

New Possibilities for Metallic Nanoshells: Broadening Applications with Narrow
Extinction Bands

by

Regivaldo Gomes Sobral Filho

B.Sc., Universidade Federal de Pernambuco, 2006

M.Sc., Universidade Federal de Pernambuco, 2010

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University of Victoria

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Supervisory Committee

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Abstract

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This dissertation comprises experimental studies on the synthesis and applications of metallic nanoshells. These are a class of nanoparticles composed of a dielectric core and a thin metallic shell. Metallic nanoshells play an important role in nanotechnology, particularly in nanomedicine, due to their peculiar optical properties. The overall objectives of the dissertation were to improve the fabrication of these nanoparticles, and to demonstrate new applications of these materials in cancer research and spectroscopy.

The fabrication of nanoshells is a multi-step process. Previously to our work, the procedures for the synthesis of nanoshells reported in the literature lacked systematic characterization of the various steps. The procedure was extremely time-consuming and the results demonstrated a high degree of size variation. In Chapter 3, we have developed

characterization tools that provide checkpoints for each step of the synthesis. We demonstrated that it is possible to control the degree of coverage on the shell for a fixed amount of reagents, and also showed important differences on the shell growth phase for gold and silver. The synthetic optimization presented in Chapter 3 led to an overall faster protocol than those previously reported.

Although the improvements presented in Chapter 3 led to a higher degree of control on the synthesis of nanoshells, the variations in the resulting particle population were still too large for applications in single particle spectroscopy and imaging. In Chapter 4, the synthesis was completely reformulated, aiming to narrow the size distribution of the nanoshell colloids. Through the use of a reverse microemulsion, we were able to fabricate ultramonodisperse silica (SiO_2) cores, which translate into nanoshell colloids with narrow extinction bands that are comparable to those of a single nanoshell. We then fabricate a library of colloids with different core sizes, shell thicknesses and composition (gold or silver). The localized surface plasmon resonance (LSPR) of these colloids span across the visible range. From this library, two nanoshells (18nm silver on a 50nm SiO_2 core, and 18nm gold on a 72nm SiO_2 core) were selected for a proof of principle cell imaging experiment. The silver nanoshells were coated with a nuclear localization signal, allowing it to target the nuclear membrane. The gold nanoshells were coated with an antibody that binds to a receptor on the plasma membrane of MCF-7 human breast cancer cells. The nanoshells were easily distinguishable by eye in a dark field microscope and successful targeting was demonstrated by hyperspectral dark field microscopy. A comparison was made between fluorescent phalloidin and nanoshells, showing the superior photostability of the nanoparticles for long-term cell imaging.

The results from Chapter 4 suggest that the nanoshells obtained by our new synthetic route present acceptable particle-to-particle variations in their optical properties that enables single particle extinction spectroscopy for cell imaging. In Chapter 5 we explored the use of these nanoshells for single-particle Surface-enhanced Raman spectroscopy (SERS). Notice that particle-to-particle variations in SERS are expected to be more significant than in extinction spectroscopy. This is because particle-to-particle SERS variabilities are driven by subtle changes in geometric parameters (particle size, shape, roughness). Two types of gold nanoshells were prepared and different excitation wavelengths (λ_{ex}) were evaluated, respective to the LSPR of the nanoshells. Individual scattering spectra were acquired for each particle, for a total of 163 nanoshells, at two laser excitation wavelengths (632.8 nm and 785 nm). The particle-to-particle variations in SERS intensity were evaluated and correlated to the efficiency of the scattering at the LSPR peak.

Chapter 6 finally shows the application of gold nanoshells as a platform for the direct visualization of circulating tumor cells (CTCs). 4T1 breast cancer cells were transduced with a non-native target protein (Thy1.1) and an anti-Thy1.1 antibody was conjugated to gold nanoshells. The use of a transduced target creates the ideal scenario for the assessment of nonspecific binding. On the *in vitro* phase of the study, non-transduced cells were used as a negative control. In this phase, parameters such as incubation times and nanoshell concentration were established. A murine model was then developed with the transduced 4T1 cells for the *ex vivo* portion of the work. Non-transduced cells were implanted in a control group. Blood was drawn from mice in both groups over the course of 29 days. Antibody-conjugated nanoshells were incubated with the blood samples and detection of single CTCs was achieved in a dark field microscope. Low levels of nonspecific binding

were observed in the control group for non-transduced cells and across different cell types normally found in peripheral blood (e.g. lymphocytes). All positive and negative subjects were successfully identified.

Chapter 7 provides an outlook of the work presented here and elaborates on possible directions to further develop the use of nanoshells in bioapplications and spectroscopy.

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Chapter 1: Overview

1.1 Motivation and General Objectives

1.1.1 Motivation

As we approach the end of another decade, it is possible to look back at the progress of nanotechnology to find a large number of publications on the synthesis and application of nanoparticles.¹ Much has been discussed in the field and, undeniably, great progress has been made, with some of the most advanced developments combining particle architecture and composition to achieve the desired physical, chemical and biological properties.^{2, 3}

In the fabrication of nanostructured particles, the use of noble metals, namely silver and gold has been favored mainly due to their plasmonic properties, and ease with which surface chemistry can be performed.¹ Among the different shapes/categories, such as nanospheres⁴, nanorods⁵, nanocubes⁶, and nanowires⁷, the early 2000's saw the onset of a peculiar type of nanoparticle with the coined name "metallic nanoshells".⁸ These nanoshells are composed of a dielectric core and a metallic shell, and their optical properties can be tuned according to the ratio between core size and shell thickness. The resulting extinction bands from nanoparticles with a range of core size/shell thickness ratio cover a wide portion of the electromagnetic spectrum, from visible to near-infrared wavelengths. This large coverage can be explored in a broad range of biomedical applications in therapy and diagnostics.⁹

The inspiring potential of these particles for bioapplications was unveiled when Halas and coworkers¹⁰ demonstrated their use for the treatment of solid tumors in mice. Nanoshells tuned to absorb light at the biological window (ranging from 770-900nm) were

able to produce enough heat to kill tumor cells upon laser irradiation. Their success was shortly followed by a Phase I clinical trial for the treatment of head and neck cancer.¹¹

In spite of this promising opportunity for lab-to-clinic translation, the fabrication of nanoshells has been permeated with serious reproducibility problems.¹² That happens largely because the classic synthetic process involves several types of colloids and surface processes, each of those carrying their own challenges. As a result, almost two decades after their first appearance, a procedure that could systematically produce relatively monodispersed particles was long overdue. Eventually, it became clear that, for certain applications, further refinement on the synthesis was still necessary to produce high quality nanoshells.¹³

For historical reasons, the use of nanoshells in bioapplications has always been associated with cancer treatment. However, it is also clear that these nanoparticles could contribute to cancer diagnostic and other bioanalytical applications.¹⁴ Gold and silver-based nanoparticles can be easily conjugated to biomolecules.¹⁵ Furthermore, if colloids with narrow extinction bands were produced, they could be suitable for use in multiplex analysis. Finally, the large extinction cross sections of nanoshells compared to their pristine gold or silver counterparts could be successfully detected even by inexpensive microscopy techniques.

1.1.2 General Objectives

The main objectives of this research project are to improve and understand the process of nanoshells' fabrication, to a point where new applications can be explored whilst seeking the widespread use of this peculiar class of nanoparticles. Over the course of 20 years, we have lacked reports that stand out regarding the reproducible and systematic synthesis of

these particles, and here we aim to establish new routes that will result in nanoshells of the highest quality, while relying only on simple labware. The methods for assessing each step of the synthesis will also be defined and will play an important role in the dissemination of the new processes. The applications we envision to derive from such methods heavily rely on the uniformity of the synthesized particles. The successful outcome for this venture includes the use of nanoshells as a suitable platform for multiplex analysis at the single nanoparticle-single cell level. Coupled with advanced microscopy techniques, such particles can enable spectroscopic studies on individual nanoparticles, showing for example, how far-field optical properties (*e.g.* scattering) can relate to near-field effects. Ultimately, we seek to establish our nanoshells as a viable platform for cell analysis, including the main stages that comprise a preclinical validation (including *in vitro* and *ex vivo* studies). According to the structure of the chapters on this dissertation, the objectives have been broken down in the following manner: (1) to establish a systematic method to fabricate and characterize gold and silver nanoshells, providing the checkpoints for the process so that researchers can track their progress and troubleshoot their obstacles (Chapter 3); (2) to optimize the synthesis to a point where nanoshells of the highest quality can be obtained with the use of only simple labware, so that researchers across the globe can reproduce our results (Chapter 4) and (3) to establish the use of nanoshells as a suitable platform for cell analysis (Chapter 4); (4) to assess the importance of their LSPR tunability, related to near-field plasmonic effects in the form of Surface-enhanced Raman scattering (Chapter 5) and finally (5) to develop and validate a nanoshell-based detection platform for circulating tumor cells (Chapter 6).

1.2 Outline

This dissertation follows the article-style dissertation format and is organized as follows:

Chapter 2 presents a brief introduction to the topics of localized surface plasmon resonance, nanoshells synthesis and surface-enhanced Raman scattering. Important considerations are made in the end about the use of nanoparticle-based platforms for application in cell analysis.

Chapter 3 is based on the following published work: A. M. Brito-Silva, R. G. Sobral-Filho, R. Barbosa-Silva, C. B. de Araújo, A. Galembeck, and A. G. Brolo, Improved Synthesis of Gold and Silver Nanoshells, *Langmuir* 2013, 29, 4366-4372.

Chapter 4 is based on the published work: R. G. Sobral-Filho, A. M. Brito-Silva, M. Isabelle, A. Jirasek, J. J. Lum and A. G. Brolo, Plasmonic Labeling of Subcellular Compartments in Cancer Cells: Multiplexing with Fine-tuned Gold and Silver Nanoshells, *Chem. Sci.* 2017, 8, 3038-3046.

Chapter 5 is based on the unpublished work: R. G. Sobral-Filho, X. Zhang, C. D. L. de Albuquerque, A. G. Brolo, SERS Excitation Wavelength and Localized Surface Plasmon Resonance: A Single Nanoparticle Study. Dissertation withheld from publication. Manuscript in preparation.

Chapter 6 is based on the published work: R. G. Sobral-Filho, L. DeVorkin, S. Macpherson, A. Jirasek, J. J. Lum, A. G. Brolo, *Ex vivo* Detection of Circulating Tumor Cells from Whole Blood by Direct Nanoparticle Visualization, *ACS Nano* 2018, 12, 1902–1909.

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Chapter 2: Introduction

This chapter provides general background information on several topics, including: Localized Surface Plasmon Resonance, Nanoshells Synthesis, Surface-enhanced Raman Scattering and Considerations for Nanoparticle-based Platforms in Cell Analysis.

2.1 Localized Surface Plasmon Resonance

When submitted to an oscillating electric field, the conduction electrons on the surface of metallic nanoparticles can undergo collective oscillations, generating a phenomenon called localized surface plasmon resonance (LSPR). The wavelength to this resonance can be determined by chemical composition, as well as the physical constraints dictated by the nanoparticle's size, shape and architecture.¹ Insight into this topic is provided here, followed by a comment on the plasmonic properties of nanoshells.

The LSPR phenomenon corresponds to the excitation of electromagnetic surface modes as resonant oscillations of the surface charge density at the boundaries of the metal nanoparticle.² One of its most comprehensible representations is illustrated in Figure 2-1, where a metallic spherical nanoparticle is submitted to a propagating electromagnetic wave.

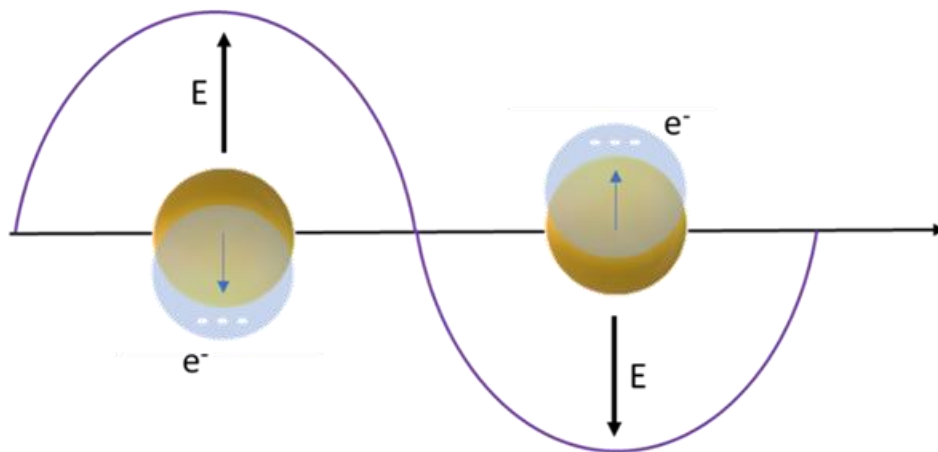


Figure 2–1 Localized surface plasmon resonance: electrons (e^-) oscillate in resonance with an incoming electric field (E).

In simple terms, the electric field associated with the electromagnetic wave causes a displacement of the conduction electrons relative to the nuclei of the surface atoms. This displacement gives rise to Coulomb attraction forces from the nuclear framework, that pull the electrons back to their initial position, establishing a coherent oscillation in the form of an electron cloud.³ A dipole is then established. To better understand the process, assumptions can be made that for particles considerably smaller than the wavelength of the incoming light, the propagating electric field is constant, and the LSPR response is governed by electrostatics rather than electrodynamics. This is usually referred as the quasistatic approximation.¹ These assumptions give rise to equations that can successfully predict the polarizability and extinction cross section of spherical particles up to 100nm in diameter.⁴

In this regime, the extinction cross-section, σ_{ext} can be defined by:

$$\sigma_{ext}(\omega) = 9 \frac{\omega}{c} \varepsilon_d^{3/2} V \frac{\varepsilon_2(\omega)}{[\varepsilon_1(\omega) + 2\varepsilon_d]^2 + \varepsilon_2(\omega)^2} \quad (2.1)$$

$$\sigma_{ext} = \sigma_{scatter} + \sigma_{abs} \quad (2.2)$$

with ω as the frequency of the incident light, c as the speed of light, V is the volume of the particle, ε_d is the dielectric constant of the surrounding medium, and ε_1 and ε_2 are the real and imaginary parts of the metal's dielectric constant, where $\varepsilon_{metal}(\omega) = \varepsilon_1(\omega) + i\varepsilon_2(\omega)$. $\sigma_{scatter}$ and σ_{abs} are the scattering and absorption cross-section of the particle, respectively. Notice from equation 2.1 that a large value of σ_{ext} , representing the resonance condition is achieved if $\varepsilon_1(\omega) = -2\varepsilon_d$ and ε_2 is small. This is also known as the Frölic condition, and it is met for gold, silver and copper in the visible and near infrared portions of the electromagnetic spectrum.⁴

In the case of spherical particles with dimensions on the same range as the wavelength of the incident light, the same assumptions from the quasistatic approach cannot be applied. Significant phase-changes will be present in the incident field over the particle volume, and to calculate and predict a scattering response from such particles to incoming electromagnetic fields, an electrodynamic approach is required. In the early years of the 18th century, German physicist Gustav Mie developed a theory for the scattering and absorption of electromagnetic radiation by a larger sphere, studying the different colors of colloidal gold particles in suspension.⁵ In his work, a full analytical solution of Maxwell's equations is presented, to obtain the spectral position and intensity of the resonances on a spherical particle. The mechanisms and assumptions detailed in Mie's theory served as the foundation for the theoretical predictions of the plasmonic properties of nanoshells. This theory was developed in 2003 and it is called the *plasmon hybridization* model.⁶ The main

assumption to this model is that metallic nanoshells are a two-interface system, where two plasmon modes are supported: 1 – the outer shell-surface mode and 2 – the inner shell-surface cavity mode. These two modes couple giving rise to a hybridized plasmon. Jain and El-Sayed developed upon this model to reach a simple mathematical expression capable of predicting the dipolar plasmon resonance of a silica-gold nanoshell of given dimensions.⁷ The expression is outlined below:

$$\varepsilon_c = -2\varepsilon_s \frac{\varepsilon_s(1-f) + \varepsilon_m(2+f)}{\varepsilon_s(1+2f) + 2\varepsilon_m(1-f)} \quad (2.3)$$

where ε_c , ε_s and ε_m are the dielectric constants of the core, shell, and medium, respectively, and f is the fraction of the volume of the core in the composite structure, so $f = (t/R + 1)^{-3}$, with t representing the thickness of the shell and R representing the radius of the core. Figure 2-2 shows some example of calculated spectra for single nanoshells with different values of f .

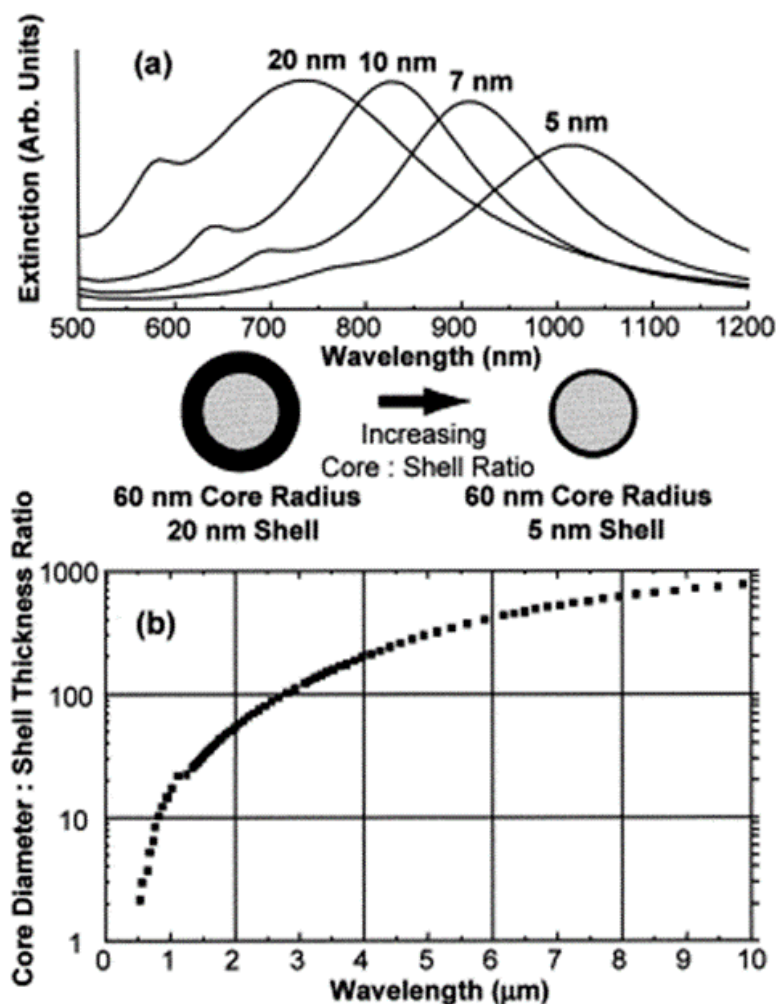


Figure 2–2 (a) Theoretically calculated optical resonances of metal nanoshells (silica core, gold shell) over a range of core radius/shell thickness ratios. **(b)** Calculation of optical resonance wavelength versus core radius/shell thickness ratio for metal nanoshells (silica core, gold shell).⁸
 [Used with permission from Reference 8]

2.2 Nanoshells Synthesis

The first experimental report on the plasmonic properties of nanoshells happened in 1998 when Oldenburg⁸ communicated some data on their LSPR tunability. Due to their complex architecture, works focusing on the fabrication aspect of nanoshells only started to appear

in the literature around 2002-2004.^{9, 10} In 2003 Hirsch, Halas and West¹¹ reported for the first time the effective use of gold nanoshells for the thermal ablative treatment of tumors, based on the efficient conversion of light into heat by the nanoshells. This was groundbreaking for the therapeutic use of nanoparticles, and prompted metallic particles for use in a variety of bioapplications.¹⁰ The technique is referred today as photothermal therapy, and three clinical trials are currently listed on the NIH database.¹²

In general, the fabrication of nanoshells is composed of several processes that can be broken down into the following checkpoints: 1) synthesis and functionalization of the silica (SiO_2) core, 2) fabrication of small gold (Au) seeds, 3) attachment of the Au seeds onto the functionalized cores and finally, 4) shell growth. Figure 2-3 illustrates the overall process.

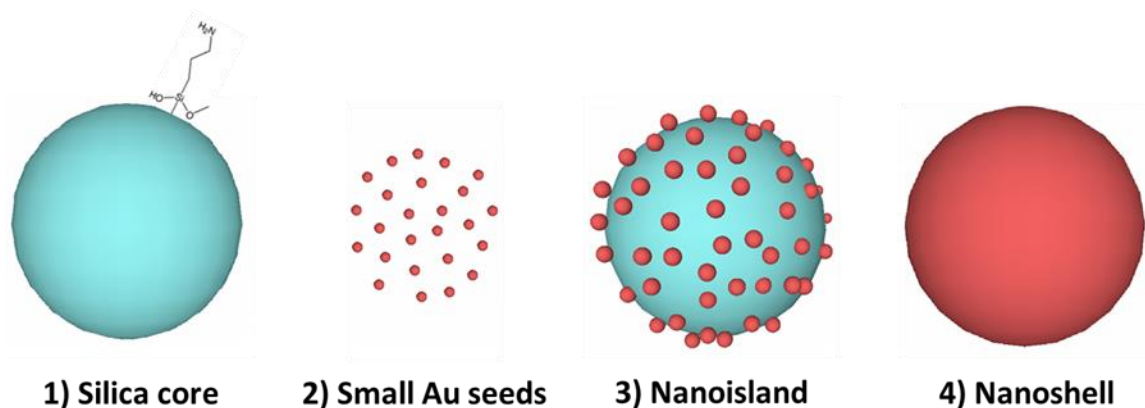


Figure 2–3 Overall process for nanoshell synthesis. Silica core shown in blue, gold seeds and gold shell shown in red.

The classical approach to nanoparticle synthesis would generally involve: 1) a precursor material in the form of ions or molecules, 2) a reducing agent or catalyst to promote nucleation and growth and 3) a stabilizing/passivating agent to prevent aggregation and

further modifications to the nanoparticles.¹³ Nonetheless, considering the increasing complexity of the nanoparticulate systems used today, we cannot afford to hold a simplistic view of the process, and, in the case of metallic nanoshells, each step may present specific factors that need to be brought into consideration when planning and designing experiments.

The SiO₂ core

In their first description, Halas and coworkers⁹ utilize the sol-gel method outlined by Stöber¹⁴ to produce the silica cores. In this approach an ethanolic solution of tetraethyl orthosilicate (TEOS) is placed under stirring and aqueous ammonium hydroxide is added to promote the alkaline hydrolysis of TEOS through a nucleophilic substitution mechanism. Details on the chemical reactions underlining the process have been extensively discussed in the literature, and Sakka *et al.*¹⁵ have performed a systematic study on the evolution of the synthesis under different conditions. The particles produced via the Stöber method are spherical, can be considered monodisperse, and the procedures to be executed in the lab are relatively simple. A variety of sizes ranging from nanometer to micrometer scale can be obtained through changes in reaction times, reagents amounts and sequential growth steps. In modern colloidal chemistry, the functionalization of SiO₂ particles is perceived as a continuation of the growth process. The molecules used in this process are usually siloxanes and the same chemical principles that drive the formation of the particles will apply to the surface modification. Different functionalities can be achieved according to the siloxane of choice, and commonly used molecules include amino-propyl trimethoxysilane (for amine-functionalized silica) and mercapto-propyl trimethoxysilane (for thiol-functionalized silica). The resulting aminated or thiolated SiO₂

cores are suitable for the further attachment of the gold seeds. A good functionalization will result in homogeneous nanoislands that will, by their turn, result in a homogeneous shell.

Small Au seeds and Nanoislands

The small gold nanoparticles used to produce the nanoislands play a fundamental role in the final product of the synthesis. The previously functionalized SiO_2 cores will be combined with the gold seeds to form the nanoislands. During the first stages of the growth phase, the small gold nanoparticles will act as sites for the deposition of gold from solution. Because they will first grow isotropically, expanding in all directions until the point of coalescence is achieved, this limits the lowest thickness that can be achieved for the final shell. The process is illustrated in Figure 2-4.

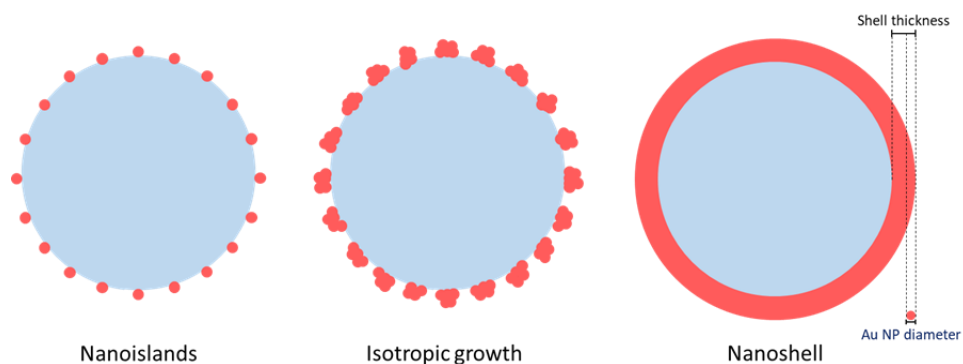


Figure 2-4 The small gold seeds (red) limit the shell thickness and determine a homogeneous coating of the silica core (blue).

It is important to notice that there are two aspects of the synthesis that are highly dependent on this process: 1) the complete coalescence of the shell – related to the

homogeneous distribution of the gold seeds onto the silica core, and 2) the final thickness of the shell – related to the initial diameter of the Au seeds. The Duff method¹⁶ was widely adopted for the synthesis of the gold seeds for the nanoislands. It comprises the reduction of gold ions in aqueous solution by tetrakis(hydroxymethyl)phosphonium chloride (THPC). The resulting particles need to undergo a ripening process that can take up to several days, to achieve a monodisperse size distribution with diameters ranging from 2-3nm. In spite of the long waiting times and the biotoxicity of THPC, this method results in gold seeds small enough to generate shells as thin as 10nm. Prasad *et al.*¹⁷ have also showed thicker shells using nanoislands made from bigger Au seeds (approx. 10nm) prepared using the Turkevich method.¹⁸

Shell growth

The final step of the synthesis consists in placing the nanoislands in a solution containing the ionic precursors of the metal of choice. Gold and silver are the most common choices in this phase, but copper nanoshells have also been synthesized.¹⁹ This portion of the synthesis lacks reports describing a systematic investigation. Several reaction systems have been used and it has been difficult to establish the guidelines for the successful growth of the metallic shell. In general, the nanoislands are placed in an alkaline gold or silver solution and a reducing agent is added under stirring. A prominent change in color indicates that growth is happening in the reaction vessel. The critical point of the growth phase is the possibility of forming new nanoparticles in suspension. This process is known as external nucleation in the sense that they are foreign to the nanoislands and, therefore, detrimental to the shell growth – newly formed nanoparticles will compete with the gold seeds for the metallic ions in solution.

In summary, several types of colloids and processes are involved in the fabrication of metallic nanoshells. Each of these brings their own challenges and complexities, indicating that they should be individually studied and well understood before the synthesis can progress. Among several reports on different approaches for nanoshell synthesis, not many have stood out in terms of reproducibility and the scientific literature has been lacking a guide for the systematic synthesis and characterization of these particles. Notable works in the field include the automation of a complex microfluidic platform to synthesize nanoshells²⁰ and the work outlined in reference 17, where nanoshells were produced using polystyrene cores as an alternative to SiO₂.

2.3 Surface-Enhanced Raman Scattering

Surface-enhanced Raman scattering consists of the amplification of the Raman signal due to intense localized fields distributed on a metallic surface.²¹ Due to the LSPR phenomenon, strong localized fields can be produced by metallic nanoparticles, and over the years, SERS has been intimately linked to these nanostructures. Its main mechanism is electromagnetic in nature.²² The local fields generated by plasmon resonances concentrate the electromagnetic field in a region near the surface of the metal, enhancing the intensity of the Raman scattering from active molecules situated within that region. These regions are called hotspots, and have been the focus of many research papers in the field.²³ A typical SERS hotspot formed by a gap between two metallic nanoparticles is shown in Figure 2-5.

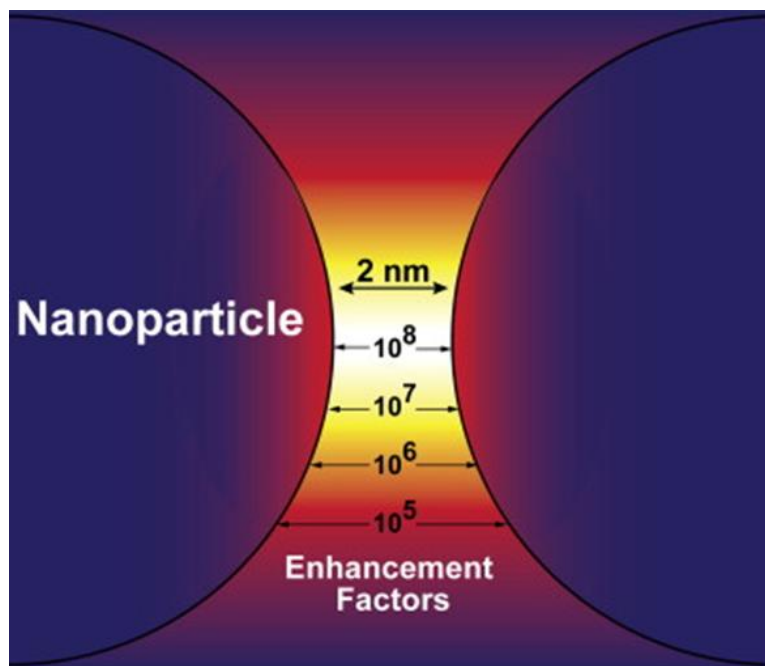


Figure 2-5 An illustration of hotspot for nanoparticle dimer and rapid change in SERS enhancement factors with respect to relative position. Nanoparticle diameter = 20nm. [Used with permission from Reference 24]

Any Raman active molecule located in a hotspot will have its Raman scattering intensity enhanced by the local field. The magnitude of this enhancement will depend on the position of the molecule respective to the hotspot, and how intense are the local fields produced by the particles. The spatial distribution of the SERS enhancement factors within a typical hotspot is displayed in Figure 2-5. A detailed view on the enhancement factors in SERS and how they can be determined can be found in Le Ru and Etchegoin's work.²⁵

The field of nanomaterials synthesis evolved substantially in the last 2 decades, and now, the development of nanoparticles that are tailored to meet specific experimental requirements has finally become a reality. The high proficiency scientists have achieved in making diverse types of nanoparticles, opens the door for more detailed studies on SERS

even at the single nanoparticle level.²⁶ Chapter 5 conveys the status of this area and mentions the most recent reports for different types of nanoparticles.

2.3.1 SERS excitation wavelength and LSPR

There are several forms to optimize the SERS efficiency of a system. Aggregating nanoparticle samples, choosing molecules with high absorption cross-sections at the excitation laser wavelength to benefit from the occurrence of resonant Raman are just a few of them.²⁷ With intense activity on the field over the last years, researchers also started to look at the position of the LSPR peak of the substrates respective to the SERS excitation wavelength. It has become a commonly accepted rule that the best SERS signal will come from excitation wavelengths that match the LSPR peak position.²⁸ This can turn out to be a problematic generalization, though, and there are several constraints that may accompany this statement.

As previously discussed, the size of a nanoparticle has a deep impact on its plasmonic properties. It has been shown, for example, that a thin film of silver nanoparticles with LSPR around 420nm will have better SERS response if excitation is performed in the red or near infrared portions of the electromagnetic spectrum.²² It is also of importance to notice that the SERS phenomenon results from local fields on the vicinity of nanoparticles – a near-field property, and the best near-field response does not necessarily relate to far-field properties, such as extinction, based solely on wavelength.²⁹ Besides, there are two contributions to the extinction of a nanoparticle: absorbance and scattering, and the way they relate to each other is also highly dependent on the size of the particle. It becomes clear then that the size of the nanoparticles is a crucial parameter for a comparative study relating SERS excitation and LSPR. In this case one of the most important aspects of the

study would be to change the plasmonic properties of the particles, whilst keeping their size constant. Metallic nanoshells appear as the ideal candidate for such a study. As figure 2-2 shows, by fine-tuning the core size and the shell thickness it is possible to control the position of the LSPR peak, therefore, two types of nanoshells could preserve a similar diameter, but have LSPR peaks situated at different wavelengths. Different excitation conditions could then be probed according to those peaks. This work is discussed in Chapter 5.

2.3.2 A good SERS substrate

Among many applications that the technique has found along the years^{30, 31}, a recurrent topic in the field is the search for “good SERS substrates”. There are many features that must be considered in the evaluation of the quality of a SERS substrate, among them: low-cost, disposable, easy to fabricate/use, reproducible, repeatable results, etc. In this scenario, single nanoparticles that can produce a SERS response tunable by the excitation wavelength might find niche applications in chemical analysis that require: 1) low detection limits that can be achieved through detection of a single entity capable of providing meaningful chemical and/or biological information; 2) low particle-to-particle variation – particles of the same type, under the same conditions, should produce a similar response; and 3) particles of the same type should produce a different response if the excitation conditions are changed. As it is mentioned in Chapter 4, single nanoparticle SERS is finding an interesting pool of applications including super resolution chemical imaging³² and cellular studies on nanoparticle endocytosis.³³

2.4 Considerations for Nanoparticle-based Platforms in Cell Analysis

2.4.1 Choosing a biomolecule

In the gradual evolution of the nanoparticle research process, one of the most challenging and exciting phases consists in the transition from development to application. In the case of applications aimed at biological systems, there are important considerations to take into account, sometimes even before the nanoparticle synthesis has been completed. Among many possibilities for the use of nanomaterials in biology and medicine, the work presented here reports on *in vitro* (cultured cells) and *ex vivo* (tissues from an organism processed outside the body) studies. Chapters 4 and 6 give examples of bioapplications that heavily rely on the specificity of biomolecules for a designated cellular target. In Chapter 4, an antibody is used to target plasma membrane receptors, and a nuclear localization signal (NLS) in the form of a peptide is used to target the nuclear membrane of breast cancer cells. Antibodies are the most common type of biomolecule used to grant nanoparticles with specificity.³⁴ They can be produced as polyclonal or monoclonal and most researchers are not aware of how important the differences in these two types³⁵ can be, and the impact such differences will have in the overall performance of a nanoparticle-based bioanalytical platform.³⁶

2.4.2 Nonspecific interactions

The term nonspecific binding refers to the adsorption of a molecule like an antibody or aptamer to sites that are not their intended target binding site. Here the term nonspecific interaction is used with the intention to achieve a broader meaning, since the same idea of specificity cannot be applied in the same way to different categories of biomolecules. The main problem with these types of interaction is the detrimental effect it will have on the

performance of the assay, which can ultimately affect clinical decisions. For example, an antibody-coated nanoparticle designed to target the free form of the prostate-specific antigen (PSA) in blood, should only detect PSA molecules, however, that is not usually the case. Due to nonspecific interactions, other proteins can also bind to the antibody-coated nanoparticles, and that can lead to a misinterpretation of the data.³⁷ In this specific case, the most common scenario would be the presence of false-positive results. In clinical settings, this is addressed through a cut off threshold and the use of positive and negative controls.³⁸

When it comes to cell analysis, this problem expands into a more complex situation. A typical human cell expresses between 10,000 – 20,000 of its approximately 30,000 genes. If the expression patterns of different human cell lines are compared, variations are seen among different cell types. However, within the same cell type (e.g. epithelial), these variations most times are subtle.³⁹ Essentially, what this means is that a cell that is so called negative for a certain target, may actually have a small expression level for that target. Therefore, in comparing a positive cell with a negative cell (as a control for example), the obtained data can only translate into differential expression levels, and there is no way to assure that the readout obtained from the negative cell is due to low expression or nonspecific interactions. Chapter 6 expands upon, and presents an alternative to this problem.

