule is determined by the geometry of its chemical bonds or symmetry components that are present in the molecule. A change in the electric field along one axis \(x\), \(y\), or \(z\), induces a change in the induced dipole moment in all three directions. Therefore, polarizability is represented by a second-order symmetric tensor (having 6 different components: \(\alpha_{xx}, \alpha_{yy}, \alpha_{zz}, \alpha_{xy}, \alpha_{xz}, \text{ and } \alpha_{yz}\)).

\[
\begin{bmatrix}
\mu_x \\
\mu_y \\
\mu_z
\end{bmatrix}
= \begin{bmatrix}
\alpha_{xx} & \alpha_{xy} & \alpha_{xz} \\
\alpha_{yx} & \alpha_{yy} & \alpha_{yz} \\
\alpha_{zx} & \alpha_{zy} & \alpha_{zz}
\end{bmatrix}
\begin{bmatrix}
E_x \\
E_y \\
E_z
\end{bmatrix}
\]

Equation 3.17

A transition of the molecule between vibrational states results in Raman scattering if one of the six different components of the molecule’s polarizability tensor changes during the vibration. The symmetry components of the molecule determine whether there will be a change in polarizability in response to the external field.

Raman scattering is of low intensity, with Raman cross sections typically on the order of \(10^{-30}\) to \(10^{-25}\) cm\(^2\) per molecule [11]. Therefore methods for enhancing its intensity such as placing the
Raman scatterer in close proximity to a plasmonic surface, as for SERS which is investigated in this thesis, have enabled Raman spectroscopy to be adapted for use with numerous applications.

### 3.5 Surface-enhanced Raman Scattering

The intensity of Raman scattering is inherently very weak: approximately 1 in $10^6$ incident photons will cause a vibrational transition in the scatterer resulting in either a Stokes or anti-Stokes Raman shifted photon. To address this shortcoming, several enhanced Raman spectroscopic techniques have been introduced \[12\] such as tip enhanced Raman scattering (TERS), coherent anti-Stokes Raman scattering (CARS), stimulated Raman scattering (SRS), and resonance Raman scattering (RRS). Surface enhanced Raman has the advantage of being experimentally more straightforward to implement, though aspects of the SERS phenomenon and quantitation of the SERS signal and enhancement factor remain areas of active research \[13\].

SERS can be accomplished from a variety of plasmonic substrates including planar surfaces of irregular roughness, or surfaces that have been functionalized with periodic features such as nanohole or particle arrays. Isolated or clustered nanoparticles of various structures and geometries are also widely used SERS substrates. There are two mechanisms that are responsible for the SERS effect: electromagnetic enhancement, and chemical enhancement mechanisms. The focus of this discussion will be for SERS from spherical metal nanoparticles with diameters sufficiently small relative to visible light wavelengths such that the quasistatic approximation is valid.

### 3.5.1 SERS Electromagnetic Enhancement Mechanism

For metallic nanoparticles irradiated with light having a wavelength near or matching the particles’ LSPR, the magnitude of the local near-field experienced by molecules at the nanoparticle surface is enhanced, as discussed above in Chapter 3.3[14]. For molecules located near the surface of a plasmonic nanoparticle, there is an enhancement of the incident field experienced by the analyte, and an additional enhancement of its Raman scattered field, both due to coupling with the induced dipole field in the metal nanoparticle. Because Raman scattering
intensity is proportional with the magnitude of the incident field \( I \propto |E_o|^2 \). SERS intensity (I_{SERS}) is proportional to the magnitude of the enhanced field at the NP surface [3,15-18]. The average magnitude of the electric field at the NP surface is the following:

\[
E_{out} = gE_o
\]

Equation 3.18

Where \( E_{out} \) is the magnitude of the enhanced field at the surface of the nanosphere, \( E_o \) is the magnitude of the incident field, and \( g \) is the field enhancement. In actuality, there is an angular dependency to the magnitude of the field enhancement with respect to the angle between the axis of propagation of the incident field and the Raman-active molecule on the surface of the sphere (maximal field enhancement for molecules located at 0 and 180°) [3]:

\[
|E_{out}|^2 = E_o^2[|1 - g|^2 + 3 \cos^2\theta (2Re(g) + |g|^2)]
\]

Equation 3.19

Where \( \theta \) is the angle between the axis of propagation of incident light, and the location of the Raman-scatterer on the NP surface. The remainder of this discussion however, will continue with the field enhancement that has been radially averaged over the surface of the sphere.

From equation 3.16, the magnitude of the Raman scattered field (\( E_R \)) is dependent upon the field at the surface of the NP that is experienced by the molecule [15,17]:

\[
E_R = \alpha gE_o
\]

Equation 3.19

The Raman scattered field is again enhanced through coupling to the nanoparticle’s dipole field. This relationship is complex and can be approximated by evaluating equation 3.18 at the Raman scattered wavelength, having a field enhancement of \( g' \). It has been addressed in more detail elsewhere [19].

\[
E_R \propto \alpha gg'E_o
\]

Equation 3.20

And therefore, SERS intensity is given by the following relation:
For small wavelength shifts in the Raman scattering, the SERS intensity can be approximated by fourth power of the enhanced field at the surface of the sphere. For larger shifts in wavelength, the Raman scattering may no longer overlap as strongly with the particle plasmon as does the wavelength of the incident field, and the relationship in equation 3.21 would be more accurate.

The SERS enhancement factor describes the magnitude by which the Raman cross-section of the scatterer is increased on the plasmonic surface compared to its Raman cross-section as an isolated molecule. The SERS enhancement factor is given by the following:

\[
EF(\lambda) = \frac{|E_{\text{out}}(\lambda)|^2 |E'_{\text{out}}(\lambda)|^2}{\varepsilon^2} = 4|g|^2 |g'|^2
\]

Equation 3.22

Where \(E_{\text{out}}(\lambda)\) and \(E'_{\text{out}}(\lambda)\) are the magnitudes of the enhanced field at the NP surface at the incident, and Raman-shifted wavelengths, respectively. To determine the enhancement factor experimentally, the intensities of the normal Raman and SERS spectra for a particular analyte can be compared:

\[
EF = \frac{I_{SERS} |C_{SERS}|}{I_{NR} |C_{NR}|}
\]

Equation 3.23

Where \(I_{SERS}\) and \(I_{NR}\) are the intensities of a particular Raman band in the SERS and normal Raman spectra, and \(C_{SERS}\) and \(C_{NR}\) are the concentrations of the Raman scatterer in the SERS and normal Raman spectra, respectively. Concentration is evaluated differently between the two types of spectra: the concentration of molecules in the SERS spectrum refers to the number of molecules adsorbed to the area of the plasmonic surface being probed, while the concentration for the normal Raman spectrum refers to the solution concentration in the laser interrogation volume.

SERS enhancement for isolated Au spheres such as those used to make SERS probes in this thesis, has been reported elsewhere to be approximately \(10^4\) [11] which is also what is observed herein (Chapters 6 and 7). SERS has been reported to enhance the intensity of Raman scattering
by upwards of 4 orders of magnitude, and there are reports ranging from $10^8$-$10^{11}$ times enhancement depending on the experimental conditions and instrumentation. SERS experiments conducted using lasers of visible wavelengths employ the coinage metals as the substrate material due to the surface plasmon resonant condition (equations 3.2 & 3.6) being met for these materials at visible wavelengths. Spherical silver nanoparticles are capable of generating greater field enhancements that can be used for SERS than gold nanoparticles of the same dimensions due to increased damping of the field in the Au NPs [20]. Despite the greater SERS enhancements reported for Ag, Au nanoparticles were used in this thesis because they are more biocompatible (Chapter 12).

There is also a distance-dependence to SERS enhancement because the intensity of the plasmon field decays exponentially perpendicular to the plasmonic surface [21]. The dipole decay law indicates that the strength of the field should decrease with distance as $r^{-3}$ where $r$ is the distance from the particle surface. After accounting for the $E^4$ field enhancement, this becomes $r^{-12}$ for an individual molecule on the nanoparticle surface. Because the enhanced field contains a volume of molecules for an absorbed monolayer, the SERS intensity decreases as $r^{-10}$ for a particle of radius, $a$.

$$I_{SERS} = \left(\frac{a}{a+r}\right)^{10}$$  \hspace{1cm} \text{Equation 3.24}

### 3.5.2 SERS Chemical Enhancement Mechanism

There is an additional contribution to the SERS enhancement factor through the chemical enhancement mechanism (accounting for 1-2 orders of magnitude in enhancement) [14, 21-23]. While electromagnetic enhancement makes a greater contribution, chemical enhancement can significantly increase the SERS enhancement factor because the electromagnetic and chemical contributions are multiplicative. Chemical enhancement occurs independently of the electromagnetic mechanism, however it is difficult experimentally to decouple the two effects for characterization.
Chemical enhancement occurs through either a change in the Raman polarizability of the adsorbed scatterer as compared to the free molecule, and/or through the formation of a charge-transfer complex between the Raman scatterer and the metal surface. For the coinage metals, the HOMO and LUMO of adsorbed organic molecules are often situated below and above the Fermi level of the metal, respectively (Figure 3.5).

![Energy level diagram](image)

**Figure 3.5**: Energy level diagram depicting SERS chemical enhancement mechanism: formation of charge transfer complex between the metal nanoparticle, and the adsorbed Raman analyte.

When this is the case, a charge-transfer complex can form whereby the molecule and metal can donate electrons (in either direction) and the energy that is required for excitation of the absorbed molecules is decreased; often corresponding to visible light wavelengths instead of the UV wavelengths required for the same species isolated in solution. This mechanism of enhancement is similar to resonance Raman whereby Raman scattering intensity is increased when the molecule is in its excited electronic state (albeit with allowed Raman modes determined by the excited state symmetry of the molecule). Additionally, adsorption of the Raman-scatterer to a metal surface can change its polarizability and different surface selection rules can contribute to altered peak presence and intensities for the SERS spectrum with respect to the normal Raman spectrum of the isolated molecule [23].
3.6 Conclusions

In this thesis, the enhanced optical properties of metal nanoparticles are exploited to image cells using the particles’ intense Rayleigh scattering in coloured dark field images, or through the collection of SERS from reporter molecules embedded in the NP coating. The presence of NPs is detected by comparison of spectral intensities from treated cell samples, or through the formation of pseudo-coloured microscopy images that indicate the location of NPs in the biological sample based on recognition of the SERS spectrum from reporter molecules near the NP surface.

3.7 References


4 General Methods and Instrumentation

4.1 Overview

This chapter contains a general description of the experimental methods used to prepare the SERS-active gold nanoparticles and the instrumentation used for characterizing them and for investigating their binding efficacy and specificity in \textit{in vitro} biological labelling experiments. An emphasis will be placed upon general methods because more detailed descriptions pertaining to individual experiments will be provided in the experimental chapters. In the first section, instrumentation methods, schematics, and principles of operation will be provided. In the second section, nanoparticle surface functionalization will be discussed including self-assembly of polymers and phospholipid bilayers, as well as the reaction mechanism for bioconjugation using EDC-sulfo-NHS zero-length crosslinkers and the structure of the antibody molecules used to direct SERS Au NPs to their cell surface protein targets.

4.2 General Instrumentation Methods

4.2.1 Raman spectroscopy

In Raman spectroscopy, inelastic light scattering from interrogated samples is collected and spectrally separated for analysis. Frequency shifts in the scattered radiation correspond to energy differences between vibrational levels of the molecules in the sample, and therefore spectra can be analyzed to obtain chemical information about the sample components [1]. The physical principles dictating Raman scattering are discussed in more detail in Chapter 3.4.

Raman spectroscopy is a useful analytical technique because it typically does not require specialized sample preparation [2,3], however Raman scattering is of low intensity. Approximately 1 in $10^6$ incident photons will be Raman scattered by a molecule and therefore these signals can be difficult to distinguish above detector noise or low-level fluorescence from glass cuvettes or biological samples. To overcome this challenge, Raman spectra can be
enhanced by localization of the analyte near a plasmonic structure as for SERS and tip-enhanced Raman scattering (TERS), or through non-linear spectroscopic techniques such as coherent anti-Stokes Raman scattering (CARS) or stimulated Raman scattering (SRS) [4,5]. Resonance Raman (RR) whereby samples are probed with a laser wavelength matching their maximum absorbance, can increase the Raman scattering cross-sections of sample molecules in an excited electronic state, but molecules in this state also return to the ground state through radiative decay and therefore intense fluorescence can overwhelm RR spectra. A much simpler approach to improving the sensitivity and signal to noise ratio of Raman spectra can be made by introducing confocal detection optics on to a standard Raman microscope [6-9].

Raman spectroscopy was used in this thesis to assess whether Au NPs had been functionalized with Raman-active dyes during the preparation of SERS probes, and to investigate whether targeted SERS probes had bound to cells in in vitro cell labelling experiments. Two different Raman microscopes were used for these purposes: a standard Raman microscope for the collection of widefield spectra from the entire illuminated sample volume, and a second with confocal detection optics for the collection of spatially resolved spectra. Samples were probed using 633 nm and 638 nm lasers for the widefield and confocal instrument respectively. Designing SERS probes to resonate at wavelengths approaching or in the NIR are beneficial for use with biological samples that do not absorb light as strongly at these wavelengths [10] thus reducing the potential for sample degradation and confounding autofluorescence.
The use of a standard Raman microscope for collection of widefield spectra was advantageous for collecting averaged SERS intensity values such as from NP suspensions in various stages of preparation, and the averaged intensity from large populations of cells treated with SERS Au NPs. A general schematic representation of this instrument is provided in Figure 4.1. The Abbe diffraction limit approximates the minimum separation distance ($\Delta x$) between two objects at which they can be resolved in an image constructed through a lens [11-13]. This limit determines the ideal spatial resolution of spectra collected using the widefield Raman microscope and is given as equation 4.1 for the x-y plane:

$$\Delta x = \frac{\lambda}{2n \sin \theta}$$

Equation 4.1

Where $\lambda$ is the wavelength of light used for imaging, $n$ is the refractive index of the surrounding medium and $\theta$ is the half angle over which the objective collects light. The denominator can also be written as the numerical aperture of the objective ($NA = n \sin \theta$). Axial resolution ($\Delta z$) is inferior, following equation 4.2:
Samples were probed using an inverted microscope outfitted with a 633 nm HeNe laser with power density of approximately 0.77 mW/μm² and lens-coupled to a Czerny-Turner configuration monochromator. Drops of NP or cells in aqueous suspension were placed on glass coverslips and probed with the 633 nm laser. Both Raman and Rayleigh scattering from the sample were collected by a 40X objective and Rayleigh scatter was removed by passing the light through an optical interference filter with low transmission at the laser wavelength (notch filter). The Raman scattering that enters the monochromator has a point of focus at the entrance slit, the beam diverges on to a collimating mirror that directs it to a holographic diffraction grating. The periodicity and angle of the grating grooves determine the wavelength range over which the grating efficiently diffracts light, as well as the divergence angle of the diffraction and therefore spectral resolution. A 1200 groove/mm grating was used most often for these experiments. A CCD detector was used to collect the wavelength resolved light.

A second spectrometer-coupled confocal inverted microscope was used that was configured to collect spectra from a defined sample volume. To introduce confocality for a typical fluorescence microscope, a pinhole aperture is placed at some point along the light collection optical train that is sized appropriately for each objective to match the dimensions of the image as it appears at the plane where the pinhole is placed. The pinhole restricts entrance of light from sample regions that are out of focus in to the detector. For the confocal Raman microscope, the pinhole was created by restricting the light scattering from the sample in two different locations before it could enter the detector: the x-axis was restricted by controlling the size of the spectrometer entrance slit, and the y-axis collection area was restricted by limiting the pixels on the CCD detector from which signals were sent for analog-to-digital conversion. This instrument was advantageous for use with cell samples that had been treated with multiple SERS dyes simultaneously because the distribution of the different particle types could be isolated from individual cells, as well as from different regions on a single cell. Additionally, the quality of the spectra were improved by excluding some of the autofluorescence from cells and endogenous Raman from the mounting medium used to prepare the cell slides for microscopy.
The confocal volume for Raman microscopy can vary from the ideal calculated volume due to the presence of non-planar structures or interfaces in the sample and unmatched refractive indices of different layers within the sample and mounting medium. Therefore it may be best to evaluate the confocal depth profile experimentally for each instrument and sample [9,14], however the ideal depth of field (h) and confocal volume (V) can be calculated using the equations 4.2 and 4.3 as follows:

\[ h = n \frac{\lambda}{NA^2} \]  

Equation 4.2

\[ V = 3.21\lambda^3 \left( \frac{f}{D} \right)^4 \]  

Equation 4.3

Where \( \lambda \) is excitation wavelength, \( n \) is refractive index of media surrounding the sample, and \( NA \) is numerical aperture of objective, \( f \) is objective focal length, and \( D \) is the diameter of the front lens of the objective.

To deconvolute spectra from mixed samples of SERS probes or cells that had been treated with multiple probes simultaneously, least-squares fitting was applied using input spectra from pure NP suspensions for each dye. Details of this fitting procedure are located in Chapter 7.

### 4.2.2 Dark Field Microscopy

Dark field microscopy collects only light that has been scattered from interrogated samples while excluding unscattered light from the detection optics. Image contrast is introduced through scattering objects appearing bright on a dark background. Dark field is a straightforward technique that is useful for imaging plasmonic nanoparticles. The large light scattering cross-sections of plasmonic NPs enable them to be visualized in dark field images despite the fact that they are smaller than the standard diffraction limit of light microscopy [15]. The NPs will scatter light most strongly at wavelengths matching their LSP and therefore their material, size, geometry, degree of monodispersity and index of refraction of the surrounding medium will
dictate the colour of light scattering imaged [16-20]. Dark field is being increasingly adopted for hyperspectral imaging of metal nanoparticles [21-24] and specialized commercial instruments are becoming widely available for this purpose.

**Figure 4.2: Schematic representation of optical configuration for transmitted light dark field microscopy.**

Dark field microscopy is similar to standard bright field transmitted light microscopy whereby samples are illuminated with a broadband halogen lamp light source. Two modifications are made to the optical components (Figure 4.2). An annular diaphragm is placed in the light path before the condenser lens assembly to attenuate light that would pass though the centre of the condenser, thus removing the zeroth order diffraction from passing through the condenser lens. This creates an inverted hollow cone of illumination with a focal point at the sample plane. If there is no sample in place, light passes unobstructed through the sample plane and diverges at an angle large enough to prevent it from entering the objective. The size of the dark field stop is matched to the numerical aperture of the objective to insure that the divergence angle is sufficiently wide. The second modification is the inclusion of an adjustable circular diaphragm in the objective lens assembly that further restricts the collection of transmitted light. The objective iris effectively reduces the numerical aperture of the objective to insure that only sample light scattering is imaged. The sample is illuminated at oblique angles using this configuration and only light that has been scattered from the sample is capable of entering the objective for imaging. The light scattering collected using a dark field microscope can also be coupled with a spectrometer to improve characterization of scattering wavelengths (14,22).
The resolution limit of dark field microscopy is the same as for standard brightfield microscopy and is given by the Abbe diffraction limit provided in equation 4.1. As can be seen from equation 4.1, spatial resolution can be improved by changing the refractive index of the medium separating the sample from the objective. For this purpose, drops of high index mounting medium and glass coverslips were used to seal samples of cells deposited on glass slides, and oil immersion condenser and objective were used for dark field imaging.

Dark field microscopy was used in this thesis to identify the presence, and ascertain the spatial distribution of SERS Au NPs in in vitro cell samples subjected to different nanoparticle treatments. The spherical Au NPs used to make SERS probes in the experiments herein have a diameter of 60 nm and a plasmon peak centered at 536 nm thus appearing green in dark field images. While the relative spatial distribution of nanoparticles that are closer to one another than the diffraction limit cannot be definitively resolved without applying complex image analysis techniques such as point spread function analysis [26,27], the plasmons of clustered particles overlap and the red-shift in resonance wavelength can be observed as a change in colour in dark field images. Interparticle distances based on colour shifts have also been reported elsewhere to be of use in the creation of plasmon rulers with range of up to 70 nm [28]. Analysis of light scattering colour in the dark field images also indicated that particle clustering had occurred upon their binding to cells in this thesis. Details of particle counting and resolution of particles from cells based on light scattering colour are provided in Chapters 6, 7 and 8.

4.2.3 Dynamic Light Scattering (DLS)

DLS, or quasi-elastic light scattering, analyzes the changes in a laser light diffraction pattern produced by passing it through a liquid suspension of micro- or nanoparticles undergoing Brownian motion [29,30]. DLS is used as a standard nanoparticle characterization technique that provides information such as particle hydrodynamic diameter, molecular weight, polydispersity and special algorithms can analyze sample motility.

DLS was used in these experiments to monitor changes in the hydrodynamic diameter of SERS Au NPs upon grafting the NP surfaces with PEG, encapsulating SERS Au NPs in phospholipids, and/or conjugating SERS probes to antibodies. Obtaining the polydispersity index of the sample
following each functionalization step gave insight into sample quality by identifying whether a significant number of particles had aggregated during the surface functionalization [31]. Two instruments were used in this thesis to carry out DLS measurements – the instrumentation for one will be described. Additionally, two types of data analysis were used to obtain hydrodynamic radii – the method of regularization [32,33] and cumulants analysis which is discussed in more detail in Chapter 7.

Monochromatic light is directed through the sample suspension and a diffraction pattern is collected by an avalanche photodiode (APD) detector positioned at 173° from the light path in the instrument used in this thesis. Light intensity fluctuations are monitored by a digital correlator at regular time intervals that are typically in the range of milliseconds. The similarity of the diffraction pattern to the previous collection is tracked by generating an autocorrelation function (29). The general form of the normalized autocorrelation function for changes in intensity over time ($g^2(\tau)$) is shown in equation 4.4 [26,35,36]:

$$g^2(\tau) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I(t) \rangle^2}$$

Equation 4.4

Where $I(t)$ is the intensity at time $t$, and $I(t+\tau)$ is intensity at some delay time, $\tau$. What is being monitored by the instrument software is the following:

$$g^{(1)}(\tau) = \exp(-\Gamma \tau)$$

Equation 4.5

Where $\Gamma$ is decay rate over each delay time:

$$\Gamma = D_T q^2$$

Equation 4.6

$D_T$ is the translational diffusion coefficient, $q$ is the difference between the incident and scattered waves, called the scattering wave vector:

$$q = \frac{4\pi n}{\lambda} \sin \left(\frac{\theta}{2}\right)$$

Equation 4.7
As time elapses, the positions of the sample particles will become significantly different from their starting positions, resulting in an exponential decay of the autocorrelation function or correlogram of $g^2(\tau)$ versus each time point. Larger particles will diffuse more slowly than smaller ones and a more monodispersed sample will display less variability in diffusion rate. The Brownian motion is manifested in the autocorrelation function as the decay time and slope. In a polydispersed sample, there will be a distribution of decay times/rates and alternative data analysis strategies can be employed to improve the resolution of this data [37].

The diffusion coefficient of the sample is obtained by fitting the autocorrelation function and algorithms have been written to take in to account oblong, branched or motile samples. Finally, the diffusion coefficient is input into the Stokes-Einstein equation to obtain particle radius:

$$r(H) = \frac{kT}{6\pi\eta D_T}$$

Equation 4.8

Where $r(H)$ is the hydrodynamic radius, $k$ is the Boltzmann constant ($1.3806488 \times 10^{-23}$ m$^2$ kg s$^{-2}$ K$^{-1}$), $T$ is solvent temperature, and $\eta$ is viscosity. The radius obtained is equivalent to the radius of a hard sphere that would diffuse at the same rate as the sample particles. The value obtained for the hydrodynamic radius is expected to be larger than the true particle radius because the motion of the particles in solution is influenced by the size of the surrounding hydration layer, and therefore is impacted by particle surface charge and choice of solvent.

4.2.4 Electrophoretic Light Scattering – Zeta Potential Measurement

Electrophoretic light scattering analyzes the changes in a laser light interference pattern produced when the light travels through a liquid suspension of micro- or nanoparticles that are moving in response to an electric field is applied across the particle suspension. The velocity of the particles is used to calculate their zeta potential.

For charged particles in suspension, the net charge at the particle surface determines the distribution of ions in the interfacial region. As a result, there is the formation of an electric double layer of ions surrounding the particle that are oppositely charged with respect to the particle surface. The electric double layer is comprised of the inner Stern layer made up of ions
that are closely bound to the particle, and an outer diffuse region of ions that are less firmly attached but still move with the particle through solution. The boundary between the diffuse layer ions and ions in the surrounding suspension is the slipping plane and the zeta potential is the electric potential that exists across the slipping plane [38]. While the measurement of zeta potential is not a direct measurement of charge at the particle surface [39], this information may be inferred from the zeta potential while taking into account the solvent in which the measurements were conducted (and hence ion concentration), as well as the applied charges used to accelerate the particle.

Figure 4.3: Schematic representation of electrophoretic light scattering instrument.

The instrument used to collect Au NP zeta potentials in this thesis worked on the principle of phase analysis light scattering (PALS) which is a modified form of laser Doppler velocimetry [40]. Particles are accelerated through suspension by application of a voltage difference across the sample cuvette and their movement is monitored by observing changes in the phase of elastic light scattering of a laser incident on sample particles in the cuvette. A general schematic of a PALS instrument is provided in Figure 4.3. Particles will accelerate toward the anode with velocity proportional to the size of the applied field, and their surface charge in the solvent of choice. The particles will move with constant velocity across the applied field because the opposing drag and electrophoretic forces result in the particles experiencing zero net force [41]. Elastic light scattering is collected during the movement and the phase of scattered radiation is shifted compared to a reference beam in proportion to the NP velocity. The direction of the applied field is repeatedly reversed by the instrument during each data collection to reduce
polarization of the electrodes through electroosmosis [42]. As shown in Figure 4.3, compensation optics are in place in order to correct for any changes in scattered beam alignment introduced by differences in sample cells, or differences in refractive index between suspension media.

The electrophoretic velocity \( U \) of the particles in the applied field is proportional to their mobility \( \mu \) and the applied field \( E \) [43]:

\[
U = \mu E 
\]

Equation 4.9

For the isotropic particles studied in this thesis, the mobility is described by a scalar. For anisotropic particles such as nanorods, mobility is best described using a second-order tensor. The particles’ mobility is used to calculate \( \zeta \)-potential and the Smoluchowski formula was used in this thesis (equation 4.11). The Smoluchowski formula is valid for use for the regime in which the product of the radius of the spherical particle \( a \) and the Debye parameter \( \kappa \) (which is the inverse of the Debye screening length) is far greater than 1 [41]. The Debye parameter \( \kappa \) for a symmetric electrolyte is given by the following:

\[
\kappa(nm^{-1}) \approx 3.3[I]^{1/2}
\]

Equation 4.10

Where \( I \) is the ionic strength in M. For 60 nm Au NPs in 10 mM NaCl, \( \kappa a \approx 10 \) and the Smoluchowski formula is valid. Using the Smoluchowski formula, zeta potential is calculated as follows:

\[
\mu = \varepsilon \varepsilon_0 \eta^{-1} \zeta
\]

Equation 4.11

Where \( \varepsilon \) is the dielectric constant of the solvent, \( \varepsilon_0 \) is the vacuum permittivity, and \( \eta \) is the viscosity of the solvent.

PALS was used in this thesis to investigate changes in Au NP zeta potential prior to and following surface coating with thiolated PEG or phospholipids. The change in zeta potential from negative to near neutral values was indicative of the presence of a charge shielding layer.
surrounding the particles[41]. This method has been used to characterize gold nanoparticle surface coating with thiolated PEG and other ligand molecules elsewhere [41,44,45]. The experimental procedure and data collected are discussed in more detail in Chapter 7.

4.2.5 Flow Cytometry

Flow cytometry collects elastic light scattering and fluorescence from micro- and nano-scale objects suspended in a flowing liquid that passes through multiple laser lines. The simultaneously collected scattering and fluorescence are spectrally resolved by a series of optical interference filters, and correlated to each sample object [46-49]. Flow cytometry has a number of both research and clinical applications, however the most common use is for studying subpopulations in mixtures of different cell types. Flow has the potential for studying any large, heterogeneous sample and for identifying sub-classifications and rare events within it. Innovations to flow cytometry include its application to routine fluorescence activated cell sorting (FACS), 17 colour excitation, imaging cytometry, mass cytometry, detection of plasmonic labels, and in vivo photoacoustic flow [50-56].

Flow cytometry was used in this thesis to investigate the binding of targeted SERS Au NPs to cells. Two types of experiments were conducted for this purpose: indirect identification of the particles was accomplished by treating NP labelled cells with fluorescent antibodies that bound to either the IgG1 antibodies conjugated to SERS probes, or to the same CD molecule target as the SERS probe being investigated. Increased or decreased fluorescence in each of these cases, respectively, indicated the presence of SERS probes on the surface of each cell. Direct identification of the particles was accomplished by substituting one of the broadband fluorescence detection optical interference filters with a narrowband filter for the collection of a major peak in the particles’ SERS spectrum. Details of these experiments are provided in Chapter 7.
A schematic diagram of a typical flow cytometer is provided in Figure 4.4. Sample particles are suspended in liquid (typically a buffer solution) and flowed through tubing to a flow cell where the sample is irradiated with multiple laser wavelengths. The suspension is surrounded by sheath fluid that travels with laminar flow around the sample stream. Changing air pressure exerted on to the sheath fluid is used to hydrodynamically focus the sample to varying sizes in focal point, or to change the velocity of sample flow. The flow rate and size of the interrogation volume are modulated to insure that sample particles pass through individually. Particles passing through the flow cell are probed with multiple lasers in tandem, and elastic light scattering and fluorescence from the samples are collected from three sets of optics: the forward small angle scatter detector is adjustable and located at 4-30° with respect to the propagation axis of the
laser(s), and two detectors are situated orthogonally to the axis of the laser propagation for collection of elastic light scattering and fluorescence. Each laser is focused on to the flow cell by two crossed cylindrical lenses to produce an elliptical beam that is sufficiently wide to probe the full width of each sample object, while remaining narrow in the direction of sample flow so that voltage pulses at the detector are short and flow rate can be maximized. For the instrument used in this thesis, the laser beam dimensions in the sensing area were 84 x 10 μm for the 488 nm laser used to collect elastic light scattering from the sample, and 72 x 9.6 μm for the 638 nm laser used to probe labelled cells for SERS.

Light from the sample then travels through a series of optical interference filters, each of which precedes a detection channel. The series of filters each have a wavelength cutoff value that increases to longer wavelengths with each subsequent filter in the series. Each filter transmits light shorter than that cut-off to a bandpass optical filter and a photomultiplier tube (PMT) detector that converts each measurement of light to a voltage pulse of varying width and height corresponding to the sample properties. Light of wavelengths greater than the filter cutoff are sequentially reflected from each short-pass filter in the series until it is eventually transmitted by a filter with a sufficiently long cutoff wavelength to the corresponding PMT.

Voltage pulses from each PMT are plotted and analyzed by the peak intensity of the pulse, the integral of the pulse peak to determine total fluorescence from each sample particle, and pulse width which is useful for the identification of multiple sample particles passing through the flow cell sensing area simultaneously. The bit depth of the ADC determines the dynamic range of flow measurements to variations in light intensity from each sample object. The instrument used in this thesis has a 20-bit ADC meaning that there are 1 048 576 separate channels between which intensity range can be distributed either linearly, or logarithmically. Because only one contrast agent that resonated with each laser wavelength was used concurrently in these experiments, spectral resolution techniques such as multichannel compensation were not required to resolve different signals [57].
4.2.6 Transmission Electron Microscopy (TEM)

In electron microscopy, samples are irradiated using a beam of accelerated electrons to exploit the wave-like properties of electrons for high resolution imaging [58-60]. In bright field TEM, image contrast is created by differential absorption or obstruction of the electron beam by sample objects in the beam path.

The electron source is often a tungsten wire through which current is passed and conduction electrons in the gun material are ejected through thermionic emission. A voltage difference is applied across the microscope to accelerate the electrons through the sample and towards the detector. Increasing the voltage difference between cathode and anode changes the angle through which the beam is focused, and consequently, the image magnification. The beam is guided and focused using solenoids as electromagnetic lenses. In order to maximize brightness of the beam and reduce background scatter from entering TEM images, the interior of the TEM and sample chamber are operated under vacuum so that the high energy electrons move with the greatest possible mean free path. A CCD or fluorescent screen can be used as detectors.

TEM was used in this thesis to investigate whether SERS Au NPs has been coated by polymer or phospholipid molecules. Samples were deposited on to carbon coated copper grids and allowed to air dry before imaging. Dense Au NPs introduced sufficient image contrast with respect to polymer and lipid coating such that microscopy stains were not required. The instrument used to collect images in this thesis was capable of a range of acceleration voltages between 50-100 keV with a 0.5 nm spatial resolution.

4.3 General Sample Preparation Methods

4.3.1 Functionalization of Gold Nanoparticles with Raman-active Dyes

There are several strategies used to incorporate Raman-active dyes in to nanoparticle coatings including the use of dyes that chemisorb or physisorb to the metal surface, as well as dyes that are covalently bound to, or closely associated with other coating molecules. The objectives for any dye incorporation method include placement of the dye molecules as close to the metal
surface as possible in order to maximize SERS enhancement, and insuring that the dye is stably attached within the coating structure so the SERS particles maintain their intensity over extended time periods [61]. For most of the experiments described in this thesis, Raman dyes were physisorbed to Au NP surfaces prior to coating with thiolated PEG or encapsulation in lipid bilayers. The spherical Au NPs were synthesized using sodium citrate for reduction and therefore negatively charged citrate capping molecules were present on nanoparticle surfaces [62]. Dyes with a positive charge at neutral pH and multiple benzene rings were found to provide the most intense SERS spectra upon adsorption to the Au NPs and this observation has also been reported elsewhere [63,64].

Additionally, dyes that absorb light maximally at wavelengths near to the laser wavelength used (632 nm or 638 nm for different instruments) produced particles with the most intense SERS spectra. Because dye molecules with an electronic transition occurring near the 632/638nm laser lines were optimal for the production of SERS Au NPs, this suggests that a surface-enhanced resonance Raman scattering (SERRS) effect was occurring [65,66]. The use of molecules with large fluorescence quantum yields as Raman dyes was also possible due to quenching of molecules’ fluorescence in close proximity to the plasmonic surface. Instead of the molecular excited state decaying through emission of a Stokes shifted photon, the excited state decays non-radiatively by transferring its energy to the plasmonic surface [68].

Au NPs were functionalized with Raman dyes by adding aqueous dye solutions to vigorously stirring Au colloid. Dropwise addition of the dye solutions was necessary in order to prevent aggregation of the particles due to rapid alteration of their surface charges in the absence of high molecular weight coating ligands such as PEG or lipids that would reduce aggregation through steric repulsion. SERS probes could be successfully produced using a number of different dyes fitting the above description: malachite green isothiocyanate, crystal violet, cresyl violet, ethyl violet, nile blue, methylene blue, and 3,3’-diethylthiadicarbocyanine. Rhodamine 6G and rhodamine B isothiocyanate could also be used to produce SERS probes, but the resultant spectra were less intense on average, likely due to the fact that their electronic transitions occur near 550 nm which is blue-shifted with respect to the probe laser wavelength(s).
4.3.2 Polyethylene Glycol Self-Assembly

Heterobifunctional polyethylene glycol (PEG) was used to coat nanoparticle surfaces in order to prevent non-specific association of particles with surfaces or solution species with particles, to sterically stabilize the particles and prevent their aggregation upon subjection to buffer salts, as well as to maintain fidelity of their SERS spectra by reducing any dissociation of dye molecules from the Au surface. PEG as a SERS nanoparticle surface coating has been reported to reduce non-specific association of particles with cells and tissues *in vitro* and *in vivo* [68]. Two different types of 5kDa molecular weight thiolated PEG molecules were used for the NP coating: one with a methoxy group on the distal end of the PEG for passivating the particle surface, and the other with a carboxylic acid functionality to facilitate conjugation to targeting proteins.

To coat the particles with thiolated PEG, ligand exchange was conducted whereby citrate capped particles were suspended in aqueous solutions of PEG-SH present in molar excess. The weakly associated citrate should exchange with PEG to form a stable gold-thiol dative bonds on the Au surface. Such Au-thiol dative bonds have been reported to have bond strengths on the order of 40-50 kcal/mol which is approaching Au-Au bond strengths [69,70]. Such ligand exchanges typically occur in two phases on planar surfaces: the first is a rapid, concentration and diffusion dependent Langmuir adsorption, and the second is slower and more complex to predict and is dependent upon inter-chain interactions, surface mobility of ligands, and chain disorder [71,72].

In polymer self-assembled monolayers, straight chain molecules can take on two different conformations depending on the density of molecules grater to the metal surface. The Flory radius ($R_F$) describes the volume taken up by the polymer for non-overlapping polymer molecules in a good solvent [73]:

$$R_F = \alpha n^{3/5}$$  \hspace{1cm} Equation 4.12

Where $\alpha$ is the length of one monomer unit (3.5Å for PEG), and $n$ is the number of repeats in the polymer chain (113 units for 5 kDa thiol-PEG). When the distance between molecules is greater than $R_F$, then PEG adopts a more diffuse mushroom configuration. As the PEG density increases and the space between adjacent molecules becomes shorter than $R_F$ the molecules take on a more extended and densely packed brush configuration on the surface, to accommodate the presence
of greater numbers of molecules [73,74]. This is of particular importance in insuring that carboxylate functionalities are exposed at the surface for bioconjugation where a more diffuse configuration could result in functional groups being buried in the PEG layer [74]. Additionally, tightly packed PEG ligand molecules deter adsorption of serum proteins to the surface of NPs when they are exposed to biological environments \textit{in vivo}.

![PEG Brush Configuration](image1)

\textbf{Figure 4.5:} Brush and mushroom configuration poly(ethylene glycol) molecules grafted on a gold surface. Molecular conformation is dependent on spacing between adjacent PEG chains.

Packing on Au NP spheres is also dependent upon particle diameter (and therefore surface curvature) as well as PEG chain length. Once bound to curved Au NPs surfaces, PEG ligands that are considered to coat the Au surface in a brush configuration are able to sample an area in space that is best described by an inverted hollow cone [75]. For example, spherical Au NPs of larger diameter, when coated in PEG-SH, were reported as being more resistant to degradation with sodium cyanide (NaCN) and flocculation with high salt conditions than smaller Au NPs coated with PEG of the same chain length. The PEG-SH molecules on smaller particles, while perhaps packed densely on the surface, have more space to sample on the outer surface and therefore do not shield the NP as well from the surrounding solution. The 60 nm Au NPs used in this thesis are considered large by the standards of this study further indicating that PEG-SH is a suitable ligand to insure their stability in solution [76].
4.3.3 Phospholipid Bilayer Encapsulation

Phospholipid bilayers were also investigated as a SERS Au NP coating in this thesis. The bilayer was comprised of three components: dioleoylphosphatidylcholine (DOPC), egg sphingomyelin (ESM) and cholesterol in a 2:2:1 molar ratio (DEC221). 2kDa PEG-1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (PEG-PE) was added to the bilayer mixture to produce “stealth liposomes” for biological targeting and this is discussed in more detail in Chapter 8.

