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Probing Membrane Receptors with Enhanced Raman Imaging

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Abstract

Our lab has shown that nanoparticles functionalized with short peptides can selectively bind to receptor proteins *in vitro*. Our results indicate that the Raman signals observed from purified receptors in surface enhanced Raman scattering (SERS) experiments match those observed with tip-enhanced Raman scattering (TERS) experiments performed on membrane receptors in intact cell membranes. Analysis of the observed Raman signals suggest the signals arise from the amino-acids in the protein receptor responsible for binding and recognition of the ligand attached to the nanoparticle probe. Further experiments show the variance in the data correlates with affinity of the nanoparticle probe with a specific receptor. This result illustrates a new approach to studying membrane receptors.

Keywords

SERS; TERS; Proteins; drug targeting

1. INTRODUCTION

Understanding the molecular interactions between proteins on the surface of cells and molecules with which they interact is fundamental for understanding biological signaling and for manipulating these signals as druggable targets.¹ Traditionally, these studies involve determining the structure of the protein receptor through x-ray crystallography, nuclear magnetic resonance (NMR), or more recently cryo-electron microscopy.²⁻⁴ These techniques provide exquisite structural information, and can probe changes in structure when ligands or drugs are included with the protein. However, these techniques are challenged when studying the protein in a living cell, require crystallization or isolation of the protein, and the interactions between proteins and other biomolecules in the cell that affect behavior are often lost. Given the importance of these protein receptors, the development of new tools can significantly impact our understanding and advance the treatment of disease.

Vibrational spectroscopy has a long history providing structural details of proteins.⁵ Structural features of proteins, such changes in secondary structure can be monitored by changes in frequency of the amide vibrational modes. Raman in particular, has been used extensively because it is not significantly affected by water. To study membrane proteins, the

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challenge has been to obtain a significant concentration of the protein within the focus of a laser to produce sufficient scattering for characterization. Thus, most spontaneous Raman studies have focused on soluble proteins at millimolar concentrations, which provide ensemble information from the proteins in solution. However, innovative approaches continue to be developed that are facilitating the use of vibrational spectroscopy to study membrane proteins.⁶

One approach that is showing increasing impact in the study of protein structure is enhanced Raman spectroscopy. The use of plasmonic materials to increase the Raman scattering from samples is enabling analysis of individual proteins and proteins in cells. Surface enhanced Raman scattering (SERS) has been used to detect proteins for some time,⁷ and the nanoscale imaging analogue tip-enhanced Raman scattering (TERS) is now providing characterization on the nanometer length scale.^{8, 9} Impressive TERS results include analysis of amyloid fibrils,^{10, 11} protein glycosylation,^{12, 13} and the work in our lab studying protein-ligand binding.^{14–18} Earlier SERS work postulated that amino acids closest to the nanoparticle gave rise to the enhanced Raman signal,¹⁹ and the improved spatial resolution associated with TERS has validated this idea. What has become contentious is understanding why certain signals associated with proteins, specifically the amide I modes, are observed sporadically,²⁰ and why there are differences in the enhanced cross-sections of the aromatic amino acids relative to their spontaneous Raman cross sections.²¹ Many of these studies use parallel SERS and TERS measurements to identify trends and provide confidence in the observed signals, an important step which helps avoid the perception of signal irreproducibility associated with early SERS studies.

The work in our lab has sought to use the signals observed from a hybrid SERS/TERS approach to characterize the molecular interactions associated with protein-ligand binding.¹⁸ Specifically the experiment involves a spherical nanoparticle probe that is functionalized with a short peptide or small molecule that will bind, preferably with high selectivity, to protein. When a TERS probe is in close (nanometer) proximity to the nanoparticle, coupling between the nanoparticle and TERS probe generates a significant enhancement of the Raman signal of the protein interacting with the nanoparticle.²² Proof-of-concept studies were performed using biotin and streptavidin as a model protein-ligand binding system. Using a streptavidin coated glass slide and a biotinylated gold nanoparticle, we were able to detect signals from the streptavidin protein when a bound nanoparticle was mapped with an gold-ball TERS tip.¹⁸ Further investigation of this system indicated that the protein detected was on the glass surface, as the observed enhanced Raman signal changed when the protein was deliberately located between nanoparticles compared to being on the surface.¹⁷ The changes observed in the TERS experiment were found to be consistent with SERS signals from aggregated nanoparticles. Differences in the electric field gradient on plasmonic structures have been shown to alter the observed enhanced Raman signal,^{23, 24} and this differential enhancement was implicated for the changes in signal observed from proteins in different locations relative to the nanoparticle-TERS tip construct. Interestingly, it was determined by examining mutant proteins that a single residue, a tryptophan, in the wild-type mutant was responsible for many of the observed frequencies in the enhanced Raman spectrum.²⁵ Tryptophan 120 is reported to be key to high affinity binding.^{26–28} From the structure of streptavidin, this residue should lie close to the nanoparticle when bound to the