It is also important to notice that the degree of specificity of a biomolecule can also be affected by the adopted conjugation process⁴⁰, and that sometimes it is possible to explore the presence of functional groups in the biomolecule itself, rather than resort to a coupling process. In Chapter 4, an antibody targeting insulin receptors on the plasma membrane of

breast cancer cells, and an NLS peptide are conjugated to gold and silver nanoshells, respectively, simply via the amine groups present on these biomolecules.

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Chapter 3: Synthesis and Characterization Checkpoints for Metallic Nanoshells

This chapter is a systematic investigation on the synthesis and characterization of gold and silver nanoshells. This Chapter is published as: A. M. Brito-Silva, R. G. Sobral-Filho, R. Barbosa-Silva, C. B. de Araújo, A. Galembeck, and A. G. Brolo, Improved Synthesis of Gold and Silver Nanoshells, Langmuir 2013, 29, 4366-4372. Copyright: Reproduced with permission.

All the experiments presented here were performed by myself and Dr. A. M. Brito-Silva. We provided equal contributions to the work – I performed the experiments for gold nanoshells and Dr. Brito-Silva performed the experiments for silver nanoshells. R. Barbosa-Silva performed the sample preparations and some of the characterization measurements.

Metallic nanoshells have been in evidence as potential multifunctional particles for optical and biomedical applications. Their surface plasmon resonance can be tuned over the electromagnetic spectrum by simply adjusting the shell thickness. Obtaining these particles, however, is a complex and time-consuming process, which involves the preparation and functionalization of silica nanoparticles; synthesis of very small metallic nanoparticles seeds; attachment of these seeds to the silica core; and, finally, growing of the shells in a solution commonly referred as K-gold. Here we present synthetic modifications that allow metallic nanoshells to be obtained in a faster and highly

reproducible manner. The main improved steps include a procedure for quick preparation of 2.3 ± 0.5 nm gold particles, and a faster approach to synthesize the silica cores. An investigation on the effect of the stirring speed on the shell growth showed that the optimal stirring speed for gold and silver shells were 190 and 1500 rotations per minute (rpm), respectively. In order to demonstrate the performance of the nanoshells fabricated by our method in a typical plasmonic application, a method to immobilize these particles on a glass slide was implemented. The immobilized nanoshells were used as substrates for the surface-enhanced Raman scattering (SERS) from Nile Blue A (NBA).

3.1 Introduction

Since first synthesized by Oldenburg *et al.*¹ in 1998, metallic nanoshells have been extensively investigated due to their peculiar plasmonic properties and vast potential for biomedical and optical applications.² The versatility of these particles and the possibility of tuning their surface plasmon resonance to the biological transparency window^{2, 3} allow their use for both *in vivo* therapy and diagnosis.⁴

Gold nanoshells have been successfully reported as contrast agents for optical coherence tomography (OCT), diffuse optical tomography (DOT), and as SERS imaging probes.⁵⁻⁷ Photothermal therapy using metallic nanoshells is one of the most developed examples of nanomedicine application.⁸ Metallic nanoshells have been used to treat many types of murine tumours.⁹⁻¹¹ Recent studies have shown encouraging results involving the use of gold nanoshells to treat high grade glioma.¹² These nanoparticles have also been used as DNA vectors, showing the possibility of their application in the development of light-triggered delivery systems for gene therapy.¹³ Nanoshell-based nanoarrays, such as polymer-coated quadrimers¹⁴ and fanoshells¹⁵, have also been fabricated. The high spectral sensitivity of the Fano resonance to the refractive index of the environment indicates that those structures can be important in chemical sensing applications.¹¹ In addition, tetrahedral nanoshell clusters were shown to provide the appropriate requirements for the generation of isotropic metamaterials in the visible range.¹⁶ High-order nonlinearity for these particles has also been reported.¹⁷

The examples described above confirm the key role played by metallic nanoshells in shaping the future of nanotechnology. However, the utilization of metallic nanoshells suffers from a major fabrication drawback, since their chemical synthesis is a complex and

time-consuming endeavor. The synthesis involves many steps related to the preparation of the different colloids, and some of them take several days to be completed. The entire process consists on the following: 1) fabrication and functionalization of silica nanoparticles; 2) synthesis of small-sized seed metallic nanoparticles (2-5 nm diameter); 3) attachment of the seeds to the silica nanoparticles; and, 4) growth of the shell in a solution commonly referred to as “K-gold”.¹⁸

In this work, key modifications in the procedure for the synthesis of metallic nanoshells will be described. This modified procedure significantly decreases the overall synthesis time. The method also provides a better degree of reproducibility and improves the nanoshell size distribution. The results presented in this contribution constitute, then, an important step forward towards the wide spread application of the nanoshell platform.

3.2 Experimental section

3.2.1 Chemicals. All chemicals were used as obtained. Tetraethyl orthosilicate (TEOS) >99.0%, (3-aminopropyl)trimethoxysilane (APTMS) 97%, chloroauric acid (HAuCl_4) 99.999%, polyvinylpyrrolidone (PVP) average $M_w \sim 55,000$ kDa, sodium borohydride (NaBH_4) >98%, formaldehyde 36.5-38% in H_2O , silver nitrate (AgNO_3) 99.9999%, anhydrous toluene 99.8%, ammonium hydroxide (NH_4OH) 28% in H_2O and Nile blue A were purchased from Aldrich. Potassium carbonate (K_2CO_3) >99% was obtained from Fluka. Square glass coverslips were obtained from VWR and cut into 1 x 2.5 cm pieces.

3.2.2 Characterization. Dynamic light scattering (DLS) and zeta potential (z-pot) was measured in a Zetatrac system (Microtrac). Extinction spectra were measured using a USB4000-UV-Vis spectrometer (Ocean Optics) Beckman Du 7500. Low resolution transmission electron microscopy (TEM) images were obtained in a Morgagni 268D (FEI)

and a LIBRA 120 Plus (Zeiss) and high-resolution TEM images were made in a Tecnai 20 (FEI). Low vacuum scanning electron microscopy images were obtained from a Quanta 200 FEG (FEI) on environmental mode. Particle counting from the transmission electron micrographs was done using the software Image Pro Plus by manual selection – five parallel gridlines were placed over each particle and the line with the largest value was used as diameter. Electron Spectroscopic Imaging (ESI) element mapping was obtained from a LIBRA 120 Plus (Zeiss). X-ray diffraction (XRD) analysis was performed in a Siemens D5000 diffractometer with Cu-K α irradiation at 1° min⁻¹. An RCT basic IKAMAG (IKA) stirring plate was used for the experiment involving different stirring rates. SERS measurements were obtained from a Renishaw inVia Raman microscope system and a He-Ne laser source at 632.8 nm using a 50x objective (NA = 0.75). The growth of the shells was conducted in V7130 Liquid scintillation vials (Sigma) or 50 mL borosilicate glass beakers with 1.5 cm and 2.5 cm magnetic stir bars respectively.

3.2.3 Preparation of the K-gold Solution. 0.0500 g of K₂CO₃ was dissolved in 197.0 mL of ultrapure water under magnetic stirring for 15 minutes. 3.750 mL of a 20.0 mM HAuCl₄ solution was added to the K₂CO₃ solution and the mixture was further stirred for 30 minutes, as the liquid became colorless. The resulting K-gold solution was stored in the dark and used within 5 days. See Appendix A for additional comments on the use of the K-gold.

3.3 Results and Discussion

3.1 Synthesis and characterization of the amino-terminated silica nanoparticles.

The preparation of the silica nanoparticles started by mixing 4.00 mL of TEOS with 50.0 mL anhydrous ethanol in a 60 mL flask. Ammonium hydroxide was added and the capped

flask was positioned in an ultrasound bath. The level of the solution inside the flask coincided with the level of water in the bath. The system was then sonicated for 2 hours. The use of the sonicator in this step significantly reduced the synthesis time, compared to the 12-24 hours required by the stirring-based methods commonly used in the field.¹⁹⁻²¹

The volume of ammonium hydroxide added ranged from 3.50 to 4.75 mL in 0.25 mL steps. It was verified that the diameter of the silica particles was controlled by the volume of ammonium hydroxide, varying from about 90 nm to 230 nm, as indicated in Table 3-1. Figure 3-1 shows a typical transmission electron image from one of the samples and the corresponding size distribution. The silica nanoparticles prepared by this procedure were stable under storage for approximately two years. The diameter of the particles was controlled by the different amounts of NH_4OH .

Table 3–1 Silica nanoparticles of different sizes (determined by both DLS and TEM).

Sample	NH ₄ OH _(aq) 28% (mL)	Diameter by DLS	Diameter by TEM
		(nm)	(nm)
1	3.50	93.5 ± 25	-
2	3.75	130.0 ± 29	-
3	4.00	146.0 ± 28	120 ± 21
4	4.25	162.0 ± 35	-
5	4.50	184.0 ± 42	-
6	4.75	226.0 ± 50	-

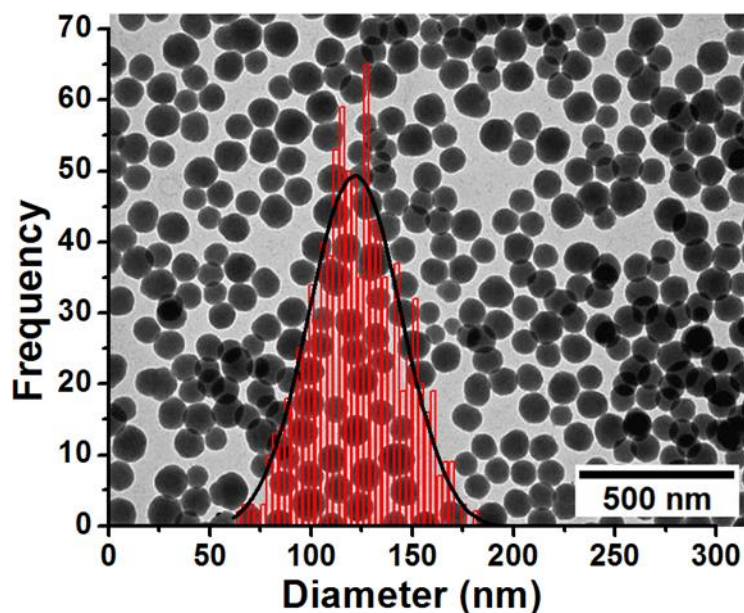


Figure 3–1 TEM image and histogram from sample 3 of Table 3-1 (Gaussian fit, $R^2=0.97$).

The next step after the synthesis of the silica particles was their functionalization using APTMS. The amount of APTMS used in the functionalization process was determined by the diameter of the particles.²² The following description is for 120 nm $\pm$ 21 nm diameter silica core (sample 3, shown in Figure 3-1). The silica nanoparticle colloid was transferred to a Petri dish containing 150.0 mL of anhydrous ethanol to allow for the unreacted ammonia gas to evaporate. The silica nanoparticles were then dried in an oven at 70°C up to the complete elimination of ethanol, following by an increase in temperature to 110°C to remove excess water. The dry particles were resuspended in toluene by stirring/sonication to a volume of 100 mL (5×10^{11} particles/mL). The resulting colloid was transferred to a round bottom flask and 200 μ L of APTMS was added under vigorous magnetic stirring. The mixture rested for 3 hours at room temperature before being heated to 100°C for 9 additional hours in a reflux system to favor the formation of siloxane bonds.²³ The amino-terminated silica nanoparticles (silica-NH₂) were cleaned 4 times by

centrifugation (4000 RCF/30 minutes) and resuspended in anhydrous ethanol to a volume of 100 mL (5×10^{11} particles/mL). The resulting colloid was stored in a capped 100 mL glass flask.

Zeta potential measurements were taken before and after functionalization. A change from -21 mV to +43 mV indicated a drastic variation in the charge distribution around the particles after the reaction with APTMS. This change in zeta potential can be attributed to the protonation of the surface amino groups that compensates the negative charge of the silanol groups²⁴ corroborating the success of the functionalization step. In order to validate the zeta potential, the functionalization was confirmed with infrared (IR) spectroscopy (data not shown).

3.2 Synthesis of (2.3±0.5 nm) gold nanoparticles.

The preparation of gold seeds with narrow size distribution (diameter ranging between 2 to 3 nm) is a key step on the synthesis of metallic nanoshells. Most authors accomplish this step by following a procedure described by Duff in 1993.²⁵ Unfortunately, the Duff method requires long aging times (days to weeks) for the suspension to reach the red-brown color characteristic of the required 2-3 nm particles. An alternative approach is to prepare gold nanoparticles using sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$) as both reducing and stabilizing agent.^{22, 26} This method is faster than the Duff approach, but it requires higher temperatures (up to 100°C) and results in particles of approximately 10 nm in diameter. These larger particles bring a critical disadvantage, since they set the minimum achievable shell thickness to about 15 nm.

Here a method that yields gold nanoparticles with average diameter of 2.3 nm (500 particles count) with a relative narrow size distribution (± 0.5 nm) is described. More

importantly, the entire process, which involves the use of PVP as stabilizing agent and NaBH_4 as reducing agent, takes up to 15 minutes to be completed. Previously described methods involving stabilization with PVP require either high temperatures²⁷ or slow light-based redox processes.²⁸ To our knowledge, there are no previous reports on the quick fabrication of small colloidal PVP-stabilized gold nanoparticles at room temperature.

In a 600 mL beaker 3.425 g of PVP-55000 were dissolved in 190.0 mL of water under magnetic stirring for 15 minutes. 4.075 mL of 20 mM HAuCl_4 solution was added to the beaker and 57.0 mL of a 5.24 mM NaBH_4 solution was rapidly added to the vortex of the PVP- HAuCl_4 mixture under vigorous stirring. A change in color from light yellow to dark brown indicated the formation of the gold nanoparticles. The stirring was maintained for additional 15 minutes, and the resulting suspension was stored in the dark. Figure 3-2 presents a TEM bright field image and a histogram of the gold nanoparticles prepared by this method. The absence of aggregates is notable from Fig. 3-2.

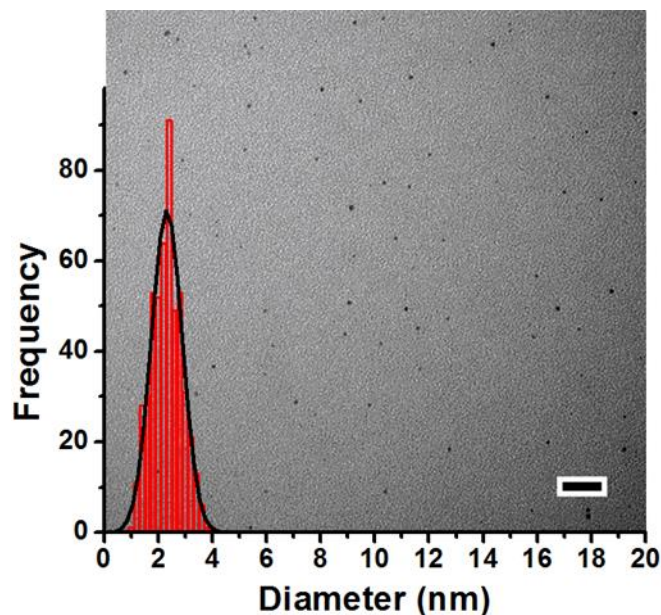


Figure 3–2 TEM and histogram of the gold nanoparticles (Gaussian fit, $R^2 = 0.96$). Average diameter is 2.3 ± 0.5 nm. Scale bar is 25 nm.

3.3 Formation of the gold-decorated silica nanoparticles.

The major advantage of the metallic nanoshell platform for plasmonic and photonic applications is that their optical properties can be controlled by the ratio between the core diameter and the shell thickness.¹ A procedure for the preparation of amino-functionalized silica cores with controlled diameters was presented in section 3.1 (see Table 3-1). Moreover, the effective control over the shell thickness requires seed gold nanoparticles with small diameters (2-3 nm) for the coating. The preparation of small diameter gold nanoparticles with narrow size distribution and minimal aggregation was described in section 3.2. In the preparation of the gold-decorated silica nanoparticles, it is very important that the gold nanoparticles are evenly distributed on the surface of the silica in order to enable the formation of uniform shells.

A procedure that ensures saturation of the silica surface with gold nanoparticles is described, as following: 30.0 mL of gold colloid was placed under magnetic stirring, and 3.00 mL of the suspension containing the amino-functionalized silica particles were added. The silica colloid was sonicated for 10 minutes before use and the mixture was stirred for 2 hours. After that, it was centrifuged (10000 RCF/10 minutes) in 2 mL eppendorf® tubes and part of the supernatant was collected for further analysis by UV-Vis spectroscopy. After that, the particles were again re-suspended in the initial amount of gold colloid. The process was repeated until the UV-Vis of the supernatant matched the spectrum of the gold nanoparticle suspension. At that point, it was assumed that the small-sized gold nanoparticles were no longer being captured by the silica-NH₂ particles. The UV-Vis analysis of the supernatant of the mixture of the silica-NH₂ and metallic nanoparticles after each centrifugation step is presented in Figure 3-3. The saturation stage was reached after about 5 centrifugation cycles. The sample was then re-suspended in water and cleaned by further centrifugations (10000 RCF/10 minutes each) before having its volume completed with water to 3.0 mL (5×10^{11} particles/mL). The resulting colloid was transferred to a capped glass vial and stored in the dark.

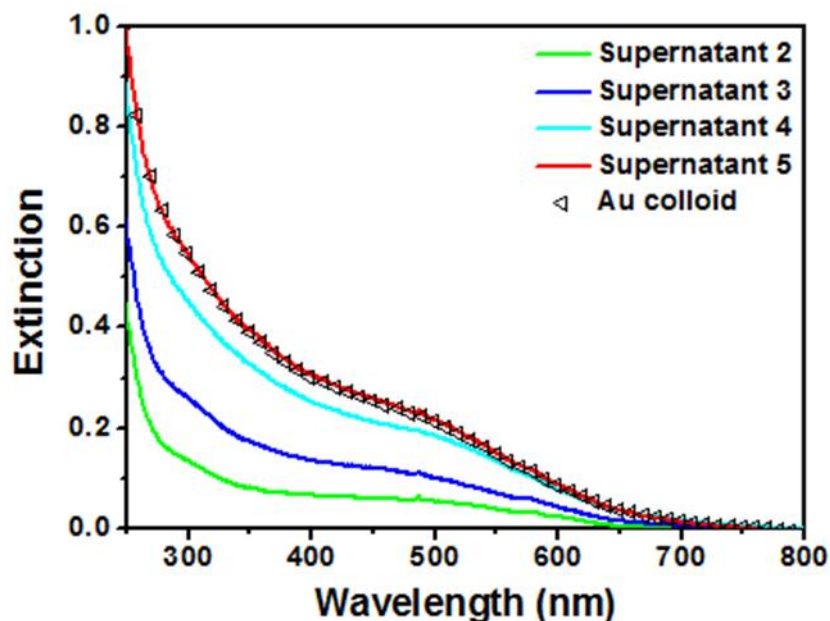


Figure 3–3 UV-Extinction spectra of the supernatants extracted after each centrifugation step.

Figure 3-3 shows a significant difference in the extinction from the second supernatant (the first supernatant was discarded due to the presence of ethanol which affected the results) relative to the original gold suspension. This fact is attributed to the attachment of the gold particles to the silica-NH₂ surface. The difference progressively becomes less significant for the subsequent supernatants, until no gold nanoparticles can be extracted by the silica-NH₂ core. After the 5th centrifugation cycle, the spectrum of the supernatant matches the Au suspension spectrum (Figure 3-3), indicating that the silica-NH₂ surface was saturated with gold nanoparticles. The gold-decorated silica colloid was used 2 months after preparation without any sign of degradation. Figure 3-4 presents a TEM image where regular distribution of gold seeds around the silica spheres is readily verified.

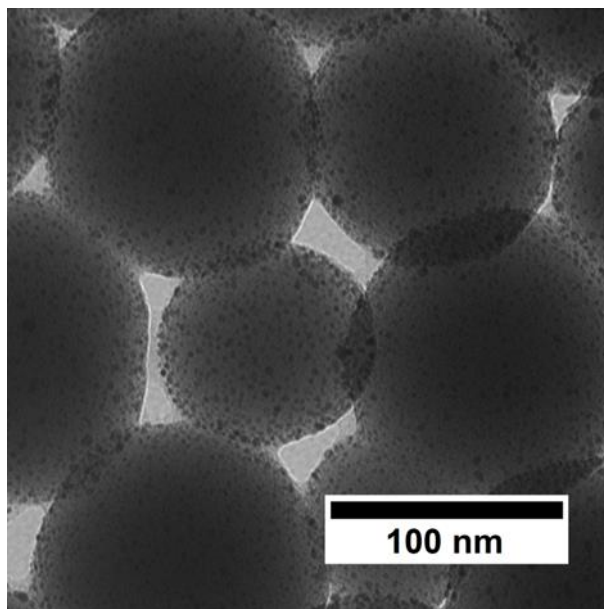


Figure 3–4 TEM micrograph of the gold-decorated silica particles.

3.4 Shell Growth and the Effect of the Stirring Rate.

Many physicochemical processes in colloidal chemistry are method- and operator-dependent.²⁹⁻³¹ In the case of nanoshell growth, it was verified that the stirring rate plays a significant role in the process reproducibility. The batch-to-batch variation was surprisingly small for the optimized stirring conditions evaluated for both gold and silver shells. The overall procedures and a proposed mechanism for the influence of the stirring rates on the nanoshell formation are described in the following sections.

3.4.1 Gold nanoshells.

The volume amounts of the reagents used to prepare the gold nanoshells are presented in Table 3-2. The K-gold solution was first placed in a glass vial. The gold-decorated silica colloid was subsequently added and the mixture was submitted to magnetic stirring at 400 rpm for 1 minute. The stirring rate was then adjusted to a pre-determined value (experiments with different stirring rates - 190, 240, 700 and 1500 rpm - were

conducted) and the reducing agent (formaldehyde) was added under stirring. Figure 3-5 presents TEM images for the sample 6 from Table 2 submitted to 190 and 1500 rpm, respectively (see section A.1.3 in Appendix A for TEM images at the other stirring rates). The mixtures were stirred for 4 minutes and then left undisturbed for 2 hours. After these 2 hours, the samples were cleaned several times by centrifugation, and had their volumes completed with water according to the values indicated in Table 3-2. The final nanoshell samples were stored in glass vials.

Table 3-2 Amounts of reagents used to grow gold nanoshells and final volume of samples to a 7.5×10^8 particles/mL colloid.

Sample	K-gold (mL)	Gold-decorated silica NP (μ L)	Formaldehyde (μ L)	Final volume (mL)
1	10.60	133	26	88.92
2	10.60	116	26	77.83
3	10.60	66	26	43.40
4	10.60	33	26	22.16
5	10.60	25	26	16.72
6	10.60	8	26	5.56
7	21.0	10	52	6.74
8	32.0	10	78	6.74

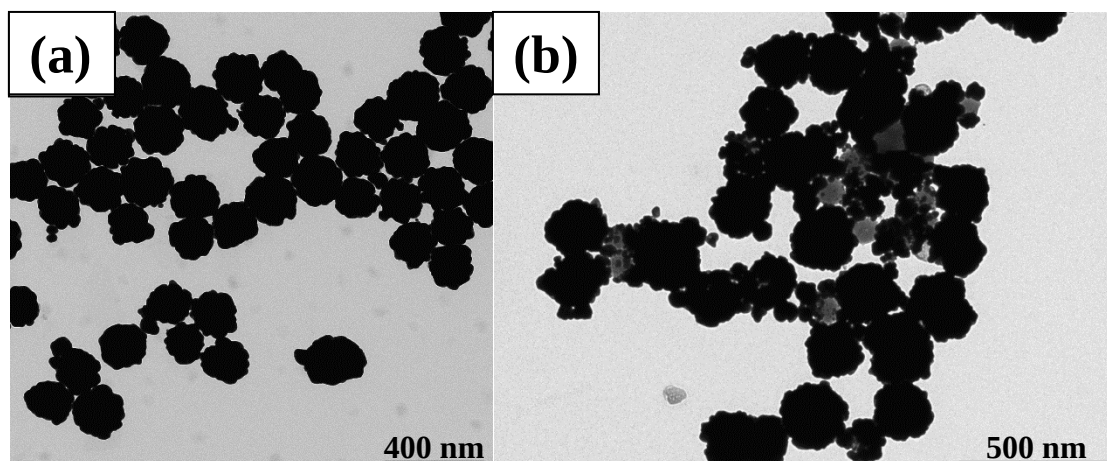


Figure 3-5 TEM images of gold nanoshells grown at (a) 190 and (b) 1500 rpm (sample 6 from Table 2).

3.4.2 Silver nanoshells.

The volume amounts of the reagents used to prepare the silver nanoshells are presented in Table 3-3. Gold-decorated silica colloid was added to a 0.15 mM AgNO_3 aqueous solution and the mixture was submitted to magnetic stirring at 400 rpm for 1 minute. The formaldehyde reducing agent was then added, and the stirring rate was adjusted to a pre-determined value (experiments involving different stirring rates - 190, 240, 700 and 1500 rpm - were performed). Ammonium hydroxide was added to the mixture and an immediate color change was observed. The samples were stirred for 30 seconds. They were then cleaned by centrifugation several times and their volumes were completed with water as indicated in table 3. Figure 3-6 presents TEM images for silver nanoshells produced using sample 6 from table 3 at two different stirring rates; 240 and 1500 rpm, respectively (see section A.1.3 on Appendix A for images from each stirring rate).

Table 3-3 Amounts of reagents used to grow silver nanoshells and final volume of samples to a 7.5×10^8 particles/mL colloid.

Sample	AgNO ₃ – 0,15 mM (mL)	Gold-decorated silica NP's (μ L)	Formaldehyde (μ L)	NH ₄ OH (μ L)	Final volume (mL)
1	10.60	133	26	50	88.92
2	10.60	66	26	50	43.40
3	10.60	41	26	50	27.76
4	10.60	33	26	50	22.16
5	10.60	16	26	50	11.10
6	10.60	8	26	50	5.56
7	21.0	10	52	100	6.74
8	32.0	10	78	150	6.74

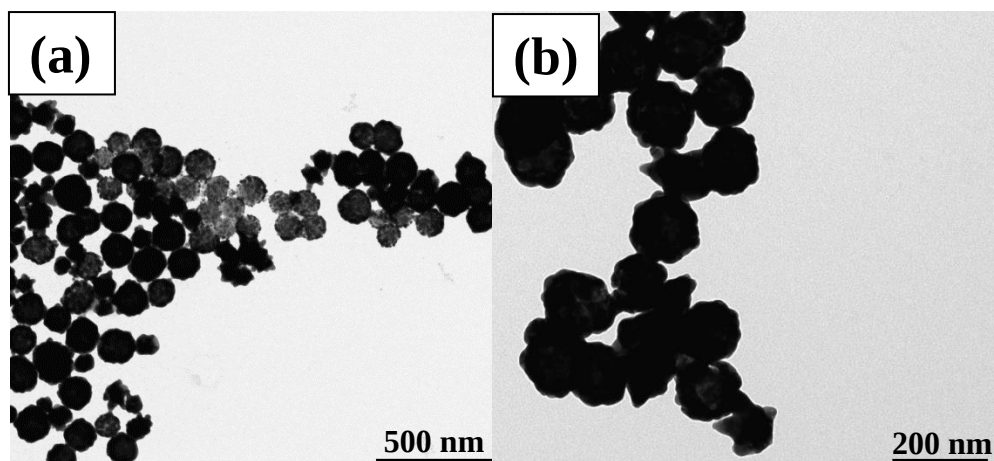


Figure 3-6 TEM images of silver nanoshells grown at (a) 240 and (b) 1500 rpm (sample 6 from Table 3).

3.4.3 Discussion on the Effect of the Stirring Rate.

The growth process for both silver and gold nanoshells was found to be highly dependent on the stirring rate. Ideally, the metallic ions from solution should deposit exclusively on the silica core, guided by the metal seeds that decorate the silica particle surface. However, the nucleation and growth of new metallic nanoparticles on the aqueous phase, “external nucleation”, is an important competitive mechanism. These newly formed metallic nanoparticles restrict the amount of metal ions available in solution for the growth of the shell, besides being a problem for the subsequent separation of the colloids. It was found that the stirring rate strongly affects the balance between these two competing processes.

Interestingly, the stirring rates that enabled a good control over the shell growth, with the smaller occurrence of external nucleation, were 190 rpm for the gold (the slowest stirring rate investigated), and 1500 rpm (the fastest stirring rate investigated) for the silver nanoshells. The TEM pictures of the Au nanoshells obtained at 190 rpm shows a

homogenous coating (Figure 3-5a), while the uncoated silica nanoparticles and residual gold fragments (evident in Figure 3-5b) are direct evidence of external nucleation when the growth was performed at 1500 rpm. Similar conclusions can be drawn from the TEM of the silver nanoshells shown in Figure 3-6. In that case, however, the nanoshells grown at 240 rpm (Figure 3-6a) present less uniform coating than the ones obtained at 1500 rpm (Figure 3-6b).

The discrepancy in the optimal stirring rate can be justified by the role of ammonium hydroxide as a growth catalyst for the silver nanoshell. The effect of the catalyst is to allow parameters such as agglomeration and Ostwald ripening - rather than concentration - to have a more pronounced effect in the final state of the colloid. The effect of the high stirring rate is to promote a better dispersion of the silver ions, favoring the catalyzed reduction of these ions onto the gold seeds at the surface of the silica nanoparticles. In reaction crystallization, ions cluster together to originate particles and these particles form agglomerates or aggregates.³² Gulrajani *et al.*³³ demonstrated that increasing the stirring rate leads to a decrease in particle size, indicating a better dispersion of colloidal elements which minimizes agglomeration.³⁴ The same applies to the silver ions clusters- increasing the stirring rate led to a better dispersion of these clusters, driving them to deposit on the gold seeds; reducing the effect of external nucleation, and promoting the growth of the shell.

There is no catalyst for the formation of the gold shell. In this case, the gold ions reduction is slow, and fast stirring favors external nucleation. A possible explanation for this is that higher stirring rates modify the shape of the vortex in the suspension, changing

the flow pattern from laminar to turbulent, and affecting the distribution of added chemicals to the reaction flask.

In summary, low stirring rates favor nanoshell formation when the ion reduction process is slow, such as for the gold nanoshell formation. On the other hand, the presence of catalysts that significantly accelerate the reduction process, such as in the silver nanoshell formation, requires the use of fast stirring rates to minimize the effect of external nucleation.

3.5 Characterization of the Shell Growth by UV-Vis Extinction Spectra.

Various gold and silver nanoshell samples were synthesized using the optimal stirring rates (190 rpm for gold and 1500 rpm for silver), as described above. Their extinction spectra were recorded and they are presented in Figure 3-7. Representative TEM images from each of the samples are also shown in Figure 3-7. Figures 3-7(b) (gold) and 7(c) (silver) show extinction spectra for the different samples obtained at different stages of the shell growth process. The TEM and UV-Vis results in Figure 3-7, numbered 1 to 8, correspond to the samples described in Tables 2 (for gold) and 3 (for silver). Samples 1 are from partially covered silica core with metallic clusters, and they present maximum extinction in the low wavelength side (about 550 nm for gold and 450 nm for silver). The metallic coverage increased from samples 1, reaching completely covered shells in samples 8. The samples with thicker shells present red-shifted surface plasmon extinction relative to samples 1. The shift in the extinction peaks for both gold and silver with the shell thickness agrees with the behavior previously described by Halas.³

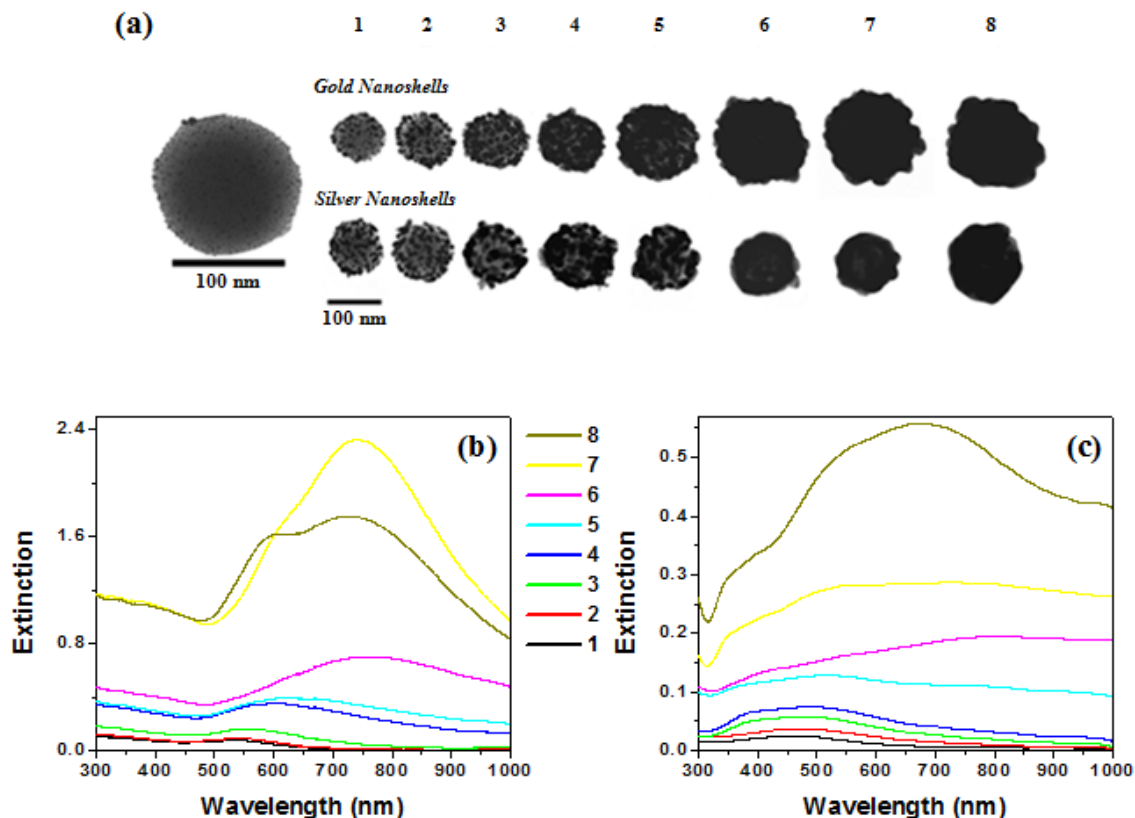
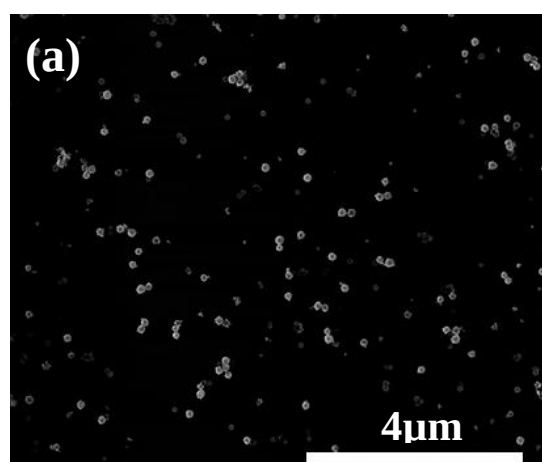


Figure 3-7 (a) - TEM images of individual gold and silver nanoshells. Sample numbers correspond to tables 2 (gold) and 3 (silver). Respective extinction spectra are shown in (b) gold nanoshells and (c) – silver nanoshells.

