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# Nanoparticle Based Surface-Enhanced Raman Spectroscopy

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**Abstract.** Surface-enhanced Raman scattering is a powerful tool for the investigation of biological samples. Following a brief introduction to Raman and surface-enhanced Raman scattering, several examples of biophotonic applications of SERS are discussed. The concept of nanoparticle based sensors using SERS is introduced and the development of these sensors is discussed.

**Keywords**. Raman spectroscopy, surface-enhanced Raman scattering (SERS), nanosensors, biodetection

## 1. Introduction

Raman spectroscopy is an optical technique that provides chemically specific information about the interrogated sample. When photons impinge on a molecule, they are scattered, and, depending on their interaction with the vibrational modes of the molecule, their frequency can be shifted as illustrated in Fig. 1a. While the vast majority of photons incident on a given bond vibration are elastically scattered (Rayleigh scattered), a small fraction of these photons interact with the vibrational mode and are shifted in frequency by the frequency of the bond vibration, as shown in the energy level diagram in Fig 1b. These Raman shifted photons can have a higher frequency (anti-Stokes) or a lower frequency (Stokes). The many vibrational modes of a molecule give rise to a spectrum that provides quantitative information about the molecular species in the probe volume.

[1]The quantitative and chemically specific information provide by Raman spectroscopy has made it useful in a wide variety of fields ranging from forensic analysis to fundamental studies of molecular interactions.[2] The non-invasive nature of the technique along with its chemical specificity make it ideally suited for biological measurements.[3, 4] Raman spectroscopy has been applied to study many different biological systems including DNA protein interactions, protein folding, and diseased tissues.[5] While Raman spectroscopy has been successfully employed to study fundamental biochemical interactions as well as being developed as a tool for *in vivo* diagnostics,[6] it is limited in its sensitivity. This low sensitivity results from the inherently low probability that a photon will be Raman scattered. Approximately 1 in $10^8$ photons that are incident on a given bond vibration are Raman scattered. These extremely low cross-sections require long integration times, and high incident power to generate Raman spectra with sufficient signal-to-noise. This has generally limited the technique to samples that are relatively concentrated.

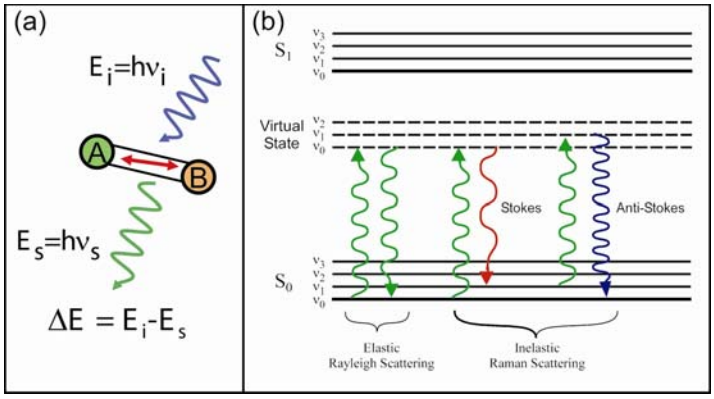


Figure 1 (a) Schematic illustration of Raman scattering. Photons incident on a given bond vibration can be Raman scattered having their frequency shifted by the frequency of the bond vibration. (b) Energy level diagram of the Raman scattering process. Most of the incident photons are Rayleigh scattered and remain unchanged in frequency. The Raman scattered photons can be shifted to a lower energy (Stokes) or higher energy (Anti-Stokes) upon their interaction with the vibrational mode of the molecule

Many biologically relevant chemicals are present in minute quantities, precluding the use of conventional Raman scattering. A technique that is often employed to circumvent the low signal inherent in Raman spectroscopy is surface-enhanced Raman scattering (SERS). In SERS, the molecule of interest is attached to a metal surface, typically silver or gold, which provides an increase in the observed Raman scattering by many orders of magnitude. This phenomenon was first discovered in 1977 by two groups working independently.[7, 8] Studying the adsorption of molecules to a roughened silver electrode, these two groups noticed and increase in the Raman scattering by 6 orders of magnitude.