protein, explaining the strong influence on the enhanced Raman signal. When put together, this work suggests the enhanced Raman signal from functionalized nanoparticles interacting with proteins can be used to assess the amino acids relevant to the binding interaction.

In this report, we will examine the ability to deduce protein structure from small peptide sequences, such as arginine-glycine-aspartic acid (RGD), to the membrane signaling proteins. We will look at the observed spectra and propose a mechanism that explains the observed signals.

2. METHODOLOGY

2.1 Materials.

Citrate capped, 80 nm diameter NanoXact gold nanoparticles (nanoComposix, San Diego, CA) were used in TERS experiments. Cyclic-RGDfC, cyclic-isoDGRFC and CisoDGRC peptides (purity > 90%) were purchased from Peptide 2.0, Inc. (Chantilly, VA). Purified human $\alpha v \beta_3$ and $\alpha_5 \beta_1$ integrin proteins (purity > 95%) was purchased from EMD Millipore Corporation (Temecula, CA). Poly-d-lysinecoated coverslips were purchased from BD BioCoat Cellware. Cell culture reagents were purchased from Thermo Fisher Scientific (Waltham, MA). Ultrapure water (18.2 M Ω cm) was produced by a Barnstead Nano-pure filtration system and used for all experiments. Other reagents were obtained from Sigma-Aldrich and used as received.

2.2 Nanoparticle synthesis.

40 nm gold nanoparticles were prepared using the previously reported method of citrate reduction of gold chloride salt.²⁹

2.3 Nanoparticle functionalization.

80 nm gold nanoparticles were functionalized with peptides by ligand exchange. Peptides were prepared at 0.05 mM concentration. 10 μ L of the peptide solution was combined with 1 mL of 80 nm gold nanoparticles (0.05 mg mL⁻¹, or 16.6 pM) providing a molar ratio of peptide to gold nanoparticles of 5×10^4 : 1. The solution was left for 24 h, centrifuged (10000 rcf, 12 min), the supernatant was removed, and the nanoparticles were re-suspended in 1mL pure water. This process removed unbound peptide. The peptide-conjugated gold nanoparticles (peptide-GNPs) were stored at 4°C until used.

2.4 Nanoparticle characterization.

Nanoparticles were characterized with UV-Vis absorption, dynamic light scattering, and zeta-potential measurements. UV-Vis measurements were performed using a UV-3100PC spectrophotometer (VWR International, Radnor, PA), coupled with a Deuterium-Tungsten halogen lamp. Dynamic Light Scattering (DLS) and Zeta potential measurements were performed using a Zetasizer Nano-ZS system (Malvern, Worcestershire, UK).

2.5 Cell sample preparation.

SW620 cells were cultured following a previously published procedure.³¹ SW620 cell were seeded on poly-D-lysine-coated coverslips for TERS experiments. After 24-h incubation,

cells were rinsed with 0.1x PBS and 100 μ L of the peptide functionalized gold nanoparticles were added to the culture medium for 2 h. Unabsorbed nanoparticles were removed by repeated rinsing with 0.1x PBS. The cells were then fixed with paraformaldehyde (4% in PBS for 10 min.), rinsed with 0.1x PBS and water, and dried before TERS experiments.

2.6 Raman Spectroscopy.

A Renishaw In Via Raman microscope with a 633 nm HeNe laser was used to obtain Raman spectra.

2.7 Tip Enhanced Raman Scattering.

TERS measurements were obtained using a Nanonics Imaging MV4000 AFM positioned on the sample stage of a dual illumination microscope as previously reported.^{21, 22} The system incorporates a home-built Raman spectrometer containing a Horiba Jobin Yvon monochromator and CCD camera. A 633 nm HeNe excitation laser was used. Radial polarization of the laser was achieved using a liquid-crystal mode converter (ArcOptix), producing a longitudinal mode at the focus that results in increased enhancement and better spatial resolution from the TERS tip³². The TERS tip is a transparent glass tip embedded with gold nanoparticles (Nanonics Imaging Ltd. Israel). TERS maps were obtained by scanning the sample stage under the TERS tip positioned in the laser focus using an acquisition time of 1s per pixel and a laser power of 0.9–1.0 mW.