3.6 Immobilization of the Nanoshells on Glass Surfaces and SERS Measurement.

A relative straightforward approach to demonstrate that the nanoshells fabricated by our method are suitable for plasmonic applications is to probe their efficiency as SERS substrates.³⁵ The preparation of SERS-active surfaces by the immobilization of nanoparticles in glass and gold films has been reported by our group^{36,37}, and the nanoshells were immobilized in APTMS-modified glass slides for the SERS measurements (the process of APTMS self-assembly on glass slides and nanoshells immobilization is described in sections A.1.4 and A.1.5 in Appendix A).

Figure 3-8 (a) shows an SEM image of gold nanoshells (sample 8 from Table 2) immobilized on a glass slide. The nanoparticle distribution over the glass surface can be controlled by adjusting both the particles concentration in the suspension and the immersion time of the slide in the colloid.^{36, 37} A high number of individual nanoshells can be identified in Figure 3-5 (left) together with small aggregates (not larger than 4 particles per aggregate). Similar particle distribution was observed from SEM pictures from other areas in the slide, indicating a relative homogenous deposition of gold nanoshells.



(b)

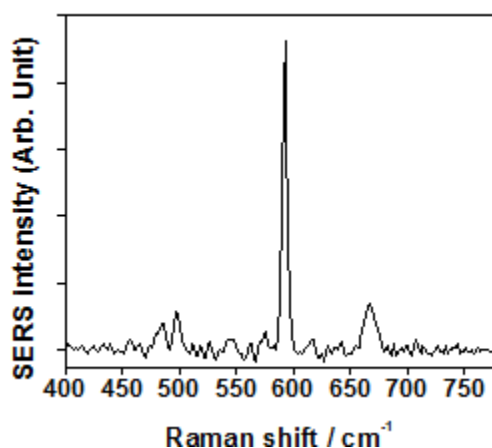


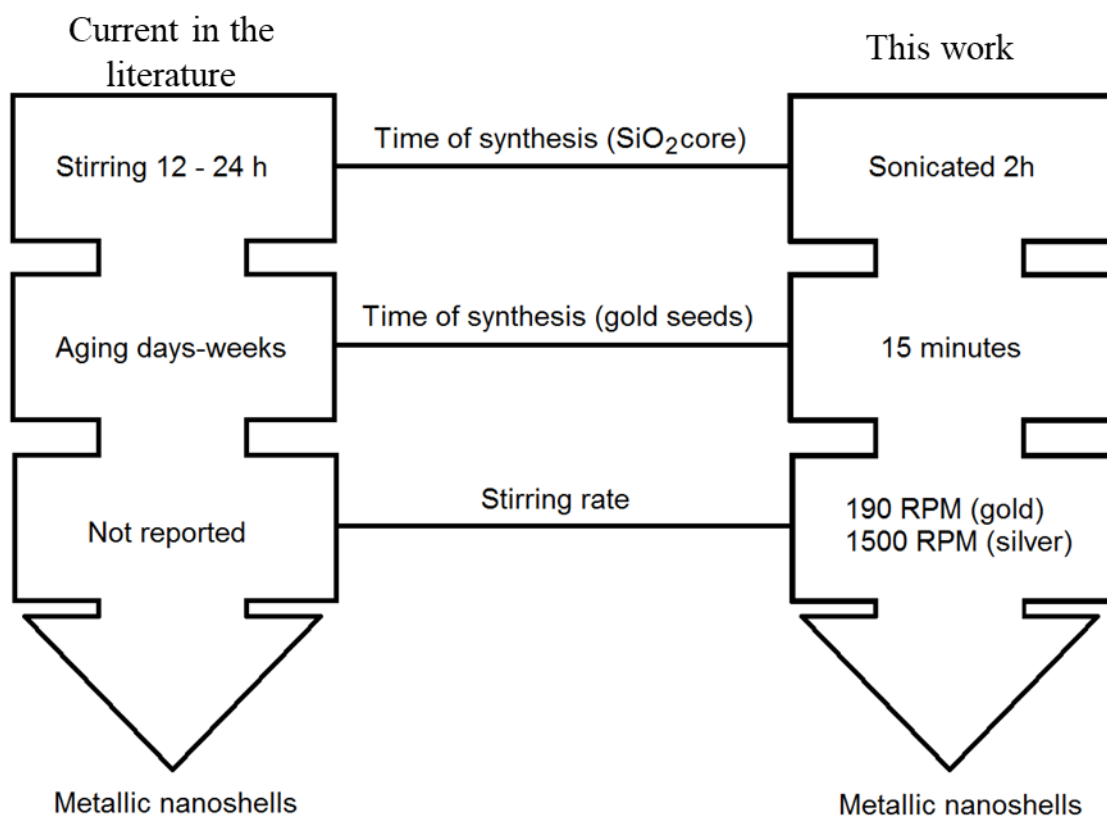
Figure 3-8 (a) – Gold nanoshells immobilized on glass slides, (b) – SERS spectra of Nile Blue-coated nanoshells immobilized on a glass substrate.

Nanoshells were coated with NBA as described in a previous work by Izumi.³⁸ The SERS spectrum adsorbed from a 1 nM NBA solution is shown in 8(b) and agrees with previous NBA SERS from single immobilized nanoshells reported by our group.³⁹ The good quality SERS from the NBA dye, shown in Figure 3-8, confirms that the nanoshells fabricated using our optimized procedure are amenable for plasmonic applications.

3.5 Conclusions

Important improvements on the synthesis of gold and silver nanoshells were presented. Scheme 1 depicts the major contributions of this work compared to methods currently described in the literature. The use of an ultrasonic bath in the fabrication of silica nanoparticles allowed the process to be accomplished in 2 hours, rather than the 12-24 hours commonly required by usual stirring-based methods.¹⁶⁻¹⁸ A fast process to synthesize 2.3 nm ($\pm$ 0.5) gold nanoparticles was also described. The use of steric stabilization with PVP decreased the synthesis time to 15 minutes, and the PVP coat was not an obstacle neither to the formation of the gold-decorated silica particles nor the subsequent growth of shells. The optimal stirring speed for shell growing was determined for both silver and gold nanoparticles. The control of the stirring is a decisive parameter to attain a homogeneous distribution of fully grown nanoshells. A method to achieve uniform immobilization of gold nanoshells onto glass slides suitable to SERS measurements was also shown.

Scheme 3-1 Summary of improvements by this method relative to those currently adopted in the literature.



Associated Content

Supporting Information presented as Appendix A. Compositional analysis. Shell growth images for different stirring rates. APTMS self-assembly on glass slides. Nanoshell immobilization.

3.5 References

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Chapter 4: Fine-Tuning Nanoshells for Multiplex Cell Analysis

This chapter shows a reformulated fabrication process for gold and silver nanoshells with narrow extinction bands. The resulting samples are suitable for multiplex analysis. A proof-of-principle experiment demonstrates the labeling of subcellular compartments in MCF-7 breast cancer cells, with two types of nanoshells. This chapter is published as: R. G. Sobral-Filho, A. M. Brito-Silva, M. Isabelle, A. Jirasek, J. J. Lum and A. G. Brolo, Plasmonic Labeling of Subcellular Compartments in Cancer Cells: Multiplexing with Fine-tuned Gold and Silver Nanoshells, Chem. Sci. 2017, 8, 3038-3046. Copyright: Reproduced with permission.

This was a collaboration with Dr. Julian J. Lum at the British Columbia Cancer Agency and Dr. Andrew Jirasek at the University of British Columbia. I performed all the experimental work. Dr. A. M. Brito-Silva and Dr. M. Isabelle helped plan the experiments, and interpret/discuss the results.

Fine-tuned gold and silver nanoshells were produced via an entirely reformulated synthesis. The new method yielded ultramonodisperse samples, with polydispersity indexes (PI) as low as 0.02 and narrow extinction bands suited for multiplex analysis. A library of nanoshell samples with localized surface plasmon resonances (LSPR) spanning across the visible range was synthesized. Hyperspectral analysis revealed that the average scattering spectrum of 100 nanoshells matched closely to the spectrum of a single nanoshell, indicating an unprecedented low level of nanoparticle-to-nanoparticle variation for this type of system. A cell labelling experiment, targeting different subcellular

compartments in MCF-7 human breast cancer cells, demonstrated that these monodisperse nanoparticles can be used as a multiplex platform for single cell analysis at the intracellular and extracellular level. Antibody-coated gold nanoshells targeted the plasma membrane, while silver nanoshells coated with a nuclear localization signal (NLS) targeted the nuclear membrane. A fluorescence counterstaining experiment, as well as single cell hyperspectral microscopy showed the excellent selectivity and specificity of each type of nanoparticle for its designed subcellular compartment. A time-lapse photodegradation experiment confirmed the enhanced stability of the nanoshells over fluorescent labelling, and their capabilities for long-term live cell imaging.

4.1 Introduction

A variety of nanomaterials with diverse chemical and physical properties have emerged in the last two decades, bringing great promise to applications in environmental, energy and health sciences.¹⁻³ Metallic nanoshells appear prominent among those. Composed of a dielectric core and a thin metallic shell, they hold great promise for bioapplications.⁴ These nanoparticles are strong scatters, enabling efficient spectroscopic responses. Their LSPR bands can be tuned throughout visible and near infrared (NIR) wavelengths by simply controlling their core size and shell thickness.⁴ However, despite this spectral tunability, efficient plasmonic multiplexing experiments based on nanoshells have not yet been outlined. The problem of multiplexing with nanoshells can be attributed to the difficulties in obtaining monodisperse colloids on a multi-step fabrication process. One potential application of metallic nanoparticles that requires multiplexing is on cellular imaging.

Recent advances have been made in intracellular probing by LSPR sensors based on nanoparticles.^{7,8} However, such sensors are very susceptible to changes in the nanoparticle characteristics, and their widespread use requires the development of uniform and well characterized colloids.^{9,10} Significant efforts have revealed progress on understanding how nanoparticles are internalized by cells and some of the physical and biochemical interactions that lead to their intracellular accumulation.¹¹ However, the use of nanoparticles as labelling and tracking agents for intracellular environments still finds itself in early stages. Developments in this area have the potential to greatly improve our understanding of cellular responses.

Considering the complexity of biological systems and cellular microenvironments, an ideal labelling experiment should allow simultaneous evaluation of several components in

the sub-micrometer range (multiplexing). Immunophenotyping of the plasma membrane is the gold standard for distinguishing between cell types and is essential as a diagnostic tool.¹² In a cell, changes in protein expression, enzymatic activity, nucleic acid production and many other processes take place intracellularly and are often related. This scenario demands the use of an efficient multiplex approach that can probe specific subcellular compartments (membranes, organelles, etc.) and components (receptors and other biomolecules). To that end, most systems investigated to date are fluorescence-based.¹³⁻¹⁵ Such cases, while extremely effective for detection, lack the stability required for long-term real-time analysis of cellular processes that happen on the range of several hours, and do not take place in stationary positions inside the cell. Plasmonic-based approaches have recently been demonstrated for intracellular probing. Gold nanorods have been 3-D imaged inside osteosarcoma cells through two-photon luminescence with precision regarding their spatial orientation¹⁶, and surface-enhanced Raman scattering measurements have been made probing the endosomal environment through the use of magnetite-metal composite nanoparticles.¹⁷ In both cases the nanoparticles were internalized through nonspecific interactions with the cells

Metallic nanoshells have fallen short for this type of application due to the broad extinction bands associated with the size disparity of the colloids, and efforts have been made to incorporate such variations into models to predict the extinction profile of nanoshells suspensions.^{22, 23} Nanoshell fabrication methods comprise a combination of multiple surface chemistry processes and colloids – each of these carrying their own potential barriers to an uniform size distribution. Such limitations have resulted in spectral overlaps that derail the effectiveness of metallic nanoshells as a multiplex platform.²⁴ In

Chapter 3, the characterization checkpoints and techniques to track the progress of the nanoshells fabrication process were presented, indicating improvements which led to a faster synthesis and control over the degree of metal coverage in a single core size.²⁵ However, that did not lead to the desired narrow extinction bands for the obtained colloids. An extensive review of the literature and several discussions within our lab, led to the decision to tackle the size distribution problem by doing dramatic changes to our previous protocols and reformulating the nanoshells synthesis to a process that would result in ultramonodisperse colloids with narrow extinction bands, enabling their use for multiplexing.

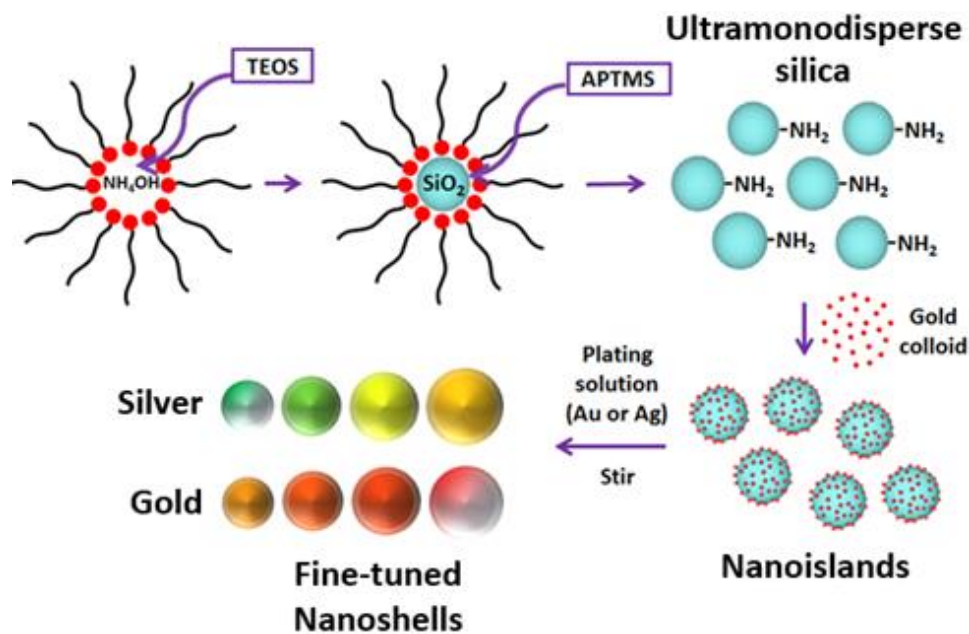
Here, single cell labelling experiments based on the scattering profiles of finely tuned gold and silver nanoshells are reported. Precise control over the two parameters that define the optical properties of these particles – core size and shell thickness – was achieved. A refined streamlined synthesis, realized using only simple labware (see Figure B-SI-1 in section B.1.1 of Appendix B), generated colloids with unprecedented quality. Highly monodisperse samples were produced, resulting in narrow extinction bands that can, in fact, be used for multiplex analysis at the single cell-single particle level. We designed nanoshell colloids that can operate in the visible range. In principle, dark field scattering images of cells labelled with colour-tuned nanoshells can be realized under white light illumination. This is a simple hardware implementation that can be easily achieved in clinical conditions. These nanoshells also show much better photostability when compared with typical fluorescence labels in cellular imaging experiments.

4.2 Results and Discussion

4.2.1 Synthesis and characterization.

The core size is one of the main factors for tuning the LSPR in a nanoshell. Silica (SiO_2) nanoparticles were used as cores. By controlling the amount of tetraethyl orthosilicate (TEOS) and reaction times, colloids with four different sizes were prepared (50, 63, 72 and 80 nm). These diameters were specifically chosen to produce nanoshells whose LSPR would operate in the visible range. A reverse microemulsion system (described below – detailed protocol available as supporting information as Appendix B) allowed a one-pot synthesis and functionalization of the SiO_2 cores. Scheme 4-1 presents the synthetic process outlined here. Ultramonodisperse samples were obtained with standard deviations ranging from 2.3–2.8 nm and polydispersity indexes (PI) as low as 0.02. These values are one order of magnitude lower than the accepted threshold of $\text{PI} = 0.2$ for monodisperse samples.²⁰ The TEM images in Figure 4-1(a, b) convey the general characteristics of the colloids showing the uniform size and shape distributions of the SiO_2 samples. Triton X-100 and n-hexanol were used as surfactants in cyclohexane. Aqueous ammonium hydroxide (NH_4OH) was contained to the inner portion of the micelles. TEOS is added to the organic phase and the process of nucleation and growth in the aqueous phase is therefore limited by the TEOS diffusion through the supramolecular structure of the micelles. This grants a slow and controlled growth of the SiO_2 particles. External nucleation in the organic phase was prevented by sequential additions of TEOS at every six hours, so that the concentration of the precursor in that phase was kept relatively low throughout the process. For the sizes evaluated, there was no disruption of the reverse microemulsion, even after long reaction times, indicating that the bending rigidity of the

micelles²¹ may also contribute to the high degree of monodispersity attained here. After the growth of the SiO₂ particles was completed, the pH of the reaction was lowered by simply opening the vial and allowing NH₃ to escape.



Scheme 4-1 Ultramonodisperse aminated silica particles are produced in a one-pot synthesis via a reverse microemulsion system. Small gold nanoparticles are then attached to the silica and the shell growth takes place under stirring in a plating solution with metal ions at low concentration (150 μM). Different SiO₂ sizes and shell thicknesses can be achieved with this method.

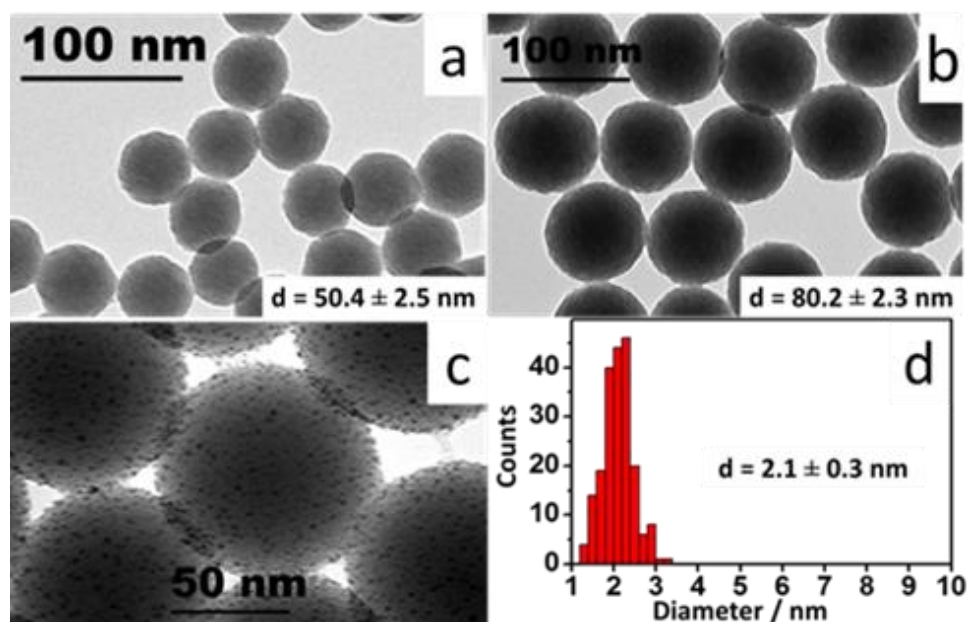


Figure 4-1 TEM images: a) and b) SiO₂ nanoparticles synthesized by reverse microemulsion; c) nanoislands at high magnification; d) Au colloid diameter histogram.

Functionalization of the SiO₂ cores was carried out with the addition of an aminosilane to the mixture for 5 minutes before breaking the micelles with ethanol. It is worth mentioning that no complex separation method was required for these samples. Special attention, however, needs to be given to the cleaning process, since several reports in the literature point out that colloids produced by a reverse microemulsion will often generate aggregated samples.³⁰ That issue was solved through a rigorous cleaning process, where the samples were centrifuged, resuspended in ethanol, and kept under vigorous sonication for 15 minutes before each cleaning cycle. Post-cleaning dynamic light scattering (DLS) measurements show that there is no aggregation in the colloids (Figure B-SI-2 in section B.1.3.1 of Appendix B). These samples can be kept in ethanol at room temperature for several months. Long term stability was assessed by TEM using a 10-month old aminated

silica sample. Figure B-SI-3 (section B.1.3.2 of Appendix B) displays the results that show the good colloidal and chemical stability of the samples produced through our method. A variety of SiO₂ sizes can be fabricated by controlling reaction parameters such as time and reagent amounts. Table B-SI-1 (section B.1.2.1 of Appendix B) shows in detail the parameters utilized for the fabrication of the diameters used in this work.

Coating the functionalized cores with a metallic shell requires the adsorption of small gold particles on the surface of the SiO₂. These nanoparticles ($2.1 \pm 0.3\text{nm}$) were prepared as previously described³¹, resulting in a colloid with $\sim 1.36 \times 10^{15}$ particles/mL, according to Au quantification by ICP-MS. A method to calculate the number of particles in the silica samples is outlined in section B.1.3.3 of Appendix B. For the SiO₂ samples prepared here, particle concentrations range from $\sim 2.4\text{-}4.1 \times 10^{12}$ particles/mL. The two colloids were mixed in a small vial at a 1:49 (SiO₂: Au) volume ratio and continuously stirred at room temperature for 3 hours in a stirring plate. The small Au particles bind to the amine groups on the silica surface to an estimated coverage of 30%, due to electrostatic repulsion between bound and free gold nanoparticles.³² These particles (gold-decorated silica nanoparticles) are referred to as ‘nanoislands’. The sample was cleaned by centrifugation-resuspension cycles in ultrapure water and had its volume reduced to the initial amount of aminated SiO₂ colloid. Figure 4-1c shows high magnification TEM images of the resulting nanoislands. A narrow size dispersion is important throughout the entire synthesis of the nanoshells, and a diameter histogram for the Au colloid is also

shown in Figure 4-1d. The uniform size distribution of the Au colloid will determine the homogeneous formation of the metallic shells in the next step of the synthesis.

Shell growth is the final step of the synthesis and is normally accomplished by adding the nanoislands to a solution containing the ionic precursors of the chosen metal. In the case of Au, a solution of gold hydroxide ($\text{Au}(\text{OH})_3$), referred as K-gold³³, is typically prepared. This solution requires aging times on the order of 12-24h, and is very sensitive to the initial pH of the water. Adjusting its final pH with acids or bases results in the addition of ions to the reaction media that can affect the stability of the colloids and promote aggregation during the growth process. We therefore decided to produce either gold or silver hydroxide (AgOH) plating solutions using a different approach, in order to standardize the coating process for these metals. HAuCl_4 (or AgNO_3) are dissolved in ultrapure water at low concentrations, in the micromolar range, and ammonium hydroxide is added to the solution in order to generate the designated hydroxide. The NH_4OH also sets the pH to an optimal range (9-10) for the shell growth.³⁴ The resulting solution is transferred to a clean vial through a syringe filter. This step will retain impurities and small gold or silver oxide particles that might form in the previous step. The appropriate amount of nanoislands is then added to the plating solution. The formation of the shells proceeded through reduction of Au (or Ag) onto the nanoislands under vigorous stirring by adding ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$) (for Au) or formaldehyde (CH_2O) (for Ag). The resulting colloid was left undisturbed and the reaction was monitored by measuring the LSPR spectrum, as showed in Chapter 3A.

The aspect of the colloids evolves with time over the course of 60-90 minutes. Even though the coating process can be stopped at any time by simply cleaning the sample, shell thickness was controlled by the amount of added nanoislands – lower amounts of nanoislands lead to thicker shells and vice-versa. It is easy to estimate the number of particles per sample through a simple calculation as shown Appendix B. The nanoshell samples were finally cleaned by centrifugation-resuspension in ultrapure water and stored at 10°C. A combination of Au and Ag nanoshells of varying thicknesses and SiO₂ diameters were produced. Figure 4-2 illustrates the whole LSPR spectral dataset for the nanoshell samples and highlights the narrow extinction bands for all colloids showed in Figure 4-2a. The reproducibility of the growth step can be assessed respective to the position of the LSPR peak. For this purpose, five Au(18nm)@SiO₂(72nm) were prepared, and the position of the peak was evaluated, showing a mean value of 676 nm ± 10 nm. In this case, variation is possibly linked to minor deviations in the concentration of the plating solution (post-filtration) and/or small changes in the number of nanoislands utilized in the growth.

The colloids prepared here have extinction maxima ranging from 550 – 685nm. The wavelength range can be expanded simply by choosing the appropriate core size and shell thickness. It is clear from Fig. 2 that the narrow LSPR characteristics of these nanoshells are suitable for multiplexing applications based solely on their spectral profiles. This attests to the exceptional high quality of the colloids prepared by the procedure reported here. Low and high magnification TEM images, displayed in figure B-SI-4 (section B.1.3.3 of Appendix B), show the good quality of the gold (or silver) coatings for these nanoshells.

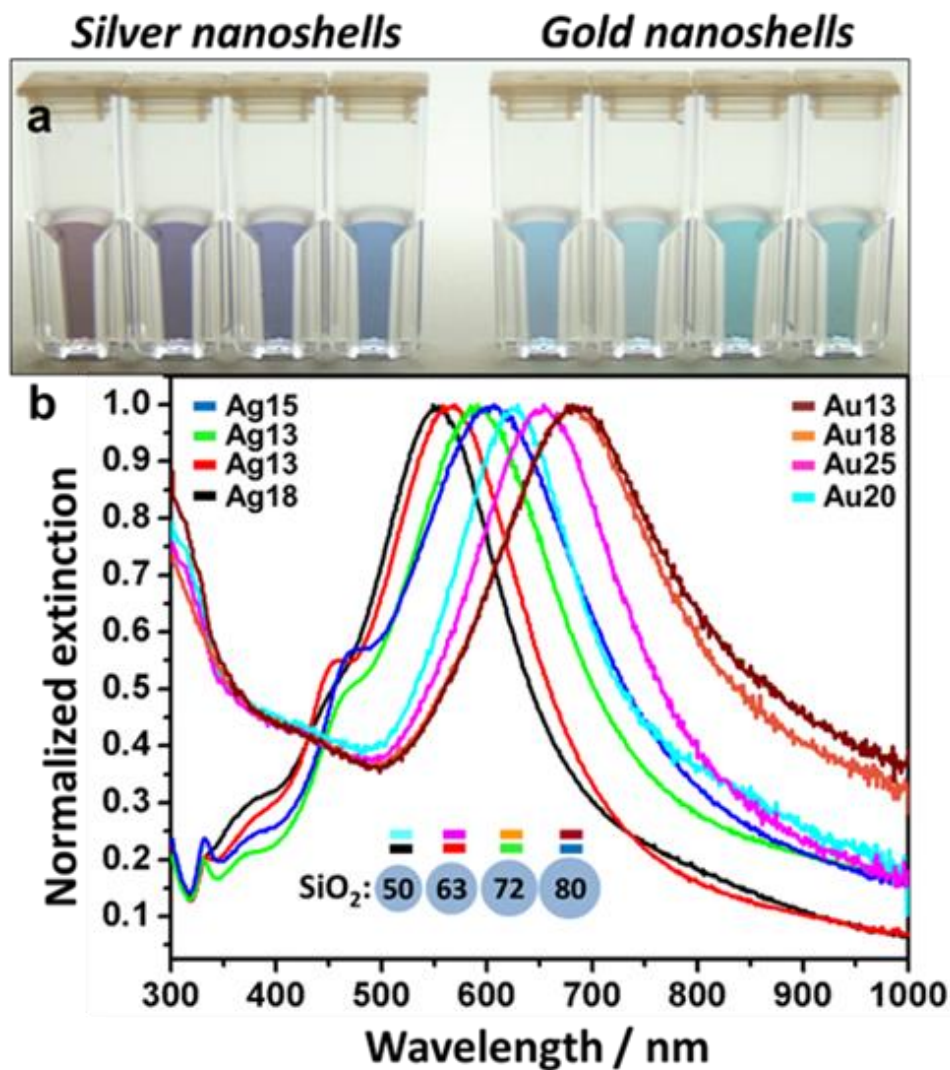


Figure 4-2 a) Fine-tuned Ag and Au nanoshells samples – cuvettes match measured spectra in 3b (left to right); b) extinction spectra for Ag and Au nanoshells – core size and shell thickness are color-coded to the curves (dimensions in nm).

4.2.2 Hyperspectral Analysis.

It is clear the distinction between the samples displayed in Figure 4-2, however, in order to assess the feasibility of effectively using these particles for multiplexing, a more in-depth analysis is required. To that end we performed hyperspectral dark field

measurements for several of the nanoshells produced here. Figure B-SI-5 (section B.1.3.4 of Appendix B) displays a hyperspectral image and the scattering spectra for isolated particles from a mixture of 4 nanoshell colloids immobilized onto an aminated glass coverslip (coverslip silanization described in detail in our previous work²⁵). The color distinction and good spectral separation between particles show the potential these colloids hold for multiplex applications. Based on that, two samples, Ag(18nm)@SiO₂(50nm) and Au(18nm)@SiO₂(72nm), were selected for the single-cell proof of principle experiment. Figure B-SI-6 (section B.1.3.5 of Appendix B) illustrates the dramatic colour separation between these particles, indicating the feasibility of achieving similar results even with the use of a regular dark field microscope (without hyperspectral capabilities). An in-depth assessment of Ag(18nm)@SiO₂(50nm) and Au(18nm)@SiO₂(72nm) was carried out by hyperspectral analysis. The results are presented in Figure 4-3, where a comparison between the measured scattering spectra for a single nanoshell and that of 100 averaged particles was performed for each of these two colloids.

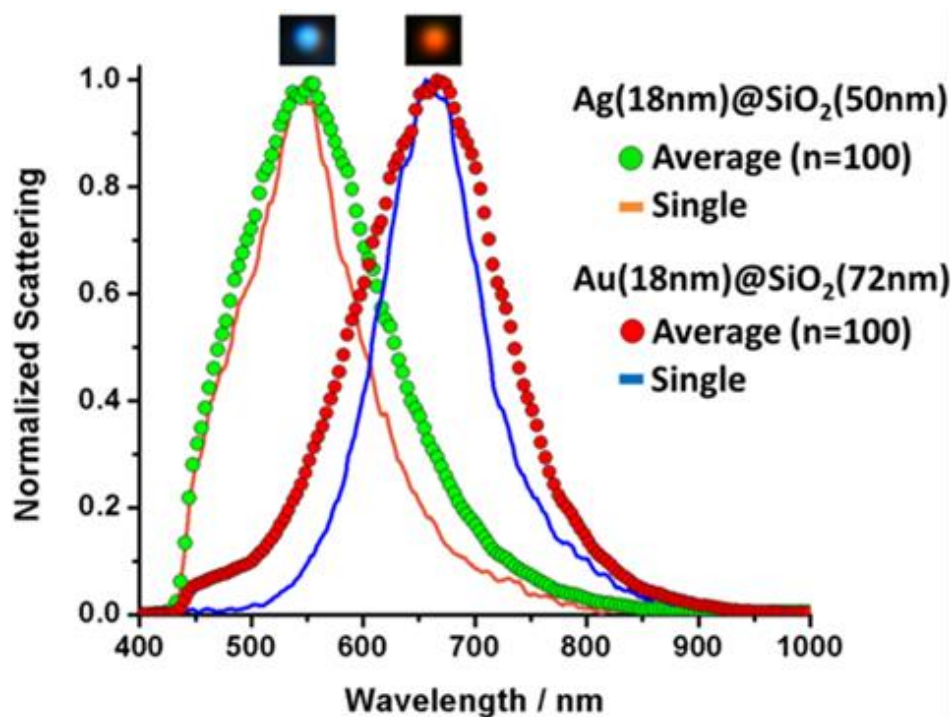


Figure 4-3 Single and averaged (n=100) scattering spectra for Ag and Au nanoshells. Inserts above each curve show individual nanoshells as seen under dark field illumination.

Hyperspectral measurements, shown in Figure 4-3, were acquired for different particles from each sample. Individual amine-modified glass slides were placed in each of the colloids in order to capture the particles for analysis. Line plots correspond to a single nanoshell and scatter plots show the averaged spectrum. Insets in Figure 4-3 show the particles as observed under white light illumination in the dark field illumination. The agreement between the LSPR peak positions is excellent in both cases, and the slightly broader average spectra (compared to that of a single nanoshell) is an indication of the optimal size dispersion of the samples. Figure 4-3 indicates the effective level of control over the LSPR bands of metallic nanoshells achieved through the wet-chemistry processes adopted here. We attribute this to the high monodispersity of the colloids produced through

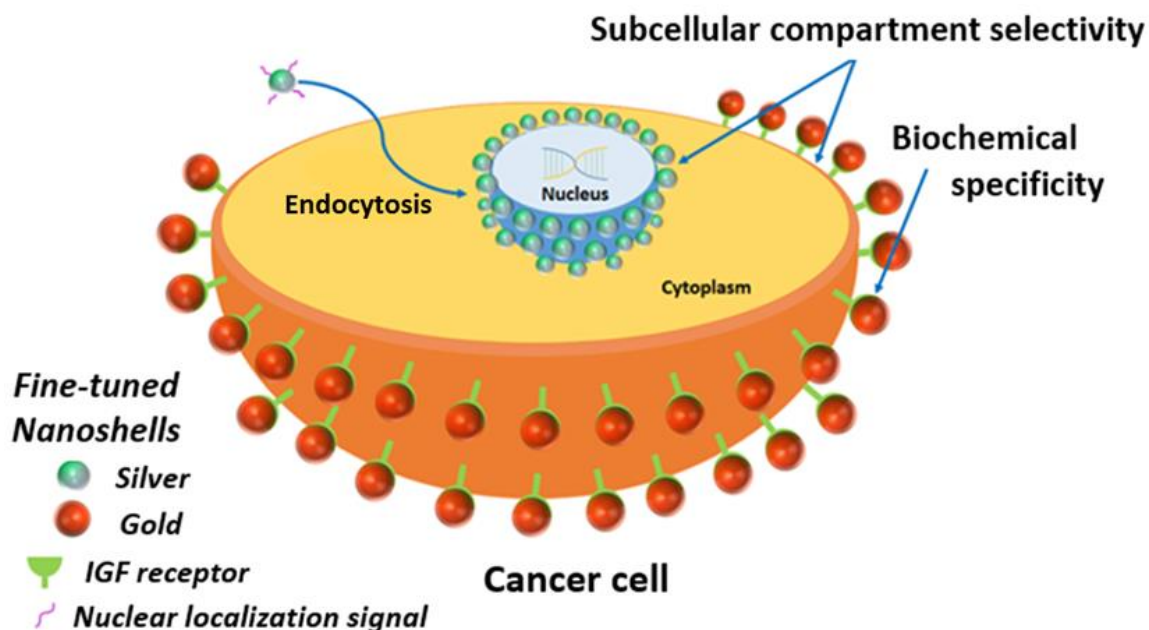
this method. Figure B-SI-7 (section B.1.3.6 of Appendix B) shows the individual normalized extinctions for each of the 100 LSPR spectra plotted on the same graph for both samples. Figure B-SI-7 provides an indication of the particle-to-particle variations.