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This large increase in the observed Raman scattering is the result of two enhancement mechanisms, an electromagnetic enhancement and a chemical enhancement.[9, 10] The electromagnetic enhancement results from a localization of the E-field through surface plasmons. This E-field enhancement has been shown to produce an enhancement of $10^6$. The chemical enhancement arises from the formation of a charge transfer complex that increases the polarizability of the molecule and produces an increase in the Raman scattering by 1 to 2 orders of magnitude.[11]

In addition to roughened metal surfaces, researchers showed early on that SERS spectra could be acquired from colloidal nanoparticles dispersed in solution.[1, 12] The colloidal particles have size dependent plasmon absorption and can be tuned to preferentially adsorb at the desired laser frequency.[13, 14]

More recently two groups working independently in 1997 showed that using colloidal nanoparticles, the Raman spectra from individual molecules could be acquired.[15, 16] Based on these observations, enhancement factors of $10^{15}$ would be required based on cross-section arguments. Calculations have shown that the E-field enhancement can approach $10^{12}$ at the junctions between two nanoparticles.[17] The remaining three orders of magnitude of enhancement have been attributed to a combination of resonance enhancement and chemical enhancement.[11] While the details of these enormous enhancements remain under study, the possibility of investigating biological systems using Raman scattering has attracted a number of researchers and resulted in many interesting biophotonic applications of SERS.[18]

## 2. Overview of Biophotonic Applications of SERS

From the very early years of SERS, researchers began to take advantage of the increase in Raman scattering to study biological samples. Cotton and Van Duyne demonstrated very early in the history of SERS that there was great potential for studying small quantities of biological materials using SERS.[19] Their work demonstrated that cytochrome C and myoglobin could be adsorbed to roughened silver electrodes and a SERS spectrum could be acquired. Since that time, there have been many biological applications of SERS over the past two and a half decades.[18] This manuscript is not intended to be a comprehensive overview of the biologically relevant SERS work, but rather an overview to give an idea of the types of applications that have been developed and the systems that have been studied using SERS.

One example which demonstrated the sensitivity attainable with SERS and biological samples was the study of single hemoglobin molecules with SERS. These experiments carried out by Käll and co-workers showed that the characteristic vibrational modes of the porphyrin moiety in the hemoglobin protein could be seen at the single molecule level.[20] While these experiments demonstrated the sensitivity attainable with SERS, they also illustrate the difficulty of practical single molecule detection using colloidal particles. Time series of spectra showed that the signal from the hemoglobin fluctuated dramatically in time.

A more robust SERS substrate has been developed by Van Duyne and coworkers that can be modified for biological applications.[21, 22] For many years researchers have attempted to develop SERS based glucose sensors. Theses attempts have fallen short due to the inability to get the glucose immobilized close to the SERS substrate. This technical hurdle was overcome recently by Van Duyne and coworkers who utilizing specially prepared SERS substrates and short chain alkanes have been

able to show that SERS can be utilized as a glucose sensor.[23] These platforms are able to detect glucose over a concentration range of 10-400 mg/dL.

An area which is currently in the forefront of biological sensing is the ability to detect DNA fragments with little or no amplification. One route to this end has been the development of SERS based DNA detection. A couple of approaches have been reported, both of which require the use of an external probe molecule, usually a laser dye, that is attached to the probe DNA. Vo Dinh and co-workers have developed a cancer gene detection assay in which the probe DNA is immobilized to a SERS substrate.[24] Upon binding the target DNA to the immobilized probe, the labeled probe DNA is added which also binds to the target DNA. The detection of the Rhodamine probe is then quantitated and can detect as few as $10^8$ copies of the gene.

A similar approach developed by Mirkin and co-workers utilizes the target DNA to bring together two metallic nanostructures.[25] Part of the complementary strand to the target DNA is immobilized on a gold substrate and the other part is attached to gold nanoparticles in solution. The DNA coated nanoparticle solution is first incubated with the target DNA and then the nanoparticles are extracted through centrifugation and washed. The resuspended nanoparticles are exposed to the DNA coated gold substrate and those nanoparticles with the target DNA will bind to the surface. The nanoparticles are labeled with a laser dye which is then detected with SERS, providing detection limits in the femtomolar range.

The approach taken for the detection of DNA also demonstrates the ability of SERS to allow for multiplexing. The narrow line widths inherent to Raman spectroscopy allow several species to be detected at one time in the same probe volume. Another application which exploits this capability is the development of SERS tags.[26] These SERS tags are metal nanoparticles coated with a SERS active molecule and then coated with a glass layer to prevent the loss of the SERS molecules in solution. Through the use of silane chemistry, these tags can be attached to proteins, etc. and utilized for biodetection. While these SERS tags are not as sensitive as fluorescent dyes, they are capable of multiplex detection of several target species in a small probe volume.