DEC221 has been reported to form supported lipid bilayers (SLBs) from unilamellar vesicles that have ruptured on negatively charged, mercaptoundecanoic acid functionalized Au(111) [77] as well as on negatively charged mica surfaces [78]. The DEC221 SLBs on planar surfaces display two phases: liquid ordered (l_0) domains that are enriched in sphingomyelin intercalated with cholesterol, and liquid disordered (l_d) regions enriched in DOPC[79]. The l_0 regions mimic cell membrane lipid rafts and can serve as a model system for studying these structures. The zwitterionic head groups of DOPC and sphingomyelin are oriented toward the outer surface and supporting hydrophilic substrate, and the hydrophobic tails are sandwiched in the bilayer interior [79].

The NP coating process was conducted by sonicating polydispersed multilamellar vesicles (MLVs) in the presence of citrate-capped spherical 60 nm Au NPs in aqueous suspension. It was expected that the formation of SLBs on a negatively charged curved nanoparticle surface would mimic the situation of charged planar surfaces. Sonication mechanically disrupts the vesicles and exposed edges subsequently close in upon themselves to reduce unfavourable interactions of hydrophobic tails with the aqueous solution, eventually producing small unilamellar vesicles (SUVs) [80]. While unilamellar vesicles of DEC221 can require 20-30 minutes of sonication to obtain SUVs, sonication in the presence of 60 nm Au NPs appeared to decrease the time required for this transition as solutions clarified in approximately 5 minutes suggesting templated vesicle formation. Templated formation of SLBs formed by sonication of unilamellar vesicles with silica nanospheres has been reported elsewhere [81,82].

Detailed experimental procedures and characterization of the lipid bilayer encapsulated SERS Au NPs are provided in Chapter 5.
4.3.4 Bioconjugation of Targeting Antibodies to SERS Nanoparticles

Conjugation of nanoparticles to targeting proteins can be accomplished using a number of different chemistries, examples of the most widely used approaches include coupling sulfhydryl to maleimide, carboxyl to amine conjugation chemistry and “click” chemistry of alkyne to an azide [83]. Molecules with the required functionalities as well as kits including the necessary coupling catalysts are widely commercially available and therefore any protein crosslinking strategy popular for use in the biosciences can be readily adopted for linking proteins to nanoparticles.

The conjugation strategy chosen was the use of zero-length crosslinkers 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysulfo-succinimide (sulfo-NHS) to catalyze the cross-linking of carboxylic acid functionalities of PEG-coated SERS Au NPs to exposed amine groups on the antibody[84,84]. Carbodiimide bioconjugation chemistry is widely used and well understood for the attachment of proteins to optical contrast agents such as fluorophores and nanocrystals. This approach is not orientation-specific for the antibodies on the Au surface because bond formation can occur between COOH-PEG and both of the N-terminal amines or ε-amines of lysine residues. The integrity of the Ig binding site for each antibody may not be maintained, but it was found through several quantification methods that multiple antibodies were bound to each nanoparticle, on average. EDC adds to the PEG carboxylic acid through its diimide group, forming an unstable ortho-acylisourea intermediate. This activated intermediate can directly form a covalent amide bond with an available amine group on the antibody via nucleophilic attack to replace the acylisourea, but the intermediate is unstable and may hydrolyze before the cross-linking occurs yielding an unreactive isourea by-product. When sulfo-NHS is present in the reaction solution, it improves reaction yield by replacing the EDC intermediate to form a more stable ester with the PEG molecule that is not as rapidly hydrolyzed. This ester is subsequently replaced by an available amine on the antibody to form a covalent amide bond, as for EDC.

EDC and sulfo-NHS were added to the particle suspension in a 2:5 molar ratio based upon methods reported elsewhere [84]. Both EDC and sulfo-NHS form short-lived ester intermediates with the carboxylic acids on PEG that are reactive toward available amines. EDC esters have shorter half-lives than those formed with sulfo-NHS, therefore it has been found that the use of
both catalysts improves the reaction yield. Although EDC-mediated amide bond formation proceeds with highest yield at pH range 4.5-7.5, it is possible to conduct this reaction in water with lower yield and decreased reaction rate outside the optimal pH range. Both heat and increased pH reduce the half-life of NHS-ester intermediates thereby decreasing the probability that each ester will bind an antibody before hydrolyzing. Salt-free medium is advantageous for use with nanoparticles that may be susceptible to aggregation in high salt concentration in buffer solutions.

In addition to conducting the conjugation chemistry in water, two additional precautions were taken to stabilize the particles during this process. During the centrifugation washing step(s), the particles are particularly vulnerable to flocculation and to irreversibly coating their storage vessel, likely due to changes in surface charge upon formation of NHS-esters and the presence of conjugated proteins. To prevent this from occurring, centrifuge tubes that were destined for use with antibody-conjugated particles were incubated in 2% BSA suspension for at least one hour, and dried prior to use. Additionally, 2kDa PEG (without terminal thiol functionality) was added to the nanoparticle suspension to a final concentration of 2% w/v in PBS for each centrifugation cycle. The 2% PEG-PBS was replaced with PBS alone immediately prior to cell labelling experiments.

4.3.5 Monoclonal Antibodies

Monoclonal antibodies were chosen as the targeting moieties for SERS Au NPs in this thesis. Other targeting molecules have been used to direct inorganic nanoparticles to their biological targets including peptides, aptamers, DNA, antibody fragments, and affibodies. Antibodies have a number of advantages, most notably their high affinity for their target antigen which is on the order of $10^{-9}$ M [86] and are commercially available for a large variety of antigens. Monoclonal antibodies are produced to respond to a single epitope of the target antigen and therefore their binding specificity should be more reproducible between batches as compared to polyclonal antibodies which vary in their epitope response. IgG1 isotype antibodies were used to target SERS Au NPs and a schematic of the IgG structure is shown in Figure 4.6.
Figure 4.6: IgG molecule with major structural elements as labelled. C\textsubscript{H}1-3: heavy chain constant domains, 1-3; V\textsubscript{H}: heavy chain variable domains; C\textsubscript{L}: light chain constant domains; V\textsubscript{L}: light chain variable domains.

IgG is the most common form of soluble immunoglobulin, making up 75% of all serum Ig [86-88]. The immunoglobulin superfamily domain is the basic subunit of many different surface bound and soluble Igs produced by immune cells. Each domain is 12-13 kDa in molecular weight, and consists of β-sheet sandwiches of 110-130 amino acids held together by a disulfide bond between two conserved cysteine residues. The full IgG structure is a heterodimer of two heavy chains and two light chains. Each heavy chain with joining hinge region is approximately 55 kDa and possesses three constant domains and one variable domain (C\textsubscript{H}1, C\textsubscript{H}2, C\textsubscript{H}3, V\textsubscript{H}1). The heavy chain constant regions determine the effector function of the antibody \textit{in vivo} while the variable region contributes to the binding specificity of the molecule. The IgG subclasses: IgG1, IgG2, IgG3, and IgG4 can be differentiated from one another based on their differences in effector function that are variations in affinity of their heavy chains for different Fc receptors and for complement. Each light chain is approximately 25 kDa, and is comprised of one constant domain, and one variable domain (C\textsubscript{L}1, V\textsubscript{L}1). The light chain can be κ or λ and both chain types are functionally identical. Each of the heavy and light chain variable domains contain three
complementarity determining regions, also called hypervariable regions, on their N-terminal ends that combine to determine the antigen specificity of the antibody molecule. The two heterodimers are held together by a disulfide bonds in the hinge region between C\textsubscript{H}1 and C\textsubscript{H}2. IgG is bivalent, thereby creating an avidity contribution to its binding strength and enabling it to cross-link antigen in vivo for removal by phagocytic cells. The different immunoglobulin isotypes, IgA, IgE, IgM, IgD and IgG vary in the structure of their heavy chain which determines their effector function.

4.4 References


5 Phospholipid Membrane Encapsulation of Gold Nanoparticles to Impart Functionality as Surface-Enhanced Raman Scattering Labels

5.1 Permissions

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5.2 Overview

The present chapter describes a set of methods for preparing ternary lipid bilayer encapsulated SERS gold nanoparticles, using an aqueous process that enables the incorporation of three different classes of Raman-active dyes into the coating structure. The mixture of DOPC, sphingomyelin, and cholesterol in a 2:2:1 molar ratio is investigated as a cell membrane model system, and this combination has been reported to strengthen the lipid bilayer. Raman reporters that were physisorbed to the charged Au NP surface, were pre-conjugated to lipid molecule head-groups, and hydrophobic reporter molecules that either encapsulated with the particle, or partitioned from aqueous solution into the hydrophobic interior of the bilayer coating, all demonstrated detectable SERS spectra. The particles were characterized using UV-Vis absorbance spectra, dynamic light scattering, and TEM imaging. Their colloidal stability was evaluated both over extended time periods, as well as in harsh acidic, and high ionic strength suspension conditions.

5.3 Introduction

Metal nanoparticles offer promise for incorporation into diagnostic imaging schemes and as therapeutic materials [1,2], and this is discussed in more detail in Chapter 11. Not only can they serve as contrast agents for optical imaging, but also as substrates for enhanced spectroscopy in order to amplify the intensity of Raman scattering from endogenous biological structures in their
vicinity, or through functionalization of their surfaces with Raman reporter molecules to produce SERS labels for cell surface receptor detection.

Gold nanoparticles, as synthesized without surface modification, are not appropriate for use directly as in vitro or in vivo labels. For example, CTAB capping molecules, used to synthesize nanorods, have been demonstrated as having cytotoxic effects. Residual borohydride and citrate molecules from sodium borohydride and sodium citrate reduction of HAuCl₄ in the synthesis of Au nanospheres, respectively, are very loosely associated with the particle and can be exchanged for interactions with amine or thiol-contained residues of serum proteins added to cell culture, and/or complement proteins or antibodies in vivo. An additional surface coating is required to render the particles biocompatible, and control of surface chemistry and charge is necessary to direct their biodistribution and ensure colloidal stability [3]. Strongly bound, or encapsulating coating molecules must provide a means through which to embed Raman reporters, or prevent dissociation of reporters bound to the particle surface [4–7]. Additionally, coating molecules should include functional groups that can be used to attach targeting moieties [8–10]. Numerous methods have been developed to address these needs, including the example of chemisorbed polymers discussed in Chapters 6, 7, and 9 of this thesis.

The development of lipid bilayer encapsulated SERS-active Au nanoparticles is presented in this chapter. The bilayer coating enables inclusion of the aforementioned functional elements required for the preparation of SERS probes destined for biological labelling applications. The bilayer is an inherently biocompatible material, methods for surface engineering are established [11] and its in vivo behaviour is well understood due to the large body of literature on liposomal contrast agents and drug delivery vehicles [12,13]. Surface charge can be modulated through selection of lipid molecules for inclusion in the vesicle [14–16], and glycans, peptides, antibodies and folate have all been covalently bound to lipid molecules anchored in the vesicle, in order to direct the vesicles to biological structural targets [13,17,18]. Additionally, Au nanoparticles functionalized with zwitterionic surface ligands have shown excellent colloidal stability in harsh solution conditions, as well as reduced non-specific association with cell surfaces and adsorption of serum proteins [2,19–21]. Presumably, any of the strategies developed to incorporate these functionalities in to drug-delivery liposomes [22,23] can be adopted for use with lipid-coated metal nanoparticles for application as SERS probes, or as contrast or therapeutic agents for other clinical applications.
A number of alternative approaches for coating metal nanoparticles with lipid species have been investigated. Thiolated lipids have been selected for chemisorption to the metal surface through strong thiol dative bonds [14]; lipid monolayers have been assembled on particles with hydrophobic surfaces [24]; and nanowires [25] and pearl necklace structures [15] have been synthesized using mixtures of phospholipids and surfactants as capping agents. While the plasmonic NP surface has been exploited to study the structure of molecules intercalated in the lipid bilayer using SERS [26], optical contrast agents, including Raman-active molecules for SERS, were not incorporated in to the coating of any of the aforementioned structures. Furthermore, three different classes of Raman-active molecules have been incorporated in to the bilayer coating reported in this chapter.

The present study describes methods for an aqueous lipid encapsulation process, which is advantageous for the production of a SERS probe intended for in vitro or in vivo labelling. The incorporation of three different classes of Raman-active species was demonstrated: 1) L-tryptophan (Trp) that likely partitions in to the bilayer structure, is interesting as a Raman reporter because it is a natural amino acid and therefore inherently biocompatible. 2) Rhodamine lissamine covalently conjugated to DSPE (Rho-PE) molecules which can then be incorporated during the lipid encapsulation procedure. Additionally, this construct is potentially useful as a hybrid label with dual Raman/fluorescence activity. 3) Malachite green isothiocyanate (MG) that is physisorbed to the Au surface prior to encapsulation, represents a standard Raman dye that has been used frequently in the literature, and elsewhere in this thesis, to produce SERS nanoparticles. All three methods are variations on the same aqueous encapsulation method.

The lipid bilayer selected for SERS particle coating was comprised of three components: dioleoylphosphatidylcholine (DOPC), egg sphingomyelin, and cholesterol in a 2:2:1 molar ratio (DEC221). This ternary lipid mixture is also notable as a particle coating because this combination has been studied elsewhere for applications as a cell membrane model system due to its propensity to form liquid ordered and liquid disordered domains that mimic the lipid rafts [27–29]. In these studies, DEC221 bilayers were formed on planar anionic gold and mica surfaces from rupture of vesicles as they settled on to the surface. Presumably, the same concept should apply to the formation of supported lipid bilayers on the surfaces of citrate-capped, anionic spherical gold nanoparticles.
The presence of the bilayer coating was interrogated indirectly as changes in hydrodynamic radius using dynamic light scattering (DLS), shifts in the particles’ LSPR upon coating using UV-Vis absorbance measurements, and directly through transmission electron microscopy (TEM) imaging. SERS activity of the particles for the case of both the Trp and Rhod-PE functionalized probes also indirectly confirmed the presence of the bilayer because both of these reporters are incorporated into the lipid bilayer structure. Additionally, encapsulated SERS particles demonstrated superior stability in ionic, and acidic conditions as compared to uncoated citrate-capped Au NPs, also indicative of the presence of a stabilizing coating on the particles.

5.4 Methods and Materials

5.4.1 Gold nanoparticles

Citrate coated Au nanoparticles were purchased from Ted Pella Inc. (Redding, CA, USA). According to the manufacturer, the particles are nominally 60 nm in diameter. To ensure that dye-association and lipid encapsulation procedures were not batch dependent, methods were developed using particles from multiple Au NP batches.

5.4.2 Lipid Preparation

Dioleoylphosphatidylcholine (DOPC), egg-sphingomyelin (ESM), and ovine cholesterol (Chol) (Avanti Polar Lipids, Alabaster, Al, USA) were combined to a 2:2:1 molar ratio to a final mass of 10.7 mg, in a 3:1 chloroform:methanol solution as the solvent as described elsewhere [30]. The lipid solution was divided into 1 mg aliquots and dried under a stream of Ar gas until the solvent had visibly dried (approximately 1 hour). The lipid films were stored overnight under vacuum to ensure removal of residual solvent. For longer-term storage, the vacuum chamber was back-filled with Ar gas, and dried lipid aliquots sealed and stored at -20°C.

To prepare lipid aliquots for Au NP encapsulation, MilliQ water was added to a final concentration of DEC221 in solution of 1 mg/mL, and aliquots were thawed in a circulating
water bath at 50°C for 30 minutes and briefly vortexed at 10 minute intervals to form multilamellar vesicles (MLVs) in suspension.

5.4.3 Lipid Encapsulation of Gold nanoparticles

Au NPs were mixed in a 1:1 volume ratio with DEC221 and sonicated in the presence of the MLV suspension for 45-60 minutes at 50°C throughout. For preparation of unilamellar vesicles from DEC221 MLVs, the suspension is sonicated under these conditions until it clarifies, indicating that that ULVs have been produced with diameters smaller than the diffraction limit of visible light (sonication typically produces ULVs < 100 nm in diameter) [31,32]. To determine whether the bilayer encapsulation of Au NPs had occurred during the preparation process, a control suspension of DEC221, prepared without Au NPs, was sonicated in tandem to act as a guide and the Au NP suspension was sonicated until the control suspension had clarified. It was observed however, that this clarifying process occurred more quickly in the presence of the particles. Modifications were made to this general encapsulation process in order to include Raman-active dyes in to the coating structure. These methods have been summarized in schematic format in Figure 5.1 and will be discussed below. To remove excess lipids from suspension, two centrifugation steps at 4500 RPM for 5 minutes each were used. The particles settled down to the bottom of the tube, the supernatant was decanted and particles resuspended in 18.2 MΩ·cm water. Encapsulated SERS Au NP suspensions were subsequently sealed and stored at 4°C until later characterization.
Figure 5.1: Schematic representation of three different methods for including Raman reporter molecules into the structure of lipid encapsulated SERS Au NPs. The Au NP is depicted by the solid yellow circle, the blue rings represent lipid layers, and the location of the dye molecules is indicated by small red hexagons. Components of the Au NP coating are not shown to scale. (From: Ip et al. *Langmuir* 2011, 27, 7024).

5.4.3.1 Preparation of Trp-labelled SERS Au nanoparticles

Because hydrophobic L-tryptophan was expected to be soluble in the MLV suspension, dessicated DEC221 aliquots were hydrated as described above with the exception that a 1mM tryptophan solution was used in place of pure 18.2 M·Ω water. The control vesicle solution for guiding sonication times was aliquoted from this MLV-Trp suspension.
5.4.3.2 Preparation of Rho-PE Labelled SERS Au Nanoparticles

A commercially available lipid molecule that had a fluorescent dye, rhodamine lissamine, covalently conjugated to the head-group portion of the molecule was used to functionalize the NPs with a Raman reporter, and conduct the encapsulation in a single step. The lipid chosen was Rhodamine-Lissamine-Phosphatidylethanolamine (Avanti Polar Lipids), and was used as received in a chloroform solution. Aliquots of Rho-PE were prepared as described for DEC221. Portions of Rho-PE that were equivalent to 1 mol% of each DEC221 aliquot were placed in glass vials, the solvent was dried under a stream of Ar gas and by vacuum, and stored at -20°C until further use. To incorporate Rho-PE with DEC221, five aliquots of Rho-PE were dissolved in 3:1 chloroform:methanol and mixed with one aliquot of DEC221. This mixture was dried as described for DEC221 above. During the preparation of Rho-PE aliquots and SERS Au NPs, the material was protected from light as much as was practical by wrapping vials in aluminum foil, and reducing ambient room light.

Three variations on the Rho-SERS Au NPs were prepared which differed at the MLV preparation stage. MLVs were suspended in either 18.2 M·Ω water, 10 mM NaCl solution, or 2 mM CaCl₂ solution. Both Au NP containing- and control vesicle solutions were sonicated in tandem as described above until suspensions had clarified. Excess lipid were removed via two rounds of centrifugation, resuspended in 18.2 M·Ω water, sealed and stored at 4°C until later use.

5.4.3.3 Preparation of MG-Labelled SERS Au Nanoparticles

A Raman reporter capable of physisorbing directly to the Au NP surface was also used to impart SERS functionality to the lipid encapsulated particles. As described in chapters 4, 6, 7, and 9, an aqueous solution of malachite green isothiocyanate was added to Au colloid and stirred for 10 min prior to lipid encapsulation, to facilitate the adsorption of the positively charged ionic dye to the negatively charged, citrate-capped 60 nm Au (forming MGITC-AuNPs). Equal parts DEC221-MLV and MGITC-Au NPs were mixed, while retaining a quantity of MUV suspension in a separate vial as a control. The nanoparticle/MUV suspension and the retained MUV suspension were sonicated in a bath sonicator for 45-60 min at 50 °C, or until the MUV
suspension without particles became clear, signifying the formation of small unilamellar vesicles. The vesicles and lipid coated particles were stored at 4 ºC until use.

5.4.4  Raman Spectroscopy

An inverted microscope (Nikon TE2000) was used to focus the CW 632.8 nm HeNe (15mW) laser beam onto the sample, in episcopic configuration. The laser beam was collimated before entrance into the optics of the objective (S Plan Fluor ELWD 40X, NA 0.6). The Rayleigh scattering from the sample at the wavelength of the laser line is blocked from entering the monochromator by a notch filter (λ > 645nm). An achromatic doublet lens (f/6.6) focuses the Raman scattered light onto the monochromator slit. The Acton SP2560 Czerny-Turner monochromator (f 6.5) had a triple grating turret (1200g/mm, 750 blaze wavelength used to collect displayed spectra). The monochromator was connected to a Princeton Instruments PIXIS BR 400 CCD detector with a 1340x400 pixel array that was Peletier cooled to 75 ºC below room temperature.

5.4.5  UV-Vis Absorbance Spectroscopy

Stock Au particles, and encapsulated SERS Au particles were placed in a 1 cm-path-length cuvette. A Cary 5000 UV-Vis spectrometer (Varian Inc. Palo Alto, CA, USA) was used to collect the absorbance spectra of the particles using 18.2 MΩ·cm water as a reference. To more directly compare maximum absorbance wavelengths between samples, spectra were normalized by shifting the baseline to zero, and scaling the data linearly so that the maximum value was 1.

5.4.6  Transmission Electron Microscopy

Five µL drops of aqueous Au NP suspension from each particle sample respectively were placed on carbon-coated copper grids, excess liquid was wicked away, and samples were allowed to air dry. Images were collected using a Hitachi H-7000 TEM operated with 75 kV accelerating voltage. A series of TEM images were analyzed to determine an average bilayer thickness
surrounding the functionalized Au NPs based on five measurements made for each NP. Analysis was conducted using ImageJ software, and length scale calibrated against the scale bar of the raw TEM images.

5.5 Results and Discussion

5.5.1 Characterization of Lipid-Encapsulated SERS Gold Nanoparticles

5.5.1.1 Trp-Labelled SERS Gold Nanoparticles

The exact location and configuration of Trp in the encapsulated SERS NP structure has not been studied and will not be discussed in this chapter. It may have co-encapsulated with the nanoparticle as hydration layers are reported to exist between planar SLBs and their substrates. However, due to its partition coefficient, the possibility that Trp partitioned into the lipid bilayer during MVL suspension cannot be discounted [33]. Additionally, when tryptophan is incorporated into membrane proteins, it has been found elsewhere to localize at the lipid-water interface [34]. Figure 5.2B shows a representative TEM image of Trp-SERS Au NPs and the lipid layer is visible as a light grey corona surrounding the dense, dark 60 nm Au NP.

Encapsulation of particles by DEC221 could be indirectly confirmed using a 3.6 nm red-shift in the particles’ visible absorbance spectrum from 535nm to 538.6nm, indicating a change in the dielectric material immediately surrounding the particle (Figure 5.2C). This observation is consistent with a red-shift observed following PEG-coating of Au NPs as reported in Chapters 6, 7, and 9. The SERS spectrum of Trp-labelled SERS Au NPs is provided in Figure 5.2D and bands observed were comparable to the SERS spectrum of Trp reported elsewhere [27] and listed in Table 5.1. Though the spectral intensity of Trp-SERS Au NPs was lower than for the other two lipid encapsulated SERS probes described herein, a solution-phase Raman spectrum of a tryptophan solution, at the same concentration at which it was added to the lipid suspension, was not detectable using the same laser power and integration time. This suggests that the observed spectrum is indicative of a significant enhancement of the Raman signal, and confirms that Trp was indeed incorporated into the coating structure.
Figure 5.2: Characterization of DEC221 encapsulated SERS Au nanoparticles with L-tryptophan as the Raman reporter molecule. A) Molecular structure of L-tryptophan. B) TEM image of encapsulated Trp-SERS Au NP. C) Visible absorbance spectra of stock, citrate-capped 60 nm Au NPs (black), and encapsulated Trp-SERS Au NPs (grey). D) SERS spectrum of encapsulated Trp-SERS Au NPs. Bands observed are consistent with reports published elsewhere [27,35] and provided in Table 5.1.

<table>
<thead>
<tr>
<th>Band (cm⁻¹)</th>
<th>Chemical Group</th>
<th>Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>1188†</td>
<td>Xanthene; C-H</td>
<td>deformation; bending</td>
</tr>
<tr>
<td>1261-1269*†</td>
<td>Xanthene</td>
<td>ring breathing</td>
</tr>
<tr>
<td>1345*†</td>
<td>Xanthene</td>
<td>ring stretching</td>
</tr>
<tr>
<td>1428</td>
<td>not assigned</td>
<td>not assigned</td>
</tr>
<tr>
<td>1505/1513*</td>
<td>Xanthene; C-N</td>
<td>ring stretching; stretch</td>
</tr>
<tr>
<td>1581†</td>
<td>Xanthene</td>
<td>ring stretching</td>
</tr>
<tr>
<td>1650</td>
<td>Xanthene; C-H</td>
<td>ring stretching; rocking</td>
</tr>
</tbody>
</table>
Table 5.1: SERS band assignments for Trp-labelled SERS Au NPs based on Chuang et al. [27]. The “band” column indicates the wave-number position of the observed peak, and bands that are further than 5 cm\(^{-1}\) away from the peak published in [27] are marked with an asterisk (*). “Chemical group” and “mode” correspond to assignments made by Chuang et al. [27] for the chemical group and type of vibrational mode for tryptophan.

5.5.1.2 Rho-PE-Labelled SERS Au Nanoparticles

An alternative dye incorporation strategy was investigated whereby lipid molecules that were covalently conjugated to a Raman reporter molecule were incorporated in to the DEC221 encapsulating layer at 5 mol\% of the total lipid mixture. This strategy facilitated simultaneous encapsulation and dye functionalization of the particles. Because dissolved lipids and vesicles can only be isolated from suspension at much higher centrifugation speeds than required for settling nanoparticles [36], it is concluded that the majority of dye molecules remaining in suspension after multiple washing steps are associated with the Au NPs, and the presence of a SERS spectrum in the finished product would be an indirect confirmation that the particles were indeed encapsulated. The structure of the rhodamine-lissamine molecule is provided in Figure 5.3A. Rhodamine-lissamine was chosen as the reporter molecule because rhodamine dyes have been shown to have large Raman cross-sections elsewhere [37–39], and the selection of a head-group labelled PE molecule ensured that the Raman reporter would be situated as close to the plasmonic NP surface as possible.

Inclusion of Rho-PE lipid molecules in to the encapsulation mixture provided an interesting avenue through which to study the impact of electrostatic charge interactions on the formation of SLBs including molecules with negatively-charged head-groups on a supporting substrate of like charge. The majority of lipids used in the NP coating are zwitterionic, however both rhodamine-lissamine head-groups and the as-purchased, citrate-coated particles have a negative charge. The findings of the present study are consistent with observations reported elsewhere [40–43]. Therefore, as discussed in Chapter 5.4.3.2, three different strategies were used to prepare Rho-PEG-labelled SERS Au NPs, that all varied in the suspension medium used to prepare Rho-PE/DEC221 MLVs. By replacement of water with ionic solutions, it was expected that the solution counterions would screen the interaction between the lipid head-group and NP charges.
Encapsulation in 10 mM NaCl will be discussed first and characterization data is provided in Figure 5.3. The rhodamine-lissamine-PE molecular structure is shown in Figure 5.3A. As for Trp-SERS NPs, the encapsulating lipid layer appears as a light grey corona in the representative TEM image in Figure 5.3B. The SERS spectrum of Rho-PE SERS Au NPs encapsulated using NaCl is provided in Figure 5.3C and peak assignments based on literature reports [44–47] are shown in Table 5.2.

A 3 nm red-shift of the nanoparticles’ LSPR wavelength was observed following encapsulation in Rho-PE/DEC221 as compared to the stock Au NPs (Figure 5.3D). This is similar in magnitude to the shift observed for both Trp- and MG- SERS Au NPs. The visible absorbance of Rho-PE SERS NPs in the presence of NaCl varied from the other lipid-encapsulated particle types however. An additional peak was observed, red-shifted with respect to the particles’ LSPR, that corresponds well to the visible absorbance of Rho-PE-containing lipid vesicles. While salt solutions can be used to aggregate nanoparticles and the presence of clusters is manifested as a double-peaked LSPR, the aggregate absorbance is significantly broader and further red-shifted from what was observed in the present study [48–50].

The centroids of log-normal fits to DLS histograms (Figure 5.3E) indicate an increase of the particles’ hydrodynamic radius from 29.8 ± 0.1 nm for stock Au NPs, to 39.9 ± 0.4 nm for Rho-PE labelled SERS Au NPs. The DLS data suggests that a larger bilayer formed around the Rho-PE SERS NPs than for MG-SERS NPs (reported below), and there are three possible explanations for this observation which will each be discussed in turn.

The structure of the lipid bilayer has been reported to depend upon the constituent molecules used [51–53]. Changes in bilayer structure would be manifested as altered rigidity and thickness, the later detected using DLS. It is feasible that inclusion of a fourth lipid species, Rho-PE, into the DEC221 bilayer could have modified the structure of the bilayer with its negatively-charged head group and saturated tail groups.

A second explanation for the apparent size increase of the encapsulating vesicle is the occurrence of osmotic swelling. The particles were encapsulated in a NaCl solution, washed, and stored in 18.2 MΩ·cm, therefore a difference in osmolarity was created between the solutions inside and outside the lipid vesicles, resulting in osmotic swelling of the encapsulation layer [54,55]. A broader size distribution was also observed for Rho-PE labelled SERS NPs prepared in NaCl
than for MG-labelled SERS NPs, and this can also be accounted for by osmotic swelling. It has been reported elsewhere that this effect is greater for larger vesicles than for smaller ones [54].

Thirdly, the ionic strength of the suspension medium used for DLS can influence the value determined for hydrodynamic radius. Unlike the other types of lipid-encapsulated SERS Au NPs described in this chapter that incorporate only zwitterionic phospholipids, the Rho-PE head-groups are negatively charged. Repulsive electrostatic interactions between NPs in suspension can impede their Brownian motion resulting in reduction of the measured diffusion coefficient by as much as 10% [56]. This effect is exacerbated by conducting the measurements in salt-free media to the extent that each decade of NaCl concentration decrease can increase the apparent hydrodynamic radius of the particles by 5 nm [57].
Figure 5.3: Characterization of DEC221 encapsulated SERS Au nanoparticles with Rho-PE as the Raman reporter molecule, prepared in Na\(^+\) containing ionic solution. A) The molecular structure of rhodamine-lissamine DSPE. B) SERS spectrum of Rho-PE labelled SERS NPs prepared using Na\(^+\) solution. The SERS spectrum is recognizable as that of rhodamine, with peak assignments provided in Table 5.2. C) Visible absorbance spectra of stock, citrate-capped 60 nm Au NPs (grey), Rho-PE SERS NPs (black), and Rho-PE containing vesicles (grey, dashed). D) DLS hydrodynamic radii histograms of stock Au NPs (grey, hatched bars) and Rho-PE labelled SERS Au NPs (black, open bars) with log-normal centroids of \(R_h = 29.8 \pm 0.1\) nm and \(39.9 \pm 0.4\) nm for stock particles and Rho-PE SERS particles respectively.

<table>
<thead>
<tr>
<th>Band (cm(^{-1}))</th>
<th>Chemical Group</th>
<th>Mode</th>
</tr>
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<td>1188(^\dagger)</td>
<td>Xanthene; C-H</td>
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<tr>
<td>1650</td>
<td>Xanthene; C-H</td>
<td>ring stretching; rocking</td>
</tr>
</tbody>
</table>

Table 5.2: SERS band assignments for Rho-PE labelled SERS Au NPs based on both Zhang et al. [46] (Ag SERS/Rhodamine B) and Jensen et al. [47] (Normal/Resonant Raman/Calculations on Rhodamine 6G). The “band” column indicates the wave-number position of the observed peak, and bands that correlate to, but are further than 5 cm\(^{-1}\) away from the peak published in [47] are marked with (*), while bands that correlate with, but are further than 5 cm\(^{-1}\) away from the peak published in [46] are marked with (†). “Chemical group” and “mode” columns indicate the chemical group and type of
vibrational mode assigned by Zhang et al. [46] and Lueck et al. [58]. Multiple assignments to a single band are separated by semicolons. Parentheses indicate location of the chemical group within the molecule.

As discussion in Chapter 5.4.3.2, two additional strategies were used to encapsulate Au NPs in Rho-PE/DEC221: the encapsulation procedure was also conducted in divalent Ca$^{2+}$ solution, and in the absence of salts (18.2 MΩ·cm water). Though a systematic characterization of the effects of various ions, charged lipid species, and particle surface charges on encapsulation is beyond the scope of this chapter, variations in the optical properties of the particles were observed, dependent upon the choice of cation present in solution.

When 18.2M·Ω water was used as the encapsulation suspension medium, Raman scattering spectra of the finished Rho-PE labelled particles did not contain any detectable Raman bands (Figure 5.4A). Additionally, the spectral baseline was shaped as an exponential decay, indicative of the presence of fluorescent species. As discussion in the methods section above, excess lipids and vesicles that were not incorporated in to the particle structure were easily removed by centrifugation due to differential sedimentation rates, therefore the fluorescent rhodamine-lissamine molecules must have been present at a distance from the Au surface whereby fluorescence could not be fully quenched nor Raman spectra enhanced. The visible absorbance spectrum of the Rho-PE sample encapsulated in water suspension exhibited a small red-shift in the particles’ LSPR consistent with observations for other particle preparations herein (534 to 536 nm), however there was no shoulder observed that would indicate the presence of a rhodamine species (Figure 5.4B). Electrostatic repulsion may have prevented a large population of Rho-PE molecules from approaching the negatively charged Au surface and being incorporated in the encapsulating bilayer structure. The large fluorescence quantum yield of rhodamine would explain the collection of fluorescence in the Raman spectrum despite the fact that only a small population of Rho-PE molecules may have been incorporated.

Divalent Ca$^{2+}$ was evaluated as a counter-ion for Au NP encapsulation as it has been known to promote vesicle fusion of zwitterionic and anionic lipid bilayer vesicles on to negatively-charged planar surfaces [28,30,40], and to bridge like charges between negatively-charged lipid head groups and surfaces [42]. Additionally, the charge shielding effects that were observed upon encapsulation in Na$^+$ solution should occur in the presence of Ca$^{2+}$. When encapsulation was
performed in 2 mM Ca\(^{2+}\) solution, intense SERS bands were observed in the spectra of finished particles (Figure 5.4C), consistent with the locations of rhodamine spectral bands reported elsewhere and are summarized in Table 5.2. Additionally, as compared to Rho-PE labelled SERS NPs encapsulated in Na\(^+\) solution, the SERS peak intensity relative to the slope of the fluorescence baseline was greater, which may indicate that a larger population of Rho-PE molecules were incorporated in to the finished particles in positions relative to the NP surface at which Raman enhancement could occur. The visible absorbance spectrum of Ca\(^{2+}\)-Rho-PE encapsulated SERS NPs contained a double-peaked absorbance, as opposed to the shoulder observed in the Na\(^+\)-Rho-PE particle spectrum (Figure 5.4D). The 536 nm peak corresponds to the LSPR of the lipid-encapsulated 60 nm Au NPs, while the second 576 nm peak coincides with the absorbance of Rho-PE in the vesicle coating. The fact that the 576 nm peak is more intense relative to the particles’ LSPR for the Ca\(^{2+}\) samples as compared to the Na\(^+\) sample is additional evidence that a greater number of Rho-PE molecules are being incorporated in to the bilayer coating when the divalent cation is used.

Figure 5.4: Characterization of Rho-PE labelled SERS Au nanoparticles encapsulated in the presence of water (A and B), and Ca\(^{2+}\) ions (C and D). A) and C) Raman spectra of Rho-PE labelled SERS Au NPs after removal of excess lipids and vesicles. B) UV-Vis absorbance spectra of stock 60 nm Au NPs (black), and water encapsulated Rho-PE SERS Au NPs (grey). D) UV-Vis absorbance spectra of stock 60 nm Au NPs (grey), Ca\(^{2+}\) encapsulated Rho-PE SERS Au NPs (black), and Rho-PE vesicles (grey, dashed).
Upon comparison of the three strategies for preparing Rho-PE SERS Au NPs, the contribution of rhodamine to the optical properties of encapsulated particles is more pronounced in the presence of salt, and varies between the monovalent and divalent cations. For colloids stabilized by repulsive electrostatic interactions, as for citrate-capped Au NPs, suspension media that screen these repulsive charges can cause the particles to flocculate, which would be manifested as a double-peaked and/or red-shifted LSPR due to overlapping plasmons between adjacent particles. The second red-shifted peak in the Na\(^+\) and Ca\(^{2+}\) encapsulated particle absorbance spectra is not consistent with what would be expected for the presence of aggregates. Dimers and trimers of spherical Au NPs exhibit LSPRs that are red-shifted by approximately 100 nm \([48–50]\).

Typically, these shifted resonance peaks are very broad due to the presence of multiple aggregate species of different sizes and conformations, and the peak of the aggregate absorption band is located around 650 nm \([48–50]\). In the UV-Vis spectra of Rho-PE labelled SERS Au NPs, the second absorbance band is centered at 576 nm, which is not consistent with a 100 nm red-shift expected for aggregated particles. Additionally, the red-shifted Rho-PE SERS NP peak is relatively narrow compared to what would be expected for the contribution of aggregates to the LSPR absorbance. Finally, absorbance spectra for rhodamine species reported elsewhere \([59,60]\) indicate a visible absorbance consistent with spectra observed herein upon incorporation of Rho-PE in to the particle coating (Figures 5.4D and 5.5D).

The variation of Rho-PE SERS NP optical properties as a function of encapsulation suspension may also be indicative of the existence of repulsive electrostatic interactions between the negatively-charged Rho-PE molecules and the anionic Au NPs surface. When encapsulation is conducted in 18.2 M\(\Omega\)-cm water, this repulsion may have prevented Rho-PE from being incorporated into the bilayer to the same extent as for the ionic solution preparations. Additionally, because some fluorescence was detected from the water encapsulated samples, a small population of Rho-PE molecules may have segregated on the outer leaflet placing rhodamine lissamine too far from the particle surface for significant Raman enhancement or fluorescence quenching to occur. When Na\(^+\) is present in the encapsulation suspension medium, the monovalent counterions shield the repulsive electrostatic interactions allowing Rho-PE to partition into the inner leaflet of the bilayer for fluorescence quenching and Raman enhancement which is consistent with the SERS spectrum of the finished particles shown in Figure 5.4C. The red-shifted shoulder of the particles’ LSPR also indicates that this charge screening may have
allowed a greater number of Rho-PE molecules to approach the Au NP surface and be included in the particle coating. When Rho-PE labelled SERS NPs are prepared in Ca\(^{2+}\) solution, even more Rho-PE molecules are included in the bilayer as evidenced by the particles’ double-peaked visible absorbance spectrum (Figure 5.4D). This is consistent with reports elsewhere whereby divalent Ca\(^{2+}\) serves as a bridge between lipid head-groups and charged planar surfaces, encouraging the formation of planar SLBs, and that charged lipids preferentially segregate between lipid bilayer leaflets to reduce electrostatic interactions with a charged surface [40–43].