4.2.3 Labeling of Subcellular Compartments.

Protein expression patterns on the plasma membrane can provide valuable information about cellular processes such as proliferation, apoptosis and senescence.³⁵ Such patterns often carry prognostic and predictive value in medical diseases and conditions, particularly those related with cancer biology. Insulin-like growth factor receptor (IGFR) expression has recently been associated with increased invasiveness, anti-estrogen resistance and induced epithelial-to-mesenchymal transition in breast cancer cells.^{36, 37} These factors can contribute to increased tumor aggressiveness and reduced sensitivity to chemotherapy, in addition to intensifying the incidence of metastasis and remittance. Moreover, many metabolic processes related to carcinogenesis take place in subcellular compartments and the importance of using nanoparticles to target intracellular processes and compartments recently been outlined.^{8,38-41}

One of the major problems in intracellular and membrane-probing with nanoparticles is to avoid nonspecific interactions between the particles and the cell. Triggering nonspecific endocytic pathways and having particles nonspecifically adsorbing to the membrane are examples of phenomena that can lead to misinterpretation. Endocytosis can happen in a variety of ways and that poses a crucial challenge in intracellular labelling.³⁰ Nonspecific pathways can allow internalized nanoparticles to be allocated to subcellular compartments (e.g. endoplasmic reticulum, Golgi complex).⁴² In order to demonstrate the feasibility of using nanoshells as a multiplex platform for cell analysis in both domains (intra and

extracellular), the nanoparticle surface was functionalized with either a NLS (nuclear localization signal) peptide or an anti-IGFR antibody. The nanoshells modified with NLS peptides are expected to be internalized and localized to the nuclear membrane. NLS are peptides capable of selectively transporting cargoes into the nucleus⁴³; therefore, they should promote entering through the nuclear pores. However, the nanoparticles used in this work are large enough to preclude nuclear internalization leading to accumulation on the nuclear membrane. The anti-IGFR antibody targets the plasma membrane. It is important to clarify that the use of NLS peptides does not necessarily mean nuclear transport.⁴⁴ Different cell lines may require different NLS peptides and for applications involving multiple cell lines, a screening step is necessary. The NLS used in this work was specifically screened for MCF-7 cells (see section B.1.1 of Appendix B).⁴⁵ Simpler than that, only cells expressing IGF receptors should be targeted by the Au-IGFR nanoshells, and a negative control was established using SKBR-3 breast cancer cells that express only low basal levels of IGF receptors. With this, we aim not only to demonstrate the capacity to target distinct subcellular compartments, but also (in the case of IGF receptors) the ability to target specific biochemical elements within such compartments. Scheme 2 summarizes and pinpoints the main features of the experiment.



Scheme 4-2 Labeling of subcellular compartments by ultramonodisperse nanoshells. Silver nanoshells bioconjugated to a nuclear localization signal (NLS) are internalized by the cells. The NLS peptide leads the particles to escape nonspecific endosomal and exocytic pathways and accumulate on the nuclear membrane. Gold nanoshells bioconjugated to an anti-IGFR antibody, target the insulin receptors (IGFR) localized on the plasma membrane of MCF-7 cells. Selectivity of the nanoshells for each subcellular compartment is evidenced, as well as the ability to target specific biochemical elements within such compartments, as showed for the insulin receptors and the antibody-coated gold nanoshells.

Ag(18nm)@SiO₂(50nm) and Au(18nm)@SiO₂(72nm) were selected for the proof-of-principle cell labeling experiment, because of the good separation in their LSPR characteristics (as shown in Figure 4-2). The Ag nanoshells were coated with the custom-made NLS (Ag-NLS) peptide while Au nanoshells were coated with the anti-IGFR antibody (Au-IGFR). Experimental details are described in Appendix B. Ag-NLS was resuspended in reduced-serum medium (opti-MEM®) and Au-IGFR was resuspended in

PBS prior to incubation. Extinction spectra before and after bioconjugation are shown in Figure B-SI-8 (section B.1.3.7 of Appendix B), where a shift in the LSPR peaks is evidenced due to the attachment of the biomolecules. Particle concentration for both samples was approximately 5×10^9 particles per milliliter. MCF-7 cells were plated onto sterile coverslips to a confluency of 30% and maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum.

4.2.4 Nanoshells Localization – Combined microscopy of individual compartments.

Individual incubations were performed with 1 mL of Au-IGFR for 30 minutes and 1mL of Ag-NLS for 3 hours at 37°C. Cells were washed with PBS after incubations, staining and then fixation protocols are described in section B.1.2.6 in Appendix B. Coverslips were washed in PBS before being mounted onto microscopy slides for analysis. In order to unequivocally confirm the cellular localization of the nanoshells, an experiment combining fluorescence, conventional dark field and hyperspectral dark field microscopy was performed. The same field of view was analyzed by all three techniques. Counterstaining with fluorescent dyes was performed for each compartment (procedures in section B.1.4 of Appendix B). Propidium iodide ($\lambda_{\text{ex}} = 535 \text{ nm}$, $\lambda_{\text{em}} = 617 \text{ nm}$) was used for staining the nucleus and Palloidin CruzFluor® 488 ($\lambda_{\text{ex}} = 493 \text{ nm}$, $\lambda_{\text{em}} = 517 \text{ nm}$) was used for the F-actin filaments on the plasma membrane. All images showed in this work were acquired in a Cytoviva Dual-Mode Fluorescence/Hyperspectral Dark Field microscope, allowing for easy interchangeable imaging modes for same-field analysis. Fluorescence filters were removed prior to dark field image acquisition. Figure 4-4 displays the obtained results.

The two inserts in Figure 4-4a and 4-4b are magnifications of the same region of interest. 4-4a shows fluorescence imaging of the nuclear staining by propidium iodide. 4-4b is a

dark field image of the same field. It is possible to notice the presence of dark spots on the magnified insert in 4-4a. The position of these spots overlaps with the position of the silver nanoshells shown on the insert in the dark field image (4-4b). Fluorescence in these particular regions has been quenched due to the presence of the silver nanoshells, corroborating the localization of the nanoshells on the nuclear membrane. Two groups of silver nanoshells are evidenced in the inserts. Arrows indicate the precise match between the relative position of the nanoshells and the dark spots. Figure 4-4c shows the hyperspectral image of the same field, where the scattering spectrum of a single nanoshell from the same group of particles is highlighted.

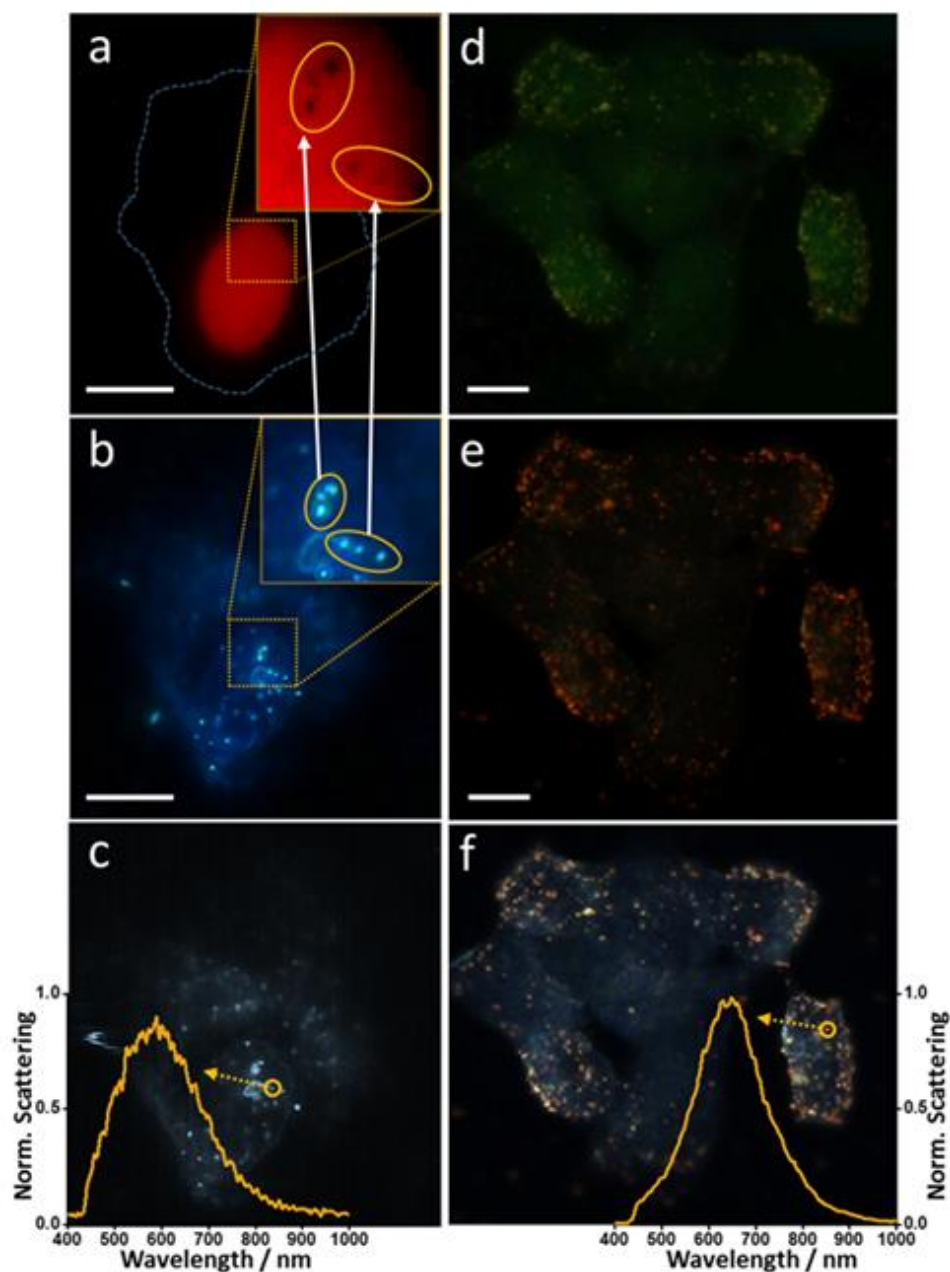


Figure 4-4 Combined microscopical (fluorescence, dark field and hyperspectral) analysis of nanoshell-labeling. (a) and (d) – fluorescence microscopy, (b) and (e) – conventional white light dark field microscopy, (c) and (f) – hyperspectral dark field microscopy. Inserts in (a) and (b) show magnified regions of interest on the nuclear membrane. Inserts in (c) and (f) evidence the scattering spectra of single silver (c) and gold (f) nanoshells. Cell membrane is outlined by dashed line in (a). Scale bar 10 μm . Measurement details in section B.1.4 of Appendix B.

In Figure 4-4d-f the counterstaining marks the inner portion of the plasma membrane. Focal depth was kept constant throughout all three measurements as to evidence the cell boundaries, while still showing the top portions of the plasma membranes. Notice that the nuclei cannot be visualized from this focal plane. The gold nanoshells can be easily seen in all three images. If compared to 4-4e and 4-4f, they are less noticeable in 4-4d, due to their much weaker ability to scatter light in this region of the spectrum, however, their visualization in this fluorescence channel is still possible due to their bigger size (diameter ~ 108 nm). They are much more evident in 4-4e and 4-4f, however, and the perfect match in their relative positions becomes evident if compared among all three images. Similar to what is showed in 4-4c, the scattering spectrum for a single gold nanoshell is also highlighted in 4-4f. This shows that with these monodispersed nanoshells, not only we are capable of selectively labeling specific subcellular compartments at the single cell level, but, with the aid of hyperspectral microscopy, also have the ability to track and select single particles within those compartments.

4.2.5 Multiple compartments at the single cell-single particle level.

Figure 4-5 shows the selectivity of each sample for the desired subcellular compartment under conventional dark field illumination.

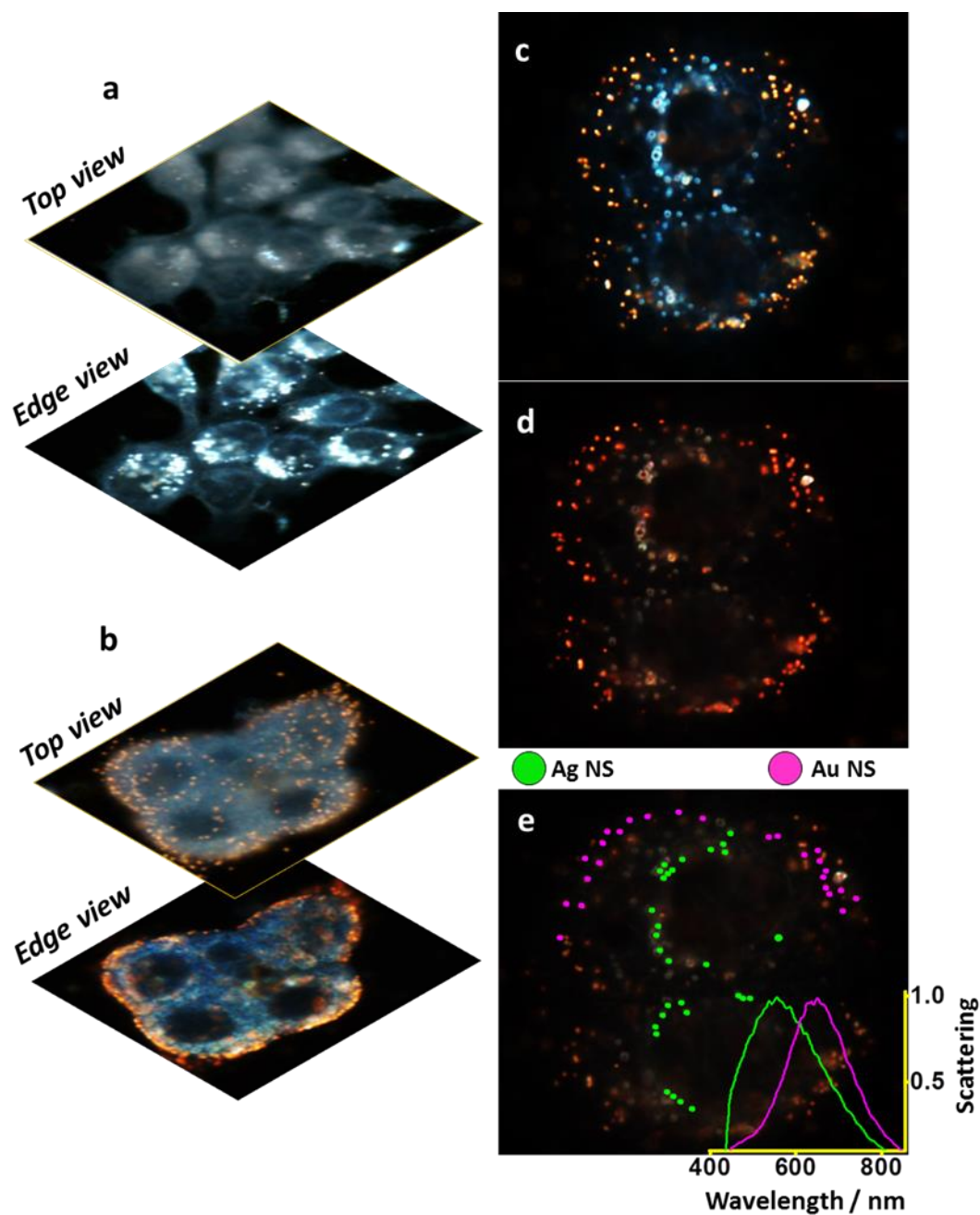


Figure 4-5 Multiplex-imaging different MCF-7 cell compartments with Ag and Au nanoshells. Different focal depths for: a) Ag-NLS – targeting the nuclear membrane b) Au-IGFR – targeting the plasma membrane; same field: c) regular dark field image, d) hyperspectral dark field image, e) Hyperspectral sorting between Ag and Au nanoshells – Ag(18nm)@SiO₂(50nm) labeling the

nuclear membrane and Au(18nm)@SiO₂(72nm) labeling IGF receptors on the plasma membrane, insert shows averaged scattering spectra for the selected nanoshells.

Different focal depths (top and edge) show that (Fig. 4-5a) Ag-NLSs accumulate in the nuclear membrane (edge view) and no particles are seen on the plasma membrane (top view). In the case of Au-IGFR (Fig. 4-5b) the nanoshells specifically target the plasma membrane and can be visualized in both top – homogeneously distributed over the cells, and edge view. Negative controls without the presence of a NLS peptide (for Ag nanoshells), and on IGFR-negative SKBR-3 cells (for Au nanoshells) are displayed in Figure B-SI-9 (section B.1.3.8 of Appendix B). Ag50 without NLS do not accumulate on the nuclear membrane. SKBR-3 cells present low expression levels of IGFR and the sparse number of gold nanoshells seen in Fig. B-SI-9 confirms the specificity of Au-IGFR for the insulin receptors on the MCF-7 cells. That also shows that nonspecific binding does not pose a problem.

It is clear from Figure 4-5a, the efficient internalization of Ag-NLS by the cells. Their overall absence from the cell membranes indicate that the Ag-NLS particles that came in contact with the cell membrane were internalized. At higher magnification (Figure B-SI-9, section 1.3.8 in Appendix B) the nanoshells are seen inside the cell surrounding the nucleus, suggesting that they have not accumulated in other subcellular compartments such as the endoplasmic reticulum and the Golgi complex. In Figure 4-5b, Au-IGFR nanoshells accumulate only on the plasma membrane and, as expected, no internalization is seen. These results attest to the efficacy of the bioconjugation process.

It is important to emphasize that sequential incubations were performed with 1 mL of Au-IGFR for 30 minutes, PBS washings followed by 1 mL of Ag-NLS for 3 hours. Cells

were then washed with PBS and fixed with 4% paraformaldehyde before being mounted onto microscope slides. Conventional (Fig. 4-5c) and hyperspectral (Fig. 4-5d) dark field images of MCF-7 cells labelled with gold and silver nanoshells are displayed. Both images were acquired from the same field of view. The image in Figure 4-5c was obtained under white light illumination with a regular CCD camera (no spectral resolution). Figure 4-5d was acquired with a spectrograph-coupled CCD camera for the hyperspectral image (microscope setup Appendix B). From Figure 4-5c, the distinction between the gold and silver nanoshells is clear even to the naked eye, demonstrating that a regular dark field microscope (without hyperspectral capabilities) can be incorporated into the workflow of this platform. In Figure 4-5e, gold (purple) and silver (green) nanoshells were sorted from the hyperspectral image based on their scattering profiles and the averaged scattering curves for each of the samples is shown on the inset. For the purpose of visualization, only 25 particles for each type of nanoshells were selected. These spectra confirm the localization and selectivity of the nanoshells, and the separation between them corroborates the multiplex capabilities of this platform.

Despite the long incubation time (3h30min total), the Au-IGFR nanoshells stay on the plasma membrane and are not internalized by the cells. In all fields evaluated both samples were selective to their designated subcellular compartment. Cell viability was determined using the Trypan Blue exclusion test.⁴⁶ Cells were analyzed 48h post-incubation, and no significant variations were seen compared to cells that did not receive nanoshells (Table B-SI-2 in section B.1.4 of Appendix B). The intracellular toxicity of pristine Ag nanoparticles has been shown to be lessened by the presence of capping agents⁴⁷. Here, we credit the absence of significant cytotoxic effects to the use of an amine-rich NLS peptide

that can act as an effective capping agent due to the strong interaction between amine groups and the metallic surface. It is important to point out that, although the results in Figure 4-5 were obtained using the nanoshells with the best spectral separation obtained in this work, other combinations from our nanoshell library could also be used. This is possible due to the relative narrow LSPR of our samples (Figures 4-2 and 4-3). For instance, Figure B-SI-10 (section B.1.3.9 of Appendix B) shows a similar experiment as in Figure 4-5, but using two nanoshells of the same metal, Au50-NLS and Au80-IGF. In that case, the colours of the two types of nanoparticles viewed from white light scattering in a dark field microscope were similar. However, their LSPR characteristics were separated enough to allow differentiation in a hyperspectral experiment.

Cell samples labelled with nanoshells can be frozen and kept, similarly to what is adopted for other techniques, such as fluorescence microscopy. One of the main advantages of using nanoshells as labels is that some of the problems that are inherent to the use of fluorophores (photobleaching, quenching, etc.) will not be observed, eliminating the need for re-labeling cell samples over time.

Long-term photostability. In order to assess how the nanoshells stand against the method of reference for cell labelling (fluorescence imaging), an experiment was performed comparing the photostability of the nanoshells with that of fluorescence staining. The plasma membrane of MCF-7 cells was double-labelled with Au-IGFR and Pallodin CruzFluor® 488. The same field of view was then kept under illumination and images were acquired at different time intervals. Figure 4-6 shows the obtained results. After 3 hours under illumination, the fluorescence image almost completely fades as the dye photodegrades (4-6a-d). This shows the superiority of the nanoshells as a platform for

long-term live imaging. Several cellular processes happen on the scale of minutes to hours. A typical cell cycle for human cell lines is on the 8-hour range. A photostable platform that can exceed the 24-hour mark, coupled to the capacity to track and identify at the single particle level will bring important progress to the field of cell analysis.

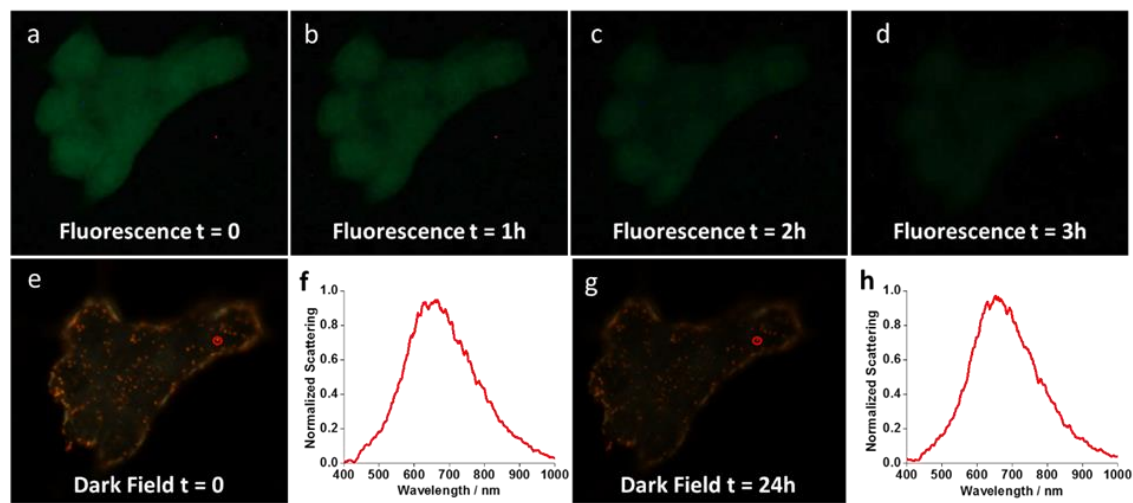


Figure 4-6 Long-term photostability of nanoshells. Time-lapse images show fluorescent phalloidin photodegrading over the course of a few hours under illumination (a-d), whereas Au-IGFR nanoshells are still active after 24h under illumination (e, g). f and h show the single scattering spectrum of the same gold nanoshell (red circular inserts in e and g) at t=0 and t=24h.

4.4 Conclusions

Hyperspectral analysis using nanoshells is potentially a powerful tool for advanced cellular investigations, such as real-time studies, nanoparticle tracking, LSPR-sensing and biomarker quantification. Due to the chemical and biological stability of noble metals, no alterations in cell activity should be expected when using nanoshells as labelling agents. However, stable and monodisperse nanoparticles must be developed for the full

implementation of dark field methods. In this work, we have reformulated the entire synthesis of gold and silver nanoshells into a simpler method that requires only basic lab equipment. The process generates ultramonodisperse particles, resulting in samples of unseen quality whose narrow spectral bands are in close agreement with that for a single particle. Several nanoshell samples were prepared having their LSPRs tuned across the visible range. Their distinctive spectral features were explored in a multiplex experiment, labelling different subcellular compartments in MCF-7 breast cancer cells at the single cell-single particle level. Ag nanoshells targeted the nuclear membrane and Au nanoshells targeted IGF receptors on the plasma membrane of MCF-7 cells. A fluorescence counterstaining experiment unequivocally showed the localization of the nanoshells in different compartments. Hyperspectral images corroborated the selectivity and specificity of the nanoshells for bioanalytical applications. Photostability of these particles was investigated against the standard fluorescent dye phalloidin and surpassed the latter by several hours, hitting the 24h mark with little degradation.

Associated content

Supplementary files shown as Appendix B. Reagents and consumables; Instruments; Methods; TEM images – ultramonodisperse non-aggregated aminated silica, colloidal and chemical stability, gold and silver nanoshells; Hyperspectral dark field microscopy; Spectral separation and sample choice for dual labeling experiment; Hyperspectral analysis – spectral variability; Spectral shift after bioconjugation; Nonspecific endocytosis and nonspecific binding - controls for the cell labeling experiment; Labeling multiple compartments with gold nanoshells only; Cell viability and fluorescence staining; Imaging parameters.

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Chapter 5: SERS from Single Nanoshell: a Study on Excitation

Wavelength Relative to LSPR Position

This chapter presents a study on the SERS excitation wavelength relative to the wavelength of the LSPR peak of two types of gold nanoshells. Several conditions are assessed at the single nanoparticle level. This chapter is a manuscript in preparation for submission. The authors and title of the manuscript are: R. G. Sobral-Filho, X. Zhang, C. D. L. de Albuquerque, A. G. Brolo, SERS Excitation Wavelength and Localized Surface Plasmon Resonance: A Single Nanoparticle Study.

This work was performed in our group. I performed all experiments, X. Zhang performed the SERS measurements and C. D. L. de Albuquerque helped with the data analysis.

Two types of gold nanoshells with narrow extinction bands were evaluated at the single nanoparticle level by SERS microscopy and hyperspectral dark field microscopy. Due to the narrow LSPR bands of the nanoshells, and their fine-tuning parameters, four conditions could be established regarding the wavelength of the SERS excitation (λ_{ex}) and the position of the LSPR peak (λ_{LSPR}). The following scenarios were evaluated $\lambda_{\text{ex}} = \lambda_{\text{LSPR}}$, $\lambda_{\text{ex}} \gg \lambda_{\text{LSPR}}$, $\lambda_{\text{ex}} > \lambda_{\text{LSPR}}$ and $\lambda_{\text{ex}} < \lambda_{\text{LSPR}}$. Significant particle to particle variations in SERS efficiency were seen for the $\lambda_{\text{ex}} = \lambda_{\text{LSPR}}$ and $\lambda_{\text{ex}} > \lambda_{\text{LSPR}}$ conditions; while consistent readings were present in the $\lambda_{\text{ex}} \gg \lambda_{\text{LSPR}}$ and $\lambda_{\text{ex}} < \lambda_{\text{LSPR}}$ conditions. A comparative study showed that the particle variations in SERS efficiency followed the same trend as variations in scattering efficiency. Our experiments agree with reports pointing to the fact that variations in scattering from single nanoshells likely arise from differences in roughness associated with

the random deposition of the gold shell over the silica core during the shell growth phase of the synthesis.

5.1 Introduction

The field of Surface-enhanced Raman Spectroscopy (SERS) has substantially evolved throughout the last few decades, with important practical applications ranging from biomedical¹ to environmental sciences.² As a plasmon-dependent spectroscopy³, the phenomenon has always relied on the quality of the substrates utilized. In the early years of SERS, common substrates would comprise roughened surfaces in the form of metallic films and nanoparticle aggregates^{4, 5} and, at that stage, theoretical studies proved very useful in clarifying basic mechanisms of local field interactions and SERS dynamics.⁶ These studies laid the foundation for discussing and understanding the phenomenon of surface-enhanced Raman Scattering.^{7, 8} Such developments have now walked hand in hand with the progress of nanotechnology, and there has come an era where it is possible to design materials that are tailored to satisfy specific SERS requirements for experiments and further applications in Physics, Chemistry and Biology.⁹ Recent advances include label-free super-resolution chemical imaging¹⁰, structural molecular analysis¹¹ and the use of near-field microscopy to spatially correlate SERS and electromagnetic hotspots.¹² Our group has demonstrated a method on the digitalization of the SERS readout for ultralow concentration analysis¹³, as well as some interesting reports pointing in the direction of single nanoparticle SERS.^{14, 15}

The classical description of SERS hotspots generated by metallic nanoparticles constitute the space between two or more particles where the local electromagnetic fields intersect.¹⁶ In this case the two fundamental requisites to be met by such samples were: 1 – having a localized surface plasmon resonance (LSPR) and 2 – being able to produce aggregates that could generate intense hotspots due to the overlapping electromagnetic fields. In this

scenario, there was no high requirement for control over features such as shape and size, and simple synthetic methods could be used to produce suitable samples.¹⁷ With the advance of colloidal chemistry, new classes of nanoparticles started to appear^{18, 19}, opening the door to more refined studies on nanoparticle SERS.

Among many papers that have looked at the effects of size on the plasmonic properties of nanoparticles, the work by El-Sayed²⁰ stands out due to the detailed studies on shape and composition of metallic nanoparticles. The optimal nanoparticle size for SERS applications was commonly accepted as approximately 50nm.^{21, 22} However, Benz *et al.*²³ recently showed that larger individual particles will have larger SERS emission, and that increasing size generally triumphs over LSPR tuning. Related to the excitation wavelength respective to the LSPR, Weber *et al.*²⁴ reported that the SERS efficiency in colloidal suspensions of nanoshells is higher when the excitation wavelength is red-shifted respective to the LSPR bands, due to the presence of small aggregates in suspension and the intrinsic roughness of the nanoshells. However, due to uncertainties associated with bulk measurements (nanoparticle quantity, minor variations in shape, size, etc.), knowledge on the plasmonic behavior of nanoparticles can be better attained through single particle approaches²⁵, and it has been very difficult for single nanoparticle studies, to isolate and compare parameters such as size and LSPR since the latter is usually modulated through variations in the former. Nie²⁶, Liz-Marzán²⁷ and Ren²⁸ independently reported the acquisition of single nanoparticle SERS with gold nanocrystals, and in a more recent work, Lin *et al.*²⁹ studied the influence of size on the SERS intensity from single gold nanorods, with excitation close to the LSPR wavelength (λ_{LSPR}). Additionally, Halas and collaborators showed that single gold nanoshells can generate SERS enhancements that are

comparable to those produced by nanospheres dimers.³⁰ Here we provide a statistical look at the SERS activity from single gold nanoshells, under different excitation conditions.

We demonstrated in Chapter 4, a new synthetic route for fabricating ultramonodisperse nanoshells of exceptional quality.³¹ In the present work, two nanoshell samples had their LSPR bands fine-tuned to 623 nm and 696 nm. In order to mitigate size effects, the diameter of the nanoshells from both samples was kept constant. By doing so, we can assess SERS behavior according to the LSPR features of the nanoshells, namely peak position and scattering intensity. We then used these two groups of nanoshells to evaluate four SERS excitation conditions: for the first group of nanoshells (N1, $\lambda_{\text{LSPR}} = 623$ nm), ON-resonance ($\lambda_{\text{ex}} = 633$ nm) and OFF-resonance ($\lambda_{\text{ex}} = 785$ nm) SERS was analyzed. For the second group of nanoshells (N2, $\lambda_{\text{LSPR}} = 696$ nm), OFF-resonance with $\lambda_{\text{ex}} < \lambda_{\text{LSPR}}$, and OFF-resonance with $\lambda_{\text{ex}} > \lambda_{\text{LSPR}}$ SERS was studied. All the single nanoshells were analyzed by hyperspectral dark field microscopy and SERS microscopy. 98 nanoshells with $\lambda_{\text{LSPR}} = 623$ nm (N1), and 65 nanoshells with $\lambda_{\text{LSPR}} = 696$ nm (N2) were probed. Each nanoshell had their scattering spectrum matched against their respective SERS spectra and a statistical analysis was performed.

5.2 Experimental section

5.2.1 Chemicals and consumables

Tetrachloroaurate (HAuCl_4), silver nitrate (AgNO_3), L-ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$), tetraethyl orthosilicate 98%, (3-aminopropyl)trimethoxysilane 97% (APTMS), polyvinylpyrrolidone (MW-55000 kDa) (PVP), ammonium hydroxide (NH_4OH) 28%, sodium borohydride (NaBH_4), Triton X-100, 1-hexanol and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich. 0.22 μm cellulose acetate syringe filters were obtained

from Sterlitech. Alpha Numeric Index Nickel TEM grids were purchased from Electron Microscopy Sciences. FisherFinest Premium coverslips were obtained from Thermo Fisher Scientific.

5.2.2 Nanoshell preparation

Nanoshells were prepared as previously detailed.³¹ Briefly, ultramonodisperse silica nanoparticles are produced and aminated in a reverse microemulsion where the diameter can be adjusted according to concentration of TEOS and reaction time. After extensive cleaning, these particles are mixed with freshly prepared, 2nm gold nanoparticles. The small gold nanoparticles attach to the surface of the silica and the resulting nanoislands are cleaned by centrifugation and dispersed in a 150 μ M plating solution of gold ions. Finally, a reducing agent is added to promote the growth of the shell. Shell thickness can be controlled based on the amount of nanoislands per reaction batch during the growth phase. Nanoshells were cleaned by centrifugation and resuspended in water before use. Here two types of gold nanoshells were used (63 nm core + 25 nm shell, $\lambda_{\text{LSPR}} = 623$ nm, and 90 nm core + 13 nm shell, $\lambda_{\text{LSPR}} = 696$ nm). Scattering spectra for both samples are shown in Figure C-SI-1 (section C.1.1 of Appendix C).

5.2.3 Self-assembly monolayer of Raman-active molecule

A 5mM solution of DTNB in acetonitrile was prepared and mixed with an equal volume of freshly made gold nanoshells to a final particle concentration of approximately 10^9 nanoparticles per mL. The mixture was kept under magnetic stirring at 400 rpm for 18 hours before being cleaned by centrifugation and resuspended in water to the same particle concentration. The SERS spectrum for the resulting TNB-coated nanoshells is showed in Figure C-SI-2 (section C.1.2 of Appendix C).

5.2.4 Sample processing and preparation for analysis

5 μ L of the TNB-coated nanoshells was dropped on a clean glass coverslip and the water was allowed to evaporate at room temperature for a period of 1 hour. An alphanumeric indexed TEM grid was placed on top of the dried nanoshells and fixated with minor amounts of fast-dry glue in one of the edges of the grid, to prevent drift.