The gold and silver nanoparticles that are commonly used in SERS are small in dimension ranging from 40 to 100 nm in diameter. These small sizes mean that the nanoparticles can be incorporated into living cells. This was first demonstrated by Manfait and co-workers in the early 1990's.[27, 28] They were able to study the complexation of a chemotherapeutic agent with DNA using SERS. More recently, the same group has utilized SERS to understand the mechanism of chemotherapeutic resistance for certain cancers.[29]

Another group has utilized SERS to characterize the distribution of certain chemicals within cells. For example by imaging with individual Raman lines of phenylalanine and DNA, Kneipp and co-workers were able to show the distribution of these two chemicals within the cell and distinguish between the cytoplasm and the nucleus.[30]

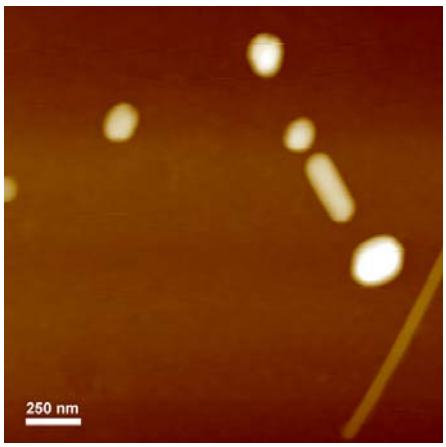


Figure 2 Atomic force microscopy (AFM) image of silver nanoparticles immobilized onto a freshly cleaved mica substrate. The nanoparticles were prepared by the reduction of silver nitrate by sodium citrate as described in reference [1].

## 3. The Development of Nanoparticle Based Sensors

The ability to incorporate nanoparticles into living cells and make localized measurements of chemical concentrations has inspired our group to develop nanoparticle based sensors. The following sections detail the development of nanoparticle based sensors using SERS.

*Preparation of Colloidal Nanoparticles*

The colloidal nanoparticles required for the sensors are produced by reducing the metal salts using a variety of reducing agents. A commonly employed preparation of silver nanoparticle first introduced by Lee and Meisel uses sodium citrate to reduce silver nitrate to produce nanoparticles with sizes in the range of 20 to 200 nm.[1] A topographic image of nanoparticles produced through this method is show in Fig. 2.

As illustrated by the AFM image, this method produces nanoparticles that vary in size and shape. More monodisperse colloidal suspensions can be produced using a seeded growth process.[31] In the seeded growth process a solution of seed particles are prepared using a fast reducing agent such as sodium citrate. The seed particles are then grown to the desired size with a milder reducing agent such as ascorbic acid. Using this seeded growth method nanoparticles can be produced with a coefficient of variation of less than 10%.

*Instrumentation*

While there are many instrument designs that allow for the detection of a Raman signal,

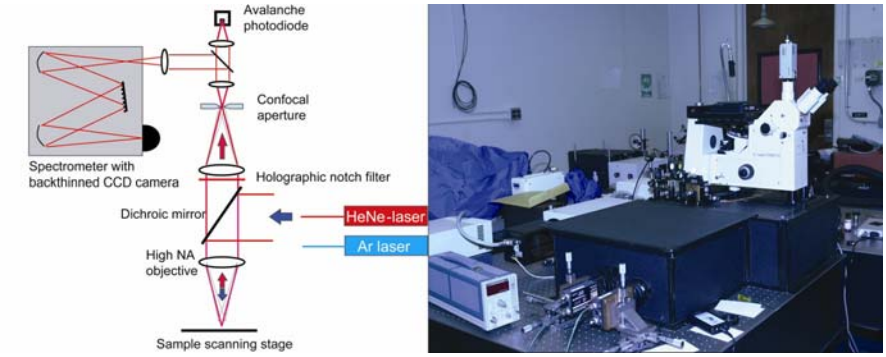


Figure 3 Schematic diagram (left) of a custom designed micro-Raman system and photograph of the system (right).

it is useful to have a versatile system that allows for both imaging and spectroscopy of individual nanoparticles. An example of such a system is shown in Fig. 3. A laser is coupled into the back of an inverted Zeiss microscope and directed into a high numerical aperture objective with a dichroic mirror. The sample is then raster scanned across the focused laser with a closed-loop XY piezo scanner. The resulting Raman scatter is collected with the same high numerical aperture objective and focused onto a 100 μm pinhole to remove out of focus scattered light. The Rayleigh scattered light is removed with a holographic notch filter and the remaining Raman scattered light is focused onto an avalanche photodiode. Once a nanoparticle is located in the image, the nanoparticle is centered in the focused laser beam and the Raman scatter is sent to a spectrograph with a back-thinned CCD camera in order to acquire a spectrum. For correlation measurements the signals from the APDs are routed through a time correlated single photon counting card. The correlation measurements are discussed in more detail in later section.