Based on the observed progression in the intensity of the Rho-PE absorbance peak at 576 nm, it is concluded that the total amount of Rho-PE molecules in the Au NP coating structure is increased when Ca\(^{2+}\) is used as the counterion [59] as compared to when Na\(^{+}\) is used, or encapsulation occurs in the absence of ions. Additionally, it is concluded that the total amount of Rho-PE located in the inner bilayer leaflet upon encapsulation in Ca\(^{2+}\) is greater than when either Na\(^{+}\) or 18.2 MΩ·cm are used.

### 5.5.1.3 MG-labelled SERS Au Nanoparticles

In addition to using bilayer-associated Raman reporter molecules to impart SERS functionality to the particles, a positively-charged dye (Figure 5.5A), malachite green isothiocyanate, was physisorbed to the negatively-charged Au NP surface prior to encapsulation with DEC221. Particle encapsulation was confirmed directly by TEM imaging. A representative image provided in Figure 5.5B shows the lipid coating as a light grey corona surrounding the 60 nm Au NP. The average thickness of this layer was determined to be 4.8 ± 0.5 nm by measurements of TEM images, and this is consistent with the size increase expected upon bilayer formation [16]. The hydrodynamic radius of the MG-SERS NPs was used to indirectly confirm bilayer encapsulation, and was observed to increase from 29.8 ± 0.1 nm for uncoated stock Au NPs to 34.1 ± 0.5 nm for the encapsulated SERS particles (Figure 5.5C).

Visible absorbance spectra (Figure 5.5D) were also indicative of Au NP encapsulation as a 2 nm red-shift of the particles’ LSPR wavelength from 534 to 536 nm would be explained by a change in the dielectric material immediately surrounding the particles, and similar observations are described for polymer-coated particles in Chapters 6, 7, and 9 of this thesis. The SERS spectrum of the final MG-SERS NPs is shown in Figure 5.5E, and band locations (Table 5.3) are consistent with those reported for MG.
SERS elsewhere [4,44,58]. The intensity of the SERS spectrum did not decrease over time following encapsulation, therefore it is concluded that the physisorbed dye was associated with the particle surface following sonication.
Figure 5.5: Characterization of DEC221 encapsulated SERS Au nanoparticles with malachite green isothiocyanate as the Raman reporter molecule. A) The molecular structure of malachite green isothiocyanate. B) DLS hydrodynamic radii histograms of stock 60 nm spherical Au NPs (grey, hatched bars) and MG-labelled SERS Au NPs (black, open bars). C) UV-Vis absorbance spectra of stock Au NPs (black), and MG-labelled SERS Au NPs (grey). Inset: plot showing full LSPR band for each sample. D) SERS spectrum of MG-labelled SERS Au NPs showing strong SERS spectrum recognizable as that of MGITC. Bands assigned in Table 5.3.

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<td>530</td>
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<td>ring deformation (in plane)</td>
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<td>Wagging</td>
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<tr>
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<tr>
<td>1364</td>
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</tr>
<tr>
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<td>stretch; stretch</td>
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</tbody>
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Table 5.3: SERS band assignments for MG-labelled SERS Au NPs based on Lueck et al [58]. The “band” column indicates the wave-number position of the observed peak, and bands that correlate to, but are further than 5 cm\(^{-1}\) away from, the peak published in [58] are marked with an asterisk (*). “Chemical group” and “mode” correspond to assignments made by Lueck et al. [58] for the chemical group and type of vibrational mode for malachite green. Where multiple assignments are made to a single band, these are separated by semicolons.
5.5.2 Colloidal Stability of Lipid Encapsulated SERS Gold Nanoparticles

As a metric for evaluating the stability of DEC221 encapsulated SERS Au NPs in long-term storage, Raman spectra of particle suspensions were monitored at multiple time-points post-production. It was concluded that degradation of the particle coating would result in the dissociation of Raman reporter molecules from their location with respect to particle surfaces, and this would be manifested as a decrease in spectral intensity. Additionally, precipitation of SERS particles from suspension would also cause a decrease in spectral intensity. Encapsulated SERS particles were suspended in 18.2 MΩ·cm water and stored at 4°C. Spectral intensity of MG-labelled particles was evaluated after 12 and 25 days of storage (Figure 5.6A), while Rho-PE-labelled particles that had been encapsulated using Ca²⁺ solution were evaluated over the course of 7 days (Figure 5.6B). Both types of particles were found to be stable in suspension over the time periods evaluated. Stability over longer time periods has not yet been tested.
Figure 5.6: Raman spectra of lipid encapsulated SERS Au NPs for evaluation of their stability in suspension over time. Spectra are offset for clarity, and collection time points for each spectrum indicated at the left. A) Spectra from MG-labelled SERS Au NPs, B) Rho-PE-labelled SERS Au NPs.

Harsh solution environments were also used to evaluate the stability of the lipid bilayer encapsulated SERS particles. Three particle suspensions were tested: control ULV suspension, stock citrate-capped Au NPs, and lipid encapsulated Au NPs. Particle concentrations were controlled to be nominally identical between the three samples. Additionally, in order to monitor the stability of the ULV control suspension, vesicles were prepared with a fluorescent nitrobenzoxadiazol (NBD)-tail-group-labelled phosphocholine lipid molecule at 0.2 mol% of the DEC221 mixture and this same suspension was also used to encapsulated Au NPs, for consistency. Because the fluorescent functionality was conjugated to the lipid tail group, it was presumed that the presence of the fluorophore would not disrupt bilayer formation during Au NP encapsulation.

Five solution conditions were tested: 5 v/v% acetic acid, 10 mM CaCl$_2$, 10 mM NaCl, and PBS (50 mM monovalent salts). The results of the colloidal stability tests are summarized in Table 5.4.

Under acid solution conditions, stock Au NPs were destabilized through protonation of their citrate surface molecules, thus eliminating the stabilizing effect of electrostatic repulsion that maintains the particle suspension at more neutral pH. This was observed as a loss of colour in the colloidal suspension. Flocculation of the lipid bilayer encapsulated Au NPs was not observed, as the solution maintained its pink colour throughout the test. This is additional indirect evidence for the presence of the encapsulating bilayer, and suggests that the encapsulated particles were not stabilized by electrostatic interaction alone. Steric interactions likely also play a role in lipid encapsulated Au NP colloidal stability, as do transient dipole interactions between the zwitterionic coating molecules on adjacent particles.

When each particle sample was suspended in 10 mM Na$^+$, or PBS, no significant effect on the stability of any of the particle samples was observed.
Divalent 10 mM Ca\textsuperscript{2+} solutions exerted a more pronounced effect on particle stability than either of the monovalent solutions. Because divalent cations are capable of bridging lipid head-groups, resulting in vesicle fusion \[28,30,40], and inducing phase segregation, it was expected that their presence would destabilize the bilayers of both NBD-labelled vesicles, and lipid-encapsulated Au NPs. This is what was observed through decrease in colour and/or clarity of both suspensions. Additionally, the 10 mM Ca\textsuperscript{2+} solution effectively screened repulsive electrostatic charges on the surfaces of the stock Au NPs, thus resulting in their flocculation and loss of colour from the particle suspension. Though both encapsulated and unencapsulated Au NPs exhibited a loss of stability due to charge screening, the induced attractive charges between zwitterionic lipid head groups and Ca\textsuperscript{2+} are transient, and so may not have had as strong an attractive interaction as Ca\textsuperscript{2+} with negatively charged citrate capping molecules.

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<th>Control 2: Lipid only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment a) Water</td>
<td>Clear, pink solution</td>
<td>Clear, pink solution</td>
<td>Greenish-yellow (NBD), faintly cloudy</td>
</tr>
<tr>
<td>Treatment b) Acetic Acid</td>
<td>Clear, pink solution</td>
<td>Clear, Colourless</td>
<td>Less colourful</td>
</tr>
<tr>
<td>Treatment c) CaCl\textsubscript{2}</td>
<td>Paler pink</td>
<td>Clear, Colourless</td>
<td>Greenish-yellow, faintly cloudy</td>
</tr>
<tr>
<td>Treatment d) NaCl</td>
<td>Clear, pink solution</td>
<td>Clear, pink solution</td>
<td>Greenish-yellow, faintly cloudy</td>
</tr>
<tr>
<td>Treatment e) PBS</td>
<td>Clear, pink solution</td>
<td>Clear, pink solution</td>
<td>Greenish-yellow, faintly cloudy</td>
</tr>
</tbody>
</table>

Table 5.4: Results of colloidal stability test. Table columns indicate particle samples tested, and rows indicate each of the treatments to which colloidal suspensions were subjected. Stability was evaluated based on changes in suspension colour and clarity over time, as indicated in the table.

5.6 Conclusions

DEC221 was successfully demonstrated for use as a SERS gold nanoparticle coating. An aqueous method for nanoparticle bilayer encapsulation was presented. Additionally, three variations on this method could be made in order to impart SERS functionality to the particle by
including three different classes of Raman reporter molecules. Two types of Raman reporters were associated with the bilayer coating: hydrophobic L-tryptophan partitioned in to the bilayer from aqueous solution, and rhodamine-lissamine-labelled DPPE lipid molecules intercalated in to the DEC221 bilayer structure. The Rho-PE-labelled SERS Au NPs also served as an interesting platform to investigate charge-dependent bilayer incorporation, and leaflet partitioning of lipid molecules with charged head-groups in the presence and absence of ions. A third class of Raman reporter was found to be compatible with lipid bilayer coating: positively charged malachite green isothiocyanate that physisorbed directly to the negatively charged Au NP surface. The physisorbed dye continued to associate with the NP surface following sonication, as observed by intense MG SERS spectra from the particles before and after encapsulation, as well as over long-term storage.

The presence of the bilayer was evaluated indirectly through changes in the particles’ hydrodynamic radii, and visible absorbance spectra before and after coating. Direct observation of the lipid bilayer was made by TEM imaging. The SERS intensity of MG-labelled SERS Au NPs and Rho-PE-labelled SERS NPs remained stable over a period of 25 days, and 7 days, respectively though longer time periods have yet to be tested. Furthermore, subjection of lipid bilayer encapsulation Au NPs imparted superior stability in acidic and high ionic strength suspension conditions, as compared to the uncoated particles.

In summary, lipid encapsulated SERS Au NPs are promising as in vitro immunolabels with demonstrated signal and colloidal stability over time, and in harsh environments. Inclusion of PEG-PE in to the DEC221 bilayer, and conjugation of the particles to antibody fragments, and whole IgG1 isotype monoclonal antibodies is investigated in Chapter 8 for B lymphocyte labelling application.

5.7 Contributions

The author prepared and characterized the SERS nanoparticles (including collection of UV-Vis spectra, Raman spectra, DLS measurements and data analysis), contributed to nanoparticle design and to the preparation of the manuscript. The author gratefully acknowledges Shell Ip for design, preparation and characterization of SERS nanoparticles. Shell Ip also co-wrote the
original publication and permitted use of the schematic presented in Figure 5.1 (from Ip et al. \textit{Langmuir} \textbf{2011}, \textit{27}, 7024). The author also gratefully acknowledges Nikhil Gunari for TEM imaging and preparation of samples; Iliya Gourevich and Niel Coombs of the Center for Nanoscale Characterization for advice on TEM sample preparation, and for TEM imaging.

5.8 References


Evaluation of SERS Labelling of CD20 on CLL Cells Using Optical Microscopy and Fluorescence Flow Cytometry

6.1 Permissions

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6.2 Abstract

Immunophenotyping of lymphoproliferative disorders depends on the effective measurement of cell surface markers. The inherent light scattering properties of plasmonic nanoparticles combined with recent developments in nanoparticle design may confer significant advantages over traditional fluorescence probes. We report and evaluate the use of surface-enhanced Raman scattering (SERS) gold nanoparticles conjugated to therapeutic rituximab antibodies for selective targeting of CD20 molecules expressed by primary human chronic lymphocytic leukemia (CLL) cells. SERS nanoparticles were prepared by adsorbing a Raman-active dye onto the surface of 60 nm spherical Au nanoparticles, coating the particles with 5 kDa polyethylene glycol (PEG) and conjugating rituximab to functional groups on PEG. The effective targeting of CD20 on CLL cells by rituximab-conjugated SERS NPs was evaluated by dark field imaging, Raman spectroscopy, and flow cytometry with both competitive binding and fluorescence detection procedures. Evidence of CD20 clustering within approximately 100 nm was observed.

6.3 Introduction

Surface enhanced Raman scattering (SERS) nanoprobes have been developed for biological detection [1]. In order to advance this technology to the clinic, there is need to characterize the chemical design of the nanoprobes and to test their ability to recognize clinical samples.
Currently, the evaluation of cell surface protein expression using fluorescence-based sensing is the predominant strategy for the diagnostic investigation of lymphoproliferative disorders [2]. The use of fluorescent probes introduces several constraints for which surface enhanced Raman scattering gold nanoparticles (SERS Au NPs) have been proposed by many as an alternative immunophenotyping solution. SERS probes are not susceptible to photobleaching upon repeated or prolonged illumination [3,4], histological stains that can quench or interfere with fluorescence signals do not interfere with SERS probes, and the narrow bandwidths of SERS vibrational spectra increase the potential for multiplexed marker detection over what can be envisioned with fluorescence [5]. Au particles have light scattering characteristics that enable their use both as SERS substrates to enhance the Raman scattering intensity from reporter molecules incorporated in the structure of a SERS probe [6-8], but also as cell labels for dark field microscopy because of their intense Rayleigh scattering [9-11].

SERS immunophenotyping initially focused on targeting the expression of a single cell surface protein [12], and the field has significantly expanded. Silica [13,14], polymer [15], and protein [16] coatings have been utilized to overcome a number of potential disadvantages of the SERS nanoprobe approach, including irregular SERS intensity of the probes, limited solubility in aqueous solution, and biocompatibility. Different targeting moieties have been employed to direct the SERS probes to their intended targets including antibodies [17,18], affibodies [19], peptides [20], and folic acid [21]. The full potential of SERS immunophenotyping lies in multiplexed detection which has been demonstrated for a number of surface receptor combinations [22,23], and in vivo targeting [6,24] has been investigated.

This chapter illustrates a set of methods to characterize SERS probes for immunophenotyping and to evaluate chemical design and effectiveness in recognizing clinical samples. Our example system is chronic lymphocytic leukemia (CLL), which is a typical model of circulating cancer and a good target for single cell analysis. We focus on the targeting of SERS probes to CD20, a transmembrane phosphoprotein expressed in conjunction with a range of B-cell lymphoproliferative disorders [25]. The particles are targeted using rituximab, an FDA approved chimeric antibody composed of murine anti-CD20 binding regions fused to human IgG [26], typically used in the treatment of CLL and non-Hodgkin’s lymphoma. This study is conducted using CLL patient derived leukocyte samples that are prepared in the same manner as for fluorescent flow cytometry immunophenotyping in order to generate the most clinically relevant
conditions for evaluating SERS nanoparticle targeting. The advantages and disadvantages of this reported SERS nanoprobe application to patient derived cells are described.

Each component in the preparation of the SERS probes herein was chosen to produce markers with efficient performance for our application. Au nanoparticles with 60 nm diameter were chosen as the SERS substrate because they have been demonstrated as providing a large SERS enhancement for use with a 632.8 nm laser [27]. An additional advantage of using 60 nm diameter Au is that this size is not associated with significant levels of non-specific uptake by cells [28]. The 5 kDa poly(ethylene glycol) (PEG) coating used to prepare the SERS probes is used widely by others for NP stability [29], water solubility [30], prevention of non-specific association with proteins and cell surfaces [31,32], and is well characterized [6,10,30,33]. The choice of 5 kDa specifically however, is an area where our NP design could be further optimized. Rituximab and other therapeutic antibodies have been successfully applied elsewhere for non-SERS based targeting of Au NPs [34,35]. The Raman signal intensity, polymer coating thickness, and conjugation to monoclonal antibodies are characterized to understand the structure, and confirm the quality of the SERS Au NPs produced for study.

Rituximab-SERS Au NPs were found to be compatible with flow cytometry, and the specificity of their CD20 binding was evaluated using this technique. The aggregation state of the SERS particles under in vitro conditions is also observed using dark field microscopy because plasmon shifts are recorded as changes in the color of the particles and could serve as a medium through which to study clustering of receptors on the cell surface. Flow cytometry also serves as a benchmark against which to compare bulk cell suspension Raman spectroscopy of SERS probe targeted cells.

6.4 Methods and Materials

The following materials were used with no further purification: 60 nm diameter Au nanoparticles, and anti-human Fc antibodies bound to 5 nm Au nanoparticles were purchased from Ted Pella Inc. (Redding, CA, USA). Malachite green isothiocyanate (MGITC) supplied by Invitrogen (Burlington, ON, Canada). Both of 5 kDa α-methoxy-ω-mercapto poly(ethylene glycol) (CH₃O-PEG-SH), and α-carboxy- ω-mercapto poly(ethylene glycol) (COOH-PEG-SH)
by Rapp Polymere GmbH (Tuebingen, Germany). 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (sulfo-NHS), and micro format bicichronicinic acid (BCA) assay by Thermo Fisher Scientific (Rockford, IL, USA). Goat anti-human Fc fluorescein isothiocyanate (FITC) antibodies by from Sigma Aldrich (Oakville, ON, Canada). FITC-anti-CD20 was supplied by Beckman Coulter (Mississauga, ON, Canada). The use of blood samples for research was approved by the Research Ethics Board at the University of Toronto.

6.4.1 Preparation of PEGylated SERS Gold Nanoparticles Conjugated to Rituximab Antibodies

Aqueous solution of MGITC dye was added to stirring gold colloid to a concentration of 33nM. 5 kDa heterobifunctional CH$_3$O-PEG-SH was added drop wise from aqueous solution to SERS-active Au NPs, and the mixture was allowed to react at room temperature for 30 minutes. Aqueous COOH-PEG-SH was added to the stirring particles to include reactive groups for conjugation to monoclonal antibodies. Rituximab antibody (Rituxan, Roche) was dialyzed against 151 mM PBS buffer to remove solution stabilizers prior to conjugation between NPs and targeting antibodies. Aqueous EDC, and sulfo-NHS were added to the stirring SERS NP mixture as described elsewhere [36,37]. Briefly, following removal of residual HS-PEG-COOH from the nanoparticle suspension, aqueous solutions of EDC and sulfo-NHS were added in 2:5 molar ratio to final concentrations of 1.5 mM and 3.75 mM, respectively. Although EDC-mediated amide bond formation proceeds at the highest yield with pH buffered in the range of 4.5-7.5, it is possible to conduct this reaction in water with potentially lower yield and slower reaction rate outside the optimal pH range. A low salt reaction medium is advantageous for use with nanoparticles that may be susceptible to aggregation in high salt concentration buffer solutions. Excess cross-linking agents were removed and rituximab antibodies (Roche) (1.78 mM) added to activated SERS NP suspension and allowed to react for 3.5 hours at room temperature. To provide negative controls, the same procedure was adopted to prepare unconjugated SERS NPs and SERS probes bound to non-specific murine IgG1 antibodies.
6.4.2 Characteristics of CLL Cell Samples Chosen for Experiment

CLL diagnosis was confirmed by hematology, morphology and typical phenotypic profile, including positive for CD5, CD19, CD20, CD23, negative for CD79b, CD10, CD103, and dim surface immunoglobulin. The blood samples were selected for the study to have a total white blood cell (WBC) count in the range of 40-80 x 10^9/L and CLL cells over 90% of WBC. Mononuclear cells were prepared by the RBC lysis technique (Immuno Prep, TQ-Prep, Beckman Coulter).

6.4.3 Preparation of CLL Cells and Labelling with SERS Nanoparticle-Rituximab Conjugates

The cells were suspended in phosphate-buffered saline (PBS) plus 1% fetal calf serum. SERS nanoparticle labelling was performed by incubation of cells with nanoparticle-rituximab conjugates at high (5.6 pM) or low (1.4 pM) concentration where noted in PBS at room temperature for 30 minutes.

6.4.4 SERS Nanoparticle Characterization

UV-Vis absorbance spectroscopy was carried out on a Cary 5000 UV-Vis spectrometer (Varian Inc., Palo Alto, CA, USA) from aqueous suspensions of nanoparticles placed in 1cm path length cuvettes. UV-Vis absorbance spectra were normalized by shifting their baselines to zero and scaling the data so that the maximum value for each plot was equal to 1. 18 MΩ cm water was used as a reference.

The hydrodynamic size of SERS Au NPs was assessed after each functionalization step via dynamic light scattering (DLS). DLS measurements were collected on a DynaPro/Protein Solutions DLS machine (Wyatt Technologies Corporation, Santa Barbara, CA, USA), and data processed using Dynamics software version 6.7.1. Three repeats of 20 accumulations each for DLS measurements were collected for each sample. Hydrodynamic radius and polydispersity index (PI) were determined using a regularization algorithm.
To further confirm successful PEG coating of the SERS NPs, scanning electron microscopy (SEM) was used (Hitachi S-5200). To prepare samples of SERS Au nanoparticles for SEM, 15 uL drops of nanoparticle suspensions were placed on carbon-coated copper grids and air dried. Images at 30 kV using 500,000 X magnification. The mean diameter of the Au NPs was confirmed using 240 independent diameter measurements of 120 different nanoparticles as imaged by SEM, with image analysis software (ImageJ). The software measurement function was calibrated using a known pixel to nanometer ratio.

6.4.5 Quantification of Rituximab Antibodies Conjugated to SERS Gold Nanoparticles

Three different methods were used to estimate the average number of rituximab antibodies conjugated to each SERS Au particle. Goat anti-human Fc FITC antibodies were used to label rituximab- and control-SERS NPs for comparison, and fluorescence measurements were collected using a spectrofluorometer (Shimadzu RF-5301PC, Columbia, MD, USA) with RF-530XPC software, in 1 cm path length cuvettes. FITC secondary antibody concentration in each nanoparticle sample was determined using interpolation on a calibration curve of fluorescence intensity with known FITC IgG concentration. Nanoparticle concentration in each sample used for fluorescence spectroscopy was determined using the visible absorbance of the nanoparticle sample and the known molar extinction coefficient provided by the Au NP manufacturer (3.531 x 10^{10} M^{-1} cm^{-1}).

For labelling in transmission electron microscopy (TEM) images, rituximab-conjugated, and control-SERS Au NPs were each incubated with secondary anti-human Fc antibodies bound to 5 nm Au nanoparticles for 2 hours at room temperature. Unbound 5 nm Au secondaries were removed via centrifugation and drops of particle suspensions were placed on to carbon-coated copper grids and air dried. TEM images were collected using a Hitachi H-7000 TEM at 75 kV with 50,000x magnification. The average number of 5 nm Au particles bound to the surface of each 60 nm rituximab-conjugated SERS Au NP was obtained as follows. The number of 5 nm Au particles visible around each 60 nm particle was counted and averaged over a series of 100 60 nm particles. This averaged value of 5 nm particles was extrapolated to account for regions
that were not visible in the two-dimensional TEM images, such as the top and bottom 60 nm particle surfaces.

Micro format bicichroninic acid (BCA) assay was used to measure protein concentration in rituximab-conjugated Au NP suspension. Samples were prepared as per assay instructions, and background colour development controlled for by conducting the assay on PEG-coated control particles, and subtracting this absorbance from values obtained using antibody-conjugated particle samples. This protein concentration value was used to determine the approximate number of antibodies in suspension, and divided by the number of nanoparticles present, determined using the 60 nm Au particle extinction coefficient as described above for fluorescence measurements.

6.4.6 Dark Field Microscopy

Dark field light scattering images were collected using a Nikon TE2000 microscope equipped with oil immersion condenser and objective (100 X, 0.5-1.25 NA) (Nikon Instruments, Melville, NY, USA). The combination of a dark field stop inside the condenser and an objective iris ensure that the sample is illuminated only at oblique angles. With this optical setup, only light scattered by the sample is capable of entering the objective for imaging by the CCD camera (DS-Fi1, Nikon).

Imaging software (Photoshop CS3) was used to develop an automated counting procedure that would identify differently colored nanoparticles in dark field images and count the green and red populations separately. The procedure included enhancing the color balance and object boundaries in the images so that the particles could be identified, and the green and red populations isolated and counted. The particles were also manually counted and tracked using Photoshop CS3 as a basis against which to evaluate the efficacy of the automated counting procedure. Cell surface area pixel intensity measurements were collected using a compilation of statistics from a consistently sized region of interest corresponding to cell location (NIS-Elements, Nikon).

It has been reported elsewhere that cells scatter light strongly in dark field images, and that this scattering is largely blue-white in character [38,39]. In order to more clearly present the dark
field images, background blue and white scattering were removed by separating the original RGB image into its constituent red, green and blue channels, and subsequently subtracting the monochrome blue channel image from the original RGB. In this manner, pixels with high intensity in the blue channel or white pixels that would have values approaching 255 in all three channels simultaneously for 8-bit images, would appear dark in the corrected images. Scattering from the gold nanoparticles is expected to appear green-gold or red in the dark field images [40-43] and therefore would still appear in corrected images because each pixel corresponding to these regions would have higher values in red and green channels than in the blue channel. To show the location of cells within the images, the brightness of the images has also been uniformly rescaled such that all pixel values have been increased by 12%. The resultant images are better representative of nanoparticle labelling, and allow for more straightforward comparison between cell samples.

6.4.7 SERS Microspectroscopy

The same inverted microscope was used to focus a 632.8 nm HeNe laser (10 mW, Melles Griot) on to drops of cell suspension through a 40 X (0.6 NA) objective. A notch filter (\(\lambda = 632.7\) nm, FWHM = 23.8 nm) was used to prevent Rayleigh scattering from entering the monochromator. Raman scattered light was collected by a Peltier cooled CCD detector with 1340x400 pixel array (PIXIS BR400, Princeton Instruments) using WinSpec32 software. Spectra of cell samples were collected from drops of cell suspension on glass coverslips using 90 second integration time.

6.4.8 Flow Cytometry

Cells were incubated with either unconjugated rituximab, or rituximab-conjugated SERS Au NPs for 30 minutes at room temperature. Secondary staining was then performed with two fluorescent antibodies: Fc-specific FITC anti-human IgG was used to detect rituximab conjugates bound to the cells; and FITC-anti-CD20 was used to measure the competitive blocking by the SERS-rituximab binding to CD20. Cells stained with secondary antibodies alone were used as controls. For each sample, at least 5000 events were collected on a Cytomics
FC500 flow cytometer (Beckman Coulter), and data analysis was performed using CSP software (Beckman Coulter) and FlowJo (Treestar, Ashland, Oregon).

6.5 Results

6.5.1 Characterization of Rituximab SERS-Gold Nanoparticles

UV-Vis spectroscopy confirmed the monodispersity of the SERS NPs after each functionalization step (Figure 6.1D). After addition of aqueous thiolated PEG to the SERS NP suspension, the formation of a polymer layer was confirmed through a 2 nm red shift in the localized surface plasmon (LSPR) absorbance of the NP suspension from 534 nm to 536 nm, and the peak location remained consistent following conjugation to rituximab antibodies. An absorbance peak did not appear in the 600-700 nm region which would have indicated the formation of particle aggregates. DLS measurements further confirmed SERS Au NP functionalization and monodispersity (Figure 6.1C). The stock citrate-coated Au nanoparticles used to prepare SERS probes in this study had a hydrodynamic radius of 33 ± 0.2 nm. Following coating the PEG, this radius increased to 39 ± 0.8 nm, and finally to 55 ± 1.6 once conjugated to rituximab antibodies. Particle diameter was confirmed by measuring particles in a series of TEM images. Mean diameter using this method was determined to be 29 ± 2 nm.
Figure 6.1: A) Schematic illustration of the preparation of rituximab-SERS gold nanoparticles. B) SEM image representative of rituximab-SERS gold nanoparticles. C) Dynamic light scattering histograms indicate an increase in particle hydrodynamic radius following each surface functionalization step. D) Visible absorbance spectra of SERS Au NPs. Inset: A 2 nm shift in the particle plasmon absorbance occurred upon PEGylation and the absorbance maximum did not shift further following conjugation to rituximab antibodies.

6.5.2 Quantification of Antibody Conjugation

FITC fluorescence from nanoparticles suspensions incubated with anti-human Fc secondary antibodies gave an average value of 80±30 antibodies per rituximab-SERS NP. In TEM imaging, anti-human 5 nm Au secondaries appear as dark grey spots within the lighter grey PEG and protein corona surrounding the dark 60 nm Au particles, and bind specifically to SERS NPs conjugated to rituximab (Figure 6.2). This estimation method gave a value of 30±5 rituximab antibodies per 60 nm SERS Au NP. The micro BCA assay gave values for total protein concentration in rituximab-conjugated nanoparticles suspension, and this antibody count was averaged over the approximate number of SERS Au NPs present yielding a value of 22±0.6 rituximab antibodies bound to each SERS probe.
Figure 6.2: TEM images of SERS Au NPs incubated with anti-human Fc 5 nm Au secondary antibodies for identification of rituximab primary antibodies on NP surfaces. A) Rituximab-conjugated SERS Au NPs. B) SERS Au NPs with PEG coating only.

6.5.3 Evaluation of CD20 Targeting of CLL Cells Using SERS Spectroscopy

The specific MGITC SERS nanoparticle spectrum was detected in cell samples labeled with rituximab-SERS Au NPs at high and low concentrations (Figure 6.3A). Positively targeted cell samples were clearly distinguishable from negative controls that used murine IgG1 non-specific SERS NPs or SERS NPs with PEG coating only (Figure 6.3A). Competitive binding experiments were performed whereby CD20 binding sites were blocked using unlabeled rituximab prior to labelling with SERS nanoparticles. A marked reduction in SERS signals was observed for those CD20 blocked samples in comparison to the non-blocked samples of equivalent nanoparticle
labelling (Figure 6.3B). No distinction could be made between the CD20 blocked and non-blocked cells treated with non-specific IgG1-SERS nanoparticles (not shown).

Figure 6.3: SERS spectra of cell suspensions in PBS following incubation with SERS gold nanoparticles. A) SERS intensity from targeted cell samples stained with high (1) and low (2) concentrations of rituximab-SERS gold nanoparticles (5.6 pM and 1.4 pM respectively), in contrast with negative controls using murine IgG1 non-specific SERS NPs (3) and SERS NPs with PEG coating only (4). B) SERS intensity in competitive binding experiments. Cell samples stained with high concentration of rituximab nanoparticles (black); cells prestained with unlabeled rituximab prior to treatment with high concentration SERS probes (dashed). SERS peak assignments are provided in Table 6.1.
6.5.4 Evaluation of CD20 Targeting of CLL Cells Using Dark Field Microscopy

Dark field imaging of fixed cell cytospin slides was used to further confirm the cell labelling by rituximab-SERS Au NPs. Cell labelling densities as observed using dark field microscopy strongly correlated to observations made using SERS spectroscopy (Figure 6.4 & 6.5). Rayleigh scattering from the SERS particles was observed in the unprocessed dark field images (Figure 6.4) however, it was difficult to differentiate from light scattered by cells. Therefore, further labelling intensity comparisons using dark field were conducted with images where all blue-white light scattering was removed, as described above (Figure 6.5).

Figure 6.4: Unprocessed representative dark field scattering images of CLL cells labelled with SERS Au nanoparticles (100x magnification). Pixel intensities have been normalized between images to account for differences in camera integration time. Top row: CLL cells subjected to varying SERS nanoparticle treatments. A) High concentration of rituximab-conjugated SERS Au NPs, B) low concentration of rituximab SERS Au NPs, and C) non-specific isotype IgG1-SERS Au NPs. Bottom row rituximab blocking: images of CLL cells treated with unconjugated rituximab prior to corresponding SERS nanoparticle labelling. D) High concentration of rituximab-conjugated SERS Au NPs, E) low concentration of rituximab SERS Au NPs, and F) non-specific isotype IgG1-SERS Au NPs.
Positively labeled cell samples using different concentrations of rituximab-bound SERS probes (Figure 6.5 A&B) exhibited corresponding densities in CD20 labelling. Images of cells treated with non-specifically targeted SERS nanoparticles did not display significant labelling of cells by SERS nanoparticles (Figure 6.5C) and can be clearly differentiated from positively targeted samples. Competitive binding experiments involved the blocking of CD20 binding sites with unlabeled rituximab prior to incubation with SERS gold nanoparticles. Rituximab-SERS particle binding was markedly reduced by the CD20 blocking (Figure 6.5D&E). Little or no cell labelling was observed by non-specific IgG1-SERS Au nanoparticles, whether or not the CD20 epitopes were masked with unlabeled rituximab (Figure 6.5C&F).

![Figure 6.5: Representative dark field scattering images of CLL cells labeled with SERS Au nanoparticles (100x magnification). The blue channel has been subtracted from original RGB images, and brightness increased to show cell location. Top row: CLL cells subjected to varying SERS nanoparticle treatments. A) High concentration of rituximab-conjugated SERS Au NPs, B) low concentration of rituximab SERS Au NPs, and C) non-specific isotype IgG1-SERS Au NPs. Bottom row rituximab blocking: images of CLL cells treated with unconjugated rituximab prior to corresponding SERS nanoparticle labelling. D) High concentration of rituximab-conjugated SERS Au NPs, E) low concentration of rituximab SERS Au NPs, and F) non-specific isotype IgG1-SERS Au NPs.](image-url)
The differences in cell labelling densities between nanoparticle treatments were quantified by plotting the distribution of the sum of pixel intensities corresponding with the location of individual cells in a series of dark field images (Figure 6.6). For each of three different nanoparticle treatments respectively, a series of 100 cells were imaged using identical microscopy and imaging settings to complete the analysis. The pattern observed was consistent with visual observations in that cells treated with rituximab-bound SERS probes were significantly more intense than those treated with non-specific IgG particles, or subjected to competitive binding experiment (Figure 6.6).

Figure 6.6: Distribution of the sum of pixel intensities corresponding to cell location in dark field images. Dashed lines show raw data; Gaussian fits represented by solid lines. Cells treated with a high concentration of rituximab-SERS NPs (black); competitive binding treatment whereby cells were blocked with unlabeled rituximab antibodies prior to treatment with a high concentration of rituximab-SERS NPs (dark grey); cells treated with non-specific IgG1- SERS probes (light grey).

6.5.5 Nanoparticle Counting and Determination of Cell Labelling Intensities Using Dark Field Imaging

The nanoparticles are expected to Rayleigh scatter light preferentially at the location of their LSPR, which corresponds to 536 nm for 60 nm diameter functionalized Au NPs, and hence
should appear as green-gold in the dark field images while the LSPR of clustered or aggregated species should red-shift. Image analysis software (Photoshop CS3) was used to objectively isolate and count these separate populations. Values calculated by the software counting procedure were compared to manual particle counts and the percentage difference between them was calculated. For a series of 8 images of high-volume anti-CD20 SERS NP cell staining at 100 X magnification, there was approximately a 3:1 ratio of green to red particles as recorded by automated counting, while this ratio was identified as 10:1 by manual counting. The average percentage difference between sets of values obtained from automated, and manual counting methods is 33% for green particles and 78% for red particles. The variation arises from the ability of the automated procedure to objectively differentiate colors, while the manual count is more efficient at discerning individual particles located in clusters in the cell samples.

6.5.6 Fluorescence Flow Cytometric Analysis

The specificity of B-cell labelling by rituximab SERS probes was further evaluated using flow cytometric assays. Fluorescence detection was undertaken using anti-human IgG (Fc specific) antibody and anti-CD20 antibody. A reduction of FITC-CD20 fluorescence (Figure 6.7A) was observed from cell samples first blocked with unlabeled rituximab prior to treatment with rituximab-SERS NPs, though the decrease in fluorescence was not as significant as for samples blocked with unlabeled rituximab (Figure 6.7B). By contrast, no decrease in anti-CD20 fluorescence was observed following pre-staining with either unconjugated SERS NPs (Figure 6.7B) or non-specific isotype IgG1-SERS NPs (Figure 6.7C).

Secondary antibody fluorescence detection of rituximab-conjugated SERS Au NPs was conducted using anti-human FITC antibody, specific for the Fc fragment of rituximab. Significant binding of the secondary was observed on cells with the primary binding of either unconjugated rituximab or rituximab-conjugated SERS Au NPs (Figure 6.7D). The fluorescence from both samples were markedly enhanced from the baseline obtained using anti-human FITC antibody alone (Figure 6.7D).
Figure 6.7: Flow cytometric assessment of SERS NP-rituximab binding. A) Changes in fluorescence intensity of anti-CD20 FITC antibody in cells pre-stained with unconjugated rituximab (dashed) or rituximab-SERS nanoparticles (solid), in comparison to cells stained directly with anti-CD20 antibody (grey). No blocking was observed following the pre-staining with either B) unconjugated SERS nanoparticles or C) IgG1-SERS particles. D) Detection of rituximab-SERS particles by secondary staining with an anti-human IgG (Fc
specific) FITC antibody. Cells prestained with unlabeled rituximab (dashed); rituximab-SERS Au NPs (solid); control cells without primary staining (grey).

6.6 Discussion

In this study, we demonstrated rituximab mediated CD20 targeting of SERS Au NPs, and the confirmation of this targeting using Raman spectroscopy, dark field microscopy, and flow cytometry analysis. It is significant to note that cell surface protein labelling using SERS probes was not only compatible with flow cytometry immunophenotyping, but could also have its efficacy evaluated using this technique.