5.2.5 Analysis

SERS measurements were performed on a Renishaw inVia Raman microscope system with a He-Ne laser source at 632.8 nm and a 785 nm diode laser, under a 100x objective (NA = 0.85). Laser consistency over time was tested with an external silicon substrate and the peak value around 520 cm^{-1} was used to normalize the photon counts. Hyperspectral measurements were performed on a Cytoviva Hyperspectral Dark Field microscope equipped with a 150W Halogen lamp with aluminum reflectors and a Headwall spectrograph-coupled CCD, under a 50x long working distance objective (NA = 0.80). TEM images were analyzed with ImageProPlus and ImageJ software.

5.2.6 Acronyms and nomenclature

For the sake of clarity, the two groups of nanoshells with $\lambda_{\text{LSPR}} = 623$ nm and $\lambda_{\text{LSPR}} = 696$ nm will be henceforth referred as N1 and N2, respectively. For N1, 633 nm and 785 nm excitation will be called ON ($\lambda_{\text{ex}} = \lambda_{\text{LSPR}}$) and OFF ($\lambda_{\text{ex}} \gg \lambda_{\text{LSPR}}$) resonance, respectively. For N2, considering that the LSPR peak is centered between the two excitation wavelengths, 633 nm and 785 nm excitation will be referred as L-OFF ($\lambda_{\text{ex}} < \lambda_{\text{LSPR}}$) and H-OFF ($\lambda_{\text{ex}} > \lambda_{\text{LSPR}}$), respectively.

5.3 Results and Discussion

5.3.2 SERS excitation and LSPR

Single nanoshells were primarily analyzed by hyperspectral dark field microscopy to assure the single nanoparticle condition. Dimers and aggregates were rare in our samples and, when present, would display a shoulder in the near infrared (NIR) region of the scattering spectrum. Single nanoshells were then assessed for their SERS activity under 633nm and 785nm excitation. In N1 the mean LSPR peak position among the 98 nanoshells evaluated was $623 \text{ nm} \pm 13 \text{ nm}$. In N2 the mean was $696 \text{ nm} \pm 12 \text{ nm}$ (histograms are displayed in Figure C-SI-3 in section C.1.3 of Appendix C). In N1, 633 nm excitation is very close to the LSPR peak (623 nm) for the single nanoshells probed, fulfilling the ON-resonance condition, and 785 nm excitation is about 150 nm OFF-resonance respective to the nanoshells LSPR peak. In N2 the LSPR peak (696 nm) is centered between the two SERS excitation wavelengths (633 nm and 785 nm). In this case 633 nm excitation is OFF-resonance at a lower wavelength, and 785 nm excitation is OFF-resonance at a higher wavelength. It is important to emphasize that, in order to eliminate size-dependent contributions to the SERS intensities, the overall diameter for both particle types (N1 and N2) was kept the same. Given their architecture, nanoshells are the ideal nanoparticles for this type of study, and due to the fine-tuning abilities demonstrated in our previous work³¹, it was possible to keep their size constant while changing their LSPR. For N1, the mean nanoshell diameter was approximately 113 nm (63 nm core + 25 nm shell, $\lambda_{\text{LSPR}} = 623 \text{ nm}$), and for N2 it was approximately 116 nm (90 nm core + 13 nm shell, $\lambda_{\text{LSPR}} = 696 \text{ nm}$). Figure 5-1 displays the SERS intensity plots for N1 and N2 under different excitation conditions.

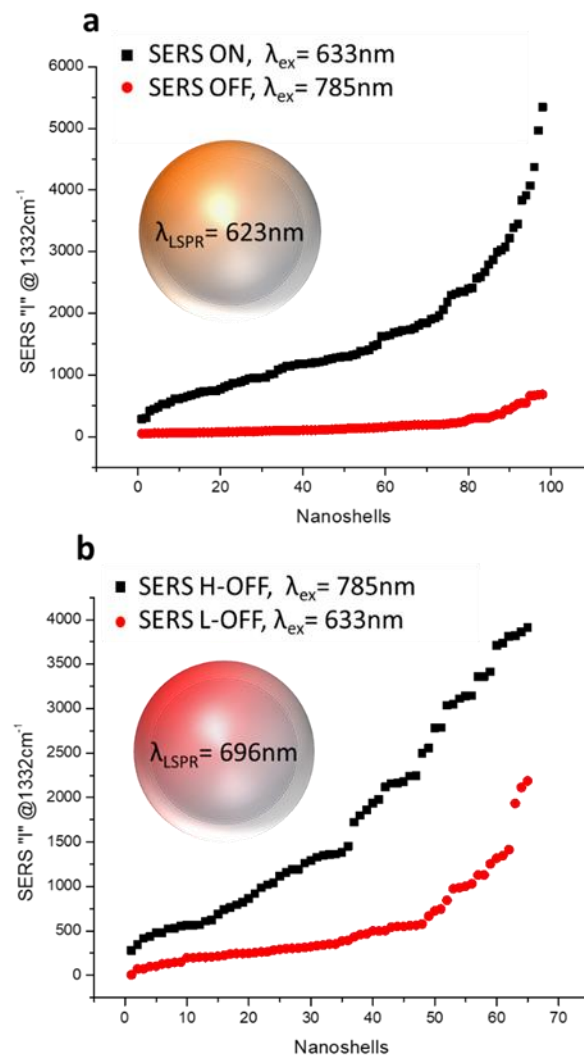


Figure 5-1 a - (▪) ON and (•) OFF resonance SERS intensity plots for single nanoshells N1; b – (▪) H-OFF and (•) L-OFF resonance SERS intensity plots for single nanoshells N2. Nanoshell insets show the mean LSPR peak for each group. SERS intensity “I” measured at 1332 cm⁻¹.

Each individual nanoshell was probed under two excitation conditions. For N1 the same area was mapped first with the 785 nm laser and then remapped with the 633 nm laser, and vice-versa for N2. For N1 nanoshells (1a), the SERS intensities obtained under the ON-resonance condition ($\lambda_{\text{ex}} = 633 \text{ nm}$, black squares) always exceeded the intensities obtained OFF-resonance ($\lambda_{\text{ex}} = 785 \text{ nm}$, red circles). For all N2 nanoshells (1b) the intensities

observed in H-OFF-resonance ($\lambda_{\text{ex}} = 785$ nm, black squares) was higher than L-OFF-resonance ($\lambda_{\text{ex}} = 633$ nm, red circles). Even though both groups N1 and N2 displayed a consistent qualitative behavior in terms of SERS intensity with changing “ λ_{ex} ”, large fluctuations in intensity can be seen in the plots from Figure 5-1. Interestingly, these variations are more pronounced ON-resonance for N1 (1a, black squares), and H-OFF-resonance for N2 (1b, black squares). In both cases, this difference can be as large as one order of magnitude in the most critical cases. In favor of a better understanding for this behavior, the SERS intensity distributions for N1 and N2 are shown in Figure 5-2.

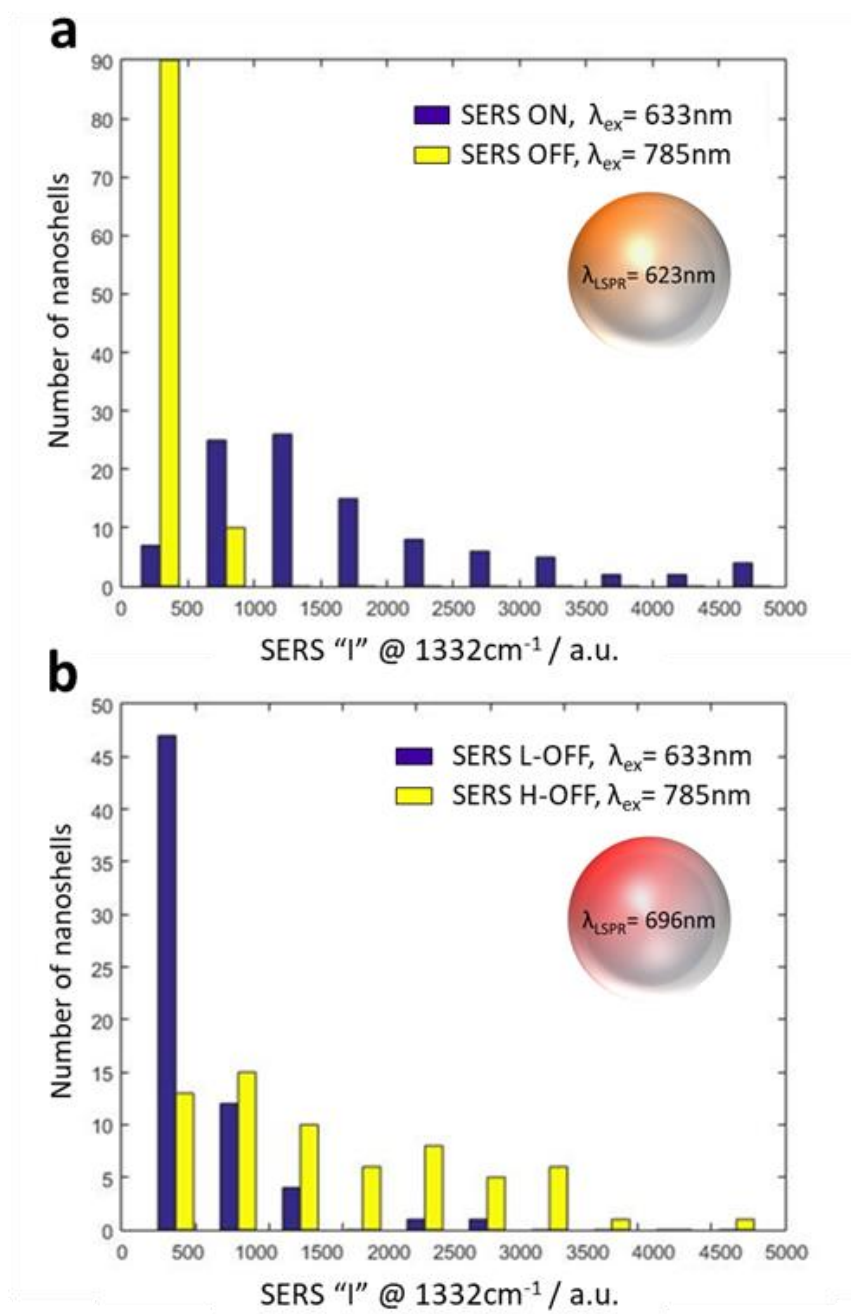


Figure 5-2 Histograms of the SERS intensity for single nanoshells. a – N1 nanoshells probed ON- (purple) and OFF- (yellow) resonance. b – N2 nanoshells probed L-OFF (purple) and H-OFF (yellow) resonance. Nanoshell insets show the mean LSPR peak for each group.

From Figure 5-2 it is easy to notice the discrepancy between the intensity distributions for $\lambda_{\text{ex}} = 633 \text{ nm}$ (purple) and $\lambda_{\text{ex}} = 785 \text{ nm}$ (yellow). In 5-2a, all nanoshells probed OFF-resonance had values lower than 1000 counts with more than 91% of the nanoshells presenting SERS intensities lower than 500 counts. This showed a better particle-to-particle consistency in terms of SERS activity for the $\lambda_{\text{ex}} \gg \lambda_{\text{LSPR}}$ OFF-resonance condition. A similar trend seems to be present for N2 under the L-OFF-resonance condition, with the majority of nanoshells presenting intensities lower than 1000 counts and only two of them showing higher values. When probed ON-resonance, the particles in N1 (2a, purple) show a similar profile to their N2 counterparts (2b, yellow) probed H-OFF-resonance.

The behavior observed in N2 H-OFF-resonance agrees well with what has been reported for bulk measurements from nanoshell colloids with λ_{LSPR} close to 700 nm.²⁴ In that case, the authors attribute the better SERS efficiency for $\lambda_{\text{ex}} = 785 \text{ nm}$ to the presence of aggregates in suspension as well as the roughness of the nanoshells. The single nanoparticle nature of our study allows us to rule out the effect of aggregates, and our data matches well with the assumption that the presence of irregularities such as gaps and tips on the metallic shell work as local hot spots within the particle surface, generating an increased SERS response to near-infrared (NIR) wavelength excitations.

Based on Figures 5-1 and 5-2, it is possible to draw the conclusion that OFF-resonance probing, under the $\lambda_{\text{ex}} \gg \lambda_{\text{LSPR}}$ condition renders the most consistent SERS readout in terms of particle-to-particle variation (5-2a, purple). Further studies were performed to look at the variations in SERS efficiency observed for the $\lambda_{\text{ex}} = \lambda_{\text{LSPR}}$ and $\lambda_{\text{ex}} > \lambda_{\text{LSPR}}$ conditions.

5.3.3 SERS and scattering efficiency

It has been demonstrated that surface roughness, even in the nanometric range, can significantly affect the performance of nanoparticle-based SERS substrates.³² Zhang *et al.*³³ have recently compared the SERS readout between coarse and smooth nanoparticles of the same diameter, showing a 55% increase in SERS efficiency with the use of coarse nanoparticles. Based on this knowledge, and on the fact that shell growth is a process that gives rise to intrinsically rough surfaces³⁴, a study on the scattering intensities for the individual nanoshells was performed. To that end, the scattering spectrum from single particles was obtained through hyperspectral dark field microscopy and matched against the SERS intensity datasets that had the most significant variations – SERS ON-resonance ($\lambda_{\text{ex}} = \lambda_{\text{LSPR}}$) for N1, and SERS H-OFF-resonance ($\lambda_{\text{ex}} > \lambda_{\text{LSPR}}$) for N2. Figure 5-3 displays the obtained results.

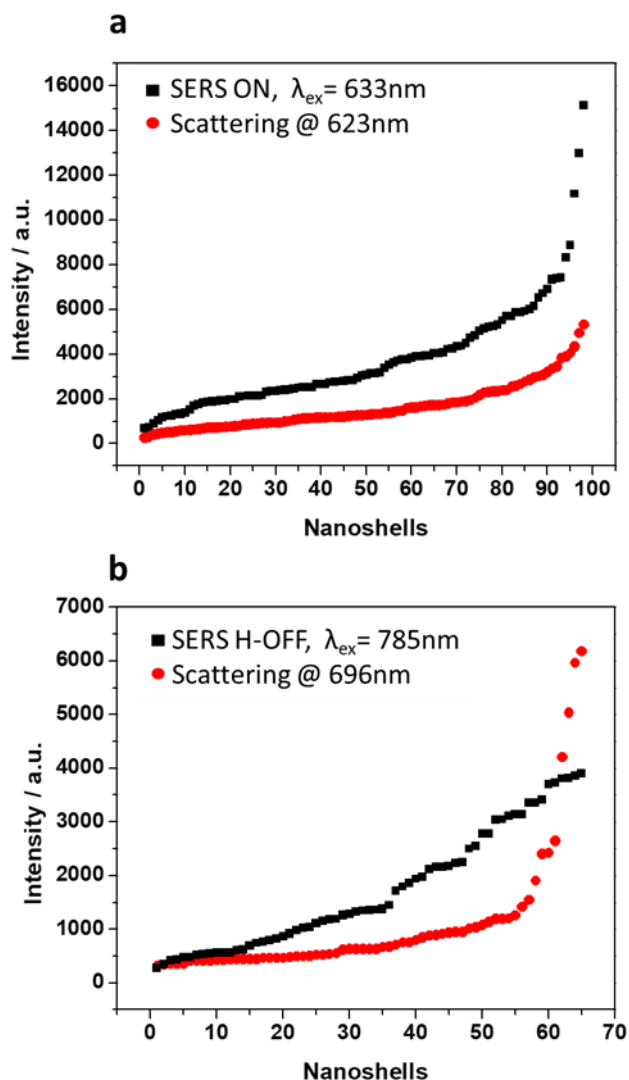


Figure 5-3 SERS and Scattering intensity plots for single nanoshells. a – N1 nanoshells (■) SERS intensity at 1332cm⁻¹ under ON-resonance condition and (●) Scattering intensity at 623 nm, b – N2 nanoshells (■) SERS intensity at 1332 cm⁻¹ under L-OFF-resonance condition and (●) Scattering intensity at 696 nm.

In both cases the SERS intensity increased following the increase in scattering. Interestingly, particle to particle variations in SERS intensity accompany the same trend of variations in scattering. In Figure 5-3a, it is clear the relationship between the SERS

intensity ON-resonance and the scattering efficiency of the nanoshells at the LSPR peak. In Figure 5-3b, the resemblance between both phenomena is also present except for 7 of the nanoshells evaluated (nanoshells 59-65). In those nanoshells, the increase in scattering actually surpasses the increase in SERS intensity. It is very likely that the variations we see in the scattering efficiency in both groups of nanoshells happen due to the differences in roughness that is inherent to the fabrication of metallic nanoshells. Although the adopted method for fabricating the nanoshells used in this study produce ultramonodisperse particles with regard to size, shell growth results in rough surfaces due to the distinct facets that the metal adopts when grown in a bulk suspension.³⁵ For gold and silver nanoshells this process happens as a one-step reaction. There are, however, three distinct phases for the completion of the shell.³⁴ Figure 5-4 provides insight into the process.

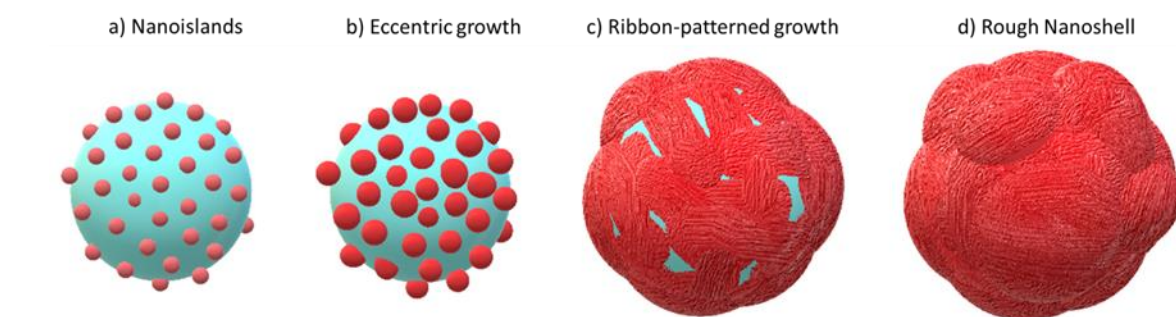


Figure 5-4 Nanoshell growth dynamics for gold and silver nanoshells grown on silica cores via colloidal chemistry. a) nanoislands, b) isotropic growth of gold seeds, c) anisotropic growth of the gold layer, d) fully coated nanoshell. Silica displayed in blue, gold displayed in red.

First, gold is isotropically deposited onto the Au seeds in on the nanoislands Figure 5-4a, resulting in the uniform eccentric expansion of the gold seeds (5-4b). The growing sites then coalesce and assume a ribbon pattern (5-4c), transitioning into anisotropic growth and

resulting in patches of gold that leave small portions of the silica surface still uncoated. These gaps are then filled by the continuous deposition of gold until the shell is complete (5-4d). For a process like this, particle to particle variations will manifest in terms of roughness. It is worth mentioning that nanoshell roughness is a feature that increases as the diameter of the core decreases, and that complete coverage is more difficult to achieve in smaller cores.³⁴ The fabrication of ultrasmooth gold nanoparticles has been presented in the literature³⁵, but to the best of our knowledge, a method that translates into ultrasmooth nanoshells has yet to be unveiled. The shell growth can be verified in detail in our previous work where samples at different stages in the process were imaged by transmission electron microscopy (TEM).³⁶

Our results show that in single nanoparticle regime, particle to particle variations in terms of scattering can dramatically affect the SERS efficiency. Because gold nanoshells are polycrystalline and therefore populated with surface imperfections, we believe such variations are associated with differences in roughness among the nanoparticles. Figure 5-5 displays a high resolution TEM image where it is possible to notice surface discrepancies from particle to particle.

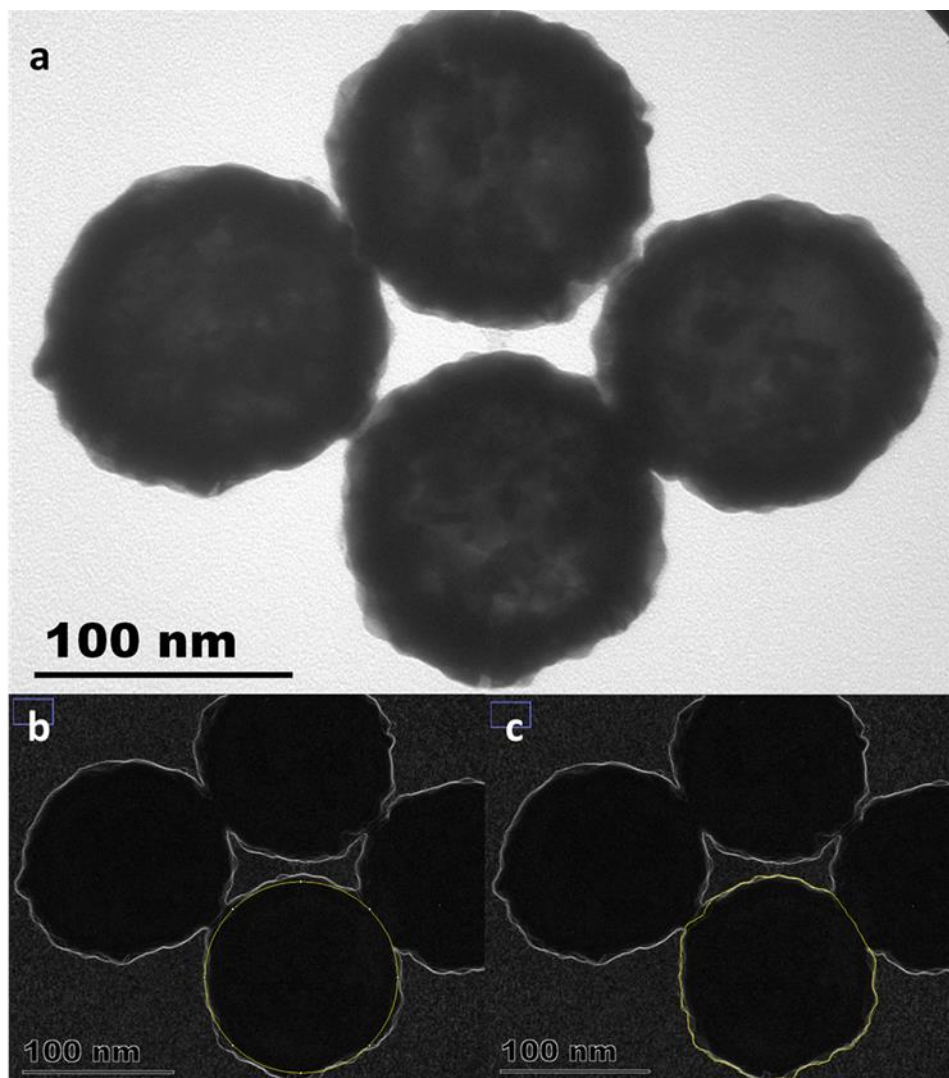


Figure 5-5 a) High magnification (500K) TEM image from N2 nanoshells, b) and circular fit for bottom nanoshell, c) edge fit for bottom nanoshell. Scale bar is 100 nm.

It is clear from Figure 5-5a the differences in roughness that are present between each individual nanoshell. Metal shell imperfections are inherent to the chemical synthesis of nanoshells³⁷ and may greatly impact their plasmonic properties. Wang *et al.*³⁸ showed that rough nanoshells have larger far-field extinction cross sections than their smooth counterparts with same size and gold mass. This behavior probably arises from two components, the increased surface area, and the distribution of hot spots within that surface.

Fig. 5-5b and 5-5c display cross-sectional circular and edge fits (respectively) for the bottom nanoshell, where the roughness of the edge line in 5-5c produces an increase in perimeter of 43 nm as compared with the circular fit circumference. This difference will be much more dramatic if the entire surface of the nanoparticle is considered. A link between scattering and SERS efficiency for nanoshells in bulk has been revealed by combining photoacoustic spectroscopy and Raman spectroscopy.²⁴ Our results confirm that link for the single particle regime. Additionally, Trügler and collaborators³⁹ demonstrated that, in gold nanorods, roughness and local protrusions up to 20 nm can have a significant impact in SERS intensity, and a decrease in SERS signal is observed with nanoparticle smoothening. The rough nature of the nanoshells surface explain the observed variations in scattering, and corroborate the hypothesis that the imperfections associated with roughness can generate local hot spots distributed on the nanoshell surface.

5.4 Conclusions

A clear correlation between scattering and SERS efficiency is reported for gold nanoshells with respect to their LSPR and SERS excitation, at the single nanoparticle level. Two groups of nanoshells presenting the same size, but different LSPR positions were probed for four SERS excitation conditions. For the nanoshells with LSPR = 623 nm (N1), 98 individual nanoparticles were probed. Excitation at 633 nm ($\lambda_{\text{ex}} = \lambda_{\text{LSPR}}$) produced larger intensities but more variation. For these particles, excitation at 785 nm ($\lambda_{\text{ex}} \gg \lambda_{\text{LSPR}}$) generated lower intensities and low variability. For the nanoshells with LSPR centered between the two excitation lines ($\lambda_{\text{LSPR}} = 696$ nm, N2), 65 individual particles were probed. 633 nm excitation ($\lambda_{\text{ex}} < \lambda_{\text{LSPR}}$) showed results similar to the observed for the $\lambda_{\text{ex}} \gg \lambda_{\text{LSPR}}$ condition, with lower intensities and lower variability. For these nanoshells, excitation at

785 nm ($\lambda_{\text{ex}} > \lambda_{\text{LSPR}}$) resulted in higher intensities and higher variations. The SERS variability for conditions $\lambda_{\text{ex}} = \lambda_{\text{LSPR}}$ (N1) and $\lambda_{\text{ex}} > \lambda_{\text{LSPR}}$ (N2) showed a direct dependence with the scattering intensities. Our results agree with studies described in the literature for bulk measurements involving gold nanoshells. The inherent roughness arising from the growth of the nanoshells seems to be the main effector to cause such variations in scattering. An interesting finding from our data is that the same type of nanoshells seem amenable for use in quantitative and qualitative measurements, according solely to the choice of excitation wavelength. For example, in a quantitative study, where particle to particle consistency is a requirement, the nanoshells could be used under the $\lambda_{\text{ex}} \gg \lambda_{\text{LSPR}}$ condition. For qualitative applications, where large SERS intensities prevail over particle to particle consistency, the $\lambda_{\text{ex}} = \lambda_{\text{LSPR}}$ can be selected.

Associated content

Supporting Information presented as Appendix C. Average scattering spectra for N1 and N2 nanoshells, SERS spectrum for a TNB-coated nanoshell, LSPR peak position histograms for N1 and N2 nanoshells.

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Chapter 6: Direct Visualization of Circulating Tumor Cells with Nanoshells

This chapter is a series of preclinical studies on the use of nanoshells as a platform for detection of circulating tumor cells. This chapter is published as: R. G. Sobral-Filho, L. DeVorkin, S. Macpherson, A. Jirasek, J. J. Lum, A. G. Brolo, Ex vivo Detection of Circulating Tumor Cells from Whole Blood by Direct Nanoparticle Visualization, ACS Nano 2018, 12, 1902–1909.

This work is a collaboration with Dr. Julian J. Lum and Dr. Andrew Jirasek at the British Columbia Cancer Agency. I performed all experiments involving nanoshells. S. Macpherson performed the Thy1.1 transduction. Dr. L. DeVorkin performed the animal work (inoculation and blood collection), and FACS measurements.

The detection of circulating tumor cells (CTCs) from blood samples can predict prognosis, response to systemic chemotherapy and metastatic spread of carcinoma. Therefore, approaches for CTC identification is an important aspect of current cancer research. Here, a method for the direct visualization of nanoparticle-coated CTCs under dark field illumination is presented. A metastatic breast cancer cell line (4T1) was transduced with a non-native target protein (Thy1.1). Positive 4T1-Thy1.1 cells incubated with antibody-coated metallic nanoshells appeared overly bright at low magnification, allowing a quick screening of samples and easy visual detection of even single isolated CTCs. The use of a non-transduced cell line as control creates the ideal scenario to evaluate

nonspecific binding. A murine metastatic tumor model with the 4T1-Thy1.1 cell line was also implemented. Blood was drawn from mice over the course of one month and CTCs were successfully detected in all positive subjects. This work validates the use of metallic nanoshells as labels for direct visualization of CTCs while providing guidelines to a systematic development of nanotechnology-based detection systems for CTCs.

6.1 Introduction

Although scientists and health professionals are steadily making progress in our fight against cancer, the number of new cases continues to rise. According to the World Health Organization (WHO) 2017 fact sheet¹, a 70% increase in the number of diagnosed cancers is expected over the next 20 years. Our ability to meet this challenge will be determined by advances in the understanding of the disease, as well as the number of new technologies at our disposal for diagnosis, prognosis and treatment.

One of the deadliest characteristics of cancer is its ability to spread to other parts of the body, a process called metastasis.² In metastasis, tumor cells escape their primary site, carried away in the bloodstream or lymphatic system, and reach a region of healthy tissue. These circulating tumor cells (CTCs) can then establish a secondary tumor in a new site. The metastasis mechanism is under heavy scrutiny by researchers across the world, but there are still several gaps in its understanding.³ Currently, CTCs are believed to be the main effector of metastasis. CTCs have been listed as a prognostic marker for breast cancer, and linked to metastatic relapse in lung, prostate and colorectal cancer.⁴ These facts underline the importance of developing methods and technologies to isolate, detect and study CTCs.

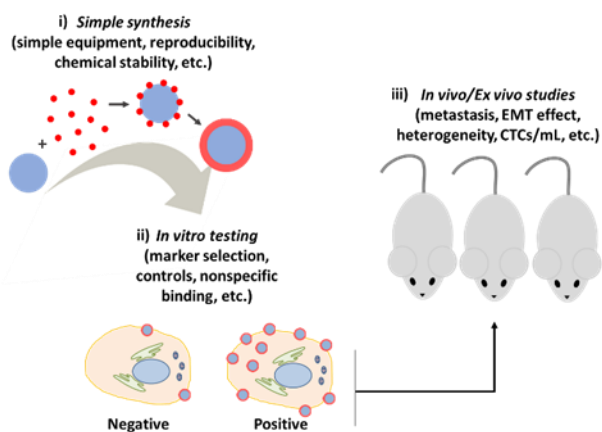
Early detection of metastatic sites, and monitoring of remissive tumors presents the same challenges as in the early detection of primary tumors – conventional diagnostic methods, such as ultrasound imaging (USI), magnetic resonance imaging (MRI), computed tomography (CT) and positron emission tomography (PET), have detection limits on the size range of millimeters. Early metastatic sites, as well as remissive tumors are only a few micrometers large and cannot be visualized using current clinical imaging tools.^{5, 6}

Considering these limitations, researchers have shifted their attention to the earlier stage of the metastatic process; focusing on the detection of CTCs themselves. In a landmark paper, Plaks and coworkers established two main routes for the development of new technologies to study CTCs: 1) Isolation and detection of CTCs, and 2) single CTC “omics”.² The present work focuses on the first route and reports on the isolation and detection of CTCs through direct visualization using a simple instrumental platform.

If a breast tumor spreads to the lungs, it will be named metastatic breast cancer and treated as breast cancer stage IV at the new site. Metastatic cancer cells, however, don't always preserve the immunochemical characteristics of the primary tumor.^{7,8} The challenge here is the identification of a metastasis as opposed to a new primary tumor. In this case, specific phenotypes on CTCs can be exploited to identify cancer patients who are at an increased risk of metastasis, even before a secondary tumor has been established. CTCs, however, are extremely rare in peripheral blood, with typical numbers ranging from 1-10 CTCs per mL in metastatic patients.⁹ They are also inherently heterogeneous¹⁰ and cell-to-cell variations may constitute a difficult challenge for any detection platform. The complexity of biological matrixes also poses a significant general problem in analytical biochemistry. However, CTC detection methods that have been developed *in vitro* often do not correlate to clinical samples, due to heterogeneity and changes in cell phenotypes that are present in a real tumor.¹⁰ Carcinomas, for example, are tumors of epithelial origin, but the tumor cells can undergo a process called epithelial-to-mesenchymal transition (EMT) that reduces the expression of potential epithelial targets, such as epithelial cadherin (Ecad).¹¹ Aiming for one of these targets, could potentially lead to false-negatives and add a degree of uncertainty on the development of new detection platforms.⁴ Due to

heterogeneity and the rarity of CTCs, *in vivo/ex vivo* validation seems to be the primary necessity when establishing a detection method.

Myung *et al.*¹⁰ listed the main issues related to the clinical translation of nanotechnology-based CTC detection platforms. 1) Complicated synthetic procedures for the nanomaterials, 2) high frequency of nonspecific binding of normal hematological cells, and 3) inconsistencies between *in vitro* and *in vivo/ex vivo* measurements due to phenotypic changes and heterogeneity. While the *in vitro* studies play an important role in the early stages of development, a more clinically-oriented perspective of the new technologies can only be achieved through *in vivo/ex vivo* studies. Scheme 1 summarizes the workflow for the systematic development of CTC detection platforms.



Scheme 6-1 Systematic development of nanoparticle-based platforms. One step at a time, the development and validation of new CTC platforms encompass: i) Simple synthetic procedures that can produce quality materials without high requirements for expertise, ii) *in vitro* testing and target selection, iii) preclinical *ex vivo* studies are the reliable way to validate the platform before clinical trials – here, factors such as cell-to-cell heterogeneity, number of CTCs per mL and the actual establishment of metastatic sites can be assessed.

An overview of nanoparticle-based CTC detection technologies reveals the recent use of surface-enhanced Raman scattering (SERS) tags for multiplexing and quantifying membrane markers on cultured cancer cells mixed with blood.¹²⁻¹⁵ SERS methods are highly accomplished in terms of sensitivity and specificity; however, only *in vitro* tests have been described. The same can be said about several reports on fluorescent and magnetic nanoparticles.¹⁰ Nevertheless, the importance of *ex vivo* validation is essential for the successful progression of any nanoparticle-based CTC detection method. For instance, magnetic ranking cytometry (MagRC) has emerged as a promising development that was validated with *ex vivo* tests. Magnetic nanoparticles were combined with a complex microfluidic chip architecture to run phenotypic profiles of CTCs based on magnetic field gradients.¹⁶ Other technologies also focused on the capture and enrichment of CTCs, and important progress has been made in that area.^{17, 18}

In Chapter 4, we reported a simplified method for the fabrication of ultramonodisperse metallic nanoshells,¹⁹ where only simple benchtop equipment was required for the synthesis. Samples of exceptional quality were easily produced. The application of those nanoshells for multiplexing, extra and intracellular labeling, was also demonstrated. Due to their large scattering cross sections, metallic nanoshells are easy to visualize in a dark field microscope, and their metallic surfaces can be readily functionalized with biomolecules. A very important aspect of the *ex vivo* study reported here is a modification to an orthotopic metastatic mammary carcinoma mouse model. The 4T1 cell line was chosen due to the ability to quickly metastasize to the lungs.^{20, 21} As mentioned previously, inconsistencies in terms of target molecule expression may happen between *in vitro* and *ex vivo* studies. Post-EMT phenotypic changes can reduce the expression levels of a certain

target protein. Besides, even if a non-tumor cell is negative for a particular target, basal levels of expression might still be present. This can lead to a misinterpretation of the results; *i.e.*, a healthy cell can be interpreted as a CTC. As a strategy to address both problems, a stable transduction of a non-native target molecule on our 4T1 cells was performed. Thy1.1, a protein that is part of a murine cluster of differentiation, was the marker of choice. In this case, the protein would be present as a cell membrane marker, but without affecting the metastatic ability of the 4T1 cells. This allowed for a comparison between the expression levels of the marker in the cultured cells *versus* the detected CTCs. Furthermore, it eliminates the possibility of misinterpreted results due to basal levels of expression in healthy cells. Therefore, a non-transduced healthy cell (hematological, epithelial, *etc.*), will have no expression whatsoever of the non-native protein Thy1.1, meaning that all the nanoshells present on the membrane of a negative cell, are there due to nonspecific interactions. This allowed an unequivocal evaluation of the performance of our particles for CTC detection.