*Unfunctionalized Nanoparticle Sensors*

The simplest way to utilize nanoparticles as sensors is to incorporate them into the medium and allow the target analyte to adhere to the nanoparticle surface. This is illustrated in the case of bacterial spores as shown in Fig 4. In conventional micro-Raman spectroscopy, the spectrum from individual bacterial spores is dominated by the calcium dipicolinate present in the core of the spore (Fig 4a).[32] The strong signal from the calcium dipicolinate makes it difficult to distinguish one spore from the next through the subtle differences in protein and lipid peaks. However, when silver nanoparticles are adsorbed to the outer coat of the spores, the signal is no longer dominated by the calcium dipicolinate in the core. Because the enhanced region is localized to a couple of nanometers from the surface of the nanoparticles, the spectra are representative of the proteins etc, in the spore coat.[33]

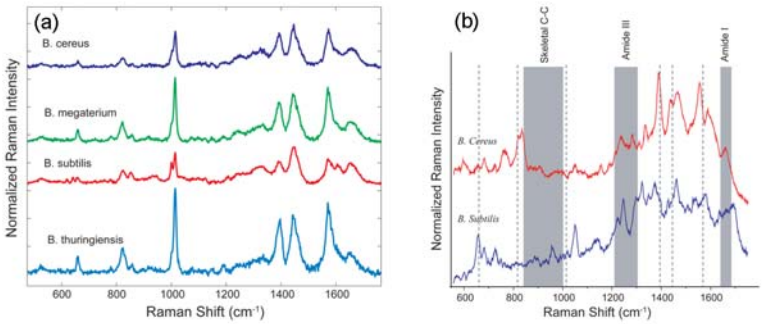


Figure 4 Conventional micro-Raman spectra of individual bacillus spores (a). The spectra are nearly identical due to the large content of calcium dipicolinate in the core of the spores. When silver nanopartilces are adsorbed to the surface of the spores (b), the spectra change significantly. The spectra are no longer dominated by the calcium dipicolinate (dashed lines).

While this technique of using nanoparticles as sensors can be useful in some cases, it is often difficult to obtain reproducible results. Moreover, the signal will be dominated by the species that adsorb most readily to the nanoparticle surface or those that are in high concentration leading to problems of an overwhelming background. In order to circumvent these problems we have begun developing functionalized nanoparticle sensors as described in the following section.

*Functionalized Nanoparticle Sensors*

Functionalized nanoparticle sensors employ nanoparticles that have been coated with a molecule that will bind to the analyte of interest. Upon binding to the analyte molecule, the characteristic SERS spectrum of the functional molecule is changed in such a way that the presence of the analyte molecule is indicated and can be quantitated, as illustrated in Fig. 5.

The use of a functional group or "probe molecule" has several advantages over non-functionalized probes. First the functional group adds a degree of specificity to the sensor by providing a specific interaction with the target analyte. Secondly, the analyte molecule does not need to be Raman active, or have a particularly large Raman cross-section. The presence of the analyte can be observed through changes in the functional group. Finally, because the surface is coated by the functional molecule, interfering molecules cannot adsorb to the particle surface and therefore the background is reduced.

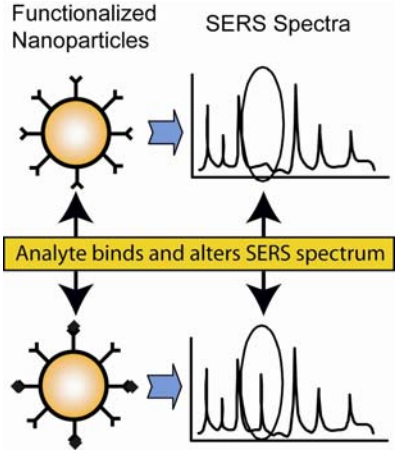


Figure 5 Illustration of the concept of functionalized nanoparticle sensors. The characteristic SERS spectrum from the functional molecule is changed upon binding to the target analyte.