The Raman reporter used in this study, malachite green isothiocyanate, was electrostatically adsorbed to the surface of colloidal gold particles. The close proximity of MGITC to the Au nanoparticle surface following adsorption resulted in SERS via electromagnetic enhancement mechanism [44]. The enhancement factor was calculated using the following equation 6.1 [45] and is discussed in further detail in Chapter 3.5.1.

\[
EF = \left( \frac{I_{SERS}}{I_{norm}} \right) \left( \frac{C_{norm}}{C_{SERS}} \right)
\]

Equation 6.1

where \( I_{SERS} \) is the intensity of the MGITC SERS spectrum at 1620 cm\(^{-1}\), \( I_{norm} \) is the intensity of the spontaneous Raman spectrum from aqueous MGITC solution at 1620 cm\(^{-1}\). \( C_{SERS} \) is the estimated concentration of MGITC adsorbed to the surface of Au NPs in suspension, and \( C_{norm} \) is the concentration of the aqueous dye solution. Adsorption of dye to Au NPs enhanced resulted in a SERS enhancement factor of \( 10^4 \) which is consistent with previously reported values for monomer particles [46]. To estimate the concentration of MGITC that adsorbed to Au nanoparticles, following the addition of dye to the colloid the solution was centrifuged, and UV-Vis absorbance of the supernatant was collected. Because the maximal absorbance peak for MGITC at 630 nm was absent, it was assumed that all dye added to the Au suspension was adsorbed to the surface of the Au nanoparticles. The concentration of Au particles in suspension was calculated using the visible absorbance spectrum of the particles, and the manufacturer’s extinction coefficient \( (3.531 \times 10^{10}) \). Spectra used for the electromagnetic SERS enhancement
factor calculation are shown in Figure 6.8. The SERS peak positions were noted for later detection and differentiation from treated cell suspensions and locations and assignments are provided in Table 6.1.

Figure 6.8: Electromagnetic SERS enhancement factor calculation. SERS spectrum of 0.043 nM SERS-active nanoparticle suspension (black, solid) and Raman spectrum of 4 uM aqueous solution of malachite green isothiocyanate (black, dotted).

<table>
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<td>1290</td>
<td>1297</td>
<td>C-C; in plane, C-C-H; in plane</td>
</tr>
</tbody>
</table>
Table 6.1: Malachite green isothiocyanate SERS band location and assignments based on resonance Raman from Lueck et al. [47].

<table>
<thead>
<tr>
<th>1362</th>
<th>1368</th>
<th>-N; stretch</th>
</tr>
</thead>
<tbody>
<tr>
<td>1487</td>
<td>1492</td>
<td>N-(CH$_3$)$_2$; bend and rock</td>
</tr>
<tr>
<td>1620</td>
<td>1619</td>
<td>-N; stretch, C-C; stretch</td>
</tr>
</tbody>
</table>

A 2 nm shift in the plasmon peak of the particles (Figure 6.1D) and an approximately 10 nm increase in their hydrodynamic radius (Figure 6.1C) were sufficient to confirm the formation of a PEG layer surrounding the SERS particles, though alternative approaches have been used elsewhere [11,24].

Bioconjugation of the PEGylated SERS Au NPs occurred through the formation of amide bonds between carboxylic acid functionalities of the PEG coating, and available ε-amine groups of lysine residues, or N-terminal amines of the rituximab antibodies [37]. The bioconjugation reaction is discussed in further detail in Chapter 4.3.4. A disadvantage of this method is that the orientation of antibody binding is uncertain and some of the anti-CD20 antibodies were likely oriented in a configuration that reduced the affinity for their CD20 targets. This fact may not limit the efficacy of anti CD20-SERS NPs as surface receptor labels however since multiple antibodies bind each nanoparticle on average, increasing the likelihood that at least one targeting moiety will be bound in the correct orientation for cell surface labelling. Multivalent targeting agents, including those for targeting rituximab, have also generated interest due to increased potency over monovalent therapeutic antibody constructs [48].

Quantification of the number of antibodies bound to the surface of each SERS Au NP was not trivial, and therefore several methods were employed in conjunction. While the absorbance and emission maxima for FITC antibodies used in secondary fluorescence labelling, located at 494 nm and 521 nm respectively, overlap with the Au NP plasmon, the 10 nm separation of the fluorophores from the nanoparticle surface prevents significant fluorescence quenching [49]. Secondary 5 nm immunogold antibody labelling of rituximab-conjugated SERS NPs [10,35] provided a lower estimate than fluorescence labelling for the average number of antibodies bound per SERS probe. Steric hindrance is introduced by the 5 nm Au particles that could prevent anti-human Fc secondary antibodies from approaching adjacent rituximab antibodies.
bound to the 60 nm Au surface. This would not have been a factor in the secondary fluorescence labelling. Additionally, some 5 nm secondary Au nanoparticles appear to fall outside of the PEG and protein corona in the TEM labelling images (Figure 6.2A) which could be suggestive of a non-specific association of 5 nm particles with 60 nm Au during the drying process of the sample deposited on to the TEM grid. This is likely not the case however, as not a single 5 nm Au particle was associated with the 60 nm SERS Au in a series of 55 particles in control images whereby SERS Au with PEG-coating only were treated with 5 nm Au secondary IgG particles.

A different approach was taken by measuring total protein concentration using a colorimetric micro-BCA assay. The BCA assay was chosen as it is highly sensitive, yields linear calibration curves, and its color development is not as susceptible as that of other protein assays to differences in sequence between proteins. Absorbance is measured at 562 nm, and increases in intensity with increasing protein concentration. Because the maximum absorbance wavelength of the assay at 562 nm overlaps with the intense 60 nm Au particle plasmon peak, the particles were removed from the assay solution prior to taking measurements. It has been reported elsewhere that the presence of the nanoparticles causes non-specific color development [11,50]. The BCA assay is sensitive to the presence of thiols in solution, and it is possible that the thiol terminated PEG coating the nanoparticles could be falsely increasing the protein concentration values. To control for this issue, the assay was also conducted on a suspension of PEG-coated particles that were not bound to antibodies.

The values for antibodies per nanoparticle determined experimentally fall within the limits of theoretical approximations of antibody densities in monolayer coating of nanoparticles [51]. The mean hydrodynamic radius of PEGylated SERS NPs, 39 nm, was used for calculating the particle surface area upon which antibodies could bind. While this value is understood to be artificially large due to the effect of particle surface charges on hydrodynamic radius, it is closer than values for unfunctionalized citrate-capped nanoparticles to the true size that interacts with approaching antibodies in solution. A random sequential adsorption model was employed to obtain antibody loading density in mg/m² on the particle surface. The RSA model assumes that the antibodies approach the particle surface randomly, under the constraint that they cannot overlap with each other on the particle surface. The individual antibodies were treated as discs with 10.5 nm diameter, with a packing efficiency of 0.547 so that the ability of antibodies to approach the surface in both end on- and side on- configurations was taken in to account. Using
this model, there is sufficient space on each Au NP surface to bind 113 monoclonal IgG antibodies.

To assess SERS probes bound to CD20 on CLL cells, SERS spectroscopy was used to detect the MGITC fingerprint spectrum from drops of cell suspension. The MGITC SERS spectrum was clearly observed from positively targeted cell samples, however a linear relationship between this spectral intensity and the amount of anti-CD20 SERS NPs was not immediately evident (Figure 6.3A). However, if the SERS intensity obtained from the sample treated with non-specific IgG1 isotype conjugated-SERS NPs was used as the background intensity level, subtracting this value from each of the positive labelling treatments yields a 1:4 intensity ratio between the treatment groups. This intensity ratio correlates linearly with the 1:4 NP volume ratio used in the low and high anti-CD20 SERS NP concentrations respectively. Flow cytometry results were indicative of a lack of CD20 receptor saturation by the concentrations of SERS NPs used in these staining procedures. As such, it would be expected that SERS intensity should rise roughly linearly with an increase in the number of anti-CD20 nanoparticles used in each cell sample, consistent with the trend observed in the SERS spectra.

Competitive binding experiments were performed to confirm the specificity of rituximab-SERS NPs binding to CD20 targets. When CD20 receptors were blocked using unconjugated rituximab antibodies, a marked reduction in intensity compared to the equivalent unmasked samples was recorded (Figure 6.3B) thus indicating the specificity of nanoparticle binding. SERS intensity collected from CD20 blocked and unblocked cells incubated with murine IgG1-SERS NP controls were equivalent which further indicates that SERS intensity obtained from the isotype control samples may be treated as low level background intensities (not shown). This background signal was a result of either non-specific association of the particles with cells in the sample, or remainder of residual SERS particles in suspension after extensive washing of the cell samples.

Evaluation of absolute receptor expression using flow cytometry requires careful calibration of the intensity of the different fluorophores used and the response from the flow cytometer to these, in addition to objective data analysis; protocols have been developed for this purpose [52,53]. Likewise, relative and absolute receptor expression could be obtained using SERS techniques, but the way in which this is evaluated will differ from fluorescence methods. A
disadvantage of the use of SERS probes for immunophenotyping is the possibility of non-linear signal increases with increasing particle binding density as a result of surface plasmon coupling between neighboring particles. This would result in non-linear increases in SERS intensity with receptor number. The detailed design of quantitative SERS immunophenotyping procedures is an important subject for further study.

Light scattering dark field images of cytospin slides were taken to ascertain the spatial location of the SERS Au nanoparticles within labeled cell samples and to further evaluate the relative degrees of cell labelling by functionalized probes (Figure 6.4). The dense cells scatter light strongly and appear blue-white in the images [38,39], while SERS NPs intensely Rayleigh scatter at the wavelength corresponding to their LSPR at 536 nm, resulting in monomer particles appearing green-gold in dark field images [41].

Some red light scattering species are visible in the dark field images and are likely aggregates or clusters of particles which could have been introduced either during the nanoparticle functionalization process, or during subjection of the particles to in vitro biological conditions causing a shift in their LSPR [42,43]. The SERS particle UV-Vis spectrum does not show an absorbance at wavelengths greater than 570 nm which would be characteristic of the formation of particle aggregates. Taken together, these observations indicate that the plasmon shift observed in dark field images is due to particles binding proximal CD20’s on the cell surface resulting in observation of receptor clustering within the separation necessary (<50 nm) to cause the plasmon shift [54].

A potential advantage of immunophenotyping with plasmonic particles as opposed to fluorophores is the potential to study receptor distributions within regions of 10s-100s nm diameter on cell surfaces. While fluorescence technologies enable visualization of receptor location, and areas that fluoresce intensely are indicative of a high density of the surface protein being labeled, information about the spacing and density of the receptors is constrained by the diffraction limit of visible light. Super-resolution fluorescence microscopy techniques are being increasingly adopted to address such questions [55], however such imaging systems are currently not widely available. Plasmonic metal nanoparticles exhibit distance dependent plasmon coupling in a predictable manner, thus red-shifting their LSPR wavelength and providing information about the distance between adjacent particles which can be collected using the
simple, inexpensive dark field microscopy technique. A disadvantage of plasmonic nanoparticles for such distance studies is sensitivity to particle aggregation prior to going down on the cell surface, which shifts the LSPR wavelength independent of cell binding. As discussed above, significant aggregation of the SERS probes in this study was not evident before their use in cell studies, and therefore color changes observed are likely the result of binding density. To ensure validity of surface receptor density studies using plasmonic particles, it is important to have controls in place to ensure that aggregation does not occur prior to surface binding.

Flow cytometry further confirmed the effective targeting of rituximab-conjugated SERS NPs to CD20 by assessing the retention of functionalized nanoparticles by CLL cells. Secondary fluorescence enhancement was observed from cells first treated with rituximab-conjugated probes (Figure 6.6D). Though the enhancement was not as significant as for unlabeled rituximab, it was markedly increased relative to the baseline level obtained using secondary antibody alone.

SERS probe binding specificity was investigated using a flow cytometric competitive assay in which CLL cells were stained with an anti-CD20 fluorescent antibody following incubation with rituximab-conjugated SERS NPs resulting in a subsequent decrease in anti-CD20 fluorescence (Figure 6.6A). This effect can be attributed to the blocking of CD20 epitopes on the cell surface by the rituximab. Similar to the findings from SERS spectroscopy, the rituximab-SERS Au NPs did not saturate CD20 binding sites to the same extent as pure rituximab, as indicated by the complete blocking of CD20 fluorescence following the incubation of cells with unconjugated antibody. In contrast, there was no decrease in anti-CD20 fluorescence following preliminary staining with controls of either non-specific isotype IgG1 control (Figure 6.6C) NPs or unconjugated NPs (Figure 6.6B).

The competitive binding assays provide strong evidence for the effectiveness and specific targeting of rituximab-conjugated SERS NPs to CD20. The disparity in targeting efficiency compared with free rituximab could be attributed to a failure to achieve CD20 saturation with the concentrations of rituximab conjugates used in this experiment. Alternatively, this effect may be an indication of steric hindrance at the cell surface caused by the blocking of neighboring CD20 binding sites by bulky 60 nm Au NPs. Receptor saturation dynamics using large SERS NPs
should be studied further, to overcome potential disadvantages of SERS nanoprobes compared with the use of molecular reporters.

A comparison of SERS technologies to conventional flow cytometric immunophenotyping was provided for leukemia detection and diagnosis, and may be useful in evaluating the SERS nanoprobes application to other systems. An important subject for future study is an understanding of the relative and absolute expression levels of cell surface proteins that takes into account the effects of plasmon shifts from particles binding separately but near each other, the effects of particle-particle contacts at the surface, and any resulting SERS enhancement above that of monodispersed SERS particles. It would also be valuable to construct SERS nanoprobes that not only report the presence of antigen because of immunochemistry but also provide enhanced spectroscopic characterization of the biological material within the plasmonic field of the bound nanoparticles.

### 6.7 Contributions

The author prepared the rituximab-SERS Au NP conjugates, and conducted NP characterization (UV-Vis, DLS, antibody quantification experiments: fluorescence, TEM, BCA assay). The author also performed the SERS measurements, dark field microscopy and dark field image colour analysis. The author wrote the paper, prepared the figures, and analyzed the data with the exception of the flow cytometry material. The author gratefully acknowledges: Edward Parker for design, undertaking, and analysis of the flow cytometric assays; Iliya Gourevich for TEM images; Mandy Koroniak for assistance with Photoshop counting protocol; and K. W. Annie Bang for analysis of flow cytometric data.

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soluble SERS labels comprising a SAM with dual spaces for controlled Bioconjugation. *Phys


7 SERS Gold Nanoparticles for Triplexed Detection of Leukemia and Lymphoma Cells and SERS Flow Cytometry

7.1 Permissions


7.2 Abstract

The labelling of cell surface receptors by fluorescent markers is an established method for the identification of cell phenotype in both research and clinical settings. Fluorescence dye labelling has inherent constraints, most notably the upper limit of labels per cell that may be probed using a single excitation source, in addition to a physical limit to the number of broad emission spectra that can be distinctly collected within the visible wavelength region. SERS labelling has the potential to mitigate these shortfalls. Herein, antibody targeted, PEG-coated surface enhanced Raman scattering (SERS) Au nanoparticles are used to simultaneously label three cell surface markers of interest on malignant B cells from LY10 lymphoma cell line. The SERS probes were characterized by multiple methods to confirm their monodispersity and functionalization with both PEG and monoclonal antibodies. The specificity of the particles’ cell labelling was demonstrated on both primary chronic lymphocytic leukemia and LY10 cells using SERS from cell suspensions and confocal Raman mapping, respectively. Fluorescence flow cytometry was employed to confirm the binding of SERS probes to LY10 over large cell populations and the particles’ SERS was collected directly from labeled cells using a commercial flow cytometer. To the best of our knowledge, this is the first demonstration of SERS flow cytometry from cells tagged with targeted SERS probes.
7.3 Introduction

Immunophenotyping is the gold standard for distinguishing between cell types and can be essential for early and accurate diagnosis of a wide range of diseases. In particular, the identification of cancerous B cells in lymphoma and leukemia presents an important problem; chronic lymphocytic leukemia (CLL) is the most widespread of hematologic malignancies with 2-6 cases per 100,000 adults per year in western countries [1]. While histological and morphological analyses of leukocyte samples can identify an overproliferation of immune cells, the combination of these methods with detailed phenotypic analysis can more clearly identify cancer subtype and stage [2]. Current diagnostic technologies predominantly rely on fluorescent markers detected using flow cytometry which provides information about the relative expression levels of cell surface proteins of interest over tens or hundreds of cells per sample. Flow cytometry is particularly valuable for CLL detection, and advances thereto could have clinical promise. Fluorescence microscopy and imaging flow cytometry also provide important information concerning the spatial distribution of labeled proteins throughout the cell. These technologies are limited, however, by the number of surface proteins that can be simultaneously imaged due to the broad bandwidths of fluorescence labels [3]. Quantum dots were developed to meet this need with narrower bandwidth emission spectra but are still limited because the bandwidths are 10’s of nanometers and cannot be overlapped [4]. Surface-enhanced Raman scattering (SERS) nanoparticles address this bandwidth issue; the narrow SERS bandwidths of 1-2 nanometers can significantly increase the number of cell labels that can be used simultaneously. SERS-based detection is also advantageous for the development of diagnostic imaging equipment because light scattering from many distinct SERS labels may be generated using a single laser wavelength. SERS probes can also be designed to scatter well at near-IR wavelengths which are not strongly absorbed by tissues [5], thus reducing the likelihood of sample damage or interference by auto-fluorescence.

Much work has been done to advance the development of SERS-based diagnostic probes by varying choice of plasmonic particle material and structure, surface coating [6-11], targeting moiety [12-16], and application [17-21]. Spectral resolution methods have been developed using probe solution and plate binding assays [22-25]. Targeted imaging of 2-3 surface proteins has been demonstrated alone [26-28] or in combination with fluorescence emission from either
traditional fluorophores [24], or fluorescent species incorporated in the SERS particle coating [29-31]. In vivo multiplexed SERS detection has been established using both targeted and untargeted probes injected into animal models [32-34].

The utility of PEG-coated SERS Au nanoparticles for imaging single surface protein CD20 on the surfaces of CLL cells in Chapter 6. Also in Chapter 6, SERS Au NPs were found to be compatible with fluorescence flow cytometric protocols [37]. While SERS has been detected from large nanoparticles in solution [38-41], and flow cytometry has been used to detect fluorescence from cells labeled with dual SERS fluorescent nanoparticles [13], we believe this to be the first demonstration of SERS flow cytometry from specifically targeted SERS probes bound to the surfaces of live cells in a clinical flow cytometer.

This chapter reports the use of SERS probes for the simultaneous detection of three surface proteins expressed by B cells and used in diagnostic immunophenotyping: CD45, CD19, and CD20. Targeted SERS probe binding and the specificity of their interaction with B cell surface molecules have been demonstrated using both LY10 lymphoma cell line, and primary CLL cells. Particle Rayleigh scattering was detected and quantified using dark field microscopy. SERS was collected from cell suspension in buffer. Triplexed labelling was resolved using confocal Raman microscopy and direct classical least squares (DCLS) fitting of the mixed spectra. Fluorescence flow cytometry confirmed the presence of the SERS probes on cell surfaces and the stability of particle binding using this diagnostic technique. Finally, specific SERS cell labelling and SERS-functionalized polystyrene (PS) microspheres were detected directly by collection of the particles’ SERS through a spectral bandpass filter using a commercial flow cytometer.

7.4 Methods and Materials

The following materials were used without further purifications: 60 nm diameter Au nanoparticles were purchased from Ted Pella Inc. (Redding, CA, USA). Malachite green isothiocyanate (MGITC) was obtained from Life Technologies (Burlington, ON, Canada); ethyl violet (EV), cresyl violet prechlorate (CV) and pararosaniline hydrochloride (basic fuchsin, BF), and 3,3’-diethylthiadicarbocyanine (DTTC) were obtained from Sigma Aldrich (Oakville, ON, Canada). 5 kDa α-methoxy-ω-mercapto poly(ethylene glycol) (CH3O-PEG-SH), and α-carboxy-
ω-mercapto poly(ethylene glycol) (COOH-PEG-SH) were obtained from Rapp Polymere GmbH (Tuebingen, Germany). 1-ethyl-3[3(dimethylaminopropyl] carbodiimide hydrochloride) (EDC), N-hydroxysulfosuccinimide (sulfo-NHS), and micro format bicinchoninic acid (BCA) assay were obtained from Thermo Fisher Scientific (Rockford, IL, USA). Monoclonal antibodies: murine anti-human CD19, murine anti-human CD45, murine anti-human CD5 and murine IgG1 isotype were purchased from Abcam (Cambridge, MA, USA). Rituxan (Roche) anti-CD20 antibodies were dialyzed against 171 mM phosphate buffered saline (PBS) to remove solution stabilizers prior to use. Streptavidin functionalized 6-8 μm diameter polystyrene microspheres were obtained from Spherotech (Lake Forest, IL, USA), and 5kDa biotin-poly(ethylene glycol)-thiol from Nanocs (New York, NY, USA).

7.4.1 Preparation of SERS Gold Nanoprobe

For each type of SERS nanoparticle, aqueous dye solutions ranging 4-20 μM, depending on dye used, were added drop wise to stirring Au colloid (0.043nM). Immediately afterward, 80 mM aqueous solution of 5kDa CH₃O-PEG-SH was slowly added to the SERS nanoparticle solutions to a final concentration of 6 mM and allowed to stir for 30 minutes. For SERS particles intended for cell labelling experiments, 80 mM 5 kDa COOH-PEG-SH solution was added to the stirring particles to a final concentration of 35 mM to incorporate reactive groups in the particle coating as conjugation sites for monoclonal antibodies. Excess PEG was removed via centrifugation (6.8 g, 5 minutes) and aqueous EDC (0.25 M) and sulfo-NHS (0.5 M) were added to the SERS particle suspension to final concentrations of 1 mM and 2 mM respectively, and allowed to react for 15 minutes. Centrifugation was used to remove excess cross-linking reagents. Monoclonal antibodies in PBS (6.7 μM) were added to the SERS probe solutions (0.43 nM) in a 1000 fold molar excess to SERS nanoparticles and incubated for 4 hours at room temperature. Following removal of unbound protein, SERS probes could be stored in PBS at 4°C for at least 1 week prior to use. For SERS Au nanoparticles intended for PS calibration bead functionalization, following dye addition to the stirring colloid, an aqueous solution of 5kDa biotin-PEG-SH and CH₃O-PEG-SH in a 1:1 molar ratio was slowly added to the particle suspensions and allowed to stir for 2 hours. Background information relevant to these procedures is available in Chapters 4.3.2 and 4.3.4.
7.4.2 UV-Vis Absorbance Spectroscopy

Visible absorbance spectra were taken from suspensions of SERS particles in 18 MΩ·cm in 1 cm path length cuvettes using a Cary 5000 UV-Vis spectrometer (Varian Inc., Palo Alto, CA, USA). Spectra were normalized by zeroing the baseline and scaling the peak absorbance to a maximum value of 1.

7.4.3 Dynamic Light Scattering and Measurement of Zeta Potential

Using a Zetasizer ZS (Malvern Technologies Ltd., Worcesthershire, UK) measurements were made on suspensions of citrate-coated AuNPs at 5.5ug/mL and PEG-coated SERS NPs at 11ug/mL in 9 mM NaCl at pH 7.2 measured at 25° C with an applied voltage of 5V. The Smoluchowski model was used for fitting [42]. The reported zeta potential values are the average of more than 20 runs for each sample. For hydrodynamic diameter and polydispersity index (PI) measurements, samples were placed in 1 cm path length cuvettes and values determined using cumulants analysis of 15 iterations for each sample. PI refers to the width of the Z-average intensity mean defined as the hydrodynamic diameter using equation 7.1::

\[ \text{PI} = \frac{2c}{b^2} \]  

Equation 7.1

where b and c are coefficients from the polynomial fit to the log of the autocorrelation function, G1 using equation 7.2 [43]:

\[ \ln(G1) = a + bt + ct^2 + dt^3 + et^4 + \ldots \]  

Equation 7.2

7.4.4 Transmission Electron Microscopy

TEM images were collected using a Hitachi H-7000 TEM at 75 kV with 50,000X magnification. Particles were mounted on carbon-coated copper grids from 5 μL drops of aqueous suspension and allowed to air dry.
7.4.5 Scanning Electron Microscopy

SERS images of nanoparticle labelled polystyrene microspheres were collected using a Hitachi S-5200 SERS with 10,000-50,000X magnification and 1kV accelerating voltage. Spheres were mounted on carbon stubs from 5μL drops of aqueous suspension and allowed to air dry before imaging.

7.4.6 Bicinchoninic Acid Assay

Assay samples were prepared according to kit instructions for advanced color development and absorbance was measured at 540 nm using a 96-well plate reader (Bio-Rad, Hercules, CA, USA). Nanoparticles were removed from the assay solution via centrifugation (three cycles at 6.8 g for 5 minutes) prior to visible absorbance measurements. To control for background color development, SERS particles without conjugated protein were simultaneously assayed and the control absorbance values were subtracted from the values for solutions of protein conjugated particles. The number of nanoparticles in each sample was calculated using visible absorbance values at 536 nm and the extinction coefficient provided by the manufacturer for 60 nm Au particles (3.531x10^{10} M^{-1}cm^{-1}).

7.4.7 SERS Enhancement Factor

The SERS enhancement factor resulting from adsorption of MG dye molecules to Au nanoparticle surfaces was calculated using equation 7.3:

$$EF = \frac{I_{SERS}}{I_{norm}} \frac{C_{norm}}{C_{SERS}}$$  \hspace{1cm} \text{Equation 7.3}

where $I_{SERS}$ is the intensity of the MG SERS spectrum at 1616 cm$^{-1}$, $I_{norm}$ is the intensity of the same peak of the normal Raman spectrum from aqueous dye solution, $C_{norm}$ is the dye solution concentration, and $C_{SERS}$ is the estimated concentration of dye adsorbed on to Au surfaces in a
SERS-active particle suspension based on the molar ratio of dye added to particles during preparation. More details about SERS enhancement factor are located in Chapter 3.5.1.

### 7.4.8 Raman Spectroscopy

Spectra were collected on an inverted microscope (Nikon TE2000, Nikon Instruments, Melville, NY, USA) and 40 X (0.6 NA) objective to focus the 632.8 nm HeNe laser onto the sample with a 1.30 μm^2 spot size. Rayleigh scattering was removed using a notch filter (λ = 632.7 nm, FWHM = 23.8 nm) and directed to the Czerny-Turner monochromator (Acton SP2560, Princeton Instruments, New Jersey, NY, USA) using an achromatic doublet lens (f6.6). A Peltier cooled CCD detector with 1340 x 400 pixel array (PIXIS BR400, Princeton Instruments) was used as the detector. Raman spectra were collected from 15 μL drops of nanoparticle or cell suspension placed on glass coverslips using 5-90 second integration times where noted.

### 7.4.9 Dark field Microscopy

Dark field scattering images were collected using the same microscope equipped with an oil immersion condenser and an objective (100X, 0.5-1.25 NA). Oblique illumination at the sample plane was accomplished using an objective iris and a dark field stop inside the condenser. Only light scattered by the sample entered the objective for imaging by the CCD camera (DS-Fi1, Nikon). The sum of pixel intensities within a consistently sized region of interest corresponding to locations of cells in a series of dark field images were collected from more than 100 cells for each treatment (NIS-Elements software, Nikon) and plotted as histograms to quantify SERS particle labelling density. Microscope and camera light intensities as well as integration settings were kept constant between all images used for Rayleigh intensity measurements. Background cell scattering is predominantly blue-white in digital images as reported elsewhere [44,45] and was therefore removed from the dark field images prior to intensity measurements by splitting the raw image in to its constituent red, green and blue channels and subtracting the blue channel intensity from the original 8-bit image. To confirm statistical significance between intensities of positive and control samples, the Wilcoxon Mann-Whitney rank sum test was used. Differences were considered significant at p < 0.05 (Igor Pro 6, Wavemetrics, Portland, OR, USA). Using
the same statistical test, pixel values from cell locations of samples labeled with non-specific IgG1 SERS were not found to be significantly more intense than those from unlabeled control sample.

7.4.10 Confocal Raman Mapping

Raman maps were collected using a Raman microscope (Renishaw InVia, Glouchester, UK) and 70 mW 638 nm diode laser. Spectra were taken in 1 μm steps in x and y directions with approximately 1 μm² laser spot size and 1 second integration time for each spectrum. Maps of the SERS label intensities were constructed using the results of direct classical least squares (DCLS) fits to each mixed spectrum over the cell surface, using individual SERS dye spectra and background Raman spectrum as inputs (WiRe 3.3 software, Renishaw). DCLS fitting determines the linear combination of component spectra that matches the mixed Raman spectrum of the sample with the lowest sum squared error; the fundamental equation of the model is the following equation 7.4:

\[ r_i = Sc_i + \epsilon_i \]  

Equation 7.4

where \( r \) is the response of the sample \( i \) at each wavelength, \( S \) is a matrix of the response of each analyte in the sample at each probe wavelength, \( c \) is the vector of all analyte concentrations in the sample that can give a response, and \( \epsilon \) is the vector of errors at each wavelength. SERS dye spectra and background Raman spectrum used as inputs were the average of 20 spectra from different locations of mapped control slides normalized to mean centre and unit variance. Therefore, the results of the DCLS fit yield the relative presence of each input dye or background spectrum in the mixed spectrum but should not be used to quantitatively estimate absolute SERS probe concentrations. A 4th order polynomial background was included in the DCLS fit to account for variance in the baseline of the mixed spectra from different locations over the cell sample. Pixel intensities of CV and EV fits to the LY10 triplex map (Figure 7.5A, 7.5C) were enhanced by a factor of 2 to resolve their locations from dense anti-CD20 MG labelling. Raman maps based on spectral intensity at 594.8 cm\(^{-1}\) were plotted with increasing pixel color intensity correlating with increasing spectral intensity at the Raman shift of interest.
To test the accuracy of the DCLS fitting procedure to estimate relative SERS NP concentrations, spectra were collected from mixed SERS NP solutions of known molar ratio as the average of 3 spectra with 50 second integration time each. A 4th order polynomial background was included in these fits to solution spectra to match the method used for DCLS mapping of cells.

7.4.11 Chronic Lymphocytic Leukemia Sample Selection

Peripheral blood samples were obtained from CLL patients during clinical follow-up. CLL diagnosis was confirmed by morphological and immunophenotypic analyses of white blood cells (WBC). Cell samples were classified as CLL upon testing positive for CD23, CD19, CD20, CD5 and negative for CD79b, CD10, CD103 with low surface density of immunoglobulin. Blood samples used for the experiments were selected to have a total WBC count in the range of 40-80 x 10^9/L and CLL cells over 90% of WBC. Mononuclear cells were isolated using the RBC lysis technique (Immuno Prep, TQ-Prep, Beckman Coulter, Mississauga, Canada).

7.4.12 LY10 Cell Line Culture

LY10 human B-cell lymphoma line was cultured in suspension in T25 flasks (Thermo Fisher) using Dulbecco’s Modified Eagle’s Medium (DME) with 15% fetal calf serum at 37°C with 5% CO₂ atmosphere.

7.4.13 Cell Labelling Using SERS Probes

Primary CLL cells or LY10 cell line were suspended in PBS with 1% fetal calf serum (FCS) added. Cells were incubated with SERS probes at a concentration of 5.6 pM at room temperature for 30 minutes. CLL cells were labeled with anti-CD45, anti-CD19 and anti-CD5 SERS probes individually, or in combination. LY10 cells were treated with anti-CD19, anti-CD45 and anti-CD20 SERS probes due to differences in surface protein expression between the two cell types. Cells were fixed in 4% formaldehyde solution in PBS for 15 minutes and either used as drops in suspension for solution SERS spectroscopy, or deposited on to glass microscopy slides using a cytocentrifuge for Raman mapping and dark field microscopy.
7.4.14  Polystyrene Microsphere Labelling Using SERS Probes

Streptavidin functionalized microspheres were suspended in solution using several repetitions of alternating vortexing and 30 second sonication in their solution as provided. The spheres were then washed using three rounds of centrifugation at 4000g for 10 minutes and the pellet was resuspended in 154 mM PBS following each round. Prior to microsphere labelling, the suspension medium for biotin-PEG-SH functionalized SERS particles was changed from 18.2 MΩ·cm water to PBS by centrifugation at 6800g and replacement of decanted water with buffer solution. PS spheres were incubated with SERS particles for 30 minutes at room temperature with agitation at 500 rpm in varying NP:bead ratios at a 150 μL total volume. Unbound particles were removed from SERS beads by 5 rounds of centrifugation at 100g and resuspension in PBS to a final concentration of nominally one million beads per mL for flow cytometry measurements. Three types of PS spheres were produced: each labelled with either MG, CV or DTCC SERS Au nanoparticles.

7.4.15  Flow Cytometry

To measure the relative expression of CD20, CD19, CD45 and CD5 in samples of CLL and LY10 cells, primary antibodies fluorescently labeled with: fluorescein isothiocyanate (FITC)-anti-CD20, phycoerythrin-cyanine 7 (PC7)-anti-CD19, phycoerythrin-texas red (ECD)-anti-CD45, and FITC-anti-CD5 were used. One million cells were suspended in PBS with 1% FCS and incubated with 1 μL of fluorescent antibody for 30 minutes at room temperature. For each sample, at least 5000 events were acquired on a Cytomics FC500 flow cytometer (Beckman Coulter, Mississauga, ON, Canada) and data plotted and analyzed using software (FlowJo, Treestar Inc, Ashland, OR, USA).

To evaluate SERS probe binding to cells using conventional flow cytometric methods, cells were subjected to a two-step labelling procedure for secondary fluorescence labelling. One million cells were suspended in PBS with 1% FCS added and incubated with either unlabeled primary antibodies raised against the surface protein of interest or the same primary antibody conjugated to SERS probes for 30 minutes at room temperature. Additional control samples were prepared.
in which SERS probes bound to non-specific IgG1 isotype antibodies or unconjugated SERS probes were used for the primary labelling. Background fluorescence intensity was established using cells treated with 5% albumin in PBS prior to labelling with fluorescent secondary antibodies. Secondary FITC-anti-mouse-IgG1 antibodies were then incubated with each of the cell samples, and FITC fluorescence was assessed from at least 5000 events for each sample.

To evaluate SERS probe labelling of LY10 cells via flow cytometry by collecting SERS directly, measurements were collected from both suspensions of LY10 cells and of PS microspheres that had been labelled with SERS nanoparticles.

For LY10 cell experiments, suspensions of LY10 cells in PBS were incubated with one positive and three control particle types: anti-CD20 MG SERS probes, anti-CD20 Au NPs without SERS dye, unconjugated MG SERS probes and unconjugated Au NPs without SERS dye, respectively, to a particle concentration of 5.6 pM for 30 minutes at room temperature. Excess particles were removed via centrifugation and cells were labeled using secondary anti-mouse IgG antibodies labeled with FITC for fluorescence flow cytometry measurements to confirm binding of SERS probes to cells. At least 20,000 events were collected for each sample using a flow cytometer (Gallios, Beckman Coulter) and analyzed using software (Kaluza, Beckman Coulter and FlowJo). An incident wavelength of 488 nm was used for forward angle scatter, side scatter and FITC fluorescence measurements. For SERS collection, the cells were probed using a 638 nm diode laser (0.07 mW/µm²) with a flow rate of approximately 10,000 cells/min and scattered light collected at 90° by replacing a broadband fluorescence filter in the instrument with a narrow bandpass filter to match the wavelength of an intense peak in the MG SERS spectrum (710 nm, 10 nm FWHM).

For all flow measurements of LY10 cells, only events recorded from live cells were used for analysis. Identification of dead cells was made by co-staining with 7-amino actinomycin D (7AAD), a fluorescent dye that can pass through membranes of dead cells and intercalate with double-stranded DNA therein. Following treatment with nanoparticle probes for SERS flow cytometry, approximately 70-80% of LY10 across all samples were living and included in data analysis. The threshold for background intensity in the SERS bandpass channel was set using LY10 labeled with 7AAD, SERS-inactive anti-CD20 Au nanoparticles, and secondary antibodies
labeled with FITC anti-IgG1 concurrently. To confirm statistical significance between intensities collected in the 710 nm SERS bandpass channel for cells labeled with anti-CD20 MG SERS NPs and anti-CD20 AuNPs without SERS dye respectively, the Kolmogorov-Smirnov test was applied using a two-tailed P value of 0.02. Additionally, log normal fits were applied to the intensity histograms for the SERS positive and negative samples (Igor Pro).

For flow measurements of SERS nanoparticle functionalized PS microspheres, the same flow cytometer and optical configuration was used with the exception that the 710/10 nm SERS filter was replaced by one of three optical interference filters that had a more narrow bandpass of 2 nm FWHM and nominally 80% transmission efficiency at the centre wavelength. Each filter had a centre wavelength corresponding to a major peak in the SERS spectrum for each type of SERS-NP labelled PS microsphere. The SERS channel for MG detection was preceded by a 711/2 nm filter, a 663/2 nm filter was used for CV labelled PS spheres, and a 693/2nm filter was used for DTCC labelled PS spheres. The probe wavelength for SERS was 638 nm and a low flow rate of 10 μL per minute was used in order to decrease the potential for multiple beads to pass through the flow cell detection volume simultaneously. To identify PS spheres in suspension, elastic light scatter in the small-angle forward, and orthogonal directions was collected using the 488 nm probe laser. To ensure that intensity events in the SERS channel were not influenced by autofluorescence from PS microspheres, a threshold for SERS positive events was set using the intensities in the SERS channel from a suspension of unlabeled PS microspheres. An additional fluorescence channel was also monitored to ensure that any background fluorescence from PS spheres did not vary between samples. A broadband 525/40 nm filter was used in this channel to collect intensity events using 488 nm as the probe wavelength. A minimum of 18,000 events were collected for each bead sample.

7.5 Results and Discussion

7.5.1 Characterization of SERS Gold Nanoprobes

Spectra of SERS probes prepared using MG, EV and CV dyes were complex but distinct (Figure 7.1A). Physisorption of positively charged MG onto negatively charged surfaces of citrate-
capped 60 nm Au NP surfaces resulted in a SERS enhancement of approximately 4 orders of magnitude compared with the intensity of the 1616 cm\(^{-1}\) band in the normal Raman spectrum of aqueous dye solution (Figure 7.1E). A comparison between the 1618 cm\(^{-1}\) band of the Raman spectrum from aqueous EV solution and EV SERS spectrum upon physisorption of the dye to Au nanoparticle surfaces revealed a 4 order of magnitude enhancement for this dye as well (Figure 7.2). Thiol terminated CH\(_3\)O-PEG-SH and COOH-PEG-SH form dative bonds with the Au surface and because there is not a significant decrease in SERS intensity following pegylation of the particles, we conclude that the polymer molecules likely do not compete significantly with the Raman dye molecules for surface binding sites. If a higher concentration of either CH\(_3\)O-PEG-SH or COOH-PEG-SH were used, or the order of addition of dye and PEG-SH were reversed, the PEG-SH could compete with dye molecules for binding sites of the Au nanoparticle surface.

TEM imaging was one of several methods used to confirm the coating of Au particles with CH\(_3\)O-PEG-SH and COOH-PEG-SH. In TEM images, the PEG layer is visible as a light grey ring surrounding the dense 60 nm Au that appears black in the image (Figure 7.1D and 7.3).