6.2 Results and Discussion

6.2.1 Ultramonodisperse gold nanoshells

Metallic nanoshells of different colors can be produced by judicious tuning of core diameter, shell thickness and metal type.¹⁹ Gold nanoshells were synthesized and characterized as previously described^{19, 22}. The core diameter and shell thickness were 80 and 15 nm, respectively. The resulting colloid had a narrow extinction band with maximum localized surface plasmon resonance (LSPR) at 680 nm (Figure D-SI-1 in section D.1.1 of Appendix D). Anti-Thy1.1 monoclonal antibody was linked to the nanoshells through EDC/NHS coupling.²³ The bioconjugated samples (Au-Thy) were kept in the fridge at

10°C for no longer than 7 days before being used. It is important to mention that the storage conditions for bioconjugated samples should follow the recommendations that assure the biomolecule stability.

6.2.2 Thy1.1 transduction

Stable transduction was achieved through viral vectors produced in 293T cells.²⁴ Briefly, viral vectors containing the DNA encoding Thy1.1 are produced in T293 cells, isolated and incubated with the 4T1 cells. The vectors are then internalized and the DNA is assimilated by the 4T1 cells genome. Parental 4T1 cells were genetically screened before transduction. Following transduction, 4T1-Thy1.1 cells were isolated and expression was confirmed by flow cytometry (Figure D-SI-2 in section D.1.2 of Appendix D). For clarity purposes, the Thy1.1 transduced and non-transduced cells will be referred to as 4T1+ and 4T1-, respectively.

6.2.3 *In vitro* testing

Preliminary testing for specificity of the anti-Thy1.1 modified gold nanoshells (Au-Thy) towards Thy1.1 proteins was performed at a concentration of approximately 5×10^9 particles per mL. Samples were incubated with 4T1+ and 4T1- cells for 35 minutes at 37°C in PBS. Figure 6-1 displays images of both samples analyzed through dark field hyperspectral microscopy.

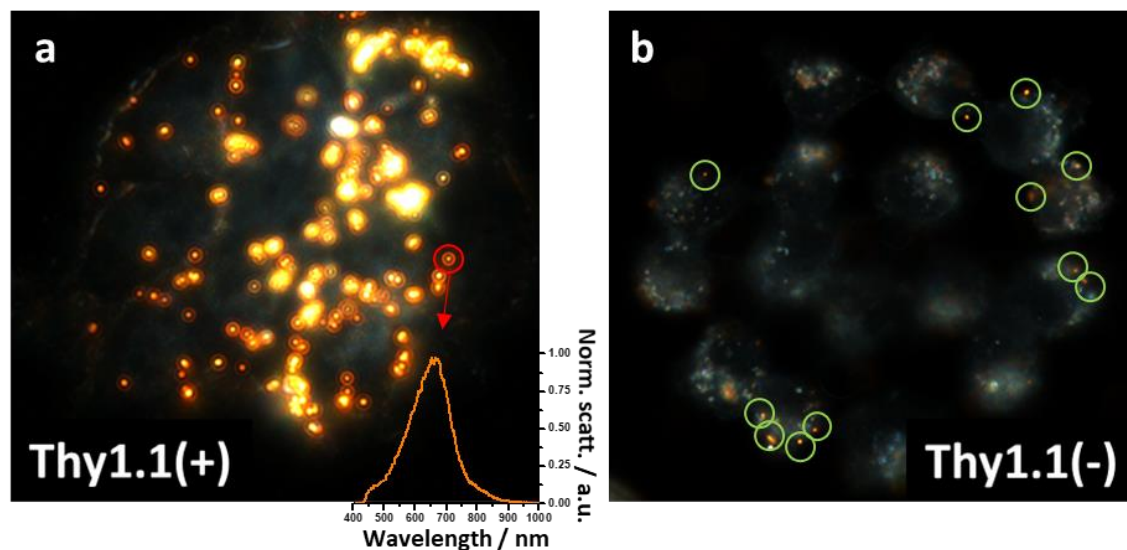


Figure 6-1 *In vitro* specificity testing for Au-Thy nanoshells. (a) – A high number of gold nanoshells labeling Thy1.1 on the membrane of 4T1+ cells. Inset shows a single nanoshell and the respective normalized scattering spectrum. Acquisition under a 100x oil immersion objective; (b) – only a few nanoshells are seen on the 4T1- cells. Nanoshells located through a digital spectral filter are circled in green. Acquisition under a 63x oil immersion objective. Both images collected at 150ms exposure.

Nonspecific adsorption is a recurrent challenge for the development of specific protein detection from nanoparticle labels. Given the complexity of the plasma membrane, non-specific adsorption can be due to several contributions (composition, net charge, membrane permeability, *etc.*).²⁵ The measurements in Figure 6-1, reflect a situation that is close to ideal from an experimental standpoint. The positive and negative cell lines share the same parental cell line batch, and, aside from the presence or absence of Thy1.1 on their membranes, should be in close proximity in terms of composition, charge and permeability. In most previous works on the application of nanoparticle as cell labels, different cell lines

were used as positive and negative controls. For instance, MCF-7 cells are positive for the receptor IGF1R – an insulin-like growth factor receptor. In this case, the recommended cell line that act as a negative control for IGF1R detection, would be the SKBR-3 cell line.¹⁹ Nonetheless, in clinical cases, different cell types might have the receptor of interest. The target protein can even be present in noncancerous tissues, as it is the case for the carcinoembryonic antigen (CEA).²⁶ The use of transduced cells modified with the protein of interest, shown in Fig. 1, significantly simplify the controls during the development of the labelling protocol. However, in order to highlight the adaptability of our platform to different targets and cell lines, an experiment based on the IGF1R receptor was performed to detect and differentiate between MCF-7 (positive) and SKBR-3 (negative – basal levels) cells. These results are displayed in Figure D-SI-3 (section D.1.3 of Appendix D).

6.2.4 Hyperspectral analysis

A Cytoviva Dual Mode Fluorescence/Hyperspectral Dark Field Microscope was used. Its ENVI software is equipped with a “particle filter” feature that allows the identification and quantification of nanoshells. The quantification was implemented using 4T1+ and 4T1- cells labelled by following the same procedure as in Figure 6-1. Equal populations (n=50) of 4T1+ and 4T1- cells incubated with Au-Thy were analyzed and the nanoshells were counted using the ENVI software. These experiments were performed to assess the feasibility of implementing an automated detection system in the near future. The average number of nanoshells per 4T1- cell was 3.25 particles/cell, with the maximum reaching 6 particles/cell. For the 4T1+ cells, the average was 32.5 particles/cell, with the minimum

measured quantity equal to 25 nanoparticles in a single isolated cell. Figure 6-2 displays some of the obtained images.

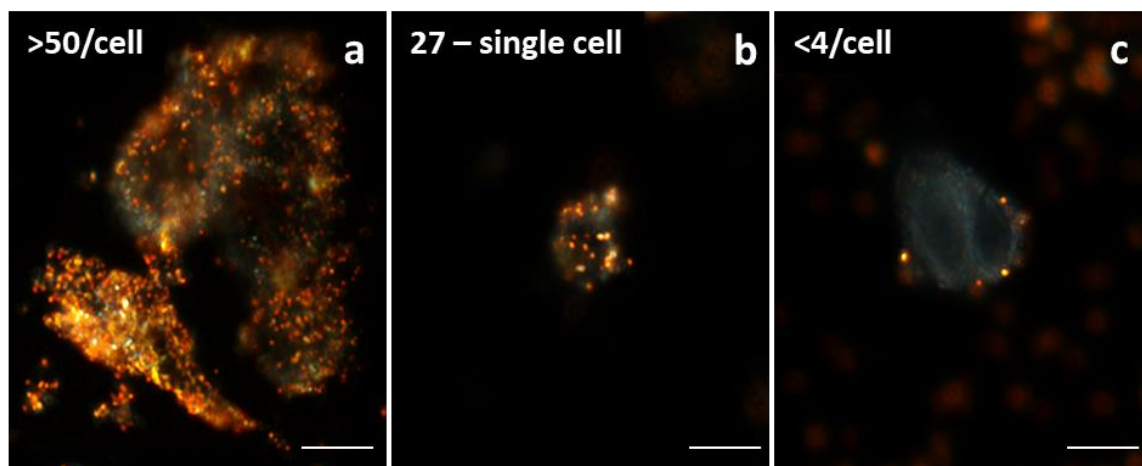


Figure 6-2 Particle quantification. (a) – 4T1+ cell cluster with high expression of Thy1.1 (>50 Au-Thy nanoshells per cell); (b) – single isolated 4T1+ cell with low expression of target protein (27 Au-Thy nanoshells); (c) – Two 4T1- cells show sparse (<4/cell Au-Thy nanoshells/cell), meaning low nonspecific binding. All images were acquired using a 100x oil immersion objective and 150ms exposure. Scale bars are 25 μm .

Figures 6-1 and 6-2 show that the distribution of Thy1.1 in a cluster is not homogeneous regarding the number of target molecules per cell. For instance, Figure 6-1a shows some cells with 56 nanoparticles/cell, while other cells in the same cluster with 5 nanoparticles/cell. The variation in the membrane protein expression can be assigned to several contributions, including differences in DNA copy numbers from the transduction, membrane cycling, and cell cycle variations within the cluster²⁷. In any case, the heterogeneous distribution of Thy1.1 does not prevent the identification of positive cells. Interestingly, Aceto *et al.*²⁸ have recently showed that CTC clusters have 23 to 50 times increased metastatic potential, when compared to single CTCs.

6.2.5 Murine 4T1 metastasis model

The 4T1+ and 4T1- cells were expanded and implanted in 6 BALB/c mice. The mice were then divided into two groups, with three of them receiving the transduced 4T1+ cells (subjects P1, P2 and P3) and the remaining three mice receiving the non-transduced 4T1- cells (subjects N1, N2 and N3). The non-transduced implantation is crucial for two reasons: 1) to allow the evaluation of tumor progression with and without the presence of the transduced target. Ideally there should be no significant difference, and all 6 tumors are expected to undergo similar growth and metastasis; 2) to evaluate cells originated from the same parental cell line, that have actually undergone all the complex physiological processes involved in their release from the primary tumor site, and fell into the bloodstream. Tumor growth was monitored daily, and blood was drawn on days 5, 10, 15 and 29 post-implantation. Tumor growth was similar for both cell lines, as expected. Figure 6-3 shows growth curves for both groups.

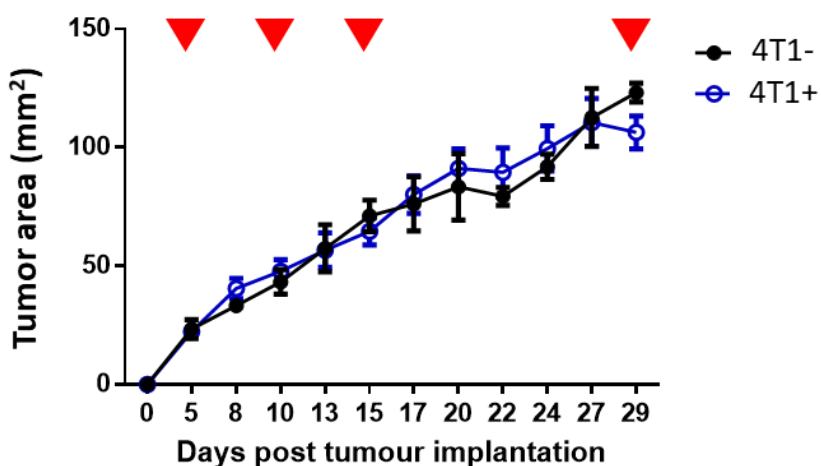


Figure 6-3 Tumor growth curves show a similar post-implantation trend for both the 4T1+ and 4T1- groups. Blood was drawn on days 5, 10, 15 and 29 post-implantation (▼). The errors are for n = 3 (3 mice in each group, as discussed in the text).

All mice were euthanized on day 29 and the tumor, lungs, lymph nodes and blood were harvested for further analysis by flow cytometry. Blood from each mouse was processed and plated onto multi-well glass slides obtained from ibidi® (Martinsried, Germany). 24 hours (at 37°C and 5% CO₂) were allowed for the CTCs to adhere to the glass surface. The wells were gently washed three times with PBS prior to incubation with the Au-Thy nanoshells. Cells were then washed and fixed with paraformaldehyde 4% in PBS. The silicone well-separator was removed and the slide was mounted for dark field imaging.

6.2.6 *Ex vivo* detection of CTCs

Mounted slides were imaged on a dark field hyperspectral microscope. A quick screening was performed for each well. Points of interest were easily identified due to the high scattering ability of the metallic nanoshells. Positive cells stood out as very bright spots under darkfield illumination even at low magnifications (Figure 6-4a).

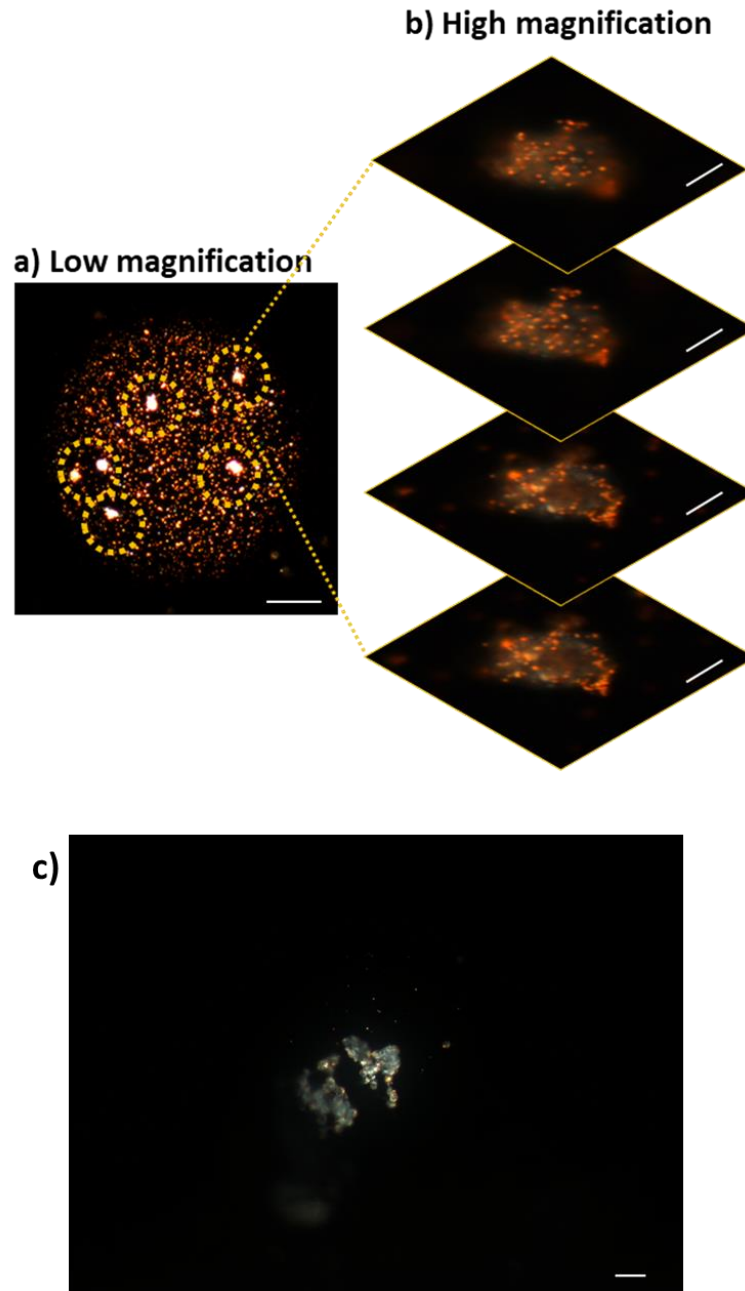


Figure 6-4 CTC visualization – (a) – CTCs appear overly bright under a 10x objective, scale bar is 25µm; (b) – different focal depths show a single isolated CTC under a 100x oil immersion objective, scale bar is 10µm; (c) negative cells imaged under 10x objective. 150ms acquisition time. Blood sample used in (a-b) obtained from mouse P1 on day 29.

Six isolated CTCs can be visualized in the low magnification field in Figure 6-4a. A Z-stack of one CTC region, acquired using a 100x oil immersion objective, is shown in Figure 6-4b. For means of comparison, 6-4c shows negative cells, also imaged under a 10x objective. Figure 6-4 demonstrates the feasibility of using this detection scheme in clinical settings, as it provides a simple way to visualize the CTCs. Figure 6-5 indicates that the number of CTCs per sample of blood increased with tumor progression, reaching a maximum at day 29 for all individuals (Table D-SI-1 in section D.1.4 of Appendix D) displays the numbers used to generate Figure 6-5). Assuming that no viable CTCs were lost in our washing steps, the minimum amount found in blood was 2 CTCs on day 5 for mouse P1 and the maximum was 32 CTCs for mouse P3 on day 29. A plot showing the accuracy of the CTC detection, including all data from the positive group, is shown in the SI file (Figure D-SI-4 in section D.1.5 of Appendix D). The nanoshell-labeled cells are clearly distinguishable, as seen in Figure 6-4, allowing the detection of even a single CTC. A more comprehensive time series could have been obtained if blood was drawn every day, but that could place the animals under excessive stress.

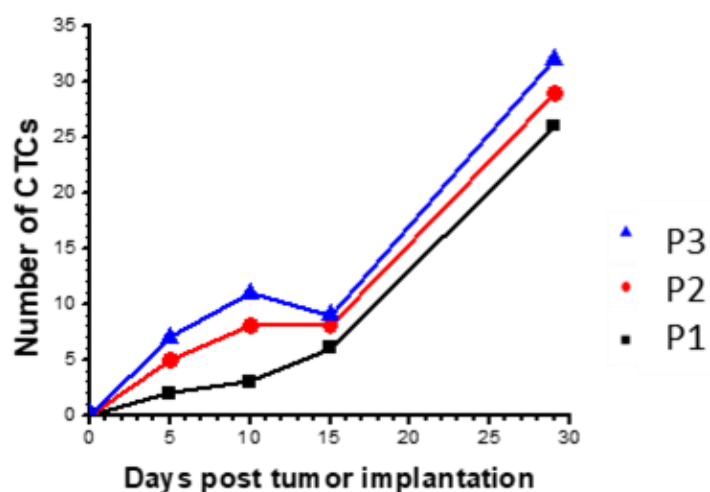


Figure 6-5 Number of CTCs detected with Au-Thy for different mice.

Minor variations in the number of CTCs from different individuals (see Figure 6-5) might be related to differences in the defense mechanisms where metastatic cells are cleared by the immune system. This assumption is in accordance with the mechanisms of the immune response involved in different stages of tumor development for the 4T1 model.²⁹ However, a study with a much larger group would be required to validate this claim.

Usually, the merit of a CTC detection system is expressed in terms of number of detected cells per volume of blood.³⁰ In our case, the lowest number of CTCs detected was 2 CTCs in 100 μ L of blood (from mouse P1 on day 5 – see Table D-SI-1 in section D.1.4 of Appendix D). Considering the low amounts of CTCs usually found in blood (1-10 CTCs/mL), certain techniques require an increase in sample volume and a preconcentration step.^{9, 30} In the experiments reported here, the cells were simply precipitated onto a small area, and further analyzed through dark field microscopy. As we clearly demonstrate in

Figure 6-4, this method allows the direct visualization and identification of even a single isolated CTC.

6.2.7 *Ex vivo* nonspecific binding

The specificity of CTC detection was also probed for *ex vivo* conditions. In contrast to *in vitro* models, the tumor microenvironment is a complex network of cells, with an extracellular matrix and signaling molecules³¹, and it is nearly impossible to re-create all these conditions *in vitro*. Even 3-D models may fall short in mimicking the physiological changes that can take place in the tumor microenvironment. Significant variations in cellular composition have been shown to happen with tumor progression in primary and metastatic breast tumors³², and, so far, the best way to do a preclinical analysis for the detection of CTCs is to work with *in vivo* models.³³

As non-adherent, most of the hematological cells will be washed away during the incubation steps³⁴ and should not pose a problem to the detection process. Nevertheless, nonspecific detection of white blood cells has been shown to introduce false negatives and complicate readout analysis.³⁵ In order to investigate the nonspecific binding across negative cells of different types (mostly hematological and epithelial), the Au-Thy nanoshell was incubated with an unwashed blood sample from mouse N3 and the results are shown in Figure 6-6.

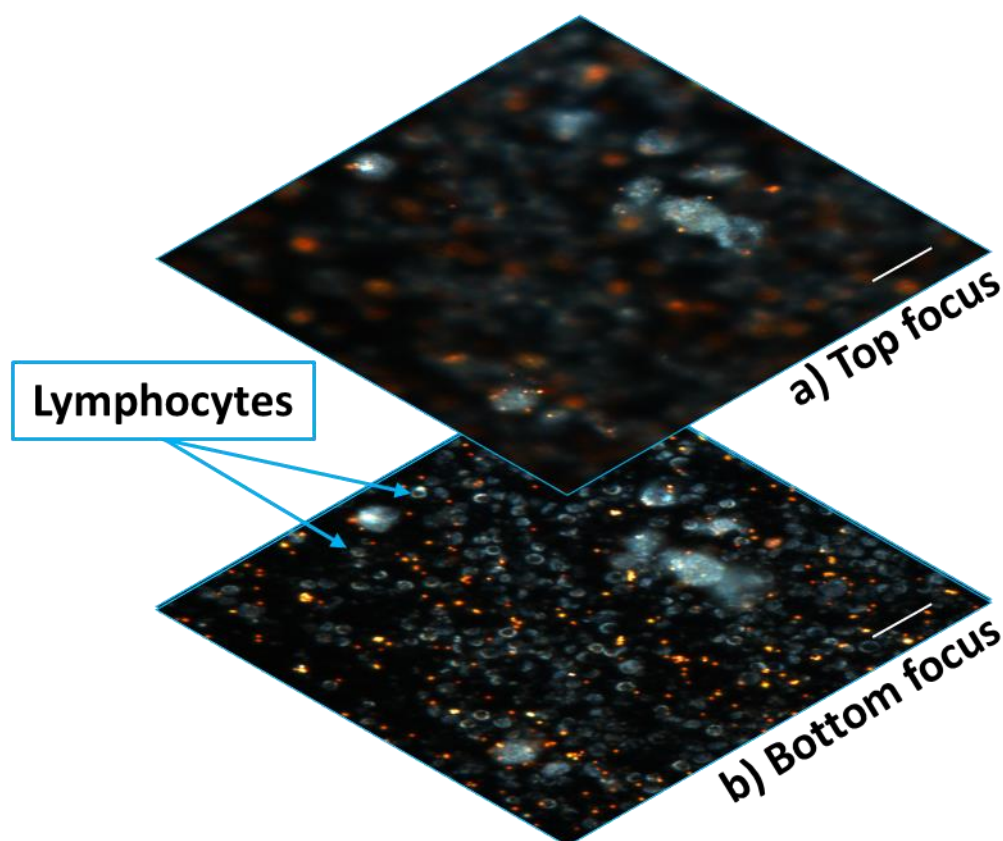


Figure 6-6 *Ex vivo* assessment of nonspecific binding across multiple cell types from an unwashed blood sample. (a – top focus) – adherent 4T1- cells, (b – bottom focus) – nonadherent hematological cells. Image acquired under a 63x oil immersion objective, 150ms. Scale bar is 50 μm .

Different focal depths are showed in Figure 6-6. The 4T1- cells adhered to the glass surface (Fig. 6a) and nonadherent hematological cells are at the bottom of the mounted slide (Fig. 6-6b). Low levels of nonspecific binding are present on the 4T1- cells. 6 nanoshells are seen on isolated 4T1- cells situated in the lower corner of the top focus view in Figure 6-6a. This number agrees well with the ones observed during the *in vitro* phase of the experiment. At the bottom focus view, Fig. 6-6b, several lymphocytes can be seen, and most of them do not show any attached nanoshells. The average nanoshell count per

lymphocyte was 0.32 nanoparticles/cell. These results attest to the good performance of our platform, and reiterate the importance of *ex vivo* probing when developing new detection systems for CTCs.

6.2.8 CTCs detection panel

All mice that received the 4T1+ tumor presented circulating tumor cells in peripheral blood. Figure 6-7 displays a CTC imaging panel for the 4T1+ group on days 5, 10, 15 and 29. Samples were screened under a 10x objective and the detected cells were imaged at higher magnification with a 100x objective.

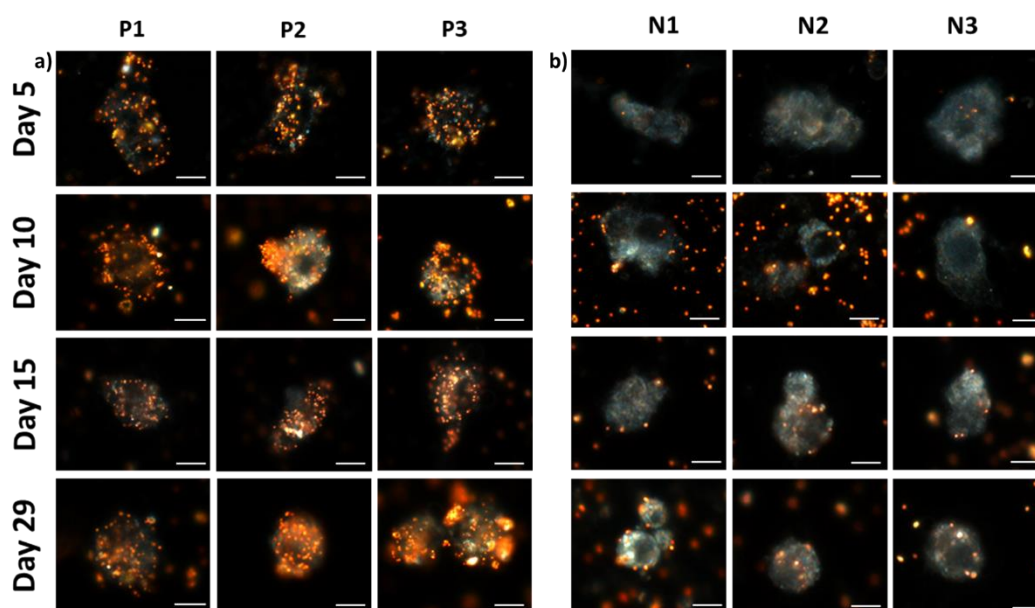


Figure 6-7 (a) 4T1+ CTCs from three mice (P1, P2, P3) and (b) 4T1- CTCs from three mice (N1, N2, N3) were incubated with Au-Thy nanoshells and imaged on a hyperspectral dark field microscope, with a 100x oil immersion objective, 150ms. Scale bars 25 μm .

The panel in figure 6-7 shows CTCs imaged from 3 mice from each group (4T1+ and 4T1-) at 4 different time points making for a set of 24 consistent samples. These results

demonstrate the low levels of nonspecific binding, and the high specificity of our platform. The establishment of metastatic sites in the 4T1+ group was confirmed by flow cytometry through the analysis of tumor tissue and lungs. All subjects in the 4T1+ group had secondary tumors in the lungs. The 4T1- group was kept as a negative control and lymph nodes and blood were also evaluated, showing that in these tissues it is not possible to distinguish between positive and negative samples through regular flow cytometry. This data is displayed in Figure D-SI-7 in section D.1.8 of Appendix D.

6.3 Conclusions

A gold nanoshell-based detection platform for circulating tumor cells has been demonstrated. We have also outlined a roadmap for the preclinical development of CTC detection systems. *In vitro* studies were performed where metastatic breast cancer cells were transduced with a target molecule (Thy1.1), and the non-transduced cells were used as a negative control. The two cell populations were incubated with our Au-Thy nanoshells and analyzed by hyperspectral dark field microscopy, being sorted and counted by a built-in ENVI-based software. Good distinction between positive and negative cells was achieved with a low number of nanoshells adsorbing to the negative cells (<6), and several (>25) nanoshells labeling the positive cells. Due to the high scattering ability of the gold nanoshells, positive cells can be easily found in a quick screening at low magnification (10x objective). Regions of interest can be further imaged at higher magnifications (63x or 100x objectives) making this system suitable for future automation. Once we learned that the platform can be used to identify differential expression of markers across cell populations, we then moved on to develop a murine model with our transduced cells, for the study and *ex vivo* validation of CTC detection. A non-transduced mice group was used

as a control. In this phase of the experiment, blood was drawn from all mice on days 5, 10, 15 and 29 post tumor implantation. Blood samples were analyzed and CTCs were successfully detected in our positive group. Nonspecific binding across cell types was evaluated and our platform showed excellent performance, making it easy not only to distinguish between our positive and negative tumor cell lines, but also hematological cells. At the endpoint of the experiment, all mice were euthanized and the establishment of metastatic sites was confirmed *via* flow cytometry. Differently from most reports on nanoparticle-based CTC platforms, by conducting experiments both *in vitro* and *ex vivo*, we increase the probability of a successful translation into the clinic for our platform. Even more than the dissemination of the platform, we expect this type of awareness to grow among researchers and reviewers in the scientific community.

Associated content

Supporting Information presented as Appendix D. Flow cytometry data, CTC detection numbers, 4T1 CTCs detection panel and multi-well incubation example.

6.4 Experimental section

6.4.1 Materials

Tetrachloroaurate (HAuCl_4), L-ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$), Tetraethyl orthosilicate 98%, (3-aminopropyl)trimethoxysilane 97%, polyvinylpyrrolidone (MW-55000), Ammonium hydroxide (NH_4OH) 28%, sodium borohydride (NaBH_4), paraformaldehyde (PFA), Triton X-100, n-hexanol, γ -Aminobutyric acid (GABA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), were purchased from Sigma-Aldrich. 0.22 μm cellulose acetate syringe filters were

obtained from Sterlitech. T-75 sterile flasks, Dulbecco's modified Eagles medium (DMEM), Phosphate buffered saline (PBS), Fetal Bovine serum (FBS), ACK lysis buffer and nonessential aminoacids solution (NEAA) were purchased from Thermo Fisher Scientific. Anti-Thy1.1 (554895) and FITC-Anti-Thy1.1 (561973) monoclonal antibodies were ordered from BD Biosciences. Anti-IGF1R (sc-713) was ordered from Santa Cruz Biotech. Multi-well 12-chamber slides were purchased from ibidi. All products were used without further purification.

6.4.2 Ultramonodisperse gold nanoshells

Synthesized as described in Chapter 4. Briefly, silica nanoparticles are fabricated and amine-functionalized by reverse microemulsion. Small (2 nm) gold nanoparticles are rapidly produced through NaBH_4 reduction, and mixed with the aminated silica at a 1:49 volume ratio for 3 hours to form the nanoislands. These particles are cleaned 5 times by centrifugation/resuspension in water (10000g/10min) and stored in the fridge. Shell growth is achieved by placing the obtained nanoislands in a plating solution with a low concentration (150 μM) of gold ions, and subsequent reduction with ascorbic acid. Resulting nanoshells are cleaned by centrifugation in water and kept in the fridge until use.

6.4.3 Bioconjugation with monoclonal antibody (Au-Thy nanoshells)

Gold nanoshells (1 mL at 2×10^{10} particles/mL) were coated with GABA (1 mL (aq.), 2mM) for 15 hours and cleaned 5 times by centrifugation/resuspension cycles (10000g/10 minutes) in ultrapure water, and bioconjugation was performed according to an earlier report.²³ The nanoshells were then redispersed in 1 mL PBS containing 5×10^{-2} mol.L⁻¹ of NHS and 2×10^{-1} mol.L⁻¹ of EDC. The reaction mixture was kept under stirring for 2 hours at room temperature and cleaned 5 times by centrifugation/resuspension (10000g/10min)

in PBS. The colloid was redispersed in 1 mL of an Anti-Thy1.1 antibody solution in PBS (20 µg/mL) and stirred for 18 hours at 10°C. The antibody-coated nanoshells were cleaned by centrifugation/resuspension (10000g/10min) 5 times in cold PBS, and kept in the fridge up to 7 days prior to use.

6.4.4 Cell cultures

4T1 cells were maintained in T-75 sterile flasks with DMEM supplemented with 10% FBS and 1% NEAA, in an incubator at 37°C and 5% CO₂.

6.4.5 Th1.1 transduction

Virus was generated using 293T phoenix cells.²⁴ Virion-containing supernatant was collected at 24 hours and 48 hours post transduction using the MSCV-Thy1.1 plasmid. For transduction, 1 mL of viral supernatant was incubated with 4T1 cells in the presence of 4 µg polybrene. After 2 hours, 1 mL of fresh media was added and cells were incubated at 37°C/5%CO₂ for 3 days. Cell culture media was completely replaced on day 2. On day 3, successfully transduced cells were analyzed *via* flow cytometry with a FITC-Thy1.1 monoclonal antibody and the Thy1.1 positive cells (4T1+) were selected using MACs[®] column (Miltenyi Biotec) magnetic bead separation as per the manufacturer's instructions. Transduced cells were maintained in the same culture conditions as the non-transduced 4T1- cells. High expression levels of Thy1.1 were observed in the transduced cells for at least 10 passages.

6.4.6 Murine 4T1+ and 4T1- metastasis models

All animal procedures were approved by the University of Victoria Animal Care Committee and were performed in accordance with the Canadian Council on Animal Care. Female BALB/c mice were purchased from Jackson Laboratories. 5×10^5 4T1- or 4T1+ tumor cells were implanted into the mammary fat pad of 6 10-week-old BALB/c mice under isoflurane anesthesia. Tumor volume was measured three times a week with digital calipers and was calculated according to the formula $\text{mm}^2 = \text{width} \times \text{area}$. To detect CTCs during tumor growth, blood was collected from the saphenous vein.

6.4.7 *In vitro* Au-Thy incubations

4T1+ and 4T1- cells were plated onto square coverslips inside 6-well chambers. Cells were washed 3 times with PBS before receiving 1 mL of the Au-Thy nanoshells suspension at a concentration of approximately 5×10^9 particles per mL. Incubations lasted 35 minutes at 37°C and 5% CO₂. Cells were then washed 3 times with PBS and fixed with 4% PFA in PBS for 10 minutes at room temperature. Coverslips were then mounted onto microscope slides for imaging.