*Nanoparticle Based pH Sensors*

In order to demonstrate the feasibility of developing a functionalized nanoparticle SERS sensor, we have developed nanoparticle based pH sensors.[34] The functional

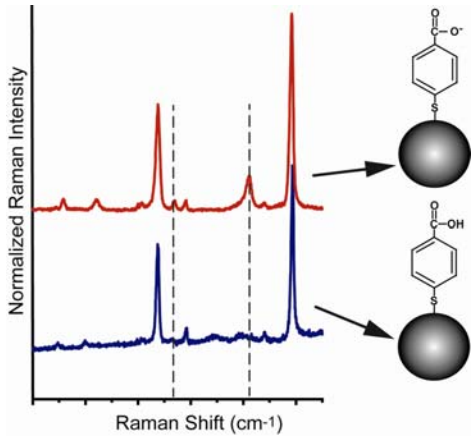


Figure 6 The characteristic SERS spectra of 4-MBA at ph 12.3 (top) and ph 5.5 (bottom). The dominate structure under these conditions are shown to the right of their respective spectra.

molecule in this case is 4-mercaptobenzoic acid (4-MBA). The Raman spectrum of the 4-MBA changes according to the state of the acid group as shown in Fig 6. As the pH is reduced and the acid group becomes protonated, the $COO^-$ stretching mode at 1430 $cm^{-1}$ decreases in intensity. The strong ring breathing modes at 1075 $cm^{-1}$ and 1590 $cm^{-1}$ are not affected by the change in pH. Figure 7 shows the pH response of a single nanoparticle cluster showing a characteristic titration curve for the nanoparticle base sensors. The 4-MBA coated nanoparticle sensors show a pH response in the range of 6-8 which is ideal for biological measurements.

The pH inside of a cell is very tightly controlled and a slight change in the pH can indicate a significant change in the biochemistry and fate of the cell. Ideally a sensor would be able to measure pH changes as small as 0.1 pH unit. In order to determine the resolution attainable with the nanoparticle pH sensors, the reproducibility of the nanoparticle based sensors was measured and is shown in Fig 8. Fig. 8 shows the response of approximately 30 nanoparticle sensors as a function of pH. As illustrated by the error bars, the resolution of the nanoparticle sensors is approximately one pH unit, which is larger desired.

In order to understand this large particle to particle viability in the measured pH, the topography and SERS response of the nanoparticle based sensors were measured simultaneously. The correlated topographic image shown in Fig 9 A and the optical image (SERS response) in Fig 9B illustrate that, under these experimental conditions, only a few nanoparticles actually yield a detectable SERS signal. Moreover, all of the nanoparticles showing a SERS response were actually clusters of nanoparticles consisting of 3-4 nanoparticles on average. An example of these nanoparticle clusters is shown in the inset of Fig. 9A which shows a zoomed in topographic image of one of the nanoparticle clusters in Fig 9A.

While some work remains to be done in the development of the nanoparticle sensors to improve their reproducibility from one nanoparticle sensor to the next, other aspects of utilizing these nanoparticle based sensors need to be addressed. The first question to be addressed is whether or not the nanoparticles will be fouled by the complicated biological matrix once these particles are incorporated into cells. This was demonstrated by incorporating the nanoparticle sensors into Chinese hamster ovary (CHO) cells by incubating the cells with the nanoparticle sensors overnight. Fig 10 demonstrates that the 4-MBA coated nanoparticles retain their robust signal upon

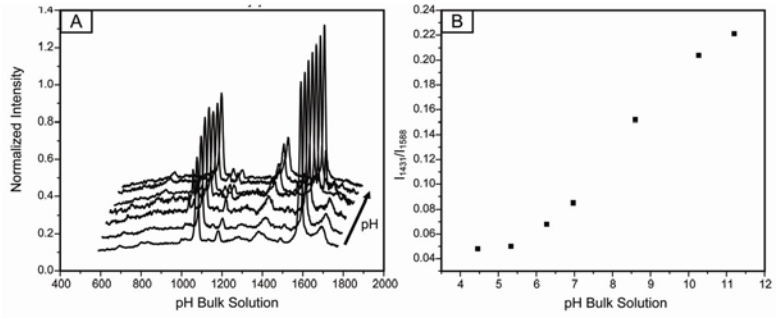


Figure 7 The normalized SERS spectra of 4-MBA as a function of pH. As can be seen in A, the $COO^-$ stretching mode increases with pH. In B the ratio of the pH sensitive $COO^-$ to the pH insensitive ring breathing mode at 1590 $cm^{-1}$ is plotted as a function of pH.