Visible absorbance measurements were used both to ascertain whether SERS probes were functionalized with CH\(_3\)O-PEG-SH and COOH-PEG-SH, as well as to confirm their monodispersity during preparation. A shift of the particles’ localized surface plasmon resonant (LSPR) wavelength from 536 to 538 nm was observed in UV-Vis spectra after coating with 5kDa CH\(_3\)O-PEG-SH (Figure 7.1B) indicating a change in the dielectric constant at the particle surface. The location of this peak remained constant following conjugation to monoclonal antibodies. For straight-forward interpretation of in vitro labelling results, monodispersed SERS probe solutions should be used for cell labelling. The formation of aggregates of spherical Au nanoparticles would be indicated by the appearance of a broad absorbance peak in the region of 700 nm [46,47]; no such absorbance was observed following each step of the SERS probe preparation.

DLS was also used to confirm nanoparticle functionalization and monodispersity (Figure 7.1C). An increase in hydrodynamic diameter from 60.7 +/- 0.1 nm for citrate-coated Au to 85.6 +/- 0.2 nm after coating with 5 kDa PEG is similar to values reported elsewhere [10,35]. The PI of the
particle suspensions decreased following PEG coating, which may have been due to the removal of trace aggregates in the stock solution following centrifugation, or the improved steric repulsion of the particles imparted by PEG coating. After conjugation of the probes to monoclonal antibodies, a small increase in the hydrodynamic diameter of the particles to 87.2 +/- 0.5 nm was observed (Figure 7.1C). The small increase in PI to 0.11 accompanied by a lack of absorbance peak in the 700 nm region for antibody-conjugated probes could indicate a non-uniform binding of protein to each particle in the suspension. Zeta potential values also confirmed the functionalization of SERS AuNPs with 5kDa CH₃O-PEG-SH, increasing from -40.4 +/- 14.2 mV for citrate-coated AuNPs to near neutral at -2.7 +/- 17.5 mV for PEG-coated SERS AuNPs (Figure 7.4).

Figure 7.1: (A) SERS spectra of 3 probe types used in in vitro labelling experiments. 1: cresyl violet; 2: malachite green isothiocyanate; 3: ethyl violet. Each is the average of 20 collections using 1 second integration time (offset for clarity). Inset: molecular structures of corresponding Raman-active dye molecules. (B) Visible absorbance spectra of SERS AuNPs after each NP functionalization step. Citrate capped Au (black; solid) LSPR wavelength shifts from 536 to 538 nm following coating with 5kDa PEG (black; dashed), maximum remains constant after conjugation to monoclonal antibodies (grey; dashed). (C) Dynamic light scattering spectra following each step in SERS NP preparation.
Hydrodynamic diameter increased from 60.7 +/- 0.1 for stock citrate Au (black; solid) to 85.5 +/- 0.2 nm after PEG coating (black; dashed). After conjugation to monoclonal antibodies, smaller and less consistent diameter increases are observed ranging from 86.3 +/- 0.2 to 88.1 +/- 0.4 nm (grey; dashed). (D) TEM image of PEG coated SERS probe. PEG coating is visible as light grey ring around dark 60 nm Au particle. (E) Comparison of Raman scattering intensities of 4 μM aqueous MGITC solution (black; dashed) and MGITC SERS Au NP suspension (black; solid). Integration time 5 seconds.

Figure 7.2: Comparison of Raman scattering intensities of 2 μM aqueous ethyl violet solution (black; dashed) and ethyl violet SERS Au NP suspension (black; solid). Integration time 15 seconds for Raman spectrum, 5 seconds for SERS spectrum.
Figure 7.3: TEM images of PEG-coated SERS probes. PEG coating is visible as a light grey ring around the dark 60 nm particles. Scale bar 100 nm.
Figure 7.4: Zeta potential distributions for 60 nm citrate coated AuNPs (black) and 5kDa CH₃O-PEG-SH-coated 60 nm SERS AuNPs (black dashed) as the averages of 22 and 68 measurements respectively. The averaged zeta potential value for citrate coated stock AuNPs is 40.4 +/- 14.2 mV, which increased after coating with 5kDa CH₃O-PEG-SH to -2.7 +/- 17.5 mV.

The BCA protein concentration assay was conducted on both antibody-conjugated SERS probe samples and control SERS probes where particles were coated with CH₃O-PEG-SH and COOH-PEG-SH only. Unconjugated Au particles also developed the colorimetric assay, therefore it was essential to distinguish background particle-based development from development due to the presence of protein in solution. This issue has also been reported elsewhere for PEG-coated Au spherical particles [48] and nanorods [49]. Though the cause is unclear, it may be due to the presence of PEG thiol groups in the sample. Antibody to particle ratios were determined using mean values from three separate batches of particles. BCA assay samples containing antibody-conjugated SERS probes exhibited increased absorbance at 540 nm as compared to control SERS probe samples with dye and CH₃O-PEG-SH/COOH-PEG-SH coating only. Protein to particle number ratio was estimated as 105 +/- 20, which is well below the theoretical maximum of 180 for a close-packed adsorbed monolayer of IgG1 molecules on the surface of a 60 nm diameter particle [50].
Figure 7.5: Assessment of DCLS fitting linearity of individual SERS probe spectra to mixed triplex solutions over a range of known molar ratios. (A) Varying CV SERS particle solution molar ratio, (B) EV, (C) MG.

DCLS fitting was used to identify the ratio of SERS particle concentrations in triplex mixtures. Spectra were collected from mixed solutions of SERS probes in known molar ratios, and DCLS was applied to assess the linearity of the fitting results (Figure 7.5). Different SERS probes typically have different signal strength, which must be accounted for to obtain mole fractions. A linear relationship does exist, however, between the percentage of total signal and the molar fraction in solution of each of the three probes (Figure 7.5). The relative brightnesses of these CV:EV:MG SERS nanoparticle probes are 3:1:1.8. To test whether DCLS was misidentifying the component spectra, Raman maps of 30 spectra were collected for each of CV, EV and MG probes individually. These pure spectral maps were fitted with all three component probe SERS spectra. For pure CV, DCLS identified the total signal as containing a 98 +/- 0.2% molar
composition as CV, for pure EV 95 +/- 2.3% of the composition was assigned as EV, and for pure MG, 96 +/- 0.5% of the composition was assigned to MG.

7.5.2 Binding Specificity of SERS Probes to CLL Cells

Anti-CD45, anti-CD19, and anti-CD5 SERS probes were tested individually for binding specificity in samples of primary CLL cells. The relative expression levels of the three cell surface molecules were evaluated using fluorescence flow cytometry and are provided in the Figure 7.6. The use of patient cells for SERS labelling provided a clinically relevant test because the cells were labeled using protocols similar to those for diagnostic immunophenotyping by flow cytometry. Primary CLL cells are complex biological samples to test and are often more difficult to analyze than standard cell lines because there is high variability in CLL cell content, surface protein expression levels, and cell robustness between samples. Based on fluorescence immunophenotyping shown in Figure 7.6, CD45 molecules are present in approximately one order of magnitude greater number on CLL surfaces than CD19 and CD5 which are expressed in similar numbers. This method is not fully quantitative however, because differences in the fluorescence quantum yields in each of the fluorophores used for labelling will impact the intensity of events recorded in each cytometer channel. The data provide an estimate of relative receptor expression and confirm that all three SERS Au nanoparticle targets are expressed by CLL cells.
Figure 7.6: Flow cytometric analysis of CD19, CD45 and CD5 expression by CLL sample used for triplex SERS staining. (A), (B) and (C) Scatter plots comparing simultaneous expression of CD5 and CD19, CD45 and CD5, as well as CD45 and CD19. (D), (E) and (F) Intensity histograms of the total expression of CD19, CD5 and CD45 throughout the CLL sample.

Raman spectra collected from drops of cell suspension showed greater relative intensities of characteristic SERS fingerprint for each probe if the cell suspension had been treated directly with an anti-CD protein probe, as compared to control samples where the corresponding CD proteins of interest were first blocked using unlabeled antibody (Figure 7.7). Because a reduction in the available binding sites for targeted SERS probes resulted in a decrease in SERS intensity from cell suspension, it is concluded that the SERS probes associated specifically with their intended surface protein targets.
Figure 7.7: Competitive binding experiments to assess the specificity of each SERS probe type for its intended surface protein target on CLL cells: (A) CD19, (B) CD45, and (C) CD5. Raman spectra were collected from drops of treated cell suspension after incubation of respective anti-CD SERS probes directly (solid lines) and after the CD molecule target was first blocked using unlabeled primary antibodies (dashed lines). Spectra of blocked cell suspensions are offset for clarity. Spectra were collected using a 90 second integration time. Also shown are SERS spectra of corresponding probe suspensions used to label cells (D) CV anti-CD19, (E) MG anti-CD45, and (F) BF anti-CD5.

Representative dark field images from CLL cells labeled with each type of SERS probe are provided in Figure 7.8, and show that positive labelling may also be differentiated from the non-specifically targeted control sample using the particles’ Rayleigh scattering.
Figure 7.8: Histograms of the sum of pixel intensities collected from identically sized areas in dark field images corresponding to B cells in samples of CLL cells. Open bars: intensities from CLL cells stained with non-specifically targeted IgG1-SERS NPs; solid bars: intensities from CLL stained with (A) anti-CD19 CV, (B) anti-CD45 MG, and (C) anti-CD5 BF. Corresponding representative dark field imaging of primary CLL cells stained using SERS Au NPs: (D) cells stained with anti-CD19 CV NPs, (E) anti-CD45 MG, and (F) anti-CD5 BF SERS NPs. (G) CLL cells stained with non-specifically targeted IgG10 SERS NPs.

7.5.3 Imaging SERS Probe Labelling of LY10

The relative expression levels of CD20, CD45 and CD19 cell surface molecules on LY10 were evaluated using fluorescence flow cytometry and are provided in Figure 7.9. As for CLL
fluorescence immunophenotyping experiments, this method provided an approximation of the relative expression levels between cell surface molecules targeted for SERS detection on LY10 cells. Based on the one parameter histograms and comparative two-parameter scatter plots in Figure 7.9, LY10 expressed CD19 in numbers approximately one order of magnitude less than for CD45 and CD20. All three SERS nanoparticle targets were expressed at levels detectable by fluorescence immunophenotyping and therefore were deemed to be suitable targets for SERS labelling.

Figure 7.9: Flow cytometric analysis of CD19, CD45 and CD20 expression by LY10 cell line. (A), (B) and (C) Scatter plots comparing simultaneous expression of CD20 and CD19, CD45 and CD20, as well as CD45 and CD19. (D), (E) and (F) Intensity histograms of total expression of CD19, CD20 and CD45 in LY10 sample. CD19 has approximately 1-1.5 orders of magnitude lower expression than CD20 and CD45.

Cell labelling on LY10 was characterized for each SERS probe type prior to triplex labelling (Figure 7.10). The LY10 cell line served as an excellent platform for testing the SERS particles since these cells are more robust than patient samples and have similar CD protein expression
levels between samples. Dark field microscopy is a widely used technique for imaging plasmonic particles and was therefore adopted to determine the location of SERS probes within labeled LY10 samples. The Au nanoparticles appear green-gold in dark field images due to intense Rayleigh scattering at their LSPR wavelength, 536 nm (Figures 7.10A, 7.10D, 7.10G) [51]. Dense cells also scatter light intensely, however this can be differentiated from particle scattering based on the predominantly blue-white character of the cells in dark field images [37,44,45]. The clustering of particles in biological samples results in plasmon coupling between adjacent particles and a red-shift of the LSPR wavelength [52,53]. Densities of cell labelling for each anti-CD SERS probe varied between CD targets as well as between positive and control samples as can be seen in the images and is quantified in the intensity histogram plots. Differences in the sum of the pixel intensities from cell locations between positive and control samples were found to be statistically significant and are plotted as histograms in Figures 7.10B, 7.10E, and 7.10H. Intensity histograms and representative dark field images of unlabeled LY10 cells as compared to the non-specific IgG1 isotype SERS probes are provided in the Figure 7.11.
Figure 7.10: Assessment of individual probe performance using LY10 B cell line. Top row: cells labeled with anti-CD45 CV SERS NPs; middle row: cells labeled with anti-CD19 EV SERS NP; bottom row: cells labeled with anti-CD20 MG SERS NPs as cell labels. (A), (D) and (G) Dark field scattering images of LY10 cells labeled with each SERS probe type respectively. (B), (E) and (H) Histograms of the sum of pixel intensities collected from identically sized areas corresponding to locations of cells in dark field images for 105 cells. Paired Wilcoxon Mann-Whitney, p<0.05 determined significance between positively- and non-specifically labeled populations. Open bars: intensities of control cells treated with non-specific IgG1-isotype conjugated SERS probes; solid color: intensities of cells labeled with each SERS probe respectively. Optical images of control cell samples are available in the Supporting Information. (C), (F) and (I) top: DCLS Raman maps of cells labeled with each type of SERS probe. Increasing color intensity shows locations where light scattering is better fit by input SERS dye spectrum using DCLS. Lower pixel intensities denote regions where the SERS spectrum being evaluated is a lesser contributor to the spectrum of light from the cell surface. (C), (F) and (I) bottom: bright field microscopy images of the cell regions corresponding to the area where spectra for the Raman maps were collected. Spectra (1), (2) and (3) correspond to highlighted pixels as noted in each Raman map.

Figure 7.11: (A) Histograms of the sum of pixel intensities collected from identically sized areas corresponding to locations of LY10 cells in dark field images for 105 cells. Open bars: intensities of untreated LY10; solid grey bars: intensities of cells labeled with non-specific IgG1-isotype SERS probes. (B) Representative dark field image of LY10 cells stained with IgG-SERS nanoparticles, and (C) no nanoparticle stain.
Representative Raman maps made of cells in each of the LY10 samples labeled with a single SERS probe are shown in Figures 7.10C, 7.10F, and 7.10I. The maps were constructed using the amplitudes of DCLS fits to each individual SERS probe spectrum and a background SERS spectrum to each cell. The maps demonstrate that the SERS intensity is strong enough to be detected from biological samples and may be positively identified using DCLS.

7.5.4 Triplex SERS Labelling of LY10

DCLS Raman maps were constructed using spectra collected from cell regions in LY10 samples that had been labeled with anti-CD20 MG, anti-CD19 EV, and anti-CD45 CV SERS probes simultaneously (Figure 7.12). Raman maps show that all three probes can be bound to the same cells, that each probe spectrum can be individually resolved from the mixture, and that the regions of best fit for each type of SERS probe also tend not to overlap with one another. Furthermore, the map regions that are best fit by each component probe spectrum are distinct from the regions of best fit by endogenous Raman scattering arising from the fixed cell slide (Figure 7.12E).

Figure 7.12: DCLS Raman maps of triplex labeled LY10. Cells were labeled with anti-CD45 CV, anti-CD19 EV and anti-CD20 MG SERS probes simultaneously. Increasing
pixel intensity denotes regions of best fit of each probe SERS spectrum to the mixed sample spectrum using DCLS analysis. (A) Areas corresponding to anti-CD45 CV SERS particles, (B) anti-CD20 MG SERS particles and (C) anti-CD19 EV SERS particles. (D) Overlay triplex SERS labelling map showing the presence of three SERS probe types bound to the same cell. (E) Bright field microscopy image of the cell region corresponding to the area where spectra for the Raman map were collected. (F) Raman map showing where the scattering is best fit to the background spectrum of the fixed cell slide, using the DCLS approach. Spectra (1), (2) and (3) correspond to highlighted pixels. Circled regions of spectrum (3) indicate regions where the mixed spectrum differs from the MG component spectrum.

The construction of Raman maps of mixed spectra based on the intensity of representative peaks from each component has been adopted elsewhere, however we found it to be less effective than DCLS fitting in our case because of peak overlap from different dyes and background scattering. Raman spectra were collected from a triplex labeled cell, and the SERS probe spectrum for CV was resolved from the sample mixture using either the intensity of a major peak in the CV spectrum (Figure 7.13A) or DCLS using the full CV spectrum (Figure 7.13B). Because the peak at 594.8 cm$^{-1}$ is intense and well isolated from bands in the spectra of MG and EV SERS probes, it was expected that the intensity and DCLS maps should correlate well in their placement of CV on the cell.

Figure 7.13: Comparison of techniques for resolving triplexed Raman mapping spectra derived from LY10 cells. (A) Intensity at 594.8 cm$^{-1}$, a major peak of the CV spectrum.
(B) Regions of best fit by CV spectrum using DCLS. Spectra (1) and (2) correspond to highlighted pixels.

While visually similar, in a few regions the two maps differ over the placement of CV. Pixel 1 (Figure 7.13) is an intense region in both of the maps and the spectrum that was collected at that point has both an intense peak at 594.8 cm\(^{-1}\) and a close resemblance to the full SERS spectrum of CV. Pixel 2 however is highlighted in the intensity map, but is not identified as having a high fitting coefficient to CV by DCLS. The spectrum obtained from pixel 2 does have a high intensity value at 594.8 cm\(^{-1}\), but this intensity originates from a high baseline of a mixed spectrum resembling MG. Alternatively, this issue could be prevented by constructing Raman maps using the difference between peak and baseline intensities. The complex spectra of the SERS probes used in these experiments would complicate the calculation of a baseline value adjacent to each band of interest. Therefore, DCLS fitting was adopted because it uses the shape of the full mixed spectrum to isolate each component.

7.5.5 Fluorescence Flow Cytometry to Confirm SERS Probe Binding to CLL Cells

CLL cells subjected to positive and control SERS probe treatments were subsequently labeled with FITC-anti-IgG1 secondary antibodies, and FITC fluorescence intensity was evaluated using flow cytometry. The benefits of measuring fluorescence intensity from secondary antibodies using flow cytometry in these experiments are twofold: 1) flow cytometry rapidly measures the light emission from thousands of individual cells thus providing an accurate distribution in density of sample labelling and 2) flow cytometry is an essential instrument for clinical immunophenotyping, and it is important to confirm the stability of the targeted SERS probe binding to cells during flow cytometric measurements. An increase in FITC fluorescence intensity for cell populations treated with anti-CD5, anti-CD19 and anti-CD45 conjugated SERS probes was observed above that obtained from the control population which had been incubated with only 5% albumin in solution before secondary antibody labelling (Figure 7.14A, 7.14B, 7.14C). This increase in FITC fluorescence was comparable to the intensity from cell samples that had been primary labeled using the corresponding unconjugated antibodies (Figure 7.14). CLL cells that were primary labeled with either unconjugated or non-specific antibody
conjugated SERS probes did not show significantly increased FITC fluorescence intensity above that of the albumin sample (Figure 7.14D). The increase in FITC fluorescence emission observed for positively targeted cell samples over emission from controls indicates that primary antibodies were successfully conjugated to the SERS probes and that the conjugated probes are present on CLL cell surfaces.

Figure 7.14: Fluorescence flow cytometry was used to evaluate binding and specificity of each SERS probe type. CLL samples were primary labeled with either unconjugated (black; dashed) or SERS probe conjugated antibodies (black; solid) against the CD surface protein of interest, followed by secondary labelling with fluorescent FITC anti-mouse-IgG1 antibodies, specific for the primary antibodies. Background fluorescence intensity was established by treating cells blocked only with 5% albumin with FITC secondary antibodies (grey; solid). (A) Changes in fluorescence intensity for CD5 targeting, (B) CD45 targeting, and (C) CD19 targeting. (D) Primary labelling controls using non-specific IgG1-SERS probes (black; solid), and SERS probes with PEG coating only.

7.5.6 Flow Cytometry for SERS Detection

Flow cytometry was employed to collect SERS directly from probes bound to LY10 cells. The spectral overlap between the optical interference filter used to collect SERS and the MG SERS
spectrum are shown in Figure 7.15. The bandpass filter had maximum transmission corresponding to the location of the most intense peak of the MG SERS spectrum when 638 nm was used as the probe wavelength.

![SERS spectrum](image)

**Figure 7.15:** Red region indicates bandpass of the optical filter used to collect MG particles’ Raman scattering in the flow cytometer. The filter having maximum transmission of 58% at 710 nm, FWHM 10 nm overlaps with the region of the MG SERS spectrum highlighted in red when 638 nm excitation is used. Solid red lines demarcate 705, 710, and 715 nm respectively.

Anti-CD20 Au nanoparticles, both with and without embedded MG Raman marker, were confirmed to have bound to LY10 based on the intensity of fluorescence emission from secondary FITC-anti IgG1 antibodies. Density plots of FITC fluorescence versus SERS channel intensity for labeled LY10 cells are provided shown in Figure 7.16. Though there were differences in the FITC fluorescence intensity between the two positively targeted samples, in both cases fluorescence intensity was greater than for LY10 first labeled with SERS-active unconjugated control nanoparticles, or 7AAD viability stain only. The distribution of FITC fluorescence events versus intensity events in the SERS channel confirm that SERS signal originates from probes bound to LY10 cells.
Figure 7.16: Flow cytometry two-parameter density plots for LY10 treated with (A) SERS-active MG anti-CD20 Au nanoparticles, (B) SERS-inactive anti-CD20 Au nanoparticles, (C) antibody-unconjugated SERS-active Au nanoparticles, and (D) 7AAD cell viability stain only. All samples were subsequently labeled with fluorescent FITC secondary antibodies specific for the Fc region of primaries conjugated to SERS nanoparticle probes.

Intensity above the baseline threshold in the SERS bandpass channel was observed only for LY10 labeled with anti-CD20 SERS-active probes indicating that the intensity from the probes prepared herein is sufficient for detection using a commercial flow cytometer. Though only 8.5% of live LY10 labeled with targeted SERS-active probes (Figure 7.17A) registered scattering events above the SERS channel threshold, this percentage is statistically significant compared to control samples where less than 1% of the population registered above the threshold for anti-CD20 SERS-inactive treatment (Figure 7.17A) and less than 0.01% of the population for LY10 labeled using untargeted particles (Figure 7.17C). Log normal fits to SERS channel intensity distributions revealed two populations within the SERS positive LY10 sample. The geometric mean of the more intense distribution was significantly shifted and could be clearly distinguished from the SERS negative log normal distribution (Figure 7.17B).
Figure 7.17: Flow cytometry single parameter histograms of intensity in the SERS bandpass channel for LY10 cells. (A) Cells labeled with anti-CD20 MG SERS-active particles (open curve), and anti-CD20 SERS-inactive particles (grey). (B) Log normal fits to SERS intensity histograms in A. The SERS positive LY10 (black, dashed) histogram is fit with two distributions having geometric means of 1230 +/- 60 and 5400 +/- 1300 respectively. The geometric mean for the SERS negative sample log normal distribution (black, solid) is 1024 +/- 5. (C) LY10 labeled with SERS-active particles lacking anti-CD20 antibody (open curve), and unconjugated anti-CD20 antibody (grey).

7.5.6.1 Flow Cytometry For Detection of SERS From Nanoparticle Labelled Polystyrene Microspheres

Flow cytometry was also used to collect SERS from PS microspheres that had been labelled with SERS-active Au nanoparticles in order to demonstrate a proof-of-concept collection of multiple SERS spectra using filter-based spectral resolution for flow cytometry. There are three advantages to testing SERS flow cytometric detection using labelled PS spheres: The first advantage is that attaching SERS nanoparticles to a larger sample object such as a microscale sphere, instead of detecting them directly from suspension, enabled intensity events to be more clearly attributed to the SERS particles and differentiated from sample debris and/or detector noise. Secondly, PS sphere labelling should in theory be more reproducible between batches than labelled cells due to potential variation in expression levels of targeted cell surface molecules between CLL donor samples, or cell line cultures. As a third advantage, the high affinity biotin-streptavidin interaction chosen to target SERS NPs to spheres eliminated the requirement for additional bioconjugation chemistry to attach targeting antibodies to Au NPs. This mitigated uncertainty in nanoparticle binding efficiency between batches that could be
introduced due to differential reaction yield or orientation of antibody binding sites relative to SERS NP surfaces. The objective of this investigation is to establish a set of methods for producing SERS calibration beads with reproducible intensities that could be used to quantify flow cytometer measurements for future SERS experiments. Additionally, determining whether SERS NPs are intense enough to be detected with very narrow bandpass filters is important to their application in higher order multiplexed labelling protocols using filter-based flow cytometry.

The filters chosen for collection of SERS from each of MG, CV, and DTCC labelled PS beads corresponded to an intense peak in each SERS spectrum. The spectral overlap between the SERS spectra and each of the optical interference filters are shown in Figure 7.18.

Figure 7.18: Spectral overlap between SERS spectra of nanoparticles used to functionalize PS microspheres with transmission wavelengths of optical interference filters used to
collect SERS in the flow cytometer. Vertical red lines on each spectrum indicate bandpass of the optical filter used to collect each SERS spectrum. A) MG SERS spectrum overlayed with transmission of 711 nm SERS bandpass filter, b) CV SERS spectrum and corresponding 663 nm bandpass filter, and C) DTCC spectrum and corresponding 693 nm bandpass filter. Each filters have maximum transmission at their centre wavelengths of approximately 80% and FWHM of 2 nm.

In order to determine whether there was a high degree of polydispersity in the SERS NP-functionalized spheres, DLS would have been the optimal method for evaluation of large sample populations. Once spheres were functionalized with large 60 nm SERS Au NPs however, they became too heavy for the cumulants algorithm to fit the correlogram describing their Brownian motion within acceptable error. Therefore, SEM imaging was used to evaluate whether biotin-SERS NPs bound to PS microspheres. Representative SEM images are provided in Figure 7.19. The pattern of NP labelling over the full PS sphere is shown in 7.19A. To confirm whether species bound to the sphere were SERS NPs, higher magnification images were also collected (Figure 7.19B). Upon closer inspection of the PS sphere surface(s), it became evident that the objects bound thereto were of dimensions consistent with the 60 nm Au NPs used to make the SERS probes.

Figure 7.19.: Representative SEM images of streptavidin-PS microspheres labelled with biotin-SERS Au nanoparticles. A) 10,000X magnification, B) 50,000X magnification.

Forward and orthogonal light scattering profiles of 488 nm laser light from PS spheres was used to differentiate the spheres from noise or sample debris for flow cytometric SERS measurements.
Unlabelled PS spheres were used to set the baseline intensity in the SERS channel for each of the three SERS dyes used. Any intensity events in the SERS channel above the baseline set by control beads were attributed to SERS from functionalized Au nanoparticles. One-parameter histograms of integral peak intensities from SERS-PS spheres in each SERS channel are plotted against histograms obtained from unlabeled spheres (Figure 7.20). For each type of SERS NP-PS bead, SERS spectra were detectable using narrow-band filter-based flow cytometry.

Figure 7.20: One parameter histograms of integral intensity in the SERS bandpass channel for each type of SERS NP-labelled PS microsphere. Intensity histograms from unlabeled control PS spheres are shown in red, and SERS-NP functionalized spheres in blue. A) MG SERS NP-labelled spheres using the 711 nm SERS bandpass filter, B) CV SERS NP-labelled spheres using the 663 nm SERS bandpass filter, and C) DTCC SERS NP-labelled spheres using the 693 nm bandpass filter.
7.6 Conclusions

PEG-coated SERS Au nanoparticle probes have been demonstrated as effective cell labels for the simultaneous detection of three surface proteins of interest on malignant B cells. Raman mapping using DCLS fits to triplex spectra resolved the locations of each probe type on the cell. The specificity of the antibody-conjugated SERS probes for their surface protein targets was also demonstrated for both LY10 cell line and primary CLL cells using dark field microscopy and Raman spectroscopy of cells in suspension, respectively. Flow cytometry was an effective method for detecting the labelling by anti-CD20 SERS probes on LY10 using both fluorescence experimental protocols as well as SERS from the probes themselves. A set of methods for the production of SERS flow cytometry calibration beads was established that will be significant in the adoption of SERS labels for quantitatively evaluating cell surface receptor expression levels. The detection of SERS from specifically targeted cells using flow cytometry is a significant step in the development of SERS immunophenotyping to improve the sensitivity and specificity of blood cancer diagnoses.

7.7 Contributions

The author prepared the bioconjugated SERS Au nanoparticles and undertook characterization measurements (UV-Vis, DLS, zeta-potential, BCA assay), treated cell samples, performed the Raman spectroscopy, confocal Raman mapping, dark field imaging, SERS flow cytometry from PS beads, all data analysis and wrote the paper. The author gratefully acknowledges Nisa Mullaithilaga for isolating primary CLL cells, preparing samples for fluorescence flow cytometry and assisting with collection of flow measurements. The author would also like to acknowledge Guisheng Yang for LY10 cell culture; Shell Ip for advice on zeta potential measurements; Annie K.W. Bang for assistance with collection of flow cytometry data; and Ilya Gourevich for EM imaging.
7.8 References


8 Dual-mode Dark Field and SERS Liposomes for Lymphoma and Leukemia Cell Imaging

8.1 Abstract

Multifunctional probes are needed to characterize individual cells simultaneously by different techniques, to provide complementary information. A preparative method and an *in vitro* demonstration of function are presented for a dual-function dark field microscopy/surface enhanced Raman scattering (SERS) liposome probe for cancer. Dark field microscopy and SERS represent new combined functionalities for targeted liposomal probes. Two methods of antibody conjugation to SERS liposomes are demonstrated: (i) direct conjugation to functional groups on the SERS liposome surface, or (ii) post-insertion of lipid-functionalized antibody fragments (Fabs) into preformed SERS liposomes. In vitro experiments targeting both the lymphoma cell line LY10 and primary human chronic lymphocytic leukemia (CLL) cells demonstrate the usefulness of these probes as optical contrast agents in both dark field and Raman microscopy.

8.2 Introduction

Multifaceted medical diagnostic imaging strategies are increasingly valuable for the diagnosis of various cancers [1,2], as a way to combine distinct tests simultaneously. In particular, multifunctional nanoparticles are promising, to exploit the capabilities of different imaging techniques and to reduce the quantity of contrast agents introduced in to the body. This chapter investigates the first demonstration of the incorporation of surface enhanced Raman scattering (SERS) contrast agents into specifically targeted stealth liposomes for imaging cell surface protein receptors. SERS nanoparticles both with and without embedded reporter molecules have demonstrated utility for the study of biological systems due to the bright and specific spectra generated by them, as has been recently reviewed [3,4]. The unique development of the targeted liposomes reported here is, they are carriers for modified gold nanoparticles (AuNPs) that impart dual mode imaging functionality for both dark field and SERS microscopy. This brings a first advantage because dark field microscopy can be used to rapidly characterize liposome binding to cells using widely available and inexpensive equipment. As a second advantage, the specific
fingerprint SERS spectra provide unambiguous identification of the probe with the potential for multiplexed labelling that can be designed for excitation and detection in the biologically significant near infrared window [5]. Furthermore, the AuNP can potentially be used as an agent for photothermal therapy [6], though that functionality was not explored in this study.

Liposomes are well established as drug delivery vehicles, and have additionally demonstrated efficacy as multifunctional probes that also act as (carriers for) contrast agents in ultrasound [7] fluorescence [8,9] magnetic resonance (MR) [10,11] computed tomography (CT) [12], and positron emission tomography (PET) [13] imaging. Because liposome technologies have been so aggressively investigated, myriad formulations have undergone development and FDA testing for clinical use since the 1990’s [14]. This had led to a number of commercialized and clinically adopted technologies such as Myocet and Depocyt, as well as a number of promising formulations currently in FDA phase I/II/III trials [15].

A major challenge of SERS labelling has been predicting and therefore tailoring the in vivo distribution of the particles over both the short and long-term in addition to reducing their toxicity profile. Because of the maturity of liposome technology in pharmaceutical applications, many chemically modified lipid species have been commercially developed to engineer the pharmacokinetic, payload uptake and release, reporting and targeting functionalities of liposomes. Furthermore, these properties and techniques of incorporating them into liposomes have been well studied in literature, making liposomes both versatile and accessible as carriers for inorganic nanoparticles in biomedical applications. The liposome formulations used to prepare SERS particles for this study are common to clinical liposomal drug delivery vehicles. Three components of the lipid formulation presented here are the constituents of clinically adopted Doxil, while the other two are used in extended release liposomes such as INX-0125 which is currently undergoing FDA trials [16].

While other nanoparticle encapsulation strategies exist such as covalent polymer attachment [17], silica encapsulation [18], and DNA coating [19,20], liposomes offer the potential to more straightforwardly incorporate the aforementioned multifunctionality, using materials whose biological interaction and behavior is well understood. Several examples exist in literature where lipids have been used to encapsulate nanoparticles [21–23]. Presently, we build upon these studies by integrating antibody targeting and stealth liposome technology to produce SERS
liposomes and demonstrate (i) the specific labelling of both lymphoma cell-line and hematologic neoplasms, and (ii) the use of these probes as contrast agents for both dark-field and SERS microscopy.

Chapter 5 of this thesis investigated the design and preparation of surface enhanced Raman scattering (SERS) liposomes consisting of a 60 nm gold nanoparticle encapsulated with a ternary lipid mixture, where the lipids both stabilize the gold nanoparticle colloid, and enable three methods of incorporating three classes of Raman reporters [21]. In this chapter, SERS activity was imparted to the liposomes by physisorbing charged Raman reporter molecules to the Au nanoparticle surface. This strategy was adopted because, of the three methods previously developed, it was designed to position the dye closest to the nanoparticles’ plasmonic surface, resulting in optimum SERS enhancement.

To capture the versatility and modularity that make liposomes advantageous, two approaches to incorporate targeting antibodies into SERS liposomes were employed. One approach involves conjugating antibody fragments to a lipid species, and incorporating them into prepared lipid-encapsulated SERS nanoparticles via post-insertion [24]. This approach is illustrated in Figure 8.1A). The other approach involves performing the conjugation chemistry directly on the surface of lipid-encapsulated SERS nanoparticles using established chemistry [25, 26]. This approach is illustrated in Figure 8.1B).
Figure 8.1: Illustration of two methods of bioconjugation. A) Antibody digestion into F(ab’)₂ and coupling of Fab’ to maleimide functionalized PEG-DSPE, and “post-insertion” into preformed SERS liposome. B) Direct conjugation of whole antibodies to SERS liposomes bearing carboxy-functionalized PEG-DPPE using established EDC/Sulfo NHS chemistry, which forms an amide bond between a carboxyl group on the SERS liposome and a free amine on the antibody. (From Ip et al. 2014, submitted).

To enable antibody-targeting functionality, several design modifications were made to the liposomal layer. First, lipid-anchored polyethylene glycol (PEG) was added to improve colloidal stability in buffer. Second, because the particles are intended for applications in buffer environments, the nanoparticles were encapsulated in buffer to minimize osmotic pressure across the lipid layer. Finally, for direct conjugation of antibodies, additional negatively charged lipids were added to help stabilize the particles during the multiple centrifugation steps required.
Prior to the consideration of SERS liposomes for in vivo experimentation or assessment of viability for future therapeutic applications, we demonstrate herein in vitro specificity through the use of SERS liposomes as optical reporters in a fashion similar to prior immunophenotyping experiments [27]. SERS liposomes bearing antibodies against B cell lineage markers useful in the diagnosis of haematologic cancers, were incubated with a lymphoma cell line as well as with primary human chronic lymphocytic leukemia (CLL) cells in vitro, with SERS liposomes bearing nonspecific isotype control antibodies serving as controls. In vitro studies are sufficient for diagnosis of haematologic cancers and we have selected markers relevant both to B cell immunophenotyping as well as having value as targets for both commercialized liposomal drug carriers [28] and therapeutic antibodies [29] in the treatment of blood cancers.

Successful targeting was observed and quantified by dark field microscopy and compared with non-specifically targeted controls. SERS functionality was confirmed by Raman mapping. The particle properties were characterized by UV-Vis and Raman spectroscopy. Conjugation was characterized by BCA assay, as well as by secondary antibody labelling with TEM and flow cytometry contrast agents.

8.3 Methods and Materials

The following materials were used without further purification: Dioleoylphosphatidyl choline (DOPC), egg sphingomyelin (ESM), ovine cholesterol (chol), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (mPEG-DPPE), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (COOH-PEG-DPPE), and dipalmitoylphosphatidic acid (DPPA) (Avanti Polar Lipids, Alabaster, AL) 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[maleimido(polyethylene glycol)-2000] (male-PEG-DSPE) (Creative PEG Works, Winston Salem, NC, USA). 60 nm diameter Au nanoparticles, 5 nm gold conjugates (immunogold) (British BioCell International, Ted Pella, Redding, CA, USA). Malachite green isothiocyanate (MG), Dylight 488-goat anti-mouse (H+L) antibodies (LifeTechnologies, Grand Island, NY, USA). 1-Ethyl-3-[3 dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (sulfo-NHS), microbicinechinonic acid (BCA) assay, murine antibody fragmentation kit, Dulbecco’s Modified Eagle’s Medium (DME) (Pierce-Thermo Scientific, Rockford, IL, USA). Immunoprep reagent
system/TQ-Prep workstation (Beckman Coulter, Mississauga, ON, Canada). Murine anti-human CD19, murine anti-human CD20, murine IgG1 isotype antibodies (Abcam, Cambridge, MA, USA).

8.3.1 Lipid Preparation

The ternary lipid mixture of DOPC, egg sphingomyelin, and ovine cholesterol at a 2:2:1 molar ratio, were prepared as 2.14 mg aliquots as previously described in Chapter 5 and reference [21].

The basis for the encapsulating lipid bilayer was prepared as described in Chapter 5, and reference [21]. DOPC, egg sphingomyelin, and ovine cholesterol in a 2:2:1 molar ratio were prepared as lyophilized 2.14 mg aliquots.

To modify bilayer coatings for use with in vitro cell samples, additional lipid species were incorporated. mPEG-modified lipids were included to reduce non-specific association of liposomes with cells, and COOH-PEG-modified lipids, and charged DPPA lipids were included in the encapsulating layer to facilitate on-particle conjugation to targeting antibodies. Aliquots of these other lipid species were prepared as for DEC221 whereby choloform solutions were aliquotted, lyophilized and stored at -20°C until use. Masses of DPPA aliquots were calculated to correspond to the same molar amount as cholesterol in each DEC221 aliquot. Each of the mPEG(2kDa)-DPPE, and COOH-PEG(2kDa)-DPPE aliquots corresponded to approximately 6 mol% of a DEC221 aliquot. For conjugated to antibody fragments, maleimide-PEG(3.4kDa)-DSPE was aliquoted in to 6.5 μg quantities.

To prepare MLVs for post-insertion of Fab-functionalized lipid molecules: one DEC221 aliquot, and one mPEG(2k) aliquot were each hydrated as previously described in Chapter 5 with modification that 2x PBS was used in place of water. After 20 minutes was allotted for hydration, the aliquots were mixed and divided in to equal 1 mL portions, and incubated for an additional 10 minutes at 50°C.