6.4.8 *Ex vivo* Au-Thy incubations

100 μL of blood was drawn from each mouse under sterile conditions. Blood samples were then mixed with ACK lysis buffer for 10 minutes, centrifuged at 1500g/5min and resuspended in supplemented DMEM twice before being plated on the multi-well chamber slide for 24 hours at 37°C and 5% CO₂ (plating volume was 150 μL per well). See Figure D-SI-8 (section D.1.7 of Appendix D) for a picture of the plated samples. After 24 hours, cells are washed 3 times with PBS – care should be taken not to completely dry the wells. A residual volume of 50 μL was always kept in the wells between washes. Cells were then

incubated with 200 μ L of the Au-Thy suspension (5×10^9 particles per mL) for 35 minutes at 37°C and 5% CO₂. Three washings with PBS were followed and the cells were fixed with 4% PFA in PBS. Three final washings were performed and the silicon separator in the multi chamber slide was removed before mounting the slide for imaging.

6.4.9 Imaging parameters

All microscopy images were acquired in a Cytoviva Dark Field Hyperspectral Microscope. For the light source, a 150W halogen lamp with aluminum reflectors (Part # L1090 by Intl. Light Tech) on a housing with analog intensity control was used. For the hyperspectral images, a Headwall spectrograph-coupled CCD was used.

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Chapter 7 Summary and Outlook

This chapter contains the summary and conclusion for this dissertation. An outlook and future direction are briefly discussed.

7.1 Summary and conclusions

In this work, we improved the synthesis of gold and silver nanoshells and expanded on their applications due to the high quality of the obtained particles.

On the work presented in Chapter 3, a systematic investigation of the synthesis was performed. One of the most important contributions to the field is that in this work, the main checkpoints for tracking the progress of the synthesis are presented. It is also shown that the degree of coverage of the silica core can be controlled by the ratio between the amounts of nanoislands and reagents used during the growth phase. A simple SERS measurement is performed using gold nanoshells as substrates, showing that these particles could work as a suitable substrate.

Chapter 4 presents a reformulation of the synthesis to address the broad size distributions usually associated with metallic nanoshells. A new strategy was presented based on the use of simple benchtop equipment. The new procedure resulted in ultramonodisperse samples of unprecedented quality. The narrow extinction bands verified in our colloids allow their use for multiplexing. A cell labeling experiments with two types of nanoshells was performed as proof of the principle. Silver and gold nanoshells were conjugated to a NLS peptide (targeting the nuclear membrane) and an anti-IGFR antibody (targeting the plasma membrane), respectively. Hyperspectral microscopy performed at the single cell-single particle level confirm the selectivity and specificity of the nanoshells.

In Chapter 5 we explore the large scattering efficiency of nanoshells and perform a SERS study at the single nanoparticle level, evaluating the SERS efficiency of the nanoparticles when the excitation wavelength is changed relative to the LSPR peak position. Two types of nanoshells were produced, and due to the fine-tuning ability granted by the method

outlined in Chapter 4, the overall diameter of the nanoshells was kept nearly constant. By changing the core size/shell thickness ratio the LSPR was tuned to be in close to 633nm and 700nm. Several conditions for the SERS excitation wavelength were then analyzed, and we found that an increase in scattering efficiency results in a similar increase in SERS intensity for some of these conditions.

Chapter 6 finally presents the application of gold nanoshells as a platform for the direct detection of CTCs. In order to evaluate the performance of this platform, a target protein was transduced onto a 4T1 breast cancer cell line. This created the ideal scenario for the assessment of the platform in terms of nonspecific binding. A series of *in vitro* studies was performed and we moved further to develop a murine metastasis model with the transduced cells. A non-transduced cell line (from the same parental line) was used as a negative control. CTC detection from whole blood was successfully performed with the nanoshells imaged under dark field illumination. All subjects (positive and negative) were correctly identified. Nonspecific binding was also assessed across different cell types. It is our hope that this work will also serve as a roadmap for the preclinical development of new nanoparticle-based platforms for cell analysis.

7.2 Outlook and future directions

The vast potential nanomaterials hold for basic studies and applications will continue to grow. From the works outlined here, interesting advances would include: studies on the endocytosis mechanisms by cancer cells, super-resolution SERS studies on the surface of individual nanoshells and the progression of the CTC detection platform into clinical trials. These studies could specifically be expanded as described below.

From Chapter 4, nanoshells of unprecedented quality were produced, and intracellular cell labeling was achieved. It would be interesting to expand upon these results, with the objective of studying nanoparticle endocytosis under different conditions. In this case, possible variables to be analyzed could be (but not limited to): pH, temperature, cell type (and cell lines) and biomolecules.

From the results obtained in Chapter 5 it is clear that, even though the nanoshells have a high degree of uniformity in terms of peak position, important particle-to-particle variations are present on surface roughness. Further studies on the topic should include: a topographic assessment of individual nanoshells by atomic force microscopy (AFM) or transmission electron microscopy (TEM); super-resolution SERS studies on individual nanoshells about the dynamics of the hotspots induced by different lasers.

Chapter 6 provides the preclinical studies necessary to the validation of nanoshells as a detection platform for CTCs. Further studies should focus on the clinical translation of this platform. This could be achieved through the analysis of whole blood from patients undergoing cancer treatment at the British Columbia Cancer Agency. A preliminary study on biomarker selection and patient inclusion/exclusion criteria would greatly improve the chance of success for such project.

Other possible ramifications include: 1) the use of nanoshells as a biosensing platform, 2) basic studies on shell composition involving alloys and other metals, 3) their use as radiosensitizers and 4) nanoshells as diffusion agents in artificial tumor models.

Appendix A

A.1 Synthesis and Characterization Checkpoints for Metallic nanoshells

Appendix A is published as Supporting Information to: A. M. Brito-Silva, R. G. Sobral-Filho, R. Barbosa-Silva, C. B. de Araújo, A. Galembeck, and A. G. Brolo, Improved Synthesis of Gold and Silver Nanoshells, Langmuir 2013, 29, 4366-4372. Copyright: Reproduced with permission.

A.1.1 Compositional Analysis

ESI data (Figure A-SI-1b) confirms the composition of the silica nanoparticles produced by this method.

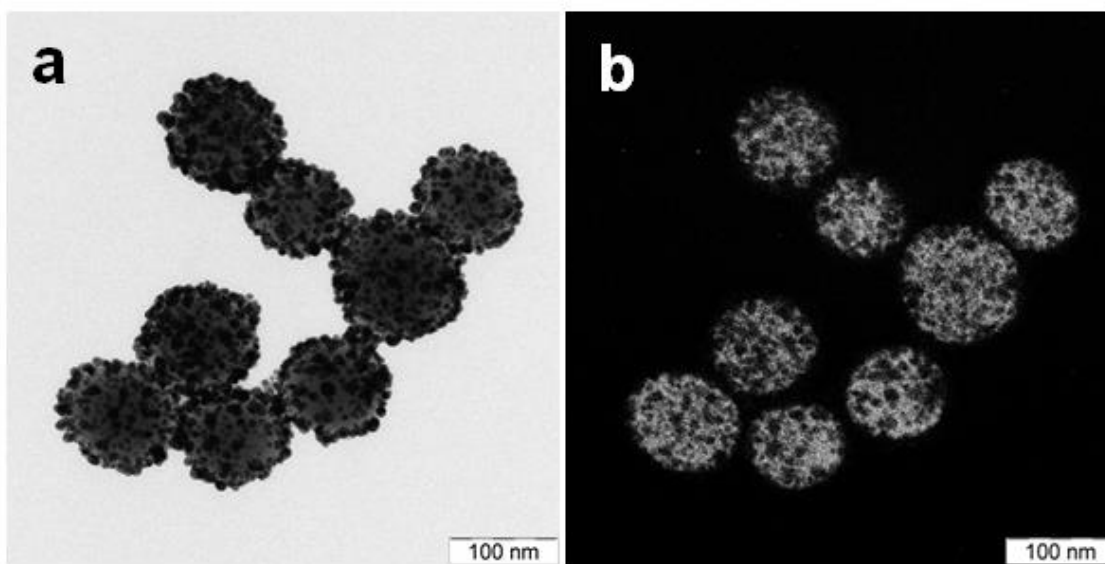


Figure A-SI-1 (a) Bright field TEM image and (b) ESI-Element Mapping for silicon using the same field as (a) – bright areas indicate presence of Si atoms.

A.1.2 X-ray diffraction of gold and silver nanoshells

The compositional analysis is complemented by X-ray diffractograms for both gold and silver nanoshells are shown below.

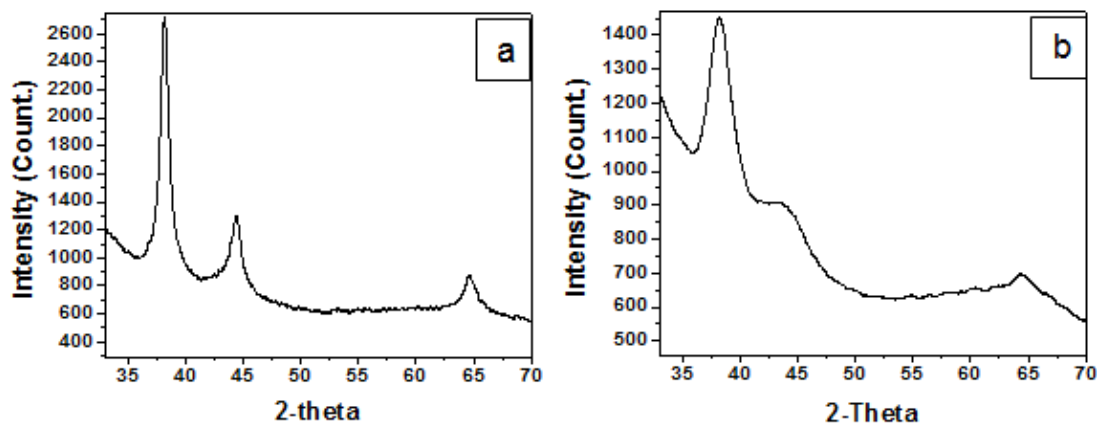


Figure A-SI-2 Diffractograms of gold (a) and silver (b) nanoshells.

The diffractogram in (a) revealed peaks at 38.20° , 44.41° and 64.54° , and in (b) at 38.28° , 43.36° and 64.46° that are relative to the planes (111), (200) and (220) of the standard cubic phases of gold and silver respectively.

A.1.3 Shell growth

Experiments to evaluate the best rotation rate to grow the nanoshells were realized. The results using samples 6 on tables 2 (for gold nanoshell) and 3 (for silver nanoshells) from the manuscript are presented. The rotation rates were set to 190, 250, 700 and 1500 rpm.

A.1.3.1 Gold nanoshells

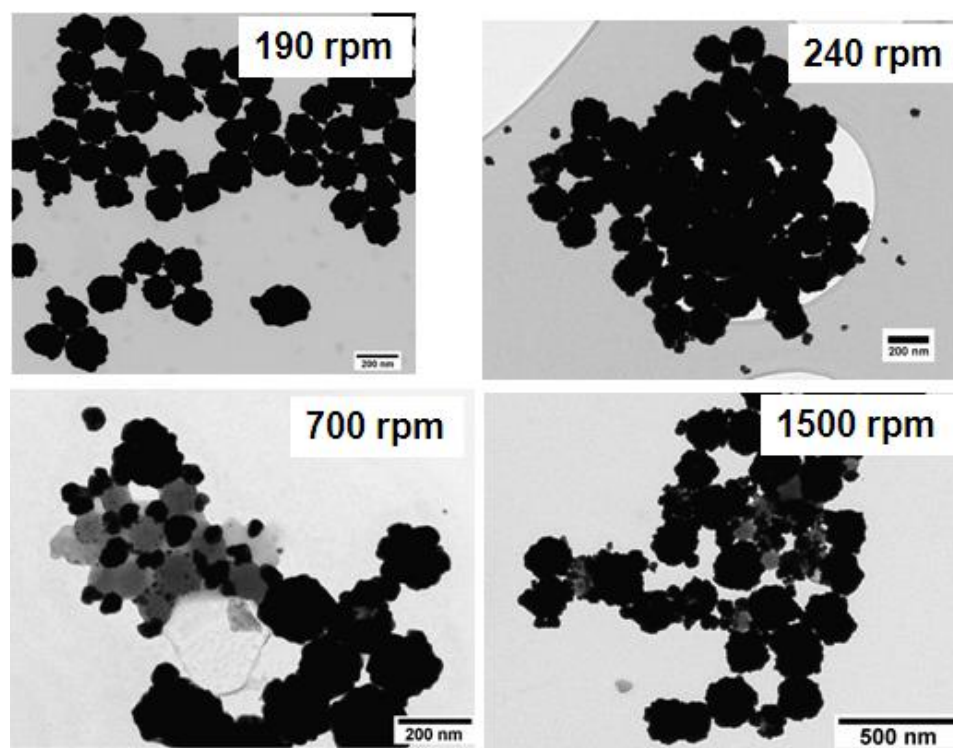


Figure A-SI-3 TEM images of gold nanoshells prepared with different stirring rates.

A.1.3.2 Silver nanoshells

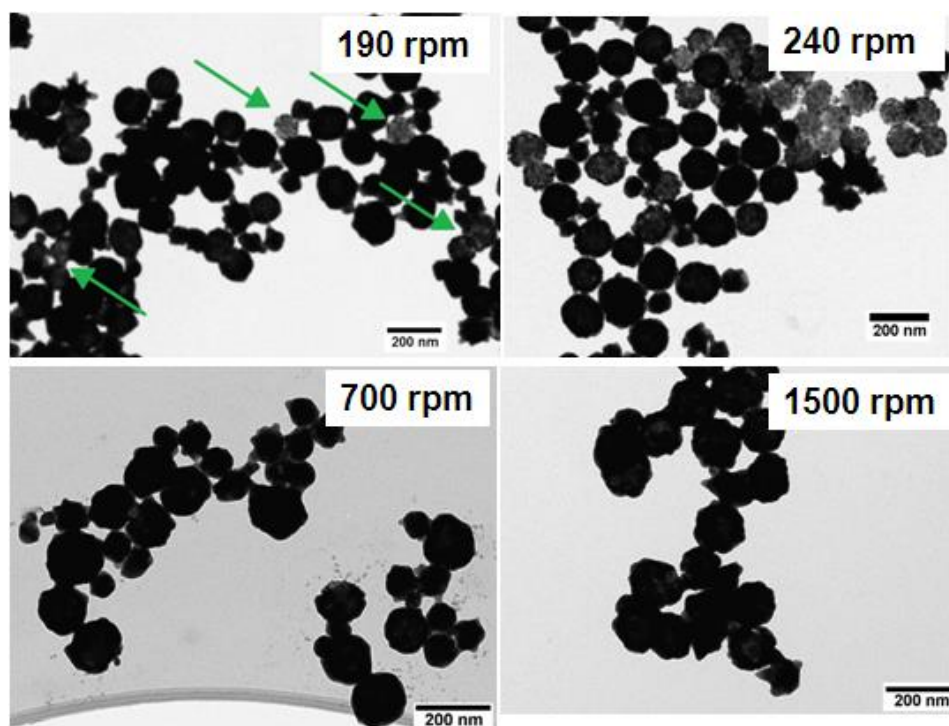


Figure A-SI-4 TEM images of silver nanoshells prepared with different stirring rates.

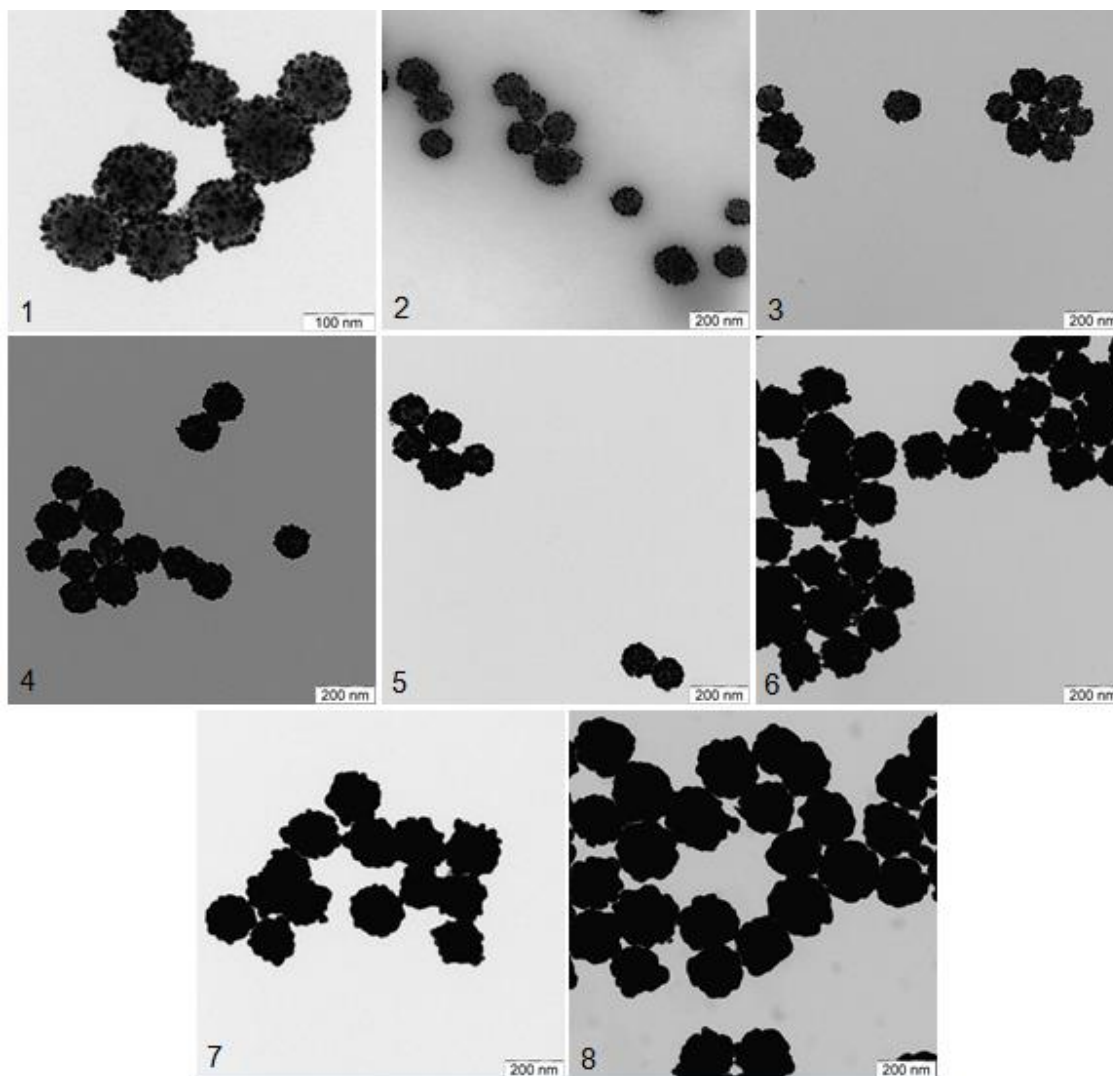


Figure A-SI-5 TEM images showing the formation gold nanoshells from the moment the process starts to the complete coalescence and thickening of shell.

The pH can also have some influence on the growth of the gold shells. High values of pH can promote external nucleation. The best range for the growth was between pH 7 and 8.

Variations in the number of particles for each colloid as well as purity grade of the reagents can generate differences in the growth process. As a way to circumvent that and assure a good degree of reproducibility we recommend a UV-Vis-NIR of the process to be performed (Figure A-SI-6).

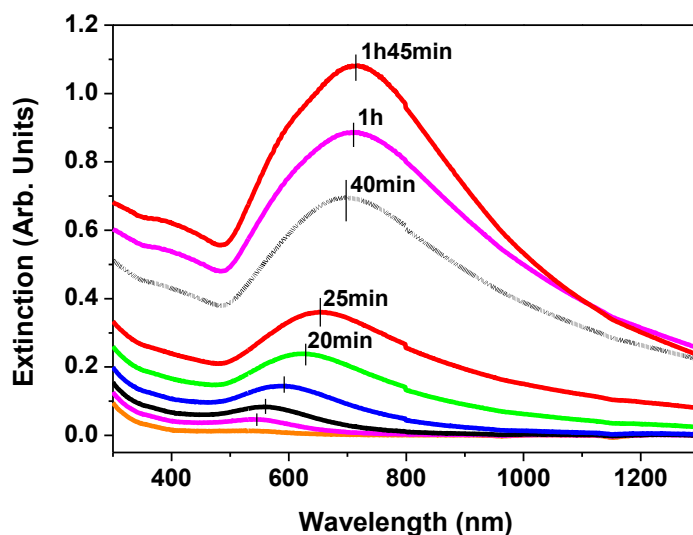


Figure A-SI-6 Growth of gold nanoshells over time as measured by UV-Vis-NIR spectroscopy.

A.1.4 APTMS self-assembly on glass slides

5 mL glass vials and glass slides (1 x 2.5 cm) were soaked in aqua-regia solution for 12 hours and thoroughly rinsed with water before drying in the oven at 100°C. The glass slides were then inserted in the vials according to figure A-SI-7. A 30% (v/v) solution of APTMS in toluene was prepared and distributed in the glass vials until the slides were completely immersed (approximately 3 mL for each vial). The glass slides were in contact to the APTMS solution for 12 hours to ensure completely self-assembly. After that the vials were abundantly rinsed with anhydrous ethanol, the slides were removed and then transferred to an identical vial containing ethanol. The slides were maintained in ethanol for 12 hours to remove any unreacted adsorbed APTMS. This process was repeated twice, and each slide was finally removed, dried in the oven at 100°C for 10 minutes and finally transferred to an empty vial. The capped vials containing the slides were stored at room temperature.

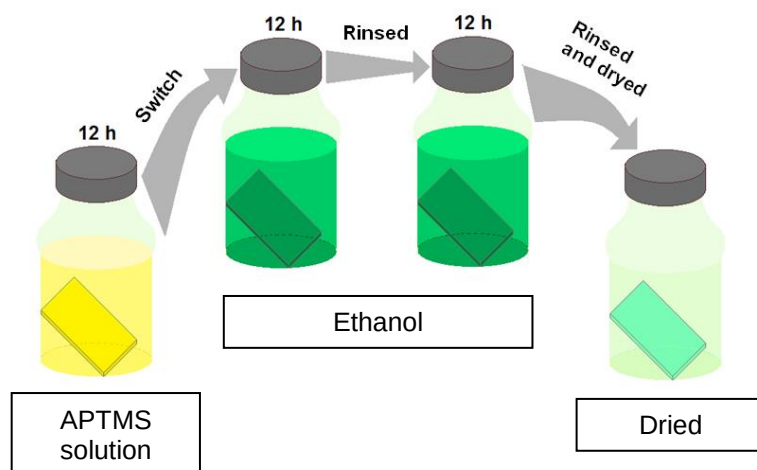


Figure A-SI-7 Process of APTMS self-assembly on glass slides.

A.1.5 Nanoshells immobilization

Amino modified glass slides, prepared as described above, were immersed in a colloid of gold nanoshells for 12 hours. The slides were then gently rinsed with water and nitrogen-dried.

Appendix B

B.1 Fine-Tuning Nanoshells for Multiplex Cell Analysis

Appendix B is published as Supporting Information to: R. G. Sobral-Filho, A. M. Brito-Silva, M. Isabelle, A. Jirasek, J. J. Lum and A. G. Brolo, Plasmonic Labeling of Subcellular Compartments in Cancer Cells: Multiplexing with Fine-tuned Gold and Silver Nanoshells, Chem. Sci. 2017, 8, 3038-3046. Copyright: Reproduced with permission.

B.1.1 Materials and instruments

Tetrachloroaurate (HAuCl_4), silver nitrate (AgNO_3), L-ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$), Tetraethyl orthosilicate 98%, (3-aminopropyl)trimethoxysilane 97%, polyvinylpyrrolidone (MW-55000), Ammonium hydroxide (NH_4OH) 28%, sodium borohydride (NaBH_4), formaldehyde 37% (CH_2O), Triton X-100, n-hexanol and propidium iodide were purchased from Sigma-Aldrich. Opti-MEM® reduced serum media, Dulbecco's modified Eagles medium, Fetal Bovine serum and Trypan Blue 0.4% solution were purchased from Thermo Fisher Scientific; custom-made NLS peptide (PKKKRKV) was purchased from Pharmaster Laboratories, Anti-IGFR antibody (sc-713) and Pallodin CruzFluor® 488 were obtained from Santa Cruz Biotechnology; 0.22 μm cellulose acetate syringe filters were obtained from Sterlitech. Notice that filters from different brands can lead to small variations in the synthesis because the quality of the cellulose used in the filters vary according to different standards in different Countries. High quality filters are recommended. Barnstead Nanopure Diamond Ultra-Pure Water system, FisherBrand FB11201 Ultrasonic bath, Fisher Scientific Accuspin® 17 microcentrifuge, Fisher

Scientific Isotemp® magnetic stirring plate, Brookhaven ZetaPals® Particle Analyzer, Varian Cary® 50 UV-Vis-NIR spectrophotometer, JEOL JEM-1400 Transmission Electron Microscope (All size determinations from TEM had 200 particles counted), Cytoviva Dual Mode Fluorescence/Hyperspectral Dark Field Microscope.



Figure B-SI-1 Only simple labware is required for the synthesis of the nanoshells. Left to right: Microcentrifuge, ultrasonic bath, 0.22 μm syringe filter, vials, stir bars and stirring plate.

B.1.2 Methods

All the synthetic methods described in this work take place at room temperature. Cell incubations happen at 37°C in an incubator at 5% CO₂.

B.1.2.1 One-batch synthesis and functionalization of aminated silica nanoparticles

In a clean glass vial, 7.5 mL of cyclohexane, 1.7 grams of Triton X-100 and 1.8 mL of 1-hexanol are placed under magnetic stirring at 800 rpm. In a separate vial, 25 μL of NH₄OH (28%) and 500 μL of ultrapure water are mixed to constitute the aqueous phase of the reverse microemulsion system. This aqueous solution is then added to the organic phase under stirring and the vial is capped. Micelles are allowed to form and stabilize for one

hour. 100 μL of TEOS is then added to the vial. Sequential additions of 100 μL of TEOS are made every six hours for bigger SiO_2 sizes (See Table B-SI-1). After the reaction time has elapsed, the vial is opened and NH_3 is allowed to escape for two hours before functionalization. The pH should then reach a range of 8-9 before silanization is performed. pH paper or pH strips can be used in this step. Performing the silanization without lowering the pH may result in aggregated samples. In this case 600 μL of a 24 nM APTMS solution in cyclohexane is added to the reaction vial and kept under stirring for 5 minutes. 15 mL of anhydrous ethanol are then added to break the micelles. The sample is centrifuged at 5000g/5min. Three to five cleaning cycles are repeated to remove excess amounts of reagents and the sample is finally resuspended in a total 30 mL of ethanol before being placed under sonication for 15 minutes for a more vigorous cleaning that will remove adsorbed molecules from the surface of the silica. Centrifugations happen at 10000g/10 now and five vigorous cleaning cycles take place before the sample can be resuspended in ethanol and stored. During the cleaning process the colloid will change from a cloudy and flocculated aspect into a stable and semitransparent suspension. The aminated silica colloids used here were kept on a benchtop at room temperature for up to 4 months. The number of silica nanoparticles per sample can be determined through a simple process as described in Table B-SI-1.

Table B-SI-1 Parameters for the synthesis of SiO₂ nanoparticles of different diameters.

100 μ L-TEOS additions*	Reaction time after last TEOS addition (hours)	Resulting diameter and standard deviation (nm)
1	24	50 ± 2.5
3	12	63 ± 2.8
3	36	72 ± 2.5
4	24	80 ± 2.3

* After the first TEOS, subsequent additions happen every 6 hours. For instance, 3 means the first TEOS addition, following 6 hours waiting, then another TEOS addition, another 6 hours waiting and a final 100 μ L addition.

B.1.2.2 Fabrication of small (2.1 ± 0.3 nm) gold nanoparticles

In a 600 mL beaker, 3.425g of PVP (MW-55000) are slowly dissolved in 190 mL of ultrapure water. 4.075 mL of a 20 mM HAuCl₄ solution (aq.) is then added to the beaker. Formation of the small gold nanoparticles is achieved by turning the stirring speed up to 1500 rpm and quickly adding 57 mL of a freshly prepared 5.24 mM NaBH₄ solution to the vortexing mixture. A quick change in color from light yellow to dark brown is verified. The resulting colloid is kept under stirring at 1500 rpm for 15 minutes before being ready for use. We recommend that this colloid be used fresh as some growth can be seen in the particles after a period of 24-48 hours.

B.1.2.3 Nanoislands formation

Typically, 500 μ L of aminated silica colloid (containing approximately 2×10^{12} particles) is sonicated and 24.5 mL of freshly prepared small-gold colloid is placed under gentle

stirring at 300 rpm. The aminated silica (in ethanol – there is no need to transfer the silica into water for this step) is then added to the vial containing the gold nanoparticles and the reaction mixture is kept under stirring for 3 hours. Saturation of the silica surface can be verified by UV-Vis spectroscopy as demonstrated in our previous work.²⁵ The formed nanoislands colloid can then be cleaned by centrifugation in water 5 times at 10000g/10min. The final product can be resuspended in 500 μ L of ultrapure water and kept in the fridge for up to one month. With longer storage periods the gold nanoparticles on the surface of the silica tend to aggregate. This will negatively affect the shell growth as the coating process will become inhomogeneous.

B.1.2.4 Shell growth

It is important to notice that the appropriate amount of nanoislands to be used in this step can change according to the desired shell thickness. Less nanoislands will produce thicker shells and vice-versa. In order to provide readers with a reference, considering the biggest SiO₂ core (80nm), 1 μ L of nanoislands colloid resulted in a 13 nm gold shell; and 5 μ L of nanoislands colloid resulted in a 15 nm silver shell. **Plating solution.** Typically, 13 mL of a 0.15 mM of HAuCl₄ or AgNO₃ is prepared in a glass vial and placed under stirring at 200 rpm. 25 μ L of NH₄OH (aq., 28%) are added and after 5 minutes the resulting solution is filtered through a 0.22 μ m cellulose acetate filter into a clean vial. For the growth of **gold nanoshells**, the appropriate amount of nanoislands is added to the gold plating solution under stirring at 200 rpm, the stirring speed is increased to 800 rpm and 90 μ L of a 10mM ascorbic acid solution is added to the mixture. Stirring is kept for 5 minutes and after that the sample is left undisturbed for 30-45 minutes before being cleaned by centrifugation-resuspension in ultrapure water at 5000g/5min. For the growth of **silver nanoshells**, 25 μ L

of formaldehyde (aq., 37%) is added to the silver plating solution at 200 rpm. The stirring speed is then increased to 800 rpm and the appropriate amount of nanoislands is rapidly added to the mixture. Stirring is kept for 10 seconds and after that the sample is left undisturbed for 30-45 minutes before being cleaned by centrifugation in ultrapure water at 5000g/5min. While the aminated silica core holds strong colloidal and chemical stability over long periods of time (Figure B-SI-3), we recommend that the bioconjugation step be performed with freshly prepared nanoshells, due to the lower chemical stability of the silver nanoshells.

Sample-to-sample variations regarding reaction time for the shell growth may happen in this step of the process, depending on factors such as shell thickness, number of particles per sample and environmental conditions such as temperature variations. In order to monitor the progress of the growth, UV-Vis-NIR spectroscopy measurements can be taken over time as to establish optimal reaction times.²⁵

B.1.2.5 Bioconjugation to NLS peptide and anti-IGFR antibody

1 mL of a 40 mM solution of the peptide was placed under vigorous stirring at 600 rpm. 1 mL of a silver nanoshells suspension – Ag(18nm)@SiO₂(50nm), containing approximately 5×10^9 particles was added dropwise. The mixture was kept under stirring for 12 hours before being cleaned by centrifugation-resuspension in ultrapure water at 5000g/10min. Prior to incubation, the NLS-coated silver nanoshells are resuspended in 1 mL of Opti-MEM[®] reduced serum media.

Gold nanoshells – Au(18nm)@SiO₂(72nm), were prepared by resuspending approximately 5×10^9 particles in 500 μ L of PBS. 500 μ L of antibody solution (60 μ g/mL) in PBS is placed under vigorous stirring and the nanoshells suspension is added dropwise.

The mixture is kept under stirring for 40 minutes and cleaned by centrifugation-resuspension in PBS at 10000g/10min. The sample is finally resuspended in 1 mL of PBS prior to incubation.

Bioconjugated samples can be kept in the fridge at 10°C if necessary before being used for cell-labeling experiments. We point to the fact though that not all biomolecules will remain optimally active at this temperature. In such case, bioconjugated samples should be prepared immediately before use. In our experiments all bioconjugated samples were prepared one day before use.

B.1.2.6 Cell culture and plasmonic labeling

MCF-7 breast human cancer cells were plated onto sterile coverslips in six-well plates, and maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum to a confluency of approximately 30%. Cell growth and labeling with nanoshells took place in an incubator at 37°C and 5% CO₂. Cell cultures were washed three times with PBS before incubations. Single incubation time for the Ag-NLS sample (Fig. 6a) was 3 hours. Single incubation time for Au-IGFR nanoshells (Fig. 6b) was 30 minutes. Sequential incubation for the multiple-target experiment was done first with the Au-IGFR sample, and after 30 minutes the cells were washed three times with PBS and the Ag-NLS sample was added for 3 hours. After three PBS washings, the cells were then fixed with 4% paraformaldehyde and mounted on microscope slides for analysis.

B.1.3 Images

B.1.3.1 Ultramonodisperse non-aggregated aminated silica

Figure B-SI-2 depicts the silica samples used in this work as well as dynamic light scattering measurements of the bare and aminated colloids (functionalized with increasing amounts of APTMS).

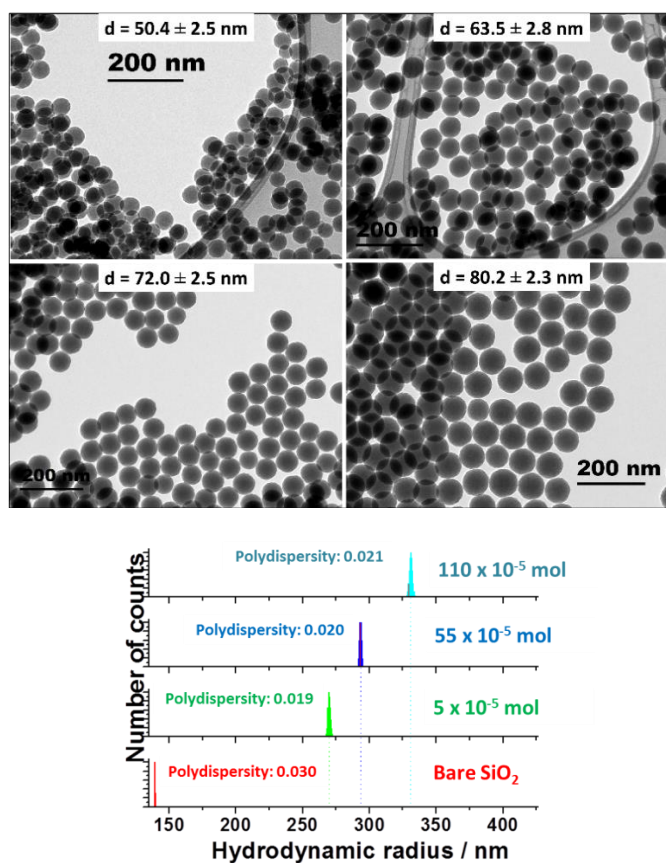


Figure B-SI-2 Ultramonodisperse SiO_2 samples as produced by the reverse microemulsion approach. Low polydispersity indexes are achieved through this method, indicating the successful fabrication of ultramonodisperse samples, and the absence of aggregation.