incorporation into the cells. It is likely that the nanoparticles were taken up by the cells through phagocytosis and that the nanoparticles are confined to lysosomes within the cell. This is supported by the pH reading from the nanoparticles which suggests that the pH in the immediate surroundings of the sensor is less than 6 which is consistent with the pH found in lysosomes.[35]

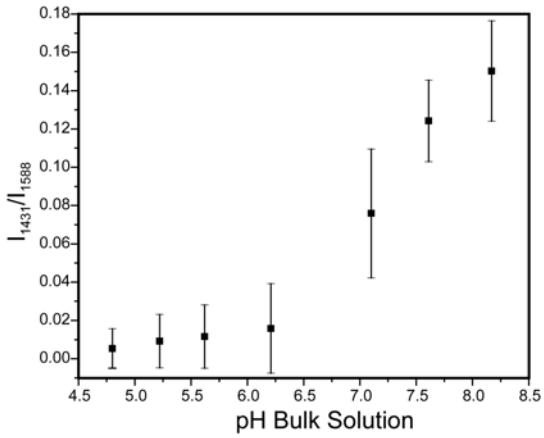


Figure 8 The intensity ratios as a function of pH for 30 individual nanoparticle sensors. The error bars represent one standard deviation.

Although passive uptake will work for a few select cells, it is necessary to

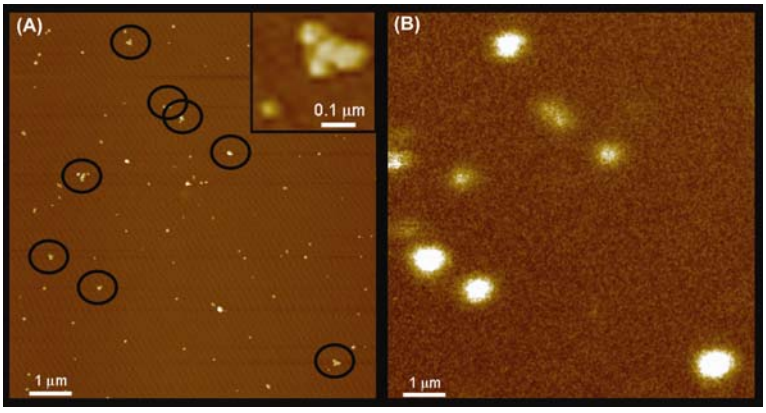


Figure 9 AFM (A) and optical (B) images of nanoparticle sensors immobilized on a glass coverslip. The circled nanoparticles in A correspond to those nanoparticles giving a strong SERS response as seen in B. Those nanoparticles giving a strong SERS signal are actually nanoparticle clusters. A zoomed in image of the nanoparticle cluster in the lower right of the AFM image is shown in the inset at the upper right.

have a reproducible, high throughput method for incorporating the nanoparticle sensors into cells. One way to incorporate nanoparticle sensors into cells that has been explored recently is through the use of small peptides. Small peptides are able to bind to endocytotic receptors on the cell surface and through endocytosis draw the nanoparticles into the cell. This has been demonstrated using a TAT peptide which is a peptide derived from the HIV virus.[36] As shown in the series of image in Fig 11 the TAT peptide coated nanoparticles are incorporated into BJ1 cells. The movies from which this image was extracted show hindered diffusion suggesting that they are compartmentalized within the cell. Current studies are aimed at understanding the fate