To prepare MLVs for conjugation of whole IgG1 antibodies on to finished SERS Au NPs directly, the following aliquots were hydrated as for DEC221 in Chapter 5, with the exception that 2x PBS was used in place of water, and added to a total volume of 2 mL: 2x DEC221, 2x
mPEG(2k)-DPPE, 2x DPPA, and 1x COOH-PEG(2k)-DPPE. After 20 minutes of incubation at 50°C and occasional vortexing, the aliquots were combined, divided in to equal 1 mL portions, and incubated for an additional 10 minutes.

8.3.2 Gold Nanoparticle SERS Functionalization

Aqueous solution of MG was added dropwise to Au colloid to a final concentration of approximately 33 nM while stirring throughout. Functionalization of the Au nanoparticles with MG was immediately evaluated by collection Raman spectra from 20 uL drops of particle suspension prior to liposome encapsulation. Procedure for collection of Raman spectra provided in subsequent sections.

8.3.3 SERS Liposome Encapsulation

MG-functionalized 60 nm Au NPs were encapsulated with liposomes as described in Chapter 5.4.3.3 with the exception that shorter sonication times were found to be effective for the formation of SERS liposomes. Sonication for 15 minutes were sufficient, and nanoparticle/lipid suspensions could be stored, refrigerated at 4°C until functionalization.

8.3.4 Antibody Functionalization

For SERS liposome functionalization with antibody fragments: Prior conjugation to lipid molecules, Fab fragments had to be produced from whole anti-CD20 IgG1 monoclonal antibodies. The first step of digestion of IgG in to F(ab’)2 fragments was accomplished using a commercial kit (Thermo Fisher Scientific, Rockford, IL, USA), without modification to the manufacturer’s instructions. To produce maFab fragments, the method of Shahinian and Silvius (2004) [30] was used with the exception that maleimide-containing liposomes were replaced by mal-PEG(2k)-DSPE in an 8-fold molar excess in reaction with F(ab’)2 fragments. The final Fab-PEG-DSPE product, once the reaction is quenched, is purified and concentrated to ~50 μL total
volume, using 10 kDa molecular weight cut-off Amicon Ultra centrifugation filters. The concentration of protein in the final product is determined by measuring the visible absorbance spectrum and using the OD at 280 nm along with a molar extinction coefficient of 83,800 M$^{-1}$ cm$^{-1}$. To incorporate Fab-PEG-DSPE in to SERS liposome coatings, ~0.42 μg of Fab solution was added to 1 mL of SERS liposomes and incubated for 5 hours at 37°C with agitation (shaker-incubator: Isotemp, Fisher Scientific, Ottawa, ON, Canada)

For SERS liposome functionalization with whole IgG1 antibodies: Bioconjugation was performed directly on to finished SERS liposomes, using EDC and sulfo-NHS mediated cross-linking of COOH-functionalities in the lipid bilayer, and available amine groups of the antibody. The procedure is modified from the methods used for PEG-coated particle conjugation (Chapters 4, 6, 7, and 9). 0.25 µg anti-CD19 antibodies were added to 0.5 mL of NHS ester activated SERS liposomes suspended in water, and incubated at ambient temperature for 4-5 hours with gentle agitation. Excess antibodies were removed by repeated centrifugation and resuspension in water. Following all washing steps, to ensure SERS liposome stability and their suitability for use as *in vitro* labels, the antibody-conjugated SERS liposomes were diluted 1:1 with 2x PBS.

### 8.3.5 SERS Nanoparticle Characterization

The colloidal stability of the particles was determined by UV-Vis spectroscopy. To determine whether significant aggregation had occurred, absorbance spectra were examined for the presence of a shoulder or second peak to the red of the localized surface plasmon resonance absorbance band [31,32]. The absence of a peak or shoulder was deemed to be sufficient for subsequent use.

SERS spectra of the particles were also collected to verify SERS activity. A 50 µL drop of the SERS liposome suspension was placed on a cover slip and probed using a Raman microscope consisting of a monochromator (Acton SP500i, Princeton Instruments, Acton, Massachusetts) connected to an inverted microscope (Nikon, etc.) employing a HeNe laser (CVI Melles Griot, Albuquerque, New Mexico) as the monochromatic source. Light scattering was collected using a CCD detector (PIXIS BR400, Princeton Instruments). The SERS spectrum of malachite green was recorded with 1 second integration time.
Antibody conjugation was assessed using several methods including the colorimetric bicinchoninic acid (BCA) protein assay, Transmission Electron Microscopy (TEM) with a secondary antibody immunogold contrast agent, and flow cytometry using fluorescent secondary antibodies.

Protein concentration from conjugated SERS liposome samples was also directly evaluated using a BCA protein assay. Because the presence of lipids [33] and Au nanoparticles [34,35] in the assay solution may also result in color development, the assay was performed on SERS liposomes both protein-conjugated, and unconjugated. The values for color development were normalized to the number of particles in each sample suspension, and absorbance values obtained from control SERS liposome suspensions were subtracted from the absorbance values obtained from protein-conjugated particle suspensions. The assay was otherwise performed according to manufacturer’s instructions. Immediately prior to absorbance measurements at 562 nm, nanoparticles were removed from the assay solution via centrifugation at 9.6 xg for 5 minutes.

For TEM characterization, an antibody-specific TEM contrast agent consisting of goat anti-mouse IgG secondary antibodies bound to 5 nm gold nanoparticles was used to visualize primary antibody conjugation to SERS liposomes. The 5nm Au NP conjugated to secondary antibodies against murine IgG will bind to the murine antibodies on the SERS liposomes and those that remain associated with the SERS liposomes after several washing steps will be visible under TEM. SERS liposomes conjugated with mouse mABs and Fabs were incubated with the immunogold labels for 20 min at room temperature (~20°C). Unbound immunogold labels were separated from SERS liposomes by repeated centrifugation and resuspension using parameters described above. Several 0.5µL drops of the resulting SERS liposome suspension were deposited on lacey carbon-coated TEM grids (Ted Pella) and allowed to dry. The TEM grids were imaged in a Hitachi H7000 TEM at 50kV accelerating potential.

Flow cytometry was also employed to characterize conjugation. Dylight 650-conjugated goat anti-mouse (H+L) secondary antibodies were employed as fluorescent probes for the presence of primary murine antibodies on the surface of the SERS liposomes. Fab conjugated SERS liposomes and unconjugated control SERS liposomes were each incubated with the fluorescent secondary antibodies for 20 min at RT to allow for binding. Unbound secondary antibodies were
removed by centrifugation using parameters described above. The secondary-fluorescently labeled SERS liposomes were diluted and run through a Gallios flow cytometer (Beckman Coulter, Mississauga, ON, Canada) and data plotted and analyzed using Kaluza (Beckman Coulter) and Flowjo (Treestar) software. A minimum of 6000 events were collected for each sample using 638 nm laser. Unconjugated SERS liposomes both with and without secondary antibody treatment were used to determine the background fluorescence intensity. Baseline forward and side scattering profiles were established using unfunctionalized stock Au NPs. SERS liposome conjugation was verified upon comparison of peak fluorescence intensity histograms between conjugated samples and unconjugated controls.

8.3.6 Cell Culture

The OCI-LY10 human diffuse large B cell lymphoma line [36] was cultured in T25 flasks (Thermo Fisher) in suspension using DME supplemented with 15% fetal calf serum in a 5% CO₂ atmosphere at 37°C.

8.3.7 Selection and Preparation of CLL Samples

Peripheral blood samples were obtained from CLL patients during clinical follow-up. Both morphological and immunophenotypic analyses of white blood cells (WBC) were used for CLL diagnosis. Cell samples were considered to be CLL upon testing positive for CD19, CD20, CD23 and CD5, testing negative for CD10, CD103 and CD79b with low surface immunoglobulin expression. Blood samples selected for this study had a total WBC count within (40-80) x 10⁹/L with CLL cells representing 70-90% of all WBC. Mononuclear cells were isolated using the red blood cell lysis technique (Immunoprep/TQ-Prep) and the cells were suspended in PBS supplemented with 1% FCS and stored at 4°C until use.
8.3.8 Flow Cytometry for Immunophenotyping of LY10 and CLL

One million cells for each sample were suspended in PBS and incubated with 1μL of each of primary murine monoclonal fluorescein isothiocyanate (FITC)-anti-CD20 and phycoerythrin-cyanine 7 (PC7)-anti-CD19 antibodies (eBioscience, San Diego, CA, USA). After 30 minutes, cells were centrifuged at 1200 xg to remove unbound antibodies and evaluated using flow cytometry. A minimum of 5000 events were acquired for each sample using instrumentation as for nanoparticle characterization described above.

8.3.9 Cell Labelling Using SERS Liposomes

LY10 cell line or CLL cells were incubated with either anti-CD20 fab- or anti-CD19-antibody conjugated SERS liposomes to a final nanoparticle concentration of 5.6 pM in PBS with 1% FCS at room temperature for 30 minutes. To evaluate the specificity of the interaction between SERS liposomes and their intended surface protein targets, additional samples of LY10 and CLL cells were also incubated with non-specifically targeted IgG1 isotype Fab-SERS liposomes or whole antibody-SERS liposomes respectively. Unbound SERS liposomes were removed using multiple centrifugation steps at 1000-1200 xg for 5-10 minutes each. For dark field microscopy and Raman mapping, treated cell samples were fixed using 4% formaldehyde solution in PBS for 15 minutes and deposited on to glass slides using a cytocentrifuge. Prior to imaging, the slides were sealed with refractive index matching fluid.

8.3.10 Dark Field Microscopy and Cellular Scattering Histograms

Dark field images were collected using an inverted microscope (TE2000, Nikon, Melville, NY) outfitted with oil immersion condenser and 100 X objective, containing a dark field stop and iris respectively. Cellular scattering intensity was analyzed as described in Chapter 7 [37]. Briefly, in dark field images, blue-white background scattering from cells [38,39] was largely removed so that intensity measurements more exclusively reflected nanoparticle scattering: each 8-bit color image was separated in to its constituent red, green and blue channels and the blue channel subsequently subtracted from the full color image. An identically sized region of interest was
placed over the location of cells in the images and used to collect the sum of the pixel intensities for each of at least 100 cells from each sample (NIS-Elements, Nikon). Intensity values were plotted as histograms for comparison of SERS liposome labelling densities between samples. Differences in particle labelling were assessed for statistical significance using the Wilcoxon-Mann Whitney rank sum test with \( p < 0.5 \) being considered significant (Igor Pro, Wavemetrics, Portland, OR).

### 8.3.11 Raman Mapping

Spectra used to construct Raman maps were collected using a confocal Raman microscope and spectrometer (InVia, Renishaw, Gloucester, UK). Bright field differential interference contrast (DIC) microscopy was used to locate the cells, and a series of spectra were collected in a \( \sim 12\mu m \times 12\mu m \) area in the vicinity of a cell by a raster scan of the motorized stage in \( 1\mu m \) steps in the \( x \) and \( y \) directions. A 638 nm laser was focused through a 40x objective to probe the sample with approximately a \( 1\mu m^2 \) spot size and 1 second integration time. Spectra from each scan were fitted with both the malachite green SERS spectrum and endogenous Raman spectrum from the microscopy slide respectively, using the direct classical least squares (DCLS) method which is also discussed in Chapter 7. Two images were constructed from the fit coefficients, where each map represents the spatial distribution of either MG or the mounting medium respectively. DCLS calculates the linear combination of component spectra that best fit the mixed sample spectrum with the lowest sum of squares error [40]. Input spectra were normalized to mean centre and unit variance with a fourth order polynomial included to account for irregular spectral baselines. Therefore, the results of the DCLS fit indicate the relative contributions of each input to the final spectrum and cannot be used to quantify the SERS liposome intensity, but instead to determine the spatial distribution of the SERS particles.

### 8.4 Results

#### 8.4.1 SERS Liposome Optical Properties

Visible absorbance measurements of the SERS liposomes were collected after encapsulation and functionalization to confirm particle monodispersity. Spectra from Fab and whole antibody conjugated SERS liposomes are shown in Figure 8.2A) along with spectra from unconjugated
and unencapsulated particles for comparison. Following encapsulation, the Localized Surface Plasmon Resonance (LSPR) maximum of the particles shifted from 536nm to 538nm due to a change in the dielectric immediately surrounding the particles. The location of this maximum did not shift further following incorporation of targeting molecules, however the spectral region between 600-700nm was monitored for the appearance of a second broad absorbance that would be indicative of particle aggregation [31,32]. No such peak was visible after SERS liposome preparation.

SERS spectra from drops of SERS liposome suspensions were collected in order to confirm that MG reporter molecules were incorporated into the nanoparticle structure. Representative spectra of conjugated and unconjugated SERS liposomes are provided in Figure 8.2B).
Figure 8.2: A) Visible absorbance spectra of Fab-conjugated (dark green), whole antibody conjugated (light green), and unconjugated (red) SERS liposomes, and spectrum of unencapsulated 60nm AuNP (black). Encapsulated particles show red shift in LSPR maximum. Absence of secondary peak in the 600-700nm region suggests minimal aggregation. B) SERS spectra of Fab (dark green) and whole (light green) conjugated SERS liposomes, and unencapsulated SERS nanoparticle (black) collected with 633nm source. The spectra are consistent with the reporter molecule MG.

8.4.2 Characterization of SERS Liposome Conjugation to Antibodies

Three complementary methods were used to confirm antibody conjugation: BCA assay, and secondary antibody labelling with TEM and flow cytometry contrast agents.

Conducting the BCA assay in the presence of SERS liposomes that were not bound to protein developed the assay colour as expected, though notably advanced colour development occurred in the presence of targeting proteins conjugated to nanoparticles. Subtraction of background absorbance values yielded solution protein concentrations in the range of the 0.5 ug/mL added to each sample for bioconjugation. Some loss of protein would be expected and is dependent upon a number of factors including the efficiencies of the coupling chemistry of COOH-2kPEG-DPPE to mAbs and post-insertion of Fab-2kPEG-DPPE into the SERS liposomes’ structure. Conversely, excess lipids inadvertently present in the assay solution along with the SERS liposomes could contribute to enhanced color development. The protein concentrations determined following background subtraction correlated to approximately 110 mAbs per SERS liposome and 1800 Fabs per SERS liposome. Assuming hexagonal close packing of non-overlapping proteins on the surface of an 80nm diameter spherical particle with proteins having hydrodynamic radii of 10 nm and 2.91 nm for each of mAbs and Fabs respectively, the theoretical upper limit to the number of proteins that could be associated with each SERS liposome is approximately 232 mAbs and 2742 Fabs. Both of the experimentally determined values are lower than their corresponding theoretical limits.

In TEM images, SERS liposomes were visible as dense, dark Au cores surrounded by a thin, gray corona of lipid molecules (Figures 8.3 and 8.4). Antibody conjugated SERS liposomes that
had been treated with immunogold secondary antibodies were decorated with small 5nm Au NPs. To discount the contribution of sample drying effects on the TEM grid, unconjugated control samples were also treated in the same fashion. A comparison of representative images clearly indicates greater association of immunogold secondaries with conjugated SERS liposomes than controls.

Figure 8.3. TEM images of mAb conjugated SERS liposomes with 5nm AuNP bearing secondary antibodies. Top row, Anti-CD45 whole mAb conjugated SERS liposomes. Bottom row, unconjugated SERS liposome controls. Scale bar: 100nm.

Figure 8.4. TEM images of Fab conjugated SERS Liposomes with 5nm AuNP bearing secondary antibodies. Top row, CD20 Fab conjugated SERS liposomes. Bottom row, unconjugated SERS liposome controls. Scale bar: 100nm.
Secondary fluorescence labelling and detection via flow cytometry qualitatively indicated that targeting molecules had been successfully attached to the SERS liposomes though precise fluorophore number could not directly correlated to the number of nanoparticles in each sample (Figure 8.5). Cytometer events could be attributed to SERS liposomes using distinctive side and small angle forward scatter intensity profiles of the nanoparticles. The distributions of peak fluorescence intensity events in the DyLight650 fluorescence channel for each of the Fab-conjugated and control unconjugated SERS liposomes were distinct from one another and the distribution from the Fab-conjugated samples were shifted to higher intensities.

![Figure 8.5](image)

Figure 8.5: Secondary antibody flow cytometry contrast agent. Dylight 650-labeled antimurine IgG secondary antibodies were used as probes for primary antibodies to confirm conjugation to SERS liposomes. Normalized Dylight 650 peak fluorescence intensity distributions of anti-CD20 conjugated SERS liposomes (grey); control unconjugated SERS liposomes (open) following treatment with fluorescent secondary antibodies. Each intensity event is reflective of multiple SERS liposomes present in the interrogation volume simultaneously.

8.4.3 SERS Liposome Labelling of LY10 Lymphoma Cells

The LY10 lymphoma cell line was used as a test case for initial targeting experiments. Flow cytometry was used to evaluate the expression of CD19 and CD20 on the cell surface of representative samples (Figure 8.6). The one-parameter histograms (Figure 8.6A and 8.6B) show that the LY10 cell line express CD20 and CD19 at levels sufficient to detect using fluorescence immunophenotyping and hence should serve as an appropriate cell model for
testing anti-CD20 and anti-CD19 SERS liposomes, respectively. The two-parameter scatter plot in Figure 8.6C shows that the expression of CD20 by LY10 is approximately one order of magnitude greater than CD19 expression.

**Figure 8.6.** Flow cytometric measurements of relative CD protein expression on LY10 cell line. Normalized histograms of integral pulse intensity of fluorescence from cells treated with (A) anti-CD20 FITC antibodies, indicating CD20 expression and (B) anti-CD19 PC7 antibodies indicating the expression of CD19. (C) Two parameter scatter plot of integral pulse intensities comparing the expression of CD20 and CD19.

Binding density of the particles to LY10 cells was assessed by dark field microscopy. Representative dark field images of cells incubated with SERS liposomes labeled with whole anti-CD19 and whole IgG isotype control mAbs are shown in Figure 8.7A) and B) respectively. The cells appear as faint grainy blue circles while the particles can be located by their intense green, red, or gold light scattering. It is qualitatively clear that images typical of specifically targeted cells contain more particles co-localized with cells than the control images. Cellular scattering intensity histograms shown in Figure 8.7C) clearly demonstrate that cells labeled with anti-CD19 SERS liposomes exhibit a statistically significant population shift towards greater intensity than for cells labeled with non-specific control SERS liposomes. The cellular scattering intensity histogram for positively targeted anti-CD19 SERS liposomes also shows a statistically significant shift to higher intensities as compared to untreated LY10 cells (Figure 8.8).
Figure 8.7: Evaluation of CD19 mAb-targeted SERS liposomes for imaging LY10 cells. Representative dark field images of cells incubated with (A) anti-CD19; (B) IgG- SERS liposomes. (C) Distributions of cellular scattering intensities for 129 LY10 cells treated with SERS liposomes bound to anti-CD19 (green) and IgG (open) mAbs. (D) Bright field micrograph of mapped cell. Raman maps constructed from DCLS fit coefficients of (E) MG SERS and (F) background Raman spectra to each LY10 cell spectrum. G) Spectrum obtained from highlighted (red border) pixel in (D).

Figure 8.8. Evaluation of mAb targeted SERS liposomes for imaging CD19 on LY10 cells: comparison to untreated control cells. Representative dark field images of (A) CD19-SERS liposome treated sample; (B) untreated cells. (C) Cellular scattering intensity histogram from CD19-SERS liposome (green) and untreated (open bars) LY10 samples.
The same cell slides used for dark field imaging were interrogated using Raman microscopy and mapping. The brightfield image used to locate the cell is shown in Figure 8.7D). Two spectral fit-coefficient maps indicating the spatial location of MG and the slide background are shown in panels E) and F) respectively. The color intensity scales indicate the value of the fit coefficients to each of the sample spectra with dark color indicating a poor fit and bright color indicating a good fit. The absence of fit correlation with background components of the slide (panel F) indicates the position of the cell. The co-localization of the MG signal with the cell confirms the tagging of the cell and the match to MG confirms the SERS signal is preserved following binding, and treatment of the sample for microscopy, including fixation. A typical Raman spectrum of the particles collected from the cell map (at the highlighted pixel in Figure 8.7E)) is shown in G).

To evaluate the targeting efficacy of fab-conjugated SERS liposomes, the same experiment was repeated using SERS liposomes bearing either anti-CD20 Fabs for positive labelling, or nonspecific isotope control Fabs to determine the extent to which the SERS liposomes non-specifically associate with cells during treatment. Dark field images of cells incubated with positive and control SERS liposomes are shown in Figure 8.9A) and B) respectively. The cellular scattering intensity histograms are plotted in Figure 8.4C). Statistical analysis of the intensity data using the Wilcoxon Mann-Whitney method indicated significant differences between these data sets, indicating a detectable difference between positives and controls. Preservation of the SERS functionality and colocalization with cells is confirmed by Raman mapping. The bright field image is shown in Figure 8.9D) and Raman fit-coefficient maps for MG and slide background are shown in panels E) and F) respectively. A representative spectrum from the Raman map is shown in Figure 8.9G).
8.4.4 SERS Liposome Labelling of Primary CLL Cells

For greater clinical relevance, targeting of SERS liposomes was tested against primary human leukocytes extracted from the blood samples of anonymous consenting donors who have been diagnosed with chronic lymphocytic leukemia (CLL). The experiments described in the previous section were repeated with primary cancer cells that were incubated with SERS liposomes bearing either whole anti-CD19 antibodies, or anti-CD20 Fabs, and washed repeatedly to remove unbound particles.

Representative dark field micrographs of the anti-CD19 whole antibodies bound to CLL cells are shown in Figure 8.10A). We observed a greater variety of cell shapes and sizes than in the LY10
samples, which is consistent with the heterogeneous nature of primary cell extracts. Green, red, and gold colored scattering was found to be localized around many cells in the anti-CD19 sample, with very little present in the negative control sample (Figure 8.10B), indicating positive binding in the anti-CD19 sample. Cellular scattering intensity histograms are shown in Figure 8.5C). The shift of the distribution towards higher scattering intensity in the positive sample is clearly evident, and was confirmed by statistical analysis.

Figure 8.10: Assessment of mAb-targeted SERS liposomes for CD19 imaging of CLL cells. Dark field images demonstrative of CLL labelling using (A) anti-CD19-; (B) IgG-SERS liposomes. (C) Cellular scattering intensity histograms for 114 cells incubated with anti-CD19 SERS liposomes (green) and IgG1 SERS liposomes (open bars). (D) Bright field image of cell used for Raman map. Spectral fit coefficient maps from a representative cell labeled with anti-CD19 SERS liposomes showing regions of best fit by (E) MG; (F) background Raman spectrum. (G) Spectrum from pixel highlighted (red border) in (E).

A bright field image of the cell interrogated by Raman mapping is provided in Figure 8.10D), and Raman fit-coefficient maps of a labeled CLL cell are shown in panels E and F. As in the case of the LY10 sample, regions not matching the mounting medium appear dark in the map in panel
F and correspond to the location of a cell. Spectra strongly matching the MG spectrum appear localized on the cell. A representative SERS spectrum from a pixel with a high fit coefficient to MG is shown in panel G).

Cell labelling experiments were repeated for SERS liposomes bearing anti-CD20 antibody fragments. These experiments were performed on a different day than the whole antibody experiments described above and therefore consist of a different distribution of cells from a different donor. Representative dark field images of primary leukocytes incubated with anti-CD20 Fab-targeted SERS liposomes and IgG isotype control Fab-liposomes are shown in Figure 8.11A) and B) respectively. In both cases, green, red, and gold scattering was observed colocalized with some cells in the sample. Differences between positive, and control samples were difficult to detect by eye. The samples were however, differentiated by cellular scattering intensity analysis, which indicated a statistically significant increase in scattering intensity distribution from the positively labeled samples as compared to controls. The histograms of cellular scattering intensities are plotted in Figure 8.11C).

Figure 8.11: Evaluation of Fab-targeted SERS liposomes for CLL cell imaging. Representative dark field images of CLL cells incubated with (A) anti-CD20-; (B) IgG-Fab SERS liposomes. (C) Histograms of cellular scattering intensities for 150 cells from anti-CD20-Fab- (green) and IgG-Fab SERS liposome (open bars) samples. (D) Bright field
image of the representative cell appearing in Raman maps. DCLS Raman maps of cell treated with anti-CD20 SERS liposomes. Regions of best fit to (E) MG SERS spectrum; (F) background spectrum. (G) Spectrum from pixel highlighted (red border) in (D).

Raman fit coefficient maps indicated similar results to those found in prior samples: Raman spectra matching the mounting medium corresponded to the image background, indicating the locations of the cells by the absence of mounting medium. Areas in the map corresponding to the cells matched the spectrum of MG. Bright field image of interrogated cell and associated maps are shown in Figure 8.11D), E), and F). Figure 8.11G) is a representative SERS spectra from a pixel with strong fit coefficient to MG as highlighted in 8.11E).

8.5 Discussion

In this thesis, SERS liposomes were demonstrated as effective diagnostic probes for the identification and imaging of cell surface proteins of interest. A great deal of literature exists on the engineering of liposome coatings, targeting strategies and the interaction of liposome contrast agents and drug carriers in biological environments thus prompting our expansion of liposome technology to dark field and SERS labelling. By taking advantage of the plasmonic properties of the encapsulated Au nanoparticles, we were able to image cell labelling using dark field microscopy to collect the particles’ Rayleigh scattering as well as with Raman mapping based on the SERS enhancement of Raman reporter molecules incorporated in to the SERS liposome structure.

The LY10 lymphoma cell line was adopted for use in this study because immortalized cell lines can serve as a robust and reliable source for large quantities of monoclonal cells[40] LY10 is specifically used in many cases to manufacture xenograft lymphoma tumor models[41,42]. Retaining the cells in suspension format was more suitable for our application as any unbound SERS liposomes could be easily removed from cell suspension following incubation with cells, and the cells exist in a format amenable to collecting flow cytometric measurements or in which the cells could be easily deposited on to glass slides in densities appropriate for microscopy imaging following SERS labelling. Additionally, activated B cell-like diffuse large B cell
lymphoma has the poorest prognosis of DLBCLs, as discussed in detail in Chapter 2 of this thesis, making it a valuable test system[44].

Primary human CLL cells were adopted for use in this study to supplement the information obtained from SERS liposome labelling of LY10 cells with a more clinically relevant example of SERS imaging. CLL samples consisted of a mixture of all WBC types (though were predominantly monoclonal neoplastic cells), and significant variation in cell count and robustness can exist between samples. In such samples, we expect to find, among a variety of leukocytes, elevated proportions of neoplastic B-lymphocytes that express CD19 and CD20 on their surfaces in varying levels.

CD19 and CD20 are important targets in immunophenotyping of WBCs. These B lineage markers are useful not only in the interrogation of samples from patients/donors in good health, but also aid in the diagnosis of B cell neoplasms[41]. Additionally, CD20 is an important (and difficult) target as the expression of this protein decreases with CLL progression[46], and it is the target surface molecule for a number of different antibody therapies for hematologic malignancies[29,47].

Experiments were conducted using both Fab and whole mAb targeted SERS liposomes in order to demonstrate the versatility of the lipid bilayer as a SERS nanoparticle coating. The intended application, and the inclusion of other functionalities will dictate which conjugation method to use. Moving forward, the modularity of the SERS liposome coating could feasibly enable modification to include contrast agents such as fluorophores, ferromagnetic species and radioisotopes.

The different methods for verifying conjugation of Fabs and mAbs did not reach a precise ratio of protein molecules to nanoparticles, however what is clear is that there are multiple targeting proteins bound to each SERS liposome on average. Similar observations were made following bioconjugation of PEG-coated SERS NPs as discussed in Chapters 6 and 7. Each of the characterization methods adopted has inherent limitations and advantages that contribute to the range of values detected. Multiple targeting proteins bound to each SERS liposome could be highly beneficial for labelling efficacy as multivalent liposomes have been adopted elsewhere for their increased binding avidity to intended targets[48,49]. Additionally, because the cross-linking chemistry used to bind whole antibodies to COOH-PEG-DSPE molecules incorporated in
the SERS liposome coating is not orientation specific, having multiple targeting antibodies bound to each nanoparticle increases the likelihood that one or many may be conjugated such that their binding activity is maintained.

Having confirmed the conjugation of antibodies, the SERS liposomes were applied to cells to assess their targeting functionality. After incubating SERS liposomes with cells, the cells were washed repeatedly by centrifugation to remove unbound SERS liposomes. The cells were then mounted onto slides so targeting could be analyzed by dark field microscopy and Raman mapping.

Dark field microscopy is a useful method by which to assess the variation in labelling efficacy between positively targeted and control SERS liposomes because many cells can be imaged quickly using simple, widely employable instrumentation. Differences in labelling density were simple to quantify by using the sum of pixel values from cell locations in the dark field images to represent the cellular scattering intensity from individual cells. Positive labelling was identified as shifts of the cellular scattering histograms to higher intensities as a result of the presence of more particles associated with each cell in the positively targeted samples. Additionally, this shift in each of the distributions was found to be statistically significant.

Though Raman maps can be generated by correlating increased colour intensity of the pixels with increasing intensity of a major peak in the spectrum of interest, we chose to employ an alternative method in this study. By using the full SERS spectrum of malachite green to identify the locations of SERS liposomes, they can be thoroughly differentiated from the background spectrum. Additionally, any variation in spectral baseline intensities typical in SERS spectra as a result of the broadband continuum[50] do not contribute to the localization of SERS liposome labels in in vitro samples. This is of significance in the potential application of SERS liposomes to study the colocalization of surface proteins or variation between surface proteins expression. The relevance of such studies using fluorescent labels are discussed elsewhere[51,52]. We have demonstrated that SERS liposomes are effective labels for imaging cell surface proteins of interest in the diagnosis of B cell leukemias and lymphomas. The adoption of liposomes as a SERS nanoparticle coating and targeting technology offers the potential addition of dark field and SERS imaging probes to the list of liposome applications that already include drug delivery, and multifunctional probes in ultrasound, fluorescence, MR, CT, and PET imaging.
8.6 Contributions

The author co-wrote the manuscript, prepared SERS liposome labelled cell samples, performed SERS measurements, dark field microscopy and analyzed SERS, dark field and flow cytometry data. The author also undertook BCA assays and developed procedures for fluorescence and TEM secondary antibody labelling. The author gratefully acknowledges Shell Ip for co-writing the manuscript, preparing fab-targeted SERS liposomes and collecting SERS liposome characterization measurements. Shell Ip also permitted use of the schematic presented in Figure 8.1 (from Ip et al. 2014, submitted). The author also gratefully acknowledges Michelle Joseph for preparing mab-targeted SERS liposomes and collecting SERS liposome characterization measurements; Nisa Mullaithilaga for isolating CLL cells and immunophenotyping; Annie K.W. Bang for assistance with flow cytometry and data analysis; Guisheng Yang for LY10 cell culture; and Ilya Gourevich for assistance with TEM imaging.

8.7 References


9 Imaging Epidermal Growth Factor Receptor on Lung Cancer Cells Using Targeting SERS Gold Nanoparticle Labels

9.1 Overview

This chapter will provide a description of how PEG-coated, antibody targeted SERS Au NPs were applied to study a lung cancer model. Epidermal growth factor receptor (EGFR) is a cell surface receptor that is highly expressed in a number of different cancers making this molecule a useful target for SERS detection. This chapter will discuss the methods used for preparing, the SERS NPs, conjugating them to anti-EGFR antibodies, and characterizing them before application to in vitro labelling. Their efficacy in binding EGFR in two lung cancer cell lines will be investigated using widefield SERS from treated adherent cell cultures, confocal Raman imaging, and dark field imaging.

9.2 Introduction

SERS probes were prepared for identifying EGFR expression in non-small cell lung cancer cell (NSCLC) lines. The importance of this study is that the narrow bandwidths of SERS spectra impart the potential for highly multiplexed biomarker imaging. Lung cancers have considerably heterogenous biomarker expression [1], therefore increasing the number of cell surface antigens that can be simultaneously detected on individual cells is important for early and specific diagnosis of this disease [1]. Additionally, lung tumors may be difficult to excise and characterize therefore multifaceted diagnostic approaches are the current standard [1] towards which SERS labelling has the potential to contribute. Additionally, investigating the binding efficacy and specificity of the SERS probes developed in this thesis for imaging another cell type is important to determining their potential for more widespread use.

Lung cancers are responsible for one third of all cancer related deaths worldwide making them the leading cause of cancer fatalities [2]. The 5 year survival rate of those diagnosed with lung cancer is as low as 16% in the United States [3]. This is partly due to the fact that lung tumors
are difficult to identify and patients may be asymptomatic thus precluding early detection in many cases. Lung cancer is predominately diagnosed in the early stages of development using spiral computed tomography which while sensitive, can lack specificity. Screening for a number of biomarkers, including EGFR, may increase the specificity of diagnosis. Notably, EGFR overexpression is present in early stage tumors making this biomarker an attractive biomarker for the development of targeted contrast agents [4]. Early identification of EGFR overexpression is also critical because such tumors characteristically have a high metabolic and growth rate and are often metastatic at time of diagnosis, treatment resistant upon recurrence and associated with a poorer prognosis [5–7]. While the focus herein is on NSCLC, EGFR is overexpressed in a number of epithelial cell tumors including breast, ovarian, head and neck, and esophageal cancers making EGFR and is thus a widely useful clinical target for diagnosis and therapy.

EGFR is a transmembrane tyrosine kinase of 170 kDa molecular weight, having four extracellular domains of which DI and DIII are ligand binding with an 40 mM affinity for epidermal growth factor (EGF) [8]. EGFR is one of a family of four c-ErbB tyrosine kinases and also is referred to as HER-1 or c-ErbB-1 [6]. In regular functioning, it plays a role in cell differentiation, proliferation and growth. Upon ligand binding, EGFR forms a homo- or heterodimer and is internalized thus inciting a signal transduction cascade beginning with autophosphorylation of the intracellular tyrosine kinase domain [6,9]. In the development of tumors, EGFR is overexpressed in combination with its soluble ligands including EGF and transforming growth factor-α (TGFα). The result is a hyperactive autocrine loop that culminates in abnormal cell growth [5,10]. Small molecule kinase inhibitors and humanized antibody therapy have been developed with the objective of disrupting this autocrine signaling loop [11].

EGFR has also been an attractive target for designing both diagnostic and therapeutic Au nanoparticle technologies. Recent examples of imaging and characterization include the study of dynamic receptor clustering and intracellular trafficking [12,13] as well as EGFR imaging using SERS-active Au nanoparticles [14]. Au nanoparticles and nanocages have also been explored for EGFR targeted photothermal destruction of overexpressing cells [15–17].

In this chapter, the performance of SERS AuNPs conjugated to anti-EGFR monoclonal antibodies will be assessed for EGFR imaging using two cell lines: EGFR positive A549 NSCLC adenocarcinoma line, and the NSCLC squamous cell carcinoma line, H520 which is
expected to be low to null for EGFR expression [18–21]. It will be demonstrated that SERS-active Au nanoparticle labels bind their intended cell surface antigen targets, and are detectable from in vitro biological tissues using several methods: Raman spectroscopy, dark field microscopy and concurrent differential interference contrast (DIC) microscopy with Raman mapping. Additionally, the specificity of cell labelling by SERS particles is demonstrated by comparing cell labelling density of anti-EGFR conjugated particles to labelling by particles bound to non-specific IgG1 isotype antibodies, and particles that were not bound to antibodies.

9.3 Methods and Materials

9.3.1 Materials

The following materials were used without further purification: 60 nm diameter Au nanoparticles (AuNPs) (British Biocell International) were purchased from Ted Pella Inc (Redding, CA, USA). Malachite green isothiocyanate (MG) was obtained from Life Technologies (Burlington, ON, Canada). Five kDa α-methoxy-ω-mercaptopoly(ethylene) glycol (CH$_3$O-PEG-SH) and α-carboxyl-ω-mercaptopoly(ethylene) glycol (COOH-PEG-SH) were purchased from Rapp Polymere (Tuebingen, Germany). 1-Ethyl-3[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS) were obtained from Thermo Fisher Scientific (Rockford, IL, USA). Rat anti-human epidermal growth factor receptor (anti-EGFR) antibodies, and A549 and H520 cells cultured on glass-bottomed culture dishes and glass slides for microscopy were provided by Gang Zheng’s laboratory.

9.3.2 Preparation of Anti-EGFR PEG-coated SERS Gold Nanoparticles

Freshly prepared aqueous MG solution (4μM) was added drop-wise to stirring Au colloid (0.043 nM) to a final concentration of 33 nM. Nanoparticles’ SERS spectrum was collected using the procedure provided below to insure SERS activity was present prior to PEG coating. Aqueous 80 mM solution of CH$_3$O-PEG-SH was slowly added to the nanoparticle suspension to a final concentration of 6 mM. The particles were stirred for 30 minutes to allow surface
functionalization to occur and excess PEG removed using three rounds of centrifugation at 6.8g for 5 minutes, each time replacing the supernatant with fresh Milli-Q water. SERS Au NPs were replaced in a round-bottomed flask and an aqueous solution (80 μM) of COOH-PEG-SH was added slowly to the suspension and stirred for 60 minutes. Excess PEG was removed using centrifugation and aqueous EDC (0.25M) and sulfo-NHS (0.5 M) were quickly added and stirred with the SERS particles for 15 minutes at room temperature. Following removal of cross-linkers from the particle suspension, 30 μg of anti-EGFR antibodies were added and the suspension was gently stirred for 4 hours at room temperature. Centrifugation was used to remove unreacted antibodies and the particles were stored at 4°C until used for in vitro labelling.

9.3.3 Optical Absorbance Spectroscopy
Optical absorbance spectra were collected after each stage of nanoparticle functionalization. Spectra were collected from particle suspensions in Milli-Q water or PBS placed in 1 cm path length cuvettes using a Cary 5000 spectrometer (Varian Inc, Palo Alto, CA, USA) with water or PBS serving as the reference correspondingly. Spectra were normalized by setting the baseline to zero and scaling linearly to a maximum value of 1.

9.3.4 Cell Culture
Cell cultures were provided by Gang Zheng lab, Department of Medical Biophysics, University of Toronto. A549 and H520 cell lines (high and low EGFR expressing, respectively) were maintained in T75cm² flasks in RPMI 1640 medium supplemented with 10% FCS, penicillin, streptomycin and L-glutamine at 37°C, 5% CO₂ atmosphere. For dark field and Raman mapping experiments, cells were plated in each well of 8-well plate microscope slides or separate glass-bottomed petri dishes prior to SERS labelling experiments for optical microscopy and Raman spectroscopic measurements respectively.
9.3.5  Cell Labelling Using SERS Gold Nanoparticles

A549 and H520 were subjected to treatment with SERS particles conjugated to anti-EGFR antibodies to evaluate positive targeting, while the non-specific interactions of SERS particles with cells was determined by incubation of cells with each of IgG1 isotype-conjugated or unconjugated-SERS Au NPs. As part of a standard procedure for immunolabelling with any contrast agent, BSA was added to the cell cultures prior to nanoparticle labelling in order to reduce non-specific association of particles with cells. Cells were incubated with SERS Au NPs for 30 minutes at room temperature. Unbound particles were removed by several washes of the culture plates with fresh PBS. Cells were incubated with SERS Au NPs for 30 minutes at room temperature. Unbound particles were removed by several washes of the culture plates with fresh PBS. Cells were fixed using 4% formaldehyde for 10 minutes and rinsed 3 times with PBS. Well-plate microscope slides were sealed using index matching fluid that contained 4′,6-diamidino-2-phenylindole (DAPI) and cover slips. These slides were used for dark field and confocal Raman imaging. Widefield Raman spectra were collected immediately after nanoparticle treatment from glass-bottomed petri dishes topped up with fresh PBS.