B.1.3.2 Colloidal and chemical stability

Samples can be kept on the benchtop for several months. A stability study was performed with a 10-month old aminated silica sample. The old sample was imaged by TEM before (Figure B-SI-3a, b) and after being used for the synthesis of nanoislands (Figure B-SI-3c).

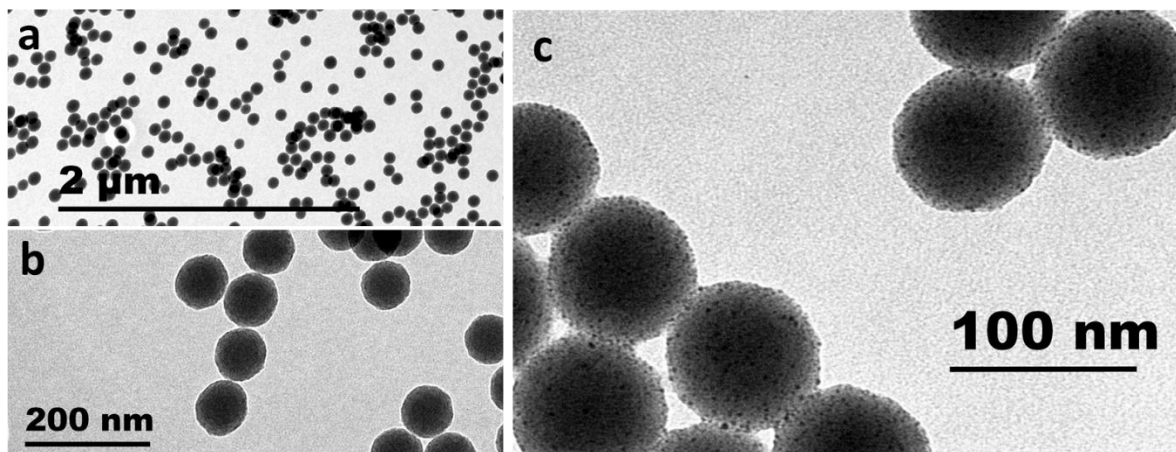


Figure B-SI-3 Long-term stability of aminated silica samples. TEM images of a 10-month old silica colloid before (a, b) and after nanoislands synthesis (c).

These images attest the great long-term stability of these colloids. B-SI-3a, b show the colloidal stability in the preserved shape and uniformity of the particles. SI-3c shows the chemical stability of the surface functionalization with APTMS.

B.1.3.3 Amount of added nanoislands and shell thickness

The appropriate amount of nanoislands to be added to the plating solution can change according to the desired shell thickness. In order to provide readers with a reference, considering the largest SiO₂ core (80nm), 1 μL of nanoislands colloid resulted in a 13 nm gold shell; and 5 μL of nanoislands colloid resulted in a 15 nm silver shell. The amount of particles per milliliter in the silica colloids can also be estimated by a simple method. 1 mL

of a silica colloid whose diameter has been determined by TEM, can be dried and have its mass measured. This mass can be converted into a volume through a simple cross-multiplication method using the density of SiO_2 . By using the mean diameter – as determined by TEM, it is possible to calculate the volume of a single silica particle. After that, dividing the total volume (as found by cross-multiplication) by the volume of a single silica particle will give the estimated number of SiO_2 particles per milliliter. The silica samples produced in this work have particles ranging from 2.43×10^{12} to 4.05×10^{12} particles per milliliter.

Figure B-SI-4 displays examples of the gold or silver nanoshells fabricated through this method.

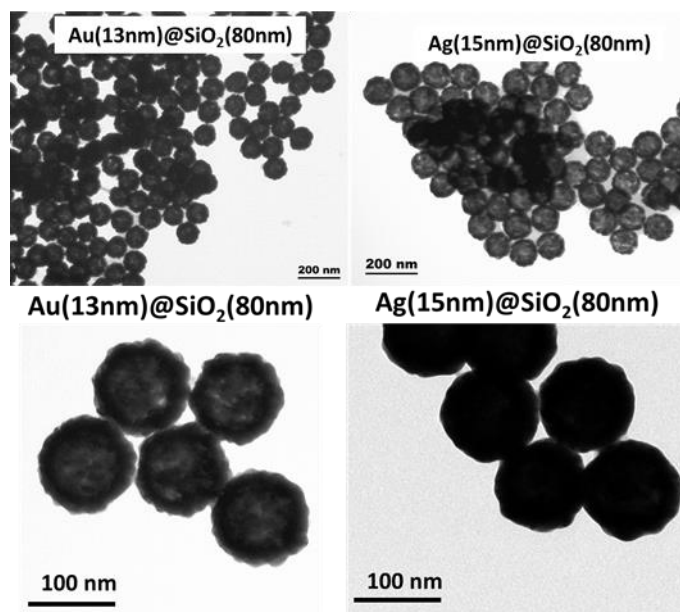


Figure B-SI-4 Gold and silver nanoshells fabricated through our process. Low magnification (top) conveys general aspect and homogeneity of the colloids. High magnification (bottom) shows the complete coverage of the silica cores by the metallic shells.

B.1.3.4 Hyperspectral Dark Field Microscopy

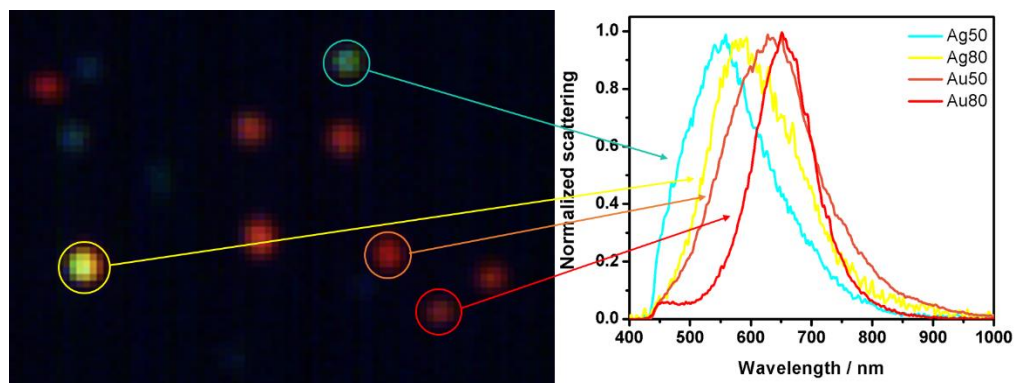


Figure B-SI-5 Hyperspectral image and scattering profiles of single nanoshells. Four samples were mixed and immobilized on an aminated glass coverslip. Hyperspectral image (left) shows isolated nanoshells and inserts pinpoint their respective scattering spectra (right).

B.1.3.5 Spectral separation and sample choice for dual labeling experiment

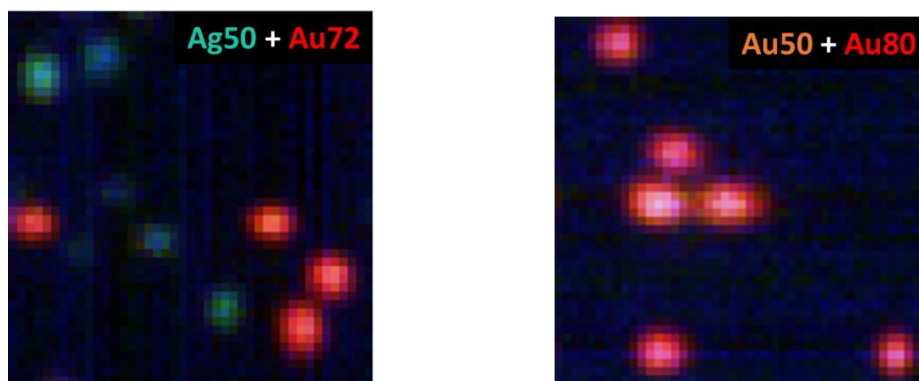


Figure B-SI-6 Hyperspectral analysis of combined particles for the dual-labeling experiment. It is clear the distinction between Ag50 and Au72 single nanoshells (left), while a good distinction between Au50 and Au80 nanoshells (right) can only be made with the use of hyperspectral analysis.

B.1.3.6 Spectral variability

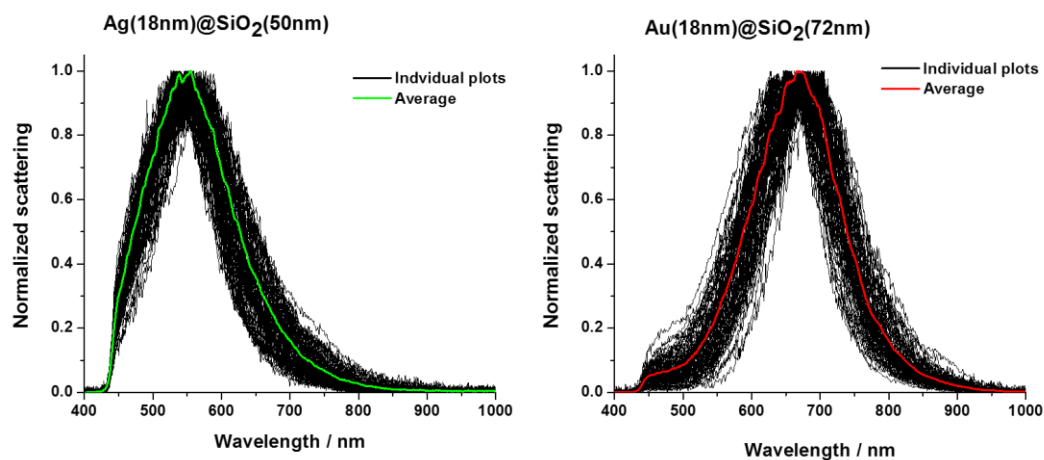


Figure B-SI-7 Individual and average plots for Ag(18nm)@SiO₂(50nm) and Au(18nm)@SiO₂(72nm).

B.1.3.7 Spectral shift after bioconjugation

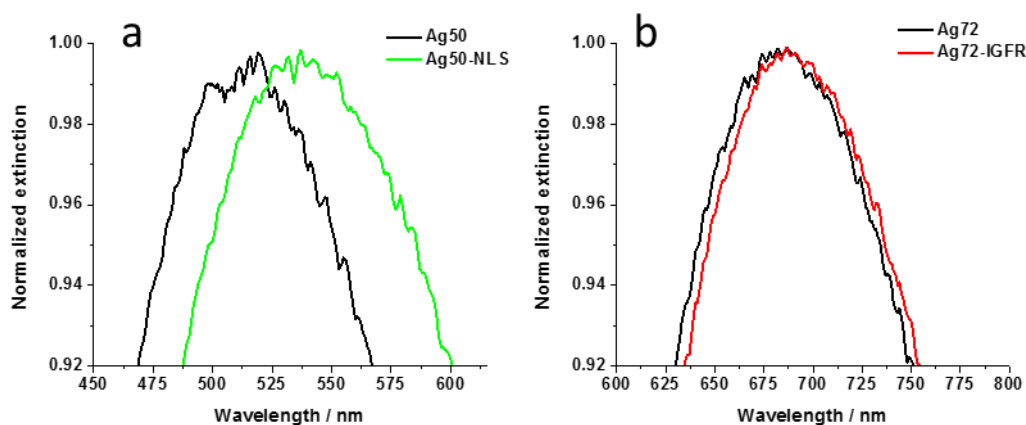


Figure B-SI-8 Spectral shift after bioconjugation. a) bioconjugation of the NLS peptide to the silver nanoshells, b) bioconjugation of the anti-IGFR antibody to the gold nanoshells.

The red shift in the LSPR of the colloids happen due to a change in refractive index on the surface of the nanoshells due to the attachment of the biomolecules.

B.1.3.8 Nonspecific endocytosis and nonspecific binding - controls for the cell labeling experiment

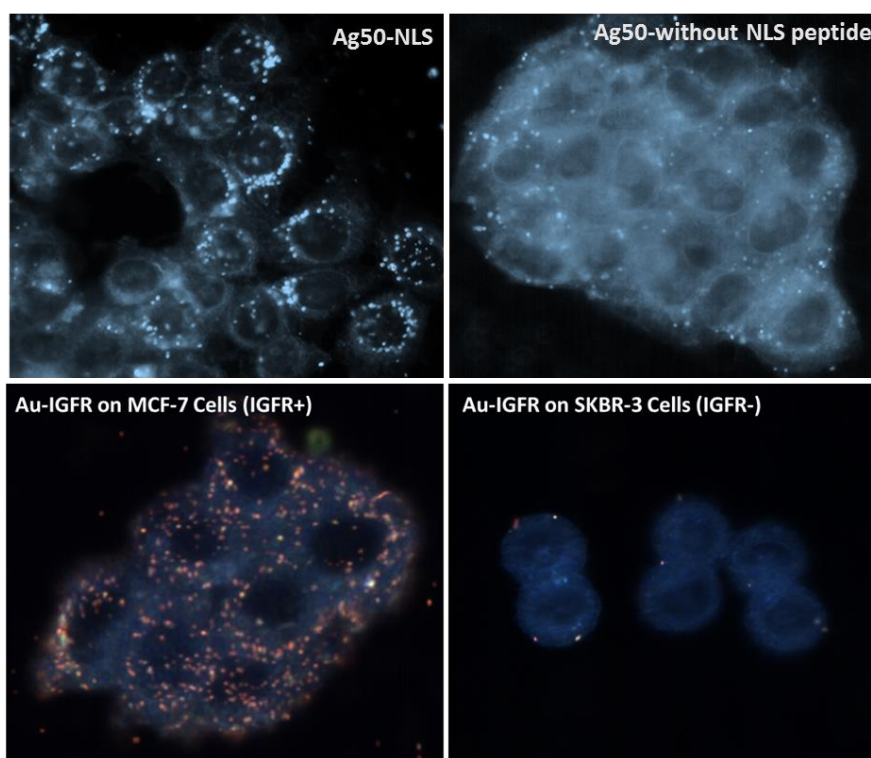


Figure B-SI-9 Negative controls. Hyperspectral images of Ag50-NLS (top left) and Ag50 without NLS peptide (top right); and Au-IGFR incubated with IGFR-positive MCF-7 cells (bottom left) and with IGFR-negative SKBR-3 cells (bottom right).

Silver nanoshells internalization and nuclear membrane labeling only happens in the presence of the NLS peptide. From SI-6 (top left), Ag-NLS nanoshells are easily visualized accumulating on the nuclear membrane, while in the absence of a NLS peptide, only a small number of particles are internalized and no selectivity for the nuclear membrane can be seen in Fig. SI-6 (top right). In the case of the anti-IGFR coated gold nanoshells (Au-IGFR), it's evident the accumulation on the plasma membrane of MCF-7 cells in Fig. SI-6 (bottom left), whereas only a few particles bind to the membrane IGFR-negative (basal

levels of expression) SKBR-3 cells in SI-6 (bottom right). This control experiment proves the effective bioconjugation and point out to the excellent level of selectivity (for specific subcellular compartments) and specificity (for the IGF receptors on the plasma membrane) of this platform.

B.1.3.9 Labeling multiple compartments with gold nanoshells only

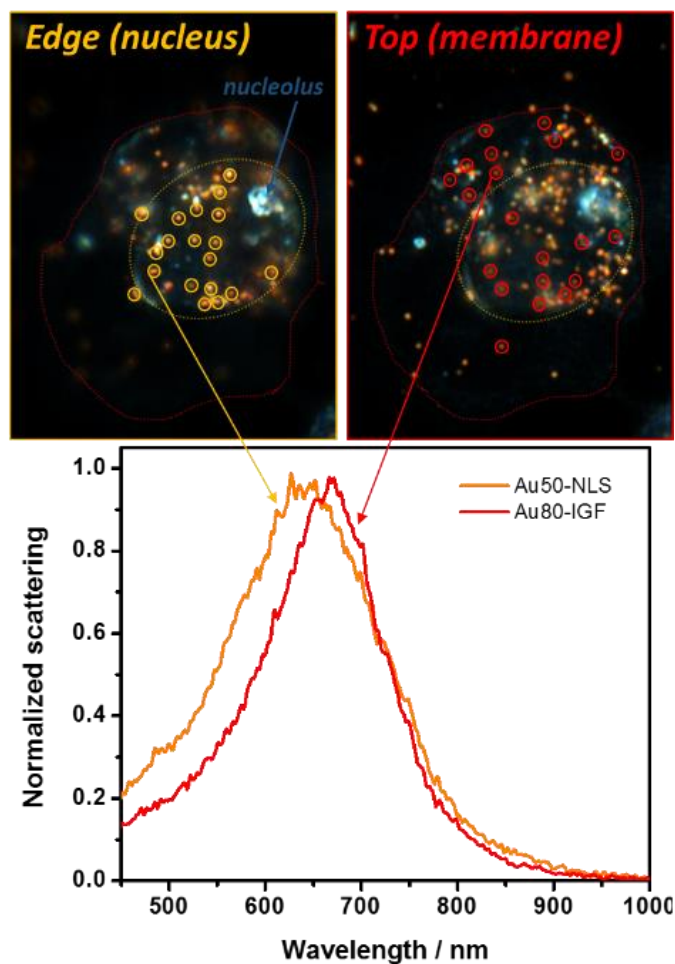


Figure B-SI-10 Dual-labeling with gold nanoshells only. Different focal depths show nucleus and membrane. Au50 nanoshells were coated with the NLS peptide and Au80 with the IGFR antibody. Due to the spectral proximity between the scattering profiles of Au50 and Au80, distinguishing between particles can only be achieved through hyperspectral microscopy. Hyperspectral sorting shows 20 nanoshells highlighted in orange (Au50) – left, and red (Au80) – right circles. Scattering spectrum for a single nanoshell is showed in the plot below the images. Nucleolus is shown by blue arrow.

B.1.4 Cell viability, fluorescence staining and imaging parameters

Cell viability 48 hours post-incubation was determined by the Trypan Blue exclusion method with the use of a hemocytometer.

Table B-SI-2 summarizes the results.

Sample	Ag-NLS	Au-IGFR	Control (no nanoshells)
Cell viability (48h post- incubation)	90.8%	92.1%	91.4%

Cells were generally kept in growth media and washed 3 times with PBS in between incubations. In the case of propidium iodide staining, cells were incubated with Ag-NLS for 3 hours, washed and stained with 1 mL of a propidium iodide solution (1 $\mu\text{g/mL}$) for 10 minutes. They were then washed and mounted onto microscope slides for analysis. For Phalloidin CruzFluor® 488 staining we followed the manufacturer's protocol.

All optical images displayed here were acquired using a Cytoviva Dual Mode Fluorescence/Hyperspectral Dark Field microscope. The imaging modes are easily interchangeable, facilitating comparative studies from the same visualization field. 60x and 100x objectives were used. While the setup is somewhat simple, they have an enclosed proprietary illumination system to which we can't gain access without damaging the setup. For detailed specifications we refer readers to the Cytoviva website (<http://www.cytoviva.com>). The light sources used in this work were a 150W Halogen

lamp with Aluminum reflectors (Part # L1090 by Intl. Light Tech) with analog voltage control for dark field measurements, and an X-Cite® 120 Fluorescence Illumination System for the fluorescence measurements. Fluorescence and conventional Dark Field images were collected using a QImaging Retiga 4000 CCD. For the Hyperspectral measurements, the detector is switched to a Headwall spectrograph-coupled CCD.

Appendix C

C.1 SERS from Single Nanoshell: a Study on Excitation Wavelength Relative to LSPR Position

Appendix C contains supporting information a manuscript in preparation for submission. The authors and title of the manuscript are: R. G. Sobral-Filho, X. Zhang, C. D. L. de Albuquerque, A. G. Brolo, SERS Excitation Wavelength and Localized Surface Plasmon Resonance: A Single Nanoparticle Study.

C.1.1 Average Scattering spectra

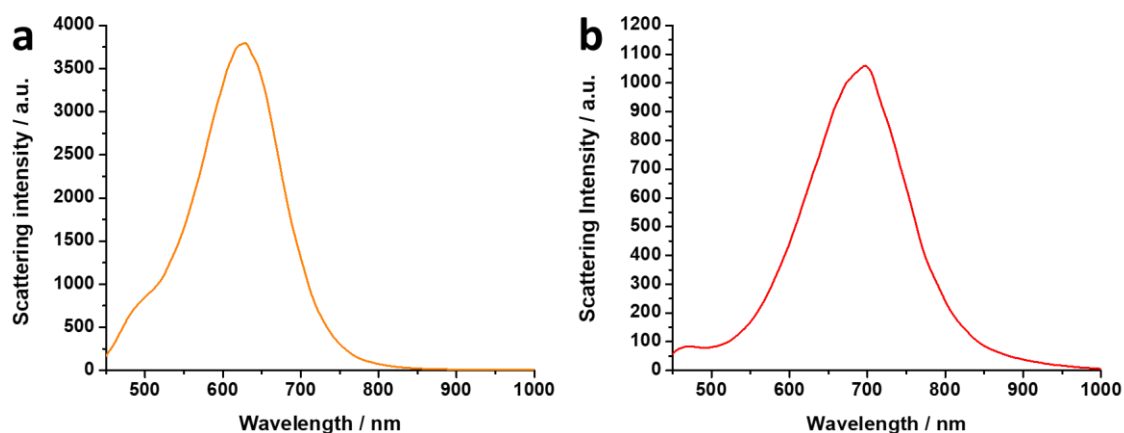


Figure C-SI-1 Average scattering spectra for a) N1 nanoshells and b) N2 nanoshells.

C.1.2 SERS spectrum for TNB

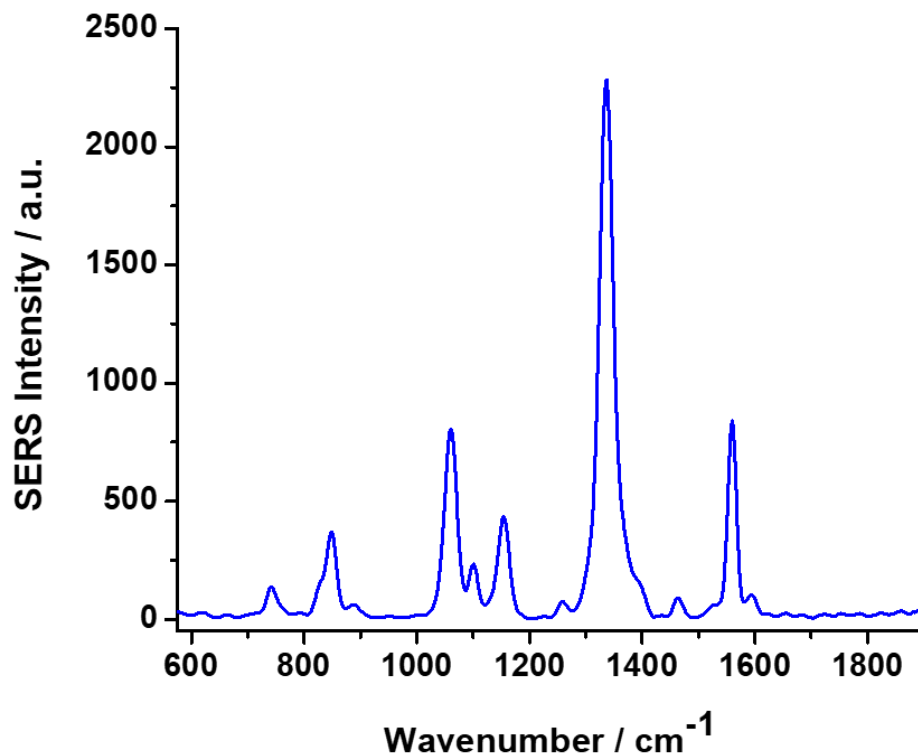


Figure C-SI-2 SERS spectrum for a TNB-coated nanoshell (obtained from an N2 nanoshell under 785nm excitation).

C.1.3 LSPR peak position histograms

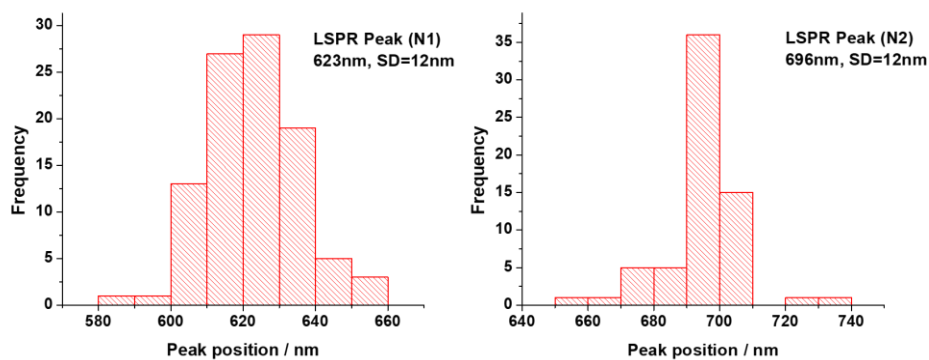


Figure C-SI-3 Peak position histograms for N1 and N2 nanoshells.

Appendix D

D.1 Directly Detecting Circulating Tumor Cells with Nanoshells

Appendix D is published as Supporting Information to: R. G. Sobral-Filho, L.

DeVorkin, S. Macpherson, A. Jirasek, J. J. Lum, A. G. Brolo Ex vivo Detection of Circulating Tumor Cells from Whole Blood by Direct Nanoparticle Visualization, ACS Nano 2018, 12, 1902–1909.. Copyright: Reproduced with permission.

D.1.1 Extinction spectrum – gold nanoshells

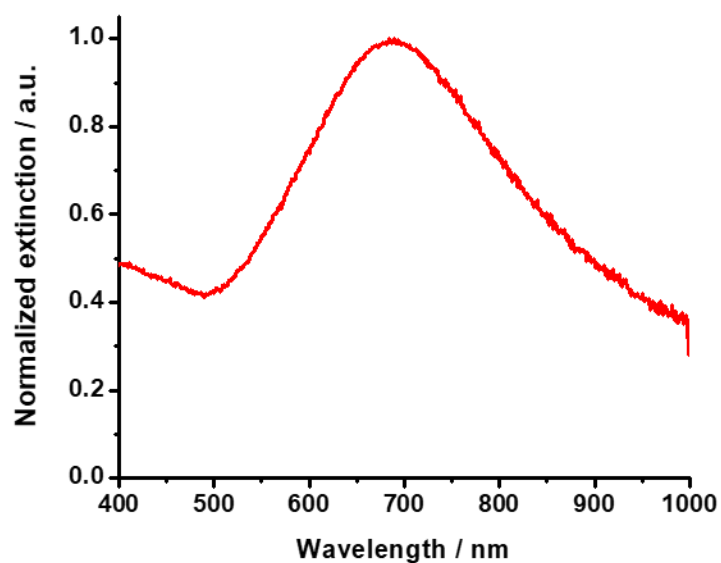


Figure D-SI-1 Normalized extinction spectrum for gold nanoshells (SiO_2 core = 80nm, Au shell = 15nm).

D.1.2 Cell selection

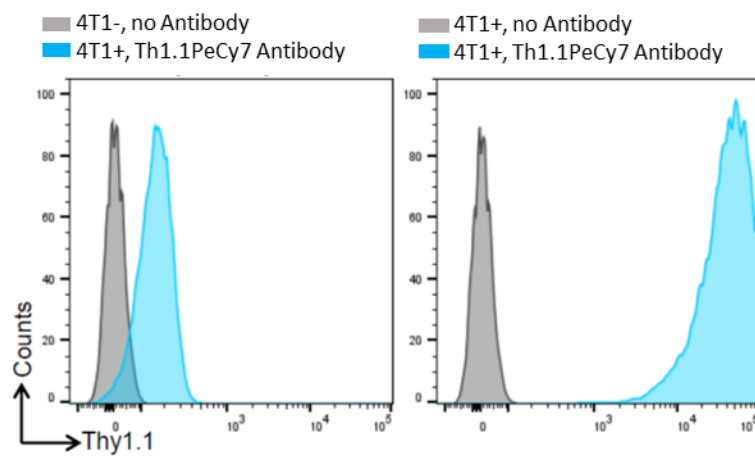


Figure D-SI-2 Thy1.1 expression on selected cells was analyzed through flow cytometry. (left) 4T1- (non-transduced) cells; (right) – 4T1+ (transduced) cells.

D.1.3 Adaptability of the platform

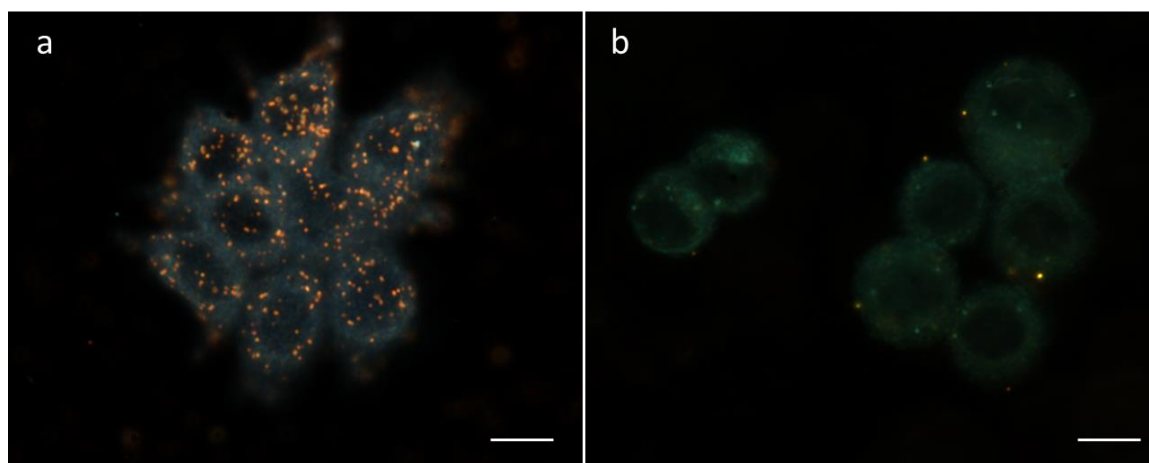


Figure D-SI-3 Adaptability of the platform to different targets and cell lines. IGF1R detection – MCF-7(+) cells shown in “a”, SKBR-3(-) cells shown in “b”. Scale bar is 25 μ m.

D.1.4 CTC detection**Table D-SI-1** CTC detection numbers for all 3 mice in the 4T1+ group.

Days post tumor implantation	Mouse P1	Mouse P2	Mouse P3
Day 5	2	5	7
Day 10	3	8	11
Day 15	6	8	9
Day 29	26	29	32

D.1.5 CTC detection numbers

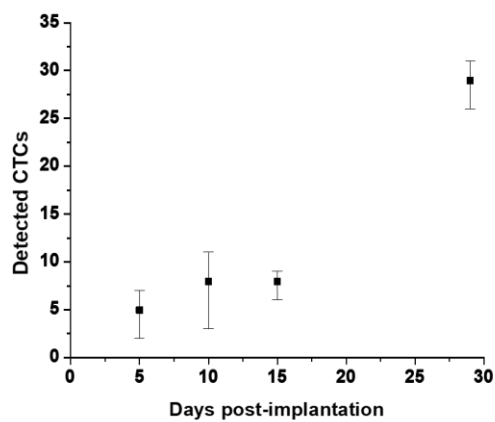


Figure D-SI-4 CTC detection for the positive group. Median values plotted. Error bars show the upper and lower number of detected CTCs from all subjects on specific time points.

D.1.6 Flow cytometry analysis

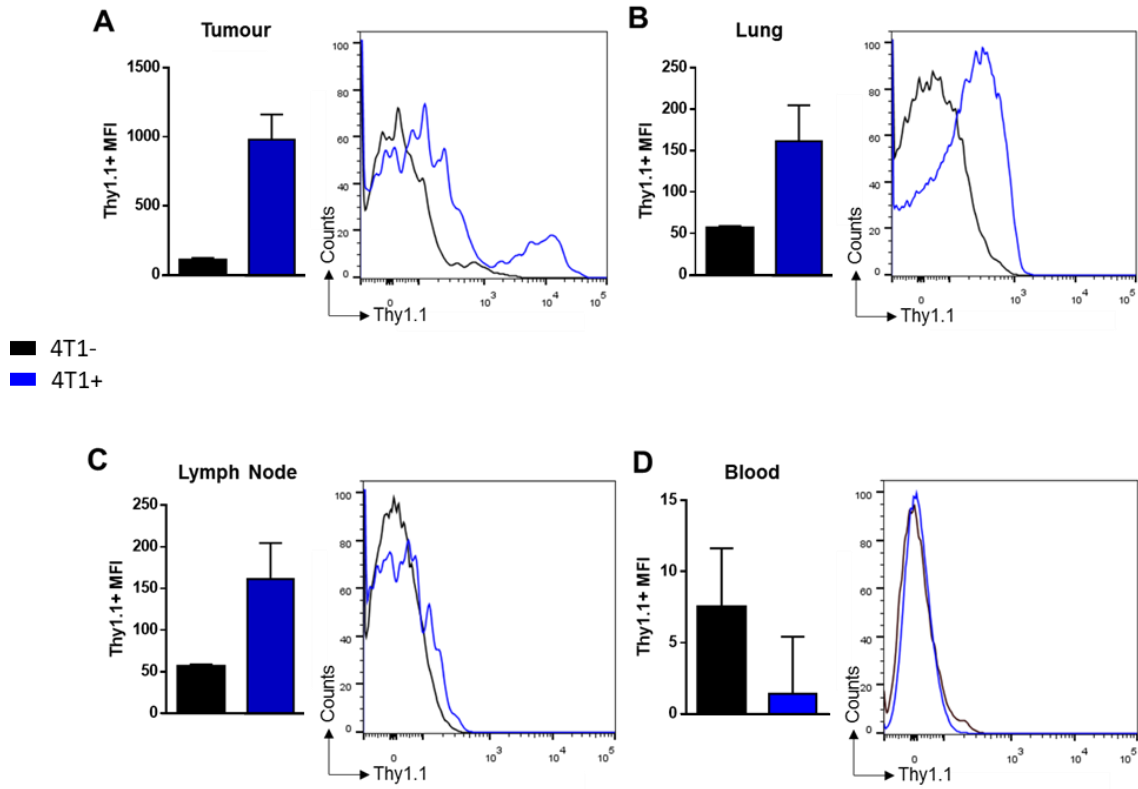


Figure D-SI-5 Flow cytometry analysis of harvested tissues confirms the establishment of metastatic sites in the lungs (B) for the positive 4T1+ group, and shows that CTCs are not discernible in blood (D) through flow cytometry. Each group had n=3.