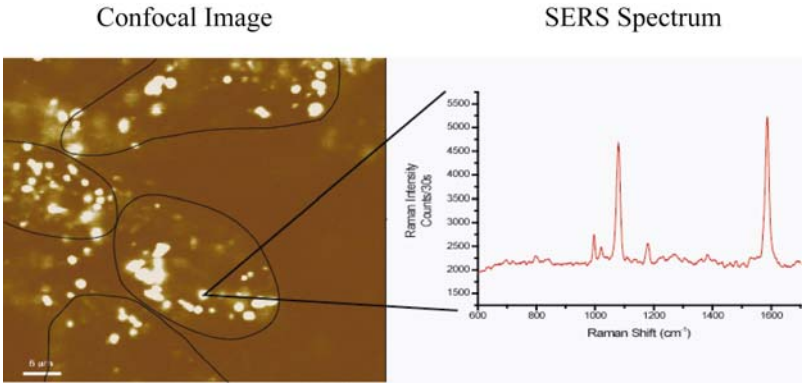


Figure 11 A confocal image of CHO cells with 4-MBA nanoparticle sensors incorporated into the cells (left) The cells are outlined in black to facilitate viewing the low contrast of the cells against the bright nanopatticles. The SERS spectrum of one of the nanoparticle sensors is shown to the right indicating that the pH around the nanoparticle snesnor is less than 6.

of the peptide coated nanoparticles once they are incorporated into the cells to determine if this is a suitable approach for high throughput nanoparticle incorporation.

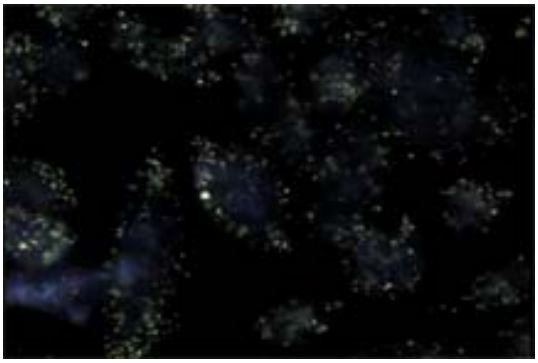

Figure 10 Dark field microscopy image of nanoparticles incoproated into BJ1 cells using TAT peptides.

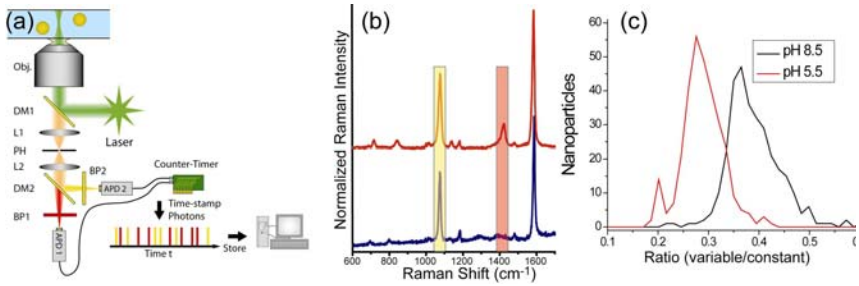


Figure 12 (a) Schematic illustration of the SERS correlation technique to measure the pH of nanoparticle sensors in solution. The spectral region of the two bandpass filters used in (a) are shown in (b). The resulting measurement of several hundred nanoparticles is shown in (c).

Finally, it is necessary to develop a method of characterizing the nanoparticle sensors while they are rapidly diffusing within the sample. In order to do this we have developed a SERS correlation technique reminiscent of fluorescence correlation spectroscopy. In the SERS correlation technique, two fast photodetectors such as avalanche photodiodes are used to individually monitor specific Raman vibrational modes. In the case of the 4-MBA coated nanoparticle pH sensors, the ring breathing mode at 1075 $cm^{-1}$ is filtered using a narrow bandpass filter and sent to one avalanche photodiode and the pH sensitive $COO^-$ stretching mode is sent to a separate avalanche photodiode. By creating a histogram of the time correlated ratios at the photodetectors, it is possible to measure the pH in a specified location within the sample.

## 4. Summary

The chemically specific and quantitative information that can be obtained through Raman spectroscopy provides a powerful tool for the investigation of biological systems. Surface-enhanced Raman scattering provides a means of increasing the observed Raman signal by many orders of magnitude allowing for detection limits that can approach the single molecule level. SERS has been proven useful for many biological applications ranging from fundamental studies to detection arrays. The colloidal nanoparticles used for SERS are small in size (40-100 nm in diameter) and can be used as sensor elements for biodetection. In order to improve on the specificity and response of the nanoparticle sensors, they are functionalized to interact with the target molecule of interest. As an example, nanoparticles can be functionalized with 4-MBA to function as a pH sensor. These nanoscale pH sensors retain their robust signal when incorporated into living cells. Finally novel detection methods such as correlation based SERS can be used to monitor the nanoparticle sensors while they are diffusing in solution.

## 5. Acknowledgements

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