9.3.6  Dark Field Microscopy

Dark field images were collected using an inverted microscope (TE2000, Nikon, Melville, NY, USA) outfitted with an 100X oil immersion (0.5-1.25 NA) objective with an adjustable iris and an oil immersion condenser having a dark field stop. In this optical configuration, samples were illuminated obliquely and only light scattering was collected by the CCD camera.

9.3.7  Raman Spectroscopy

The same inverted microscope was used to illuminate samples using a 632.8 nm HeNe laser through a 40X (0.6 NA) objective. The laser was 1 mW at the focal plane with a 1.30 μm² spot size. Light scattering from the sample was passed through a notch filter to remove Rayleigh scatter and spectrally separated with an f6.5 Czerny-Turner monochromator (Acton SP2560) and 1200g/mm grating. Spectra from cells were collected with 60 second integration time using a Peltier cooled CCD camera (PIXIS BR400, Princeton Instruments, Trenton, NJ, USA). Spectra
from nanoparticle suspensions were collected from 20 μL drops on a glass cover slip with 5 second integration time. For interrogating cell samples, Raman spectra were collected from glass bottomed petri dishes at the plane of optical focus. Spectra from cell lines were background subtracted and baseline flattened for presentation except where noted otherwise.

Nanoparticle suspensions were also characterized using a 785 nm laser on bench-top Raman instrument (DeltaNu, US BioSolutions Inc, Weston, FL, USA). Suspensions were placed in cylindrical glass vials and spectra collected with 1 second integration time.

9.3.8 Raman Imaging and Spectral Analysis

A549 cells cultured and SERS labelled on well plate slides were also interrogated by Raman imaging. Spectra were collected using an inverted microscope connected to a spectrometer (InVia, Renishaw, Gloucester, UK) in 1μm steps along the x-y plane using a 638 nm laser (2.4 mW at focal plane) with 50 msec integration time. Over 300 spectra were collected for each map and analyzed in two different ways to identify and compare SERS labelling between samples. Pseudocolour Raman images were created by correlating pixel intensity to fit coefficient for each spectral component in to spatially resolved A549 sample spectra using direct classical least squares (DCLS) fitting (WiRe 3.3 software, Renishaw). MG SERS and cell background spectra were the components used. These images were overlayed with differential interference contrast (DIC) microscopy images to determine whether there was colocalization between cells and MG SERS intensity.

For comparisons of SERS labelling density between samples, a linear baseline was drawn between 1563 and 1632 cm\(^{-1}\), and the area between signal and this baseline was calculated for 14042 spectra from anti-EGFR SERS AuNP treated cells, 5136 spectra from IgG1-SERS particle samples, and 990 spectra from A549 cells that were not treated with SERS particles. To account for the difference in sample sizes, histograms were normalized to % of total spectra for each sample yielding values between 0 and 1. For spectra without significant features over the range integrated, noise aberrantly contributed negative integral intensity values to the distributions. To remove the influence of noise from comparison of spectral intensities between positive and control samples, the integral intensity histogram from untreated A549 cells was subtracted from
each of the anti-EGFR and IgG1-SERS AuNP treatment histograms and resulting distributions plotted for comparison.

To assess statistical significance between each of the three positive and control samples, the Wilcoxon-Mann Whitney rank sum test was applied to integrated signal to baseline values for each of anti-EGFR SERS NP treated, IgG SERS NP treated and untreated A549 cells with differences considered significant at p<0.05 (Igor, Wavemetrics, Portland, OR, USA).

9.4 Results and Discussion

9.4.1 Characterization of SERS Gold Nanoparticles

The SERS spectrum of malachite green was detectable almost immediately after addition of dye solution to the stirring Au colloid and there was no change in the number or location of spectral peaks following functionalization of the particles with CH3O-PEG-SH and subsequent conjugation of anti-EGFR or IgG1-isotype antibodies (Figure 9.1). The SERS spectrum of the functionalized particles was also collected using a 785 nm laser as the probe. The peak locations were consistent with spectra obtained using 638 nm excitation, however the relative intensities of the peaks changed (Figure 9.2). While SERS from the nanoparticles was intense enough to be detected using a 785 nm laser, the shorter probe wavelength(s) were chosen for in vitro experimentation because 632 and 638 nm overlap with the particles’ LSP and the absorbance band of the MG dye (Figure 9.3A).
Figure 9.1: Raman spectra from drops of malachite green SERS AuNP suspensions after each step of particle functionalization: Au NPs functionalized with malachite green Raman active dye (grey); malachite green SERS AuNPs after coating with 5kDa CH3O-PEG-SH (black, dashed); PEG-coated SERS Au NPs following conjugation to monoclonal anti-EGFR antibodies (black). Spectra were collected using a 633 nm laser and 5 second integration time. For clarity, spectra are normalized by intensity and offset.
Figure 9.2: Raman spectrum of MG functionalized SERS Au nanoparticles collected using a 785 nm laser as the probe wavelength.

![Figure 9.2: Raman spectrum of MG functionalized SERS Au nanoparticles collected using a 785 nm laser as the probe wavelength.](image)

Figure 9.3: Normalized extinction spectra after each step of nanoparticle functionalization. (A) Citrate capped 60 nm diameter spherical Au nanoparticles (grey, solid), CH\textsubscript{3}O-PEG-SH coated SERS NPs (black, dashed), and SERS NPs conjugated to monoclonal anti-EGFR antibodies (black, solid). Normalized optical absorbance spectrum of MG aqueous solution (grey, dashed). (B) Area of absorbance spectrum over which 2 nm red-shift of LSPR occurs upon SERS NP coating with PEG.

To determine whether SERS Au NPs had been coated with 5kDa PEG-SH, optical absorbance spectra were collected before and after the PEG coating (Figure 9.3). A 2 nm red-shift from 536 nm to 538 nm in the maximum LSPR wavelength was observed indicating a change in the relative permittivity of the medium immediately surrounding the Au particles (Figure 9.3B). Following conjugation to monoclonal anti-EGFR antibodies, there was no further plasmon shift observed. The absence of a broad absorbance peak between 600 and 700 nm [22,23] indicated that there was not a large population of aggregated particles in the prepared suspension.

9.4.2 Evaluation of EGFR Targeting Using SERS Gold Nanoparticles

To image EGFR using SERS, Au nanoparticle probes were conjugated to monoclonal antibodies raised against the extracellular domain of this membrane spanning glycoprotein. The specificity
of the interaction between anti-EGFR SERS Au NPs and their target antigen was investigated by comparing cell labelling densities between samples treated with anti-EGFR SERS Au NPs, and non-specifically targeted IgG1 isotype-SERS AuNPs and untargeted SERS particles with a CH$_3$O-PEG-SH coating only.

As an additional control experiment, each particle type was tested in two non-small-cell cancer models: A549 adenocarcinoma line which expresses EGFR, and the H520 squamous cell carcinoma line that has been reported to be low to null for EGFR. However, the selection of these two lines for comparison by optical imaging and spectroscopy from adherent culture was complex because the two cell lines exhibit different growth characteristics on culture plates. A549 are epithelial cells and therefore grow and spread in ordered monolayers that are simple to wash and image, however the H520 line tend to cluster and stack reducing optical image clarity and creating relative inconsistency of cell number in a given laser focal volume. Additionally, imaging results from H520 treated with anti-EGFR SERS particles were variable with a tendency toward significantly higher intensities than for H520 incubated with IgG1- and PEG-SERS AuNPs in the same labelling pattern as for A549 (not shown). This suggests that the H520 cells used in these studies may indeed have expressed EGFR. Long term cell cultures are vulnerable to genetic drift and inadvertent aberrant clone selection [24]. EGFR expression is reported specifically in H520 elsewhere [25]. Because western blotting or fluorescence immunohistochemistry for EGFR did not accompany each culture sample for SERS experiments, an emphasis will be placed herein on reporting SERS results from A549 cells.

Three methods were used evaluate EGFR labelling by SERS Au NPs, each providing complementary information. Widefield Raman spectroscopy was a rapid and straightforward method for collecting SERS intensity from treated cells on their culture plates. In the absence of a confocal pinhole in the microscope detection optics, out-of-focus light scattering has a contribution to the acquired spectra resulting in spatially averaged measurements. The spectra collected in this manner were analyzed for the presence of MG SERS from the nanoparticle labels (Figures 9.4 and 9.5). More intense SERS spectra were detected from A549 treated with anti-EGFR SERS Au NPs than from cells incubated with non-specific IgG1- and untargeted SERS NPs. Though MG SERS was visible from H520 cells targeted by anti-EGFR SERS AuNPs, these spectra were less intense than those collected from A549 subjected to an equivalent treatment (Figure 9.4). Furthermore, comparable SERS intensity was observed upon
collecting spectra from multiple locations on the same sample plate for A549 cells treated with anti-EGFR SERS Au NPs (Figure 9.5).

Figure 9.4: Raman spectra from cell culture subjected to different SERS Au NP treatments. High EGFR expressing A549 cell line incubated with anti-EGFR conjugated SERS AuNPs (black, solid); IgG1 isotype conjugated SERS AuNPs (black, dashed); and SERS AuNPs with PEG coating only (grey, dashed). Low EGFR expressing H520 cell line incubated with anti-EGFR conjugated SERS AuNPs (grey, solid). Spectra were collected using a 633 nm laser with 60 second integration time.
Figure 9.5: Raman spectra collected from different locations of A549 cell culture that was incubated with SERS Au NPs conjugated to anti-EGFR antibodies. Spectra were collected using 633 nm laser with 60 second integration time. No spectral processing was applied.

Because widefield spectra could not be directly correlated to optical images, confocal Raman mapping was undertaken to study the colocalization of SERS intensity with cells. The individual spectra collected in 1 µm steps along sample plane were analyzed using two methods. To unambiguously identify the particle location, pseudocolour images were constructed from DCLS fit coefficients for each component [26] (Figure 9.6A). Comparison between DIC images and Raman maps show that SERS particles were colocalized with cells (Figure 9.6C). Regions of best fit to cell background spectra most strongly correlated to cell nuclei in overlay images (Figure 9.6C). The slide mounting medium contained DAPI nuclear stain and though the maximum excitation wavelength of this fluorophore is located at 358 nm, some fluorescence emission was generated by the dye upon interrogation with the 638 nm laser.
Figure 9.6: Raman imaging of A549 cell line treated with anti-EGFR conjugated SERS Au NPs. (A) Raman maps determined by direct classical least squares fitting of MG SERS spectrum (green) and background fluorescence spectrum (blue) in to spectra collected from A549 cells. Pixel intensity increases linearly with fit coefficient for each component. (B) Spectrum collected from highlighted pixel in (A). (C) DIC microscopy image of mapped cell area overlayed with DCLS Raman maps. Spectra were collected with 638 nm laser and 50 msec integration time.

To characterize the specificity of anti-EGFR conjugated SERS particles for their cell protein targets, a linear baseline was drawn between 1563 cm$^{-1}$ and 1632 cm$^{-1}$ for each spectrum obtained using Raman mapping, and the area between signal and baseline calculated. This region was selected because it contains an intense peak in the MG SERS spectrum when 638 nm is used as the probe wavelength. When SERS particles were not detected, this spectral region contained mainly noise with a high fluorescence background. Where this was the case, integrating the area under the peak often yielded negative values which were removed from the
intensity distributions by subtracting the normalized distribution from unlabelled cells from sample distributions of cells treated with SERS particles (Figure 9.7). The SERS signal distribution shifted to higher intensities for A549 treated with anti-EGFR targeted SERS NPs as compared to cells treated with IgG1 isotype conjugated particles. Furthermore, when the Wilcoxon Mann Whitney rank sum test was applied to the raw integral intensity values from each sample, A549 treated with anti-EGFR SERS particles displayed statistically significant increases in intensity of the 1616 cm\(^{-1}\) peak compared to both the IgG1-SERS NP and untreated cell samples. Differences in the distribution of intensities for this spectral feature from samples of IgG1-NP treated and untreated A549 were not considered statistically significant.

Figure 9.7: Normalized intensity distributions of spectral region between 1563 and 1632 cm\(^{-1}\) calculated from A549 Raman maps of anti-EGFR targeted cells (black, open bars) and IgG1-SERS NP treated cells (grey). Intensity values from untreated A549 cells were subtracted from each of the distributions. Inset: Normalized intensity distributions prior to subtraction: untreated A549 cells (black dashed line), anti-EGFR targeted cells (black, open bars), and cells incubated with IgG1-SERS NP (grey).
Lastly, the particles’ intense Rayleigh scattering was used to evaluate cell labelling density using dark field microscopy. Using this widefield microscopy technique, large numbers of cells can be simultaneously evaluated for the spatial distribution of plasmonic particles therein. Though cells scatter light intensely and appear blue-white in the dark field images(27,28), particles scatter light most strongly at their LSPR wavelength and can be differentiated from cells based upon their colour in the images (Figure 9.8A, C, E, & G). Nanoparticles are visible in the raw dark field images but in order to further discriminate particle scattering from that of cells, the colour images were separated in to their constituent red, green and blue channels, and the blue channel subtracted from the original colour image. This method evaluates the ratio of pixel values between the three channels which is useful for isolating white from green and red scattering originating from Au particles though image contrast is greatly decreased as a secondary consequence. The differences in labelling densities between specifically and non-specifically targeted samples are shown in the raw images (Figures 9.8A, C, E, & G) as well as the corresponding processed images (Figures 9.8B, D, F, & H). From both raw and subtracted images, there is a trend of decreased cell labelling by SERS particles from anti-EGFR conjugated, IgG1 isotype conjugated and cells incubated with untargeted particles. Images from untreated A549 cells are provided to show the appearance of light scattering originating from cells (Figures 9.8G and H).
Figure 9.8: Dark field imaging of A549 cell line subjected to different nanoparticle treatments. (A) Incubation with anti-EGFR SERS AuNPs; (C) IgG1-isotype SERS AuNPs; (E) PEG-coated SERS AuNPs without targeting moieties; and (G) cells not treated with SERS particles. Dark field images were split into their constituent red, green and blue
channels, and the blue channel subtracted from each of the raw images in the leftmost column to remove light scattering from cells. (B) Subtracted image of anti-EGFR SERS Au NP treatment corresponding to (A); (D) IgG1-SERS AuNP treatment from (C); (F) unconjugated SERS AuNP treatment from (E); and (H) subtracted image from untreated cells corresponding to (G). Three examples of light scattering from SERS AuNPs as they appear in raw and subtracted images are highlighted in (A) and (B). 100X magnification with an additional 1.5X zoom were used to collect the images.

9.5 Conclusions

In this chapter, anti-EGFR conjugated SERS AuNPs have been demonstrated as optical labels for imaging EGFR in non-small-cell lung cancer cell lines. The SERS particles bound cells with specificity for their cell surface antigen targets as was demonstrated using Raman spectroscopy, Raman mapping combined with DIC microscopy, and dark field microscopy.

Because EGFR has a tendency to be endocytosed upon ligand binding [29], future study of particle uptake kinetics would be valuable so that labelling protocols and nanoparticle size could be tailored to suit either surface or intracellular targeting. Additionally, investigation into the particles’ labelling efficacy in primary patient tissues would determine whether the anti-EGFR SERS Au NPs have the potential for clinical use. Finally, to take full advantage of using SERS labelling technologies, nanoparticle targeting could also be expanded to include such biomarkers as heat shock protein 90 (hsp90), histone deacetylase (HDAC) and phosphoinositide 3-kinase (PIK3) which are all diagnostic and/or therapeutic targets for which small molecule and antibody inhibitors are in various stages of clinical trial [8,11].

9.6 Contributions

The author prepared and characterized the SERS nanoparticles, conducted Raman spectroscopy measurements, dark field imaging, and data analysis. The author gratefully acknowledges Natalie Tam for supplying cell cultures, and treating cells with Au NPs in some experiments.
The author also gratefully acknowledges Shell Ip for his advice during the development of widefield Raman spectroscopy protocols.

9.7 References


10 Plasmonic Nanoparticles for Labelling Cells and Tissues

10.1 Permissions

10.2 Overview
Metal nanoparticles have been used to study biological systems by taking advantage of their localized surface plasmon resonance for SERS enhancement of spectra from species located near the particle surface in order to learn about their structure, environment, or to produce SERS-active optical labels. While the investigation of metal nanoparticles for SERS has been the focus of this thesis, they may also serve as contrast agents for use with other imaging modalities.

This chapter contains a brief literature review pertaining to the application of metal nanoparticles in a variety of different detection schemes for labelling cells and tissues. A discussion of surface functionalization, and bioconjugation methods will be provided, along with examples of in vitro and in vivo imaging. Finally, literature pertaining to studies of NP toxicity and biodistribution will be reviewed. Myriad protocols have been developed for the synthesis of noble metal nanoparticles, and several papers are recommended [1–5].

10.3 Introduction: Plasmonic Noble Metal Nanoparticles for Biological Imaging
Two aspects of the application of inorganic nanoparticles for optical imaging are responsible for their increased development and widespread use: 1) the potential to improve detection sensitivity
[6–11] and 2) improved specificity for biomarker targeting in biological systems due to their easily modifiable surface properties [12–31].

There are numerous strategies for using plasmonic noble metal NPs for biomedical diagnostic applications including their use for scannometric or colourimetric detection of ions or biological solution species [32]. Other applications for plasmonic biosensing include the determination of glucose or lactose concentrations [33], the detection of anthrax spores [34] or of Alzheimer’s biomarkers [35]. However, the focus of this text will be the application of plasmonic noble metal NPs to cellular and tissular labelling. The specific, early detection of cells bearing particular disease biomarkers, as for cancer cells, could enable early diagnosis and treatment, long before pathological changes occur.

There are several advantages to using NPs as either passively or directly targeted optical contrast agents. The first advantage is that their optical resonance can be tuned to the NIR where tissue and water do not strongly absorb light [36], by using a number of different strategies, some of which are discussed herein. The second advantage is that there exists the potential for multiplexed detection of several cell surface markers simultaneously, due to the narrow optical resonances of the plasmonic nanoparticles of narrow emission bands of SERS spectra. Thirdly, as a result of the dual components of the particles’ extinction (discussed in more detail in Chapter 3), the ability of metal NPs to simultaneously efficiently absorb and scatter light at wavelengths corresponding to their LSPR enables them to serve as dual imaging and therapy agents [37–39].

Light scattering from illuminated plasmonic particles enables imaging of biological targets to which they have been directed, using any modality that relies on the collection of scattered light. Examples of such imaging modalities include optical coherence tomography (OCT) where metal NPs increase optical contrast [40–42], optoacoustic imaging [43–45], in addition to the methods discussed in this thesis: dark field microscopy [37,46–54], as well as Raman and SERS [55–59]. Depending on the design and materials of the nanoparticle, plasmon decay may occur principally through heat emission, which has been shown to reach temperatures useful for hyperthermal destruction of cells in a well localized region in the immediate environment surrounding the particles [37,38,40,47–49,60–65]. The physical properties that dictate the use of noble metal nanoparticles for imaging (specifically LSPR, the quasi-static approximation for determining the
extinction of an electric field by small NPs, and the enhanced electromagnetic field that can exist at their surfaces) are discussed in detail for spherical particles in Chapter 3. Biological targets, such as cancer cells and tissue, can be reliably imaged using metal nanoparticles, and subject is the focus of this chapter.

10.4 Tuning Metal Nanoparticle Surface Plasmon Resonance

The physical principles that give rise to the unique optical properties of metal nanoparticles are discussed in detail in Chapter 3 however there are two notable motivations for using metal NPs: tunability to achieve optical resonance in the NIR, and the possibility for highly multiplexed imaging.

The potential for a particular type of metal NP to be used as a diagnostic or therapeutic agent is determined by its LSPR wavelength, and the magnitude of the absorption and scattering components of its extinction coefficient. Particles that are more efficient light scatterers are valuable for diagnostic imaging applications, whereas more efficient absorbers that convert this energy to heat are promising for targeted therapy methods. Strategies for achieving tunability of some commonly used nanostructures will now be discussed.

Spherical NPs are advantageous for use because they have facile synthesis methods, are readily commercially available, and have already been adopted for biomedical diagnostic imaging strategies [43,55,59]. A major disadvantage of using spherical particles is that despite significant changes in their diameter, they do not exhibit great variability in the location of their surface plasmon resonant wavelength. Over a diameter range of 20-80 nm, the location of the SP for Au nanospheres shifts from 520 nm to only 580 nm [66]. Because their tunability range is so restricted, their potential in multiplexing applications is limited where a variety of distinct plasmon wavelengths is required, or where detection in the NIR is essential. One feature that is tunable however, is the relative absorption and scattering components of their extinction. With increasing diameter, the relative contribution to extinction by scattering also increases significantly [66,67] and this effect is clearly shown in Figure 10.1C.
Nanoparticles composed of different core and shell materials have more highly tunable LSPR wavelengths. Nanoshells may be comprised of different combinations of two plasmonic metal components [56,68] or a dielectric silica core with a plasmonic shell, as for silica-gold nanoshells [69]. Nanoshells are similar to nanospheres in so far as increasing the total diameter of the particle increases its SPR wavelength and scattering cross-section, and total optical cross-sections of nanoshells tend to be equivalent to that of metal nanospheres composed of a single material [66]. Nanoshells have an additional method through which their SPR may be tuned: the capacity to manipulate the core-shell thickness ratio. Changes to the core-shell thickness ratio can relocate the SPR wavelength through visible to NIR wavelengths [66,69]. For example, the SPR wavelength for a silica core-gold shell NP with a core radius of 60 nm can be manipulated through 300 nm by decreasing the shell thickness from 20 to 5 nm [69]. The extinction efficiency is plotted as a function of wavelength for this system in Figure 10.1B, and clearly displays the range of nanoshell tunability.

Metal nanorods are another nanostructure with a highly tunable LSPR in addition to the advantageous property of possessing extinction coefficients that are an order of magnitude higher than those of nanospheres or nanoshells, on a per-micron basis [66]. An important consequence of this property is that a nanorod would have absorption and scattering cross-sections equivalent to a nanosphere with a greater effective size. The anisotropy of nanorods give them two surface plasmon resonant wavelengths: for Au, one SPR corresponding to the transverse axis of the particle corresponding to approximately 528 nm, and a longer, more dominant resonant wavelength that corresponds to the particles’ longitudinal LSPR [70]. The later resonance may be tuned by modifying the length:width, or aspect ratio of the nanorods. The effect of varying aspect ratio of nanorods SPR is shown graphically in Figure 10.1A. The total extinction coefficient of nanorods increases with increasing particle size, but is independent of aspect ratio [66].
Figure 10.1: Tunability of surface plasmon resonant wavelength for three different nanostructures. A) Surface plasmon resonant wavelength for Au nanorods as a function of increasing nanorod aspect ratio. B) Surface plasmon resonant wavelength for silica core-gold shell nanoparticles as a function of core-shell thickness ratio. C) Ratio of scattering ($C_{sca}$) to absorption ($C_{abs}$) cross-sections of gold nanosphere extinction as a function of nanoparticle diameter. Reproduced with permission from Jain, P.K. et al. Noble metals on the nanoscale: Optical and photothermal properties and some applications in imaging, sensing, biology, and medicine. *Accounts of Chemical Research* 41: 1578-1586 (2008). Copyright © 2008, American Chemical Society. Reprinted with permission.

Hollow gold nanocages also have highly tunable SPR wavelengths and optical properties that may be manipulated through variation of the nanostructure’s dimensions [41,42,60,71]. While
nanocage synthesis is beyond the scope of this chapter [3,71], Skrabalak et al. [39] illustrate the tunability of SPR wavelength and variability of scattering and absorption cross sections by changing nanocage edge length while leaving cage thickness constant, as shown in Figure 10.2. Increasing the total size of the nanocage also increases the total extinction of the particles as well as the relative contribution to this extinction by scattering, as for the previously described nanostructures [39]. Porosity of the hollow Au nanocage surface is another parameter that impacts the optical properties of these nanocrystals. As porosity of the nanocage surface increases, both absorption and scattering cross sections of the nanocage decrease [39].

Figure 10.2. The variation in gold nanocage optical properties as a function of nanomaterial dimensions. A) Tunability of the SPR wavelength of 30 nm length sacrificial Ag nanocube templates as a function of different volumes of HAuCl₄ in a galvanic

10.5 Surface Modification and Functionalization

Ag and Au NPs are advantageous for use with biological samples because they are often synthesized with polar citrate [72] or CTAB surface-capping ligands [73] and thus do not require ligand exchange and solvent transfer steps to transfer them to aqueous solution, as for other inorganic nanoparticles such as quantum dots. Direct protein-particle bioconjugation is possible, whereby a cysteine-terminated protein or peptide is adsorbed to the surface through the formation of a thiol-metal dative bond. The spectral characteristics of metal nanoparticles can also be manipulated through the incorporation of Raman reporter molecules into the surface coating to achieve narrow bandwidth SERS spectra.

An advantage of using metal nanoparticles over other inorganic nanoparticles as contrast agents for biological imaging despite similarities in surface functionalization methods is that synthesis in aqueous conditions enables more facile surface functionalization with hydrophilic ligands, and the possibility for *in situ* bioconjugation. Metal NPs can be synthesized in aqueous reaction conditions using aqueous precursors and therefore may be stabilized using hydrophilic surface ligands such as cetyl trimethylammonium bromide (CTAB) and citrate [54,73].

NP surface functionalization, while advantageous for adapting NPs to various imaging applications, is also necessary in order to ensure their stability and monodispersity upon changes in temperature, salt concentration and pH, and to prevent non-specific interaction with biological samples. An example of a widely used surface coating is poly(ethylene) glycol (PEG), which has been demonstrated experimentally to accomplish these tasks. Particles coated with PEG are resistant to aggregation, have longer blood circulation times, and have a reduction in cellular uptake as compared to uncoated nanoparticles of the same material(s) [38,48,50,55,56,74–77].
Surface modification also enables the inclusion of chemical functionalities for attatching targeting moieties to direct the NPs to their intended biological target for imaging contrast. An example of this is the specific antibody targeting of gold nanoparticles to molecules expressed on cell surfaces as discussed in Chapters 6, 7, 8, and 9 of this thesis. As an alternative to targeting specific biomarkers, NP surface properties can be modified to enhance their uptake in to specific tissues upon being injected into the bloodstream [15,47,51,55,78,79]. This property is advantageous for tumour targeting whereby the enhanced permeability and retention (EPR) effect caused by leaky vasculature surrounding tumour tissues enables passive accumulation of NPs therein. Biotargeting strategies for NPs have largely been adopted from existing approaches used in biological assays taking advantage of specific molecular interactions in order to direct the NP to its target. Several examples of this are targeting NPs using single-stranded DNA which will then bind its complementary strand via hybridization [34,80]; receptor-ligand interactions such as biotin-avidin [23]; and peptide and antibody targeting of NPs to their specific antigen [38,39,48,49,52–57,60,61,74,81]. In order for a particular targeting strategy to be successful, the interaction of the targeting molecule must be highly specific, the interaction must have sufficiently high affinity such that the interaction between molecules is stable enough for imaging. Additionally, the method used to couple the NP to targeting molecules must preserve its affinity for the complementary molecule.

10.6 In Vitro Studies

10.6.1 Dark field microscopy

The large light scattering cross sections of noble metal nanoparticles make them ideal for use as contrast agents for scattering based imaging modalities. Dark field microscopy is an example of one such imaging modality, and is described in more detail in Chapter 4.2.2. All of the nanostructures discussed in Chapter 10.4 have been used as optical labels for dark field and the following section will provide some examples of this application.

Dark field scattering microscopy was used by Halas and coworkers to image cancer cells that had been labelled with immunotargeted silica-gold core-shell NPs [38,48,50]. Anti-human epidermal growth factor receptor (anti-HER2) antibodies were conjugated to NIR-resonant
nanoshells to target them to the human breast cancer SK-BR-3 cell line that overexpress EGF on their surfaces. As for the experiments described in this thesis, the degree of targeting specificity of the nanoshell was evaluated by comparing positively targeted samples to cell labelling by nanoshells attached to non-specific anti-IgG antibodies for which the corresponding antigen is not present on SK-BR-3 cells. Cells were incubated with anti-HER2 and anti-IgG tagged nanoshells, respectively, for 1 hour, washed thoroughly, and scattering from biological samples was assessed using dark field microscopy. Figure 10.3 shows the degree of nanoshell binding. To further confirm that this light scattering originated from nanoshells, the samples were subsequently silver stained [48]. After imaging, a NIR laser that was resonant with the nanoshell surface plasmon was used to irradiate the labelled cell samples for selective photothermal ablation of EGF-overexpressing cells.

Figure 10.3: Immunolabelling of epidermal growth factor on SKBR3 breast carcinoma cells. A) (left to right: dark field scattering images of unlabeled SKBR3 cells, SKBR3 incubated with anti-immunoglobulin G (anti-IgG)-tagged nanoshells, and SKBR3 incubated with anti-HER2 nanoshells. B) Calcein staining to show cell viability following laser irradiation. C) Silver staining of smaples to show the degree of nanoshell binding. From Loo, C., et al. Immunotargeted nanoshells for integrated cancer imaging and therapy. *Nano Letters* 5, 709-711 (2005). Copyright © American Chemical Society. Reprinted with permission.
El-Sayad and coworkers used a similar approach to undertake biological imaging using NIR-resonant Au nanorods as contrast agents for dark field microscopy. Gold nanorods were synthesized using seed-mediated growth method [73], capped with CTAB, and coated with 5 kDa mPEG-SH to prevent aggregation of the nanorods, and to make them more biocompatible. Coated nanorods were targeted to the cell surface protein of interest for imaging using anti-EGFR monoclonal antibodies. The specificity of the anti-EGFR nanorods for their biological targets was evaluated by incubating them with both a nonmalignant cell line (HaCat) as well as two malignant cell lines with high EGFR expression: HOC313 and HSC3 [37]. Dark field microscopy images clearly differentiated the malignant from the nonmalignant cells based on differential nanorod binding. Scattered red light, corresponding to the location of the nanorod surface plasmon, specifically surrounded cells of the malignant line, while the nonmalignant line displayed nonspecific scattering. Representative images are provided in Figure 10.4. As for the previous example, irradiation of cells with a NIR [37] or visible [49] laser that resonated with the nanorods’ longitudinal plasmon resulted in selective photothermal ablation.

Figure 10.4: Dark field microscopy images of three different cell lines treated with anti-EGFR Au nanorods. A) Low EGFR expressing HaCat nanomalignant cell line, and EGFR expressing B) hematopoietic stem cell (HSC), and C) human ovarian carcinoma (HOC) cells. From Huang X, et al. Cancer cell imaging and photothermal therapy in the near infrared region by using gold nanorods (2006). Journal of the American Chemical Society 128, 2115-2120. Copyright © American Chemical Society. Reprinted with permission.

The narrow plasmonic resonances and ease of tunability of resonant wavelength make metal NPs excellent candidates for multiplexed immunolabelling and imaging. Dark field microscopy may also be used to image light scattering from multiple types of plasmonic particles, each with a different surface plasmon resonant wavelength, simultaneously.
An example of one such multiplexed detection scheme is the investigation by Hu et al. who used dark field microscopy to image human pancreatic cancer cells, of MiaPaCa cell line, that overexpress transferrin, claudin 4, and mesothelin receptors on their surfaces [46]. The plasmonic contrast agents used for imaging were gold nanorods and silver nanoparticles that were rendered biocompatible by coating with one of two polyelectrolyte coatings: either positively charged poly(diallyldimethylammonium chloride) (PDDAC), or negatively charged poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate) (PEDT/PSS). Transferin was conjugated to the cationic PDDAC-coated particles through electrostatic attraction, whereas the anticlaudin 4 and antimesothelin antibodies were conjugated electrostatically to anionic PEDT/PSS-coated gold rods and silver particles. Dark field microscopy was used to differentiate the resonant scattering of the two plasmonic nanoprobes, and a representative image is shown in Figure 10.5.

**Figure 10.5.** Dark field scattering image of human pancreatic cell line, Panc-1, tagged with two plasmonic nanoparticle labels for simultaneous imaging based on their differential LSP resonant wavelengths. Inset are dark field scattering images of Au nanorods with resonance in the red region of the visible spectrum (top right), blue-resonant Ag nanoparticles (bottom left). From Hu, R. et al. Metallic nanostructures as localized plasmon resonance enhanced scattering probes for multiplex dark field targeted imaging of cancer cells. *Journal of Physical Chemistry* 113, 2676-2684 (2009). Copyright © American Chemical Society. Reprinted with permission.

An alternative approach to multiplexed dark field imaging was taken by Yu et al by incorporating a spectral imaging system in to the dark field microscopy setup. The plasmonic
contrast agents used were Au nanorods with distinctly separated resonant wavelengths having aspect ratios of 1.5, 2.8, and 4.5 respectively [54]. The CTAB-capped nanorods underwent ligand exchange with 11-mercaptopoundecanoic acid (MUDA) to improve their biocompatibility and each type of nanorod was conjugated to a different antibody with affinity for CD44, CD24, or CD49f cell surface antigens, respectively. This combination of biomarkers was used to differentiate between two human breast cancer cell lines, MDA-MB-436 and MDA-MB-231, and one nonmalignant epithelial cell line, MCF10A. Dark field images were taken and extinction spectra analyzed to evaluate the presence of different combinations of nanorods with different LSPR wavelengths, present on the cell samples simultaneously. The identification of CD44⁺/CD24⁻ cells is significant as these have cancer stem cell properties and maintain tumour growth.

10.6.2 SERS

The enhanced electromagnetic field at the surface of plasmonic metal NPs may be exploited to enhance the intensity of Raman scattering from species in close proximity to the surface. The SERS phenomenon is discussed in detail in Chapter 3.5. The advantages of SERS-based biological detection using either the characteristic spectra of Raman reporter molecules, or the enhancement of endogenous Raman spectra from biological molecules, have been investigated at length in this thesis.

El-Sayed and coworkers collected the SERS spectra of functionalization molecules attached to NIR-resonant Au nanorods in order to distinguish between HSC human carcinoma and nonmalignant epithelial cell line HaCat, based on differential nanorod binding of the two cell types. The nanorods preferentially gathered within cells of the malignant line and aligned so that their surface plasmons overlapped, thus enhancing the intensity of Raman scattering from surrounding molecules. Two different targeting methods were investigated in separate publications: (1) gold nanorods covalently conjugated to nuclear localization signal peptide (SV40 virus NLS peptide) via thioalkyl-triazole linking click chemistry [53]; and (2) PSS coated nanorods with anti-EGFR monoclonal antibodies attached via noncovalent interactions [52]. For the immunotargeting study, SERS spectra were collected from more than 20 cells for each of the normal and cancerous lines following incubation with anti-EGFR tagged nanorods. For the HSC
cancerous cell line, 90% of cells showed strong SERS from each of the nanorod functionalization molecules (CTAB, PSS, and anti-EGFR antibodies) and 10% of cells showed intense SERS from CTAB only. This result can be contrasted with the spectra collected from the normal cell line where 80% did not have a detectable SERS spectrum and 20% of cells had low intensity spectra of CTAB capping molecules only. Representative spectra from this study are provided in Figure 10.6. Dark field images were also able to distinguish between the malignant HSC and normal HaCat cell lines.

![Figure 10.6. Representative SERS spectra from Au nanorods for detection of cancer cells.](image)

A) SERS of anti-EGFR antibodies conjugated to CTAB-capped Au nanorods; B) SERS from CTAB-capped Au nanorods; C) Bulk Raman spectrum of PSS powder; D) SERS spectrum of monoclonal anti-EGFR antibodies obtained through subtraction of (B) and (C) from (E) and E) SERS of the final system consisting anti-EGFR-PSS-CTAB Au nanorods incubated with the HSC cell line. From Huang, X., et al. Cancer cells assemble and align gold nanorods conjugated to antibodies to produce highly enhanced, sharpt, and polarized surface Raman spectra: A potential cancer diagnostic marker. *Nano Letters*, 7, 1591-1597 (2007). Copyright © American Chemical Society. Reprinted with permission.

When click chemistry was used to conjugate Au nanorods to NLS peptides, dark field images showed Au nanorods located in the nuclei and cytoplasm of both cell lines. Single cell Raman spectra showed enhancement of peptide peaks, as well as cytoplasmic molecular species. The
differential spectral enhancements were used to distinguish malignant and nonmalignant cell lines, and representative spectra from this study are provided in Figure 10.7.

An alternative strategy for biological detection using SERS is embedding a Raman reporter onto the NP surface coating thus introducing a specific fingerprint spectrum for clear identification of the NP probe and the potential for multiplexed imaging. Kneipp et al. synthesized SERS particles with a Raman reporter, indocyanine green, adsorbed to the surface of commercial 60 nm Au NPs [58]. Human serum albumin (HSA) was used to coat the NPs to prevent aggregation and to stabilize the dye molecules on the metal surface. The SERS enhancement of indocyanine green was evaluated from both Au and Ag nanoparticles in this study and were found to be comparable, hence Au was chosen for biological experiments because it is more biocompatible than Ag [58]. Indocyanine green SERS particles were incubated with rat prostate carcinoma cell line cultures, and SERS was collected using multiple excitation lines at 680, 786, and 830 nm following removal of excess nanoparticles. The uptake of SERS particles by cells was independently evaluated using light and electron microscopies. The SERS spectra obtained from treated cells contained bands specific to indocyanine green in addition to bands that were assigned to the DNA backbone and C-N ring stretching modes of DNA and RNA bases (Figure 10.8). The SERS enhancement from plasmonic Au NPs was found to be significant enough such that lower laser power densities and shorter irradiation times could have been employed for the same spectral collection, thus protecting the surrounding untagged cells.
Figure 10.8. Representative SERS spectra from single cells following incubation with indocyanine green (ICG) SERS particles. A 832 nm laser was used as the probe wavelength. A) Trace A is of indocyanine green probe in solution, as synthesized; trace B is of indocyanine green probes internalized by a cell, trance C shows peaks attributed to the cell, derived by subtracting B from A. Traces D and E are other examples of spectra obtained from cells incubated with ICG SERS particles. Spectral peaks markers with an asterisk in parentheses are from particles, which bands marked with an asterisk originate from ICG particles. B) Left: bright field microscopy image of cells with the cell chosen for Raman mapping indicated in black; right: spectral imaging of ICG particles inside cell, intensity monitored by 1147 cm\(^{-1}\) ICG line, and the product of two ICG lines at 1147 and 945 cm\(^{-1}\). From Kneipp, J., et al. Optical probes for biological applications based on surface enhanced Raman scattering from indocyanine green on gold nanoparticles.
The SERS spectrum of targeted plasmonic metal nanoparticles may also be used to determine the presence of one or more cell surface markers, without simultaneously detecting or analyzing enhanced spectral bands from surrounding biological molecules. Qian et al. synthesized SERS particles by adsorbing organic dyes malachite green isothiocyanate (MGITC) and diethylthiatricarbocyanine (DTTC), respectively, onto 60 nm diameter spherical Au NPs [55]. Single-chain fragment (ScFV) antibodies were covalently conjugated to carboxylic acid terminated PEG molecules using EDC/NHS mediated cross-linking, which is discussed in more detail in Chapter 4.3.4 of this thesis. The remaining adsorption sites on the particles were coated using an excess of thiol-terminated PEG to create a barrier that would prevent the dissociation of Raman reporter molecules from the particle surface, and also to prevent aggregation of the particles. To evaluate the specificity of the NPs for their biomarker targets, control particles were also conjugated to nonspecific anti-IgG antibodies. The Tu686 EGFR expressing human head and neck carcinoma cell line was used as the imaging target. Results from the Tu686 line were contrasted with those from the human non-small-cell carcinoma line, NCI-H520 which does not express EGFR on its surface. DTTC embedded Au NPs were used for in vitro labelling. ScFv- and IgG-tagged NPs were incubated with each cell culture respectively, and SERS intensity evaluated following removal of unbound particles. Figure 10.9 shows distinctive SERS from EGFR-positive tumour cells incubated with immunotargeted DTTC SERS probes.

For Raman-active molecules located in the interstices of two or more plasmonic NPs, SERS enhancement is markedly increased due to the overlap of plasmons from neighbouring particles, and a resultant increase in the magnitude of the electromagnetic field in this region. While much effort has been directed toward assembling NP aggregated in a controlled manner, this strategy presents a host of challenges. Lee et al. attempted to exploit this effect for single molecule SERS, by synthesizing hollow gold nanospheres, the centre of which would act as the junction between two particles, thus enhancing the electromagnetic field surrounding the Raman reporter molecules adsorbed to the particle surface [57]. The hollow gold nanospheres were used to
image the expression of human epidermal growth factor, HER2, on MCF7 cells. HER2 is an attractive biomarker target because its expression is characteristic of breast cancer [57]. The hollow nanospheres were synthesized as described by Schwartzberg et al. [82] whereby cobalt templates were coated with gold under a purging N₂ atmosphere. Once this N₂ purging was ceased and ambient conditions reestablished, the cobalt centre was fully dissolved, leaving hollow gold nanoshells remaining. The hollow nanospheres were 45 nm in diameter with a wall thickness of 15 nm. The Raman reporter used for detection was crystal violet which was adsorbed to the Au nanosphere surface. Anti-IgG monoclonal targeting antibodies were attached to hollow Au nanospheres using EDC/NHS mediated cross-linking of carboxylic acid groups of dihydrolipoic acid (DHLA) surface coating ligands with the monoclonal antibodies. Antibody-DHLA conjugates were then grafted to the Au NP surface via the two thiol terminal groups of DHLA. The remaining nanosphere surface area was coated with mercaptoethanol to prevent nonspecific adsorption of other molecules. Raman maps were collected from MCF7 cells successfully labelled with targeted Au nanospheres, and spectral intensities were compared with Ag NPs used in the same labelling strategy (Figure 10.10). The intensity distribution was much narrower for gold nanospheres than for silver NPs, and the SERS mapping image of MCF7 cells with the former appeared much more homogeneously illuminated.

Figure 10.10. Raman mapping images of MCF7 cells incubated with anti-IgG-conjugated A) hollow gold nanospheres, and b) silver nanoparticles. C) Histograms of peak intensity ratios of spectra collected from individual image pixels. From Lee, S., et al Surface-enhanced Raman scattering imaging of HER2 cancer markers overexpressed in single

10.7 *In Vivo* Studies

10.7.1 Optical Coherence Tomography

OCT may be used for deep tissue imaging in order to accurately and noninvasively identify the location of tumours and other tissue abnormalities. To improve image contrast and identify specific tissue regions of interest, targeted contrast agents are often used. Current technologies for this purpose include microbubbles filled with water or oil. Because of the microscale dimensions of these particles, they encounter difficulties entering the microcirculation and surrounding tissues [39,48]. Plasmonic metal NPs, with large absorption and scattering cross-sections, are of a size scale for which these steric difficulties are less of a concern.

An example of the application of plasmonic metal NPs as OCT contrast agents is the investigation by Gobin *et al.* of silica-gold nanoshells for this purpose [40]. The nanoshells were synthesized with a dielectric silica core, and gold shell, and were subsequently coated with 5kDa thiol-terminated PEG as per a procedure referred to earlier in the chapter [69]. Tumour xenografts were prepared using cultured colon carcinoma cells implanted into mice. The PEGylated nanoshells were passively delivered to the tumour by the EPR effect following injection into the mouse tail vein [83]. As controls, OCT images were also collected of healthy tissue injected with PBS and nanoshells, as well as of tumour tissue injected with PBS alone. Figure 10.11 shows that the OCT contrast for tumor cells injected with nanoshells was increased as compared to the OCT backscattering signal from any of the control test groups.
Targeted metal NPs have also been used as optical contrast agents for OCT imaging. Xia and coworkers have employed targeted gold nanocages for this purpose [41], and more recently, as therapeutic agents for photothermal therapy of tumour cells [60,61]. Gelatin tissue models were used to evaluate OCT optical contrast imparted by the nanocages [41,42], and *in vitro* cell studies were used to evaluate the potential of nanocages for selective cell targeting [42]. At small nanocage edge lengths of less than 40 nm, LSPR wavelengths in the NIR, of approximately 800 nm, were achieved by Chen *et al.* [42], which are excellent for biological imaging. Au nanocages were conjugated to monoclonal anti-HER2 antibodies via activated succinimidyl propionyl poly(ethylene glycol) disulfide and targeted to breast cancer cells.
overexpressing HER2 on their surfaces. SEM imaging was used to compare the binding specificity of bioconjugated nanocages with that of spherical Au NPs, and it was concluded that they were equally efficient for this purpose. Enhancement of optical contrast for OCT was also evaluated using gelatin models doped with a low concentration of Au nanocages and TiO$_2$ to mimic the inherent background scattering from biological tissues. Au nanocages were found to be effective contrast agents for OCT as areas of low nanocage concentration can be clearly differentiated from unlabeled areas in representative OCT images (Figure 10.12).

Figure 10.12: Evaluation of Au nanocages as optical contrast agents for OCT imaging. A) OCT image of TiO$_2$-embedded gelatin tissue model. The right and left sides of the image differ in that the left side contains a 1nM concentration of nanocages, while the right side of the tissue does not contain nanocages. B) OCT signal intensity as a function of tissue model depth for nanocage-containing, and nanocage-free sections of gelatin tissue model. From Chen, J. Gold nanocages: Bioconjugation and their potential use as optical imaging

10.7.2 In Vivo SERS

The utility of SERS for selective identification of cancer cells has also been demonstrated *in vivo*. On such example of *in vivo* SERS is the investigation by Qian *et al.*, whereby xenograft tumour models were prepared by implanting Tu686 human head and neck tumour cells in to mice. Anti-EGFR tagged and control untagged NPs were injected in to the tail veins of mice [55]. The PEG-coated particles were embedded with malachite green as the Raman reporter, and SERS spectra were collected by focusing a 785 nm laser onto the tumour site, as well as on the liver and leg to serve as controls. SERS probes that were not conjugated to targeting antibodies were also injected in to the mouse tail vein and directly to the tumour site to evaluate whether any benefit could be derived from specific nanoparticle targeting. SERS spectra collected from the tumour site for groups subjected to targeted particle injection were significantly more intense than SERS from the control group that had been treated with unconjugated particles. SERS intensities of spectra taken from the liver and spleen of both groups indicated that particles had accumulated in those regions, regardless of whether targeting proteins were grafted to the NP surface (Figure 10.13).

![Figure 10.13: Raman spectra collected from tumour and liver of xenograft mice that had received tail vein injections of A) anti-EGFR targeted SERS nanoparticles, and B) untargeted SERS nanoparticles. From Qian, X. *In vivo* tumor targeting and spectroscopic](image-url)

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Au/Ag core-shell NPs were also used by Lee et al. for in vivo targeting of HEK293 human embryonic kidney cells that overexpress surface biomarker, phospholipase Cγ1 (PLCγ1) [62]. Because PLCγ1 is overexpressed in multiple types of cancer cells, including colorectal and breast carcinomas, it is an attractive target for diagnostic imaging. While gold is a popular choice for in vitro and in vivo biomedical applications due to its inert surface chemistry and relative biocompatibility, silver has the ability to further enhance SERS spectral intensity by a factor of 10-1000. Rhodamine 6G was used as the Raman reporter, and adsorbed to Au NPs, followed by subsequent coating with BSA to improve particle stability. The Ag shell was then applied over top of this structure, and the final 30 nm diameter Au/Ag nanoshells were coated in thiol-terminated mPEG. EDC/NHS mediated cross-linking chemistry was used to conjugate ortho-pyridyl-sulfide-polyethyleneglycol-N-succinimidyl propionate (OPSS-PEG-NHS) to anti-mouse IgG antibodies, and the resultant molecule was used to functionalize the Ag surface in the same manner as for mPEG-SH. The SERS spectrum of rhodamine 6G embedded in the particle structure was sufficient to distinguish between populations of healthy cells, and those baring the tumour biomarkers. The SERS intensity images based upon the 1650 cm$^{-1}$ peak of the R6G spectrum in Figure 10.14, clearly show SERS particle labelling differences between normal cells and those expressing cancer cell surface markers. Spectra were acquired sequentially across the surface of the cell in 3 μm increments, and SERS intensities mapped and overlaid with brightfield images (Figure 10.14). An intense peak at 1542 cm$^{-1}$ was observed during the Raman mapping which is not characteristic of R6G SERS. This extra peak was assigned to an enhanced spectrum of molecules in the cellular environment, in close proximity to the particles. This is consistent with the findings of Kneipp et al. [58,59]. The ability of anti-IgG antibody-conjugated nanoshells to selectively bind PLCγ1 cell surface receptors has been visualized in Figures 10.14A and 10.14D where the anti-IgG labeled quantum dots were incubated with healthy and cancerous cells, respectively.
Figure 10.14: Comparison of Raman and fluorescence imaging for the detection of PLCγ1 surface receptors. A) and D) Bright field and fluorescence images of normal and cancerous cells tagged with anti-IgG-labelled quantum dots. B) and E) Bright field and Raman mapping images of normal and cancerous cells targeted with anti-IgG-labelled Au/Ag nanoshell SERS probes. C) and F) Raman depth intensity mapping images overlayed on bright field micrographs of the cells, with corresponding Raman spectra on the right. From Lee, S. et al. Biological imaging of HEK293 cells expressing PLCγ1 using surface-enhanced Raman microscopy. Analytical Chemistry 79: 916-922 (2007). Copyright © American Chemical Society. Reprinted with permission.

SERS for multiplexed detection schemes in vivo has the potential to become a powerful diagnostic tool. Zavaleta et al have provided an example of how this may be accomplished through the detection and spectral resolution of 10 different SERS NP labels [84]. Spectra were collected from NPs that had been deposited at superficial tissue depth through subcutaneous injection of 10 different SERS labels, and at greater tissue depths following non-specific accumulation of 5 different SERS labels following tail vein injection. Commercially available Au-core, silica-shell NPs with Raman-active molecules embedded in the silica layer, were used for this study. Raman mapping showed the location of each of the 10 subcutaneously injected
probes, and spectra analysis enabled the successful separation of the 10 different signals with minimal interference between the respective channels. SERS spectra from labels that had passively accumulated in the liver were used to estimate the concentration of each marker located therein.

### 10.7.3 Photoacoustic Imaging

Plasmonic nanoparticles have also been demonstrated as effective contrast agents for multiwavelength photoacoustic imaging by Mallidi et al. [45]. Because photoacoustic imaging uses NIR probe wavelengths, this technique enables tissue depths in the range of centimeters to be successfully imaged. To evaluate the potential of Au NPs as photoacoustic contrast agents, an *ex vivo* tumour model was prepared using subcutaneous gelatin implants within *ex vivo* mouse tissue. Four implants were used: (1) human epithelial carcinoma cells (A431 keratinocyte line) incubated with selectively targeted 50 nm spherical Au NPs conjugated to anti-EGFR antibodies, (2) A431 keratinocytes incubated with unconjugated control Au NPs, (3) A431 cells that were not treated with Au NPs, and (4) A431 that was treated with a NIR absorbing dye for control of contrast during the photoacoustic imaging technique. Au NPs were conjugated to anti-EGFR antibodies via hydrazide PEG dithiol heterobifunctional linkers, and incubated with A431 cells [45]. Untargeted Au NPs coated with mPEG only were incubated with A431 cells in the same manner. Each sample was suspended in gelatin and injected subcutaneously in the abdominal region of a euthanized mouse. Photoacoustic images shown in Figure 10.15 identify the light-scattering contrast provided by selectively targeted Au NPs. Ultrasound imaging (Figure 10.15A) was used to locate hyperechoic regions in the tissue for comparison. At longer wavelengths where the optical absorbance of tissue is decreased, greater contrast was apparent in the implant containing anti-EGFR tagged Au NPs incubated with A431 cells. The targeted samples could be clearly differentiation from controls using photoacoustic images. This study as well as other related investigations [44,51] highlight the potential value of plasmonic NPs to this medical diagnostic imaging technique.
Figure 10.15: Evaluation of Au NPs as contrast agents for photoacoustic imaging. A) Ultrasound image of gelatin implants. Highlighted regions: A431 with anti-EGFR NPs (red), control A431 cells (white), A431 with untargeted Au NPs (green), A431 stained with NIR dye. B-F) Photoacoustic images of the same implant region obtained using different wavelengths as noted in each image. From Mallidi, S. Multiwavelength photoacoustic imaging and plasmon resonance coupling of gold nanoparticles for selective detection of cancer. *Nano Letters* 9:2825-2831 (2009). Copyright © American Chemical Society. Reprinted with permission.

10.8 Toxicity

Much research is directed toward the synthesis of new nanostructures as well as novel ways to incorporate them into medical diagnostics. As a result of these efforts, the potential toxicity of noble metal NPs is an important topic for further investigation. Toxicity will be defined as the reduction of viability or introduction of morphological and/or genetic defects. Whether or not a nanostructure is toxic is determined by a complex interplay between biodistribution, cellular uptake of the particles, and their behaviour upon uptake. *In vitro* studies are a valuable tool for understanding the uptake mechanisms of NPs by cells as well as their distribution within the cell, interactions with species therein and whether the particles degrade over time. Zebra fish are becoming a popular *in vivo* system to mimic the potential behaviour of NPs prior to moving...
toward studies in higher-order organisms [85]. However, mammalian studies must eventually be conducted as they take into account additional complex biological reactions that cannot be investigated in any other manner, in particular the immunological response [86], in addition to absorbance, distribution, metabolism and excretion of NPs by mammals [87,88].

Two facets of in vitro and in vivo biological interaction should be examined in the exposure of cells and organisms to noble metal NPs: the inherent toxicity of the NP coating material, the NP constituent material, and the combination thereof, as well as the NP in vitro and in vivo uptake, distribution, and accumulation. The uptake and accumulation of a nanostructure does not necessarily infer that it has toxic effects, however the propensity of nonspecific uptake and accumulation must be considered in determining whether metal NPs are safe for biological applications. Specific delivery and uptake of NPs not only improves the accuracy of diagnostic measurements, but also safety for use as photothermal therapeutics. The size, composition, surface chemistry and surface charge all impact the distribution and subsequent toxic effects of nanoparticles [89,90].

10.8.1.1 In Vitro Study

The influence of nanoparticle surface coating and charge on eventual cell uptake and distribution has been the focus of a number of valuable studies. The distribution behaviour of Au nanorods was investigated by Huff et al. using the KB human oral epithelial tumour cell line [91]. Cationic CTAB molecules were present on the nanorod surfaces following synthesis and were incubated with KB cultures in serum-free growth media without further surface modification. Rapid, non-reversible uptake of the cationic CTAB-coated nanorods by KB cells was observed and upon further investigation using two-photon luminescence microscopy, the nanorods had compartmentalized as aggregates within cellular organelles [91]. When a ligand exchange of CTAB for bis(p-sulfonatophenyl)phenylphosphine (BSP) or hydrophilic mPEG-NH₂ was conducted, cellular uptake was significantly reduced for both of the alternative coatings: mPEG-coated rods displayed only 6% of the uptake level of CTAB-coated equivalents, and cell uptake significantly decreased for BSP coated nanorods as well. The growth and morphology of KB cells was also monitored for 5 days following nanorod exposure and despite the fact that uptake had been high for CTAB coated rods, there were no major changes to cell viability during this
time period. The nonspecific uptake of CTAB-coated rods could be a matter of concern for applications were specific targeting of contrast agents is required.

A thorough characterization study was conducted by Hauck et al. using multiple combinations of polyelectrolyte coatings commonly used to coat nanorods in order to modulate the particles’ zeta potential [149]. As for the investigation by Huff et al. [91], the newly synthesized rods were coated with CTAB capping molecules and these were layered with poly(4-styrene sulfonic acid) (PSS) followed by either or poly(allylamine hydrochloride) (PAH) or poly(diallyldimethyl ammonium chloride) (PDADMAC). A fifth group of CTAB, PSS, and PDADMAC functionalized rods were coated with an additional PSS layer. Though isolated molecules of each component of the coating layer had a high toxicity profile, when used as nanorod coatings, over 90% of the cells observed were found to be viable following exposure as determined using Trypan Blue stain. Human cervical HeLa cells were incubated with Au nanorods over a 6 hour period with multiple treatments of varying concentration. The PSS-coated rods having the most negative zeta potential (−69.5 mV), exhibited the lowest amount of cell uptake, while the PSS/PDADMAC-coated nanorod sample had the highest rate of uptake by cells as well as the most positive value for zeta potential (+37.1 mV). The other coating combinations exhibited uptake levels that fell between these two extreme values. The Trypan Blue viability stain monitors only whether cells are living, without providing additional information as to their long term viability, or the presence of stress responses. In order to address this, Hauck et al. used a global gene expression experiment to detect whether changes in gene expression occurred following exposure of HeLa cells to PDADMAC-coated nanorods. Only 0.35% of the 10 000 genes assessed by this test exhibited any alteration, and of the alterations detected, all consisted of downregulation of the genes in question.

10.8.1.2 In Vivo Study

Griffitt et al. conducted a small-scale in vivo characterization of the toxic effects of nanocopper, nanosilver and nanotitania particles by examining the morphological and transcriptional responses of zebrafish gills following exposure [85]. In order to decouple the effects of nanoparticle constituent material and structure, zebra fish were also exposed to dissolved metal solutions in concentrations that would be present in water during the NP exposure. Following
exposure to each of these materials, gills were evaluated for changes in expression of particular genes, as well as morphological irregularities. Uptake and distribution was found to be variable between the different metal NP species. Silver exhibited the greatest level of full body distribution of both the dissolved metal constituent material, as well as for the full nanostructure. Dissolved and nanostructure copper caused thickening of the zebrafish gills. Each of the three different metals elicited distinct changes to the gene expression profiles of zebra fish as well. It was thus concluded that the toxicity of nanostructures of a particular materials cannot be inferred from the cytotoxic profile of similarly structured nanoparticles composed of a different material.

An alternative approach to using zebra fish to study in vivo nanoparticle toxicity was used by Bar-Ilan et al. whereby zebra fish embryos were subjected to treatment with colloidal Au and Ag nanoparticles with a range of diameters: 3, 10, 50 and 100 nm [92]. Zebra fish egg water, which is used to maintain embryo survival, was spiked with increasing concentrations of each type of nanoparticle and a scoring system applied to evaluate the severity of deleterious morphological effects, or impacts on embryo survival. Toxicity for colloidal gold was found to be relatively low and consistent across the size range studied, however all sizes of Ag colloids exerted cytotoxic effects.

Cho et al. used 6 week old mice as their in vivo model for investigating the toxic effects upon exposure to 13 nm PEG-coated Au nanoparticles [77]. NPs were introduced by tail vein injection and mice were euthanized at 5 minute, 30 minute, 4 hour, 24 hour, and 7 day time points post-injection. The presence of the PEG coating was found to have resulted in long blood circulation times of approximately 30 hours. Additionally, there was long-term accumulation of PEGylated Au NPs in the liver and spleen for up to 7 days following injection, as well as acute liver inflammation and apoptosis of cells therein. Overall however, a slow, but eventual, clearance of Au NPs was observed despite temporary accumulations in the brain, testis, kidneys and lungs.

Balogh et al. evaluated the in vivo behaviour of gold composite nanodevices (CNDs) in mouse tumour models [89]. CNDs were composed of Au NPs of varying size that were conjugated to poly(amidoamine) dendrimers with differently charged groups. CNDs differ from the other NPs discussed in this review is so far as they were not functionalized using chemisorbed or physisorbed surface ligands, but instead were trapped in the dendrimers to facilitate
Several different systems were tested: 5 nm Au having an acetamide terminated surface of neutral charge, 5 and 11 nm Au with negatively charged carboxylate functionalities on the surface, as well as positively charged amine capped 5 and 22 nm diameter Au. During their investigation, Balogh et al. monitored the degree to which the CNDs remained in the mouse circulation, the biodistribution of the CNDs, their accumulation in organs and tissues as well as efficiency with which the CNDs were excreted. Biodistribution and accumulation were evaluated by euthanizing mice at several different time points post CND injection and extracting blood samples, as well as tissues of the brain, lung, liver, heart, kidney, pancreas, and spleen for analysis. In order to evaluate excretion rates, mice were housed in metabolic cages. The results of this investigation correlated well to what was discovered by the other studies discussed in this chapter. Particle biodistribution, organ deposition, blood circulation times and excretion were all found to be strongly influenced by particle size and surface charge. The smallest devices, specifically 5 nm CNDs, elicited decreased immune response, and did not accumulate in the organs to the same extent as the larger devices. The longer blood circulation times exhibited by the smaller devices also indicates that they are not significantly taken up by immune cells or the spleen. Larger, 22 nm, CNDs accumulated to a greater extent in the lung, heart, and liver as compared to the smaller particles. Surface charge also had an influence on the fate of the particles post-injection. Positively charged 5 nm CNDs had the longest blood circulation times, and highest excretion rates, though did show accumulation in the kidneys. The in vivo behaviour of neutral and negatively charged CNDs differed from that of the positively charged particles in so far as they did not remain in the circulation for as long, and also tended to accumulate in the liver. Upon extraction and analysis, the brain, muscle, and pancreas were not identified as locations of significant particle deposition for any of the sizes or charges of CNDs. The authors concluded that the 5 nm CNDs were advantageous for in vivo applications due to the fact that they did not display significant nonspecific accumulation in the organs, nor did they elicit an immune response of the same magnitude as that of the larger particles. The small CNDs were therefore concluded to be the most promising for use as a specifically-targeted diagnostic imaging platform, once bound to targeting moieties.
10.9 Conclusions and Future Perspectives

Undoubtedly, the ability to design multiplexed NIR resonating imaging platforms is compelling motivation for adoption of metal nanoparticle diagnostic technologies. Furthermore, narrow bandwidth SERS-active metal nanoparticles are attractive for their potential to increase the number of biomarkers that can be simultaneously identified with multiplexed detection platforms. The importance of this concept is best represented by the need to reduce non-specific biomarker labelling. Many \textit{in vivo} imaging platforms are tested using tumour xenograft animal models. This is problematic for the evaluation of labelling specificity because often a human tumour is implanted in the animal tissue, and thus the biomarker of interest is not expressed by any other cells in the host animal. This can result in the false conclusion that targeting is highly specific. As Diagarajane \textit{et al.} \cite{78} points out, this does not represent the clinical situation in which the tissue surrounding the tumour likely expressed the same biomarker as the tumour (though this can be to a varying extent). Multiplexed labelling platforms would enable simultaneous detection of combinations of biomarkers that are expressed in different ratios or combinations by healthy and diseased tissues. A suitable library of SERS labels may have to be refined by evaluating different dye combinations and nanoparticle sizes to ensure that surface molecule targets are bindable and ratios resolvable in an accurate manner. Additionally, smaller targeting moieties may yield improved NP binding densities, such as the replacement of large monoclonal antibodies with low molecular weight affibodies, or peptides.

A second challenge of future development of metal nanoparticles for cell and tissue labelling is the more systematic characterization of \textit{in vitro} and \textit{in vivo} toxicity. Thus far, there has been a lack of standardization of certain nanoparticle toxicity tests. Many \textit{in vivo} studies were evaluated as a component of a larger, unrelated study. Additionally, different \textit{in vivo} models are used, and a variety of variables measured to determine toxicity. Short-term acute toxicity also may not be reflective of longer-term effects of nanoparticle deposition in organs when they are not effectively excreted. Before significant advances can be made toward the clinic, the long-term toxic effects of metal nanoparticle-based labelling technologies must be understood, as well as methods for mitigating these toxic effects, identified.
10.10 Contributions

The material presented in this chapter place an emphasis on contributions made by the author. The author gathered references, and wrote the first draft of the publication for the subject matter related to metallic nanoparticles, and has adapted that text from the original publication, for this chapter. The author gratefully acknowledges Shell Ip for organizing the references, format and preparation of the final draft of the original publication. The author also gratefully acknowledges Gilbert Walker for guiding the information gathering and organization of information for original publication. The contributions from C.T. Nguyen do not appear in this chapter.

10.11 References


11 Summary of Thesis and Future Work

11.1 Summary of Thesis

It was demonstrated that spherical gold nanoparticles could form the basis for preparing SERS probes; both PEG, and lipid bilayers were demonstrated as effective SERS particle coatings; the antibody-conjugated SERS probes were found to bind specifically to their cell surface antigen targets using three in vitro tumour models.

The nanoparticle coating was required to either incorporate or prevent dissociation of Raman reporter molecules, to reduce non-specific association of nanoparticles with solution molecules or cells, and to stabilize the colloidal particles in buffer solutions. Additionally, the coating molecules included functional groups for attaching targeting antibodies.

SERS nanoparticle probes that were functionalized with thiol-terminated poly(ethylene glycol) could be prepared using a series of positively-charged Raman dyes, with the eventual goal being the establishment of a library of SERS probes for multiplexed in vitro immunophenotyping. The SERS functionality was confirmed by collecting Raman spectra of the finished nanoparticles. Functionalization with thiol-terminated PEG was evaluated indirectly by observing changes in the particles’ UV-Vis absorbance spectra, DLS hydrodynamic radius, and zeta potential, upon coating as compared to unfunctionalized stock gold nanoparticles. TEM imaging enabled direct visualization of the PEG coating layer. Monoclonal IgG1 isotype antibodies with reactivity for human B lymphocyte cell surface antigens, were covalently attached to the PEG-coated SERS probes. Standard assays for protein detection and quantification carry inherent limitations, and often need to be modified to account for interference introduced by the presence of metal nanoparticles. Therefore, the results of three different quantification methods were compared to obtain averaged values for the number of antibodies that had been bound to each SERS probe. The colourimetric BCA assay was used to measure the amount of protein present in a suspension of SERS nanoparticles, and secondary antibodies bound to two different contrast agents were used to indicate the presence of their target antigen (antibodies) on the SERS probe surfaces.
SERS gold nanoparticle labelling could be interrogated using multiple methods: Raman spectra collected from solutions and fixed microscopy slides, dark field imaging, and flow cytometry. The specificity of the antibody-conjugated SERS probes for their cell surface antigen targets was demonstrated through a number of control experiments that compared the labelling of positively targeted samples to cells treated with non-specifically targeted particles. Additionally, competitive binding experiments whereby target antigens were blocked prior to nanoparticle treatment, repeatedly demonstrated that SERS probes bound primarily to the antigen for which their targeting antibodies had reactivity.

SERS Au nanoparticles were evaluated using clinically relevant conditions whereby malachite green functionalized SERS probes conjugated to therapeutic anti-CD20 rituximab antibodies were evaluated as labels using primary human CLL cells, for Raman and dark field microscopy detection, in Chapter 6. Also notably, CD20 is known to cluster in lipid rafts upon ligand binding, and this clustering was observed through shifts in the particles’ LSPR, indicating plasmon overlap between adjacent particles. Such plasmon rulers offer promise for studying biological structures separated by distances smaller than the diffraction limit of light microscopy using straightforward instrumentation, and biocompatible contrast agents.

Three different SERS probes, each conjugated to a corresponding monoclonal antibody, were evaluated for simultaneous binding efficacy and specificity using LY10 lymphoma cell line, and primary CLL cells in Chapter 7. Triplexed labelling of the LY10 cell line was demonstrated by least-squares fitting of spatially resolved, mixed SERS spectra from antibody-targeted SERS probes bound to different CD molecule targets, simultaneously. The linearity of this spectral resolution method with the combination of cresyl violet, ethyl violet, and malachite green SERS probes was demonstrated. Wide field dark field images also enabled localization and quantification of SERS probe binding from large cell populations.

SERS probes were also demonstrated as robust, bright labels for flow cytometric detection. Not only were rituximab-SERS gold nanoparticles effective and specific labels for detecting CD20, but they were demonstrated in Chapter 6 as being compatible for use with fluorescent competitive binding, and secondary antibody labelling assays using flow cytometry. The particles’ SERS was also detected using a narrowband optical interference filter corresponding to the wavelength of an intense peak in the SERS spectra of three different dyes, respectively.
Integral intensity histograms indicated that anti-CD20 MG SERS probes were bound to their LY10 cell surface protein targets, and that biotin-labelled MG, CV, and DTCC SERS probes were bound to streptavidin-functionalized PS microspheres, in Chapter 7. Incorporation of SERS particles into flow cytometric assays is a significant advance in the development of SERS probes for clinical immunophenotyping applications.

A second SERS nanoparticle coating method was demonstrated, consisting of the encapsulation of 60 nm diameter gold nanoparticles in a ternary lipid bilayer that was a mixture of DOPC, egg sphingomyelin and cholesterol in a 2:2:1 molar ratio. This zwitterionic coating enabled incorporation of three different classes of Raman reporters molecules: charged malachite green isothiocyanate for physisorption to the nanoparticle surface, hydrophobic tryptophan that was added to the MLV suspension during encapsulation, and rhodamine lissamine that was covalently conjugated to the head group of one of the incorporated lipid molecule species. Additionally, Rho-PE inclusion in the bilayer was modulated by the presence of ions in the encapsulation solution, as well as by the choice of ion. The lipid-encapsulation of SERS probes was demonstrated to have occurred using the sample methods as for characterizing the PEG-coated SERS probes discussed above. Lipid encapsulated gold nanoparticles also demonstrated stability in acidic and ionic solutions as compared to the stock citrate-capped gold nanoparticles lacking a phospholipid coating. The development of these particles is discussed in Chapter 5.

The efficacy of liposome-encapsulated SERS gold nanoparticles as contrast agents for SERS and dark field immunophenotyping was demonstrated in Chapter 8. SERS functionality was imparted to the particles using a physisorbed Raman reporter, malachite green isothiocyanate. Using established liposomal conjugation chemistry, SERS liposomes were functionalized with either antibody fragments, or whole IgG1 isotype monoclonal antibodies specific for CD20 and CD19, respectively. The efficacy and specificity of each particle type binding to their cell surface targets was evaluated using two cell models: the LY10 cell line, and samples of primary human CLL cells. The intensity of SERS spectra for both particle types was sufficient for detection of cell binding using Raman microscopy, and Rayleigh scattering from the particles was used to identify and quantify their binding using dark field microscopy.
11.2 Suggestions for Future Work

11.2.1 Overview

Moving forward, there are two principle directions that could be pursued in the further development and *in vitro* labelling application of monoclonal antibody-targeted SERS gold nanoparticles, both with a clinical focus. SERS probes could be further developed as contrast agents for higher-order multiplexed flow cytometry immunophenotyping of B cell neoplasms. Additionally, the potential for antibody-targeted SERS gold nanoparticles to act as plasmon rulers could be exploited to provide insight into the mechanism(s) by which therapeutic antibodies induce apoptosis in B cell neoplasms.

11.2.2 Multiplexed Immunophenotyping Contrast Agents

As discussed in Chapter 2, contrast agents for immunophenotyping that could increase the upper limit of simultaneously detected biomarkers offer great promise for improving the specificity of disease diagnosis. This is a particularly important concept for flow cytometric detection. Although there are a number of alternative flow cytometry labelling approaches and the applications for fluorescence flow are being continuously developed [1], the great majority of clinical flow cytometers still rely on detection of relatively few fluorescent labels concurrently. Because fluorescence emission spectra are very broad, the upper limit for the number of spectra that can be resolved within the visible region of the electromagnetic spectrum is quickly reached; particularly without extensive modification made to the detection optics of the flow cytometer.

Narrow bandwidth SERS spectra are promising labels for flow cytometry. Presumably, a greater number of SERS labels should be able to be simultaneously detected and resolved within a given spectral bandwidth, as compared to fluorescence technologies. From an instrumentation development standpoint, a SERS cytometer is advantageous because all resolvable SERS labels can be designed to resonate with a single laser wavelength.

In order to move SERS technologies to the clinic, an analytical study of the linearity of their intensity upon labelling must be conducted. A standard for SERS labels akin to the MESF scale
for fluorophores should be established. Factors such as plasmon overlap between plasmonic nanoparticles that cluster upon binding [4], and variations in intensity between SERS particles of a single batch could both contribute to non-linearity of spectral intensity. Additionally, the dynamic range of relatively large SERS particle labels could be more narrow than small fluorophores due to potential steric hindrance of nanoparticles binding adjacent cell surface molecules. A direct comparison of the determined detection limit and dynamic range of SERS immunolabels with those of fluorophores, is essential for clinical adoption so that SERS intensity data can be used to accurately determine both relative and absolute biomarker expression levels.

Fluorescence flow cytometry calibration beads are used to calibrate intensity measurements to account for differing quantum yields between fluorophores, and spill-over of signal between channels. This strategy could be used to calibrate SERS immunolabels, and calibration beads would need to be produced for this purpose. Preliminary results for the development of SERS flow calibration beads are presented in Chapter 7. This is an essential first step toward quantifying these systems for both research and clinical application.

11.2.3 SERS Gold Nanoparticles as Plasmon Rulers

Imaging the landscape of cell surface receptor expression in complicated by the fact that these structures are smaller, and separated by distances closer together than the diffraction limit of standard light microscopy. As discussed in Chapter 1, a number of methods have been developed to address this limitation in order to image both the structure, and location of individual receptors. Most require complex instrumentation that is not widely available, or they limit the information that can be obtained because measurements cannot be collected from live cells. Noble metal nanoparticles offer promise as plasmon rulers for imaging cell surface dynamics from live cells, using more straightforward instrumentation.

In Chapter 6 of this thesis, rituximab-conjugated SERS gold nanoparticles were evaluated for labelling CD20 surface proteins on primary human CLL cells. Dark field light scattering images indicated that the colour of the particles’ light scattering, and therefore their LSPR wavelength, red-shifted upon cell binding. This red-shift was determined not to be the result of exposure to in
*vitro* biological conditions because the rituximab-conjugated particles used to label the cells were composed mainly of monodispersed particles as determined using their visible absorbance spectra and hydrodynamic radii, and PEG-coated particles had demonstrated colloidal stability in buffer solutions over periods of days, yet significant populations of clustered particles were present on labelled cells. This observation is notable because CD20 has been known to cluster into lipid rafts upon ligand binding, and this may have been observed using dark field microscopy with plasmonic labels bound to CD20.

Moving forward, this strategy could be used to study the mechanism of B cell apoptosis upon binding of therapeutic antibodies to CD20. CD20 has proven to be an advantageous target molecule for antibody-based leukemia and lymphoma therapies. This cell surface molecule is expressed by all mature B cells, and changes in CD20 expression level are used to stage disease progression. Multiple therapeutic antibodies have been developed for use with CD20, however their mechanism of action is not always fully understood, and treatment outcomes are not always consistent between patients. This is because the immune response is highly complex with many interrelated components; it is not immediately clear by what mechanism(s) apoptosis is being affected. Variability in the efficacy of anti-CD20 antibody therapies can be explained in part by different CD20 epitope specificities of the antibody therapies [5,6]; therapeutic antibodies that have the highest affinity for the epitope expressed by a neoplastic B cell clone should have the most potent therapeutic effect. However, the Fc fragment of the monoclonal antibody also plays a role by determining its effector function, and therefore by what means an immune response is recruited against the bound cell. The different anti-CD20 antibody therapies are believed to each act through a different combination of antibody-dependent-cell-mediated cytotoxicity (ADCC), compliment-mediated cytotoxicity (CDC), and antibody-dependent cellular phagocytosis (ADCP) [7,8].

The apoptotic mechanism of rituximab is believed to involve programmed cell death upon hypercrosslinking of neighbouring CD20 receptors incited by ligand binding, and individual complement proteins (for example, C1q for rituximab), may facilitate the crosslinking through binding antibody Fc regions though this is not certain [9–12]. Dark field imaging and correlating Rayleigh scattering spectra of anti-CD20 rituximab-conjugated plasmonic particles could be used to individually assay molecules that could be mediating bound receptor hypercrosslinking. The presence of individual complement proteins in cell suspensions under physiologic conditions
could change the rate or magnitude of plasmon overlap of clustered particles. Additionally, comparisons could be made to determine whether there is variation in the molecules that mediate crosslinking for different therapeutic antibodies. This information could be useful in the design of therapeutic antibodies; perhaps the most potent therapies, or therapies that are effective for a particular disease type or stage, recruit a specific compliment protein.

11.3 Final Remarks

The B lymphotype cancer models used to study the feasibility of SERS-active nanoparticles as immunolabels in this thesis could be replaced by any other type of normal or cancerous cell in order to obtain information about cell structure and function, or disease onset. In fact, this was demonstrated in part through investigating anti-EGFR SERS gold nanoparticles as contrast agents for lung cancer detection using A549 adenocarcinoma cells. Potential applications for SERS technologies are wide reaching for both fundamental studies of native cellular and/or protein structure, or in vitro and in vivo biological imaging. SERS-based nanoparticle technologies for both fundamental, and clinical applications have been developed in this thesis.

11.4 References