2.8 TERS data analysis.

TERS spectra and maps were analyzed using multivariate analysis techniques. TERS spectra of three different peptides bound with $\alpha_v\beta_3$ were analyzed by principal component analysis (PCA). PCA was performed using PLS toolbox (Eigenvector).

3. RESULTS AND DISCUSSION

Our approach to using enhanced Raman to investigate membrane receptors in biological cells is shown in Figure 1. Fig. 1A depicts the functionalization of gold nanoparticles with peptides. In Figure 1, we show the cyclic peptide, RGDfC, which is known to bind to integrin receptors. The cysteine in the peptide forms a gold-thiol bond and displaces citrate molecules on the nanoparticle. The functionalized nanoparticles are incubated with living cells in culture. After 2 h, the cells with nanoparticles are removed, fixed, and examined with TERS. Using a 1 s acquisition time, significant Raman scattering is only observed when the TERS tip is in close proximity, a few nanometers, to the nanoparticles on the surface of the cell. Previous work shows that the spectrum obtained from the TERS tip – nanoparticle interaction can be correlated to the receptor that binds the nanoparticle to the cell surface.¹⁵

The origin of the selectivity observed in the TERS signal arises from the amino acids found in the binding region of the protein. Figure 2 shows an overlay of the crystal structures of the extracellular domains of the two receptors based on the crystal structures 1LG3 ($\alpha_v\beta_3$) and 3VI4 ($\alpha_5\beta_1$) in the protein databank. Given that both receptors are known to bind the RGD amino acid motif, the strong agreement in the extracellular domain is not surprising.

However, what is interesting are the slight differences in the structures. Based on our earlier work identifying tryptophan as a key amino acid residue in the binding between biotin and streptavidin,²⁵ we have highlighted all the tryptophan residues in each structure. Changes in orientation for the indole side chain are evident which will alter the intensity of vibrational modes interacting with the optical excitation. The summation of all these small changes among the tryptophan and other enhanced residues is credited with causing the variation in the enhanced Raman signal that enables differentiation of which specific protein receptor is binding to the functionalized nanoparticle.

The ability to assign the spectrum observed to protein region with which the functionalized nanoparticle is interacting suggests a new way to study the interactions between ligands and receptors. In particular the variance observed in the enhanced Raman spectrum provides information about the binding site on a cell.¹⁴ Figure 3 illustrates how the differences in the observed TERS spectrum can be used to determine the specificity of ligand for a particular protein receptor. Similar to the differences in protein structure affecting the observed Raman signal from a single ligand; using different ligands, the variance in the observed Raman signal can inform about ligand specificity.

In Figure 3, the single clusters observed for cyclic iso-DGRfC and cyclic RGDfC suggest a single binding site on the cellular membrane. The 3 distinct clusters observed from CisoDGRC suggests less specific binding. These trends in binding were validated by determining the diffusion coefficient of each probe using single particle tracking methods.¹⁴

Given that the observed Raman spectrum arises from the molecules involved in binding, the spectrum may provide a means to perform localized structural analysis of the protein. To explore this possibility, it is important to first understand the origin of the enhanced signals. The accepted understanding of enhanced Raman suggests that the electric field provides enhanced scattering from molecules near the nanostructures. This enhancement is expected to decrease proportionally to the extent of the electric field. There are now a number of well validated studies that demonstrate the distance of the enhancement is not related to the geometric shape or size of the plasmonic structure. Particularly in TERS, models of enhancement correlated to spatial resolution break down to provide impressive results.^{31, 32} Given the controversy in the interpretation of enhanced Raman spectra of protein, perhaps an alternate model of enhancement is required.

Figure 4 shows the spontaneous Raman spectrum of tryptophan in both the powder form and in solution. What is evident is that the major bands are present in both spectra. In solution, there is additional broadening and lower intensity modes are not as apparent due to lower concentration of molecules in the focal volume. Figure 4C shows the SERS spectrum of tryptophan on gold nanoparticles in solution. The spectrum in Fig. 4C more closely resembles the spectrum of the solid; however, there are significant differences. All three spectra were recorded using 633 nm excitation. There is decent agreement among the observed frequencies, but the relative intensities vary between the SERS and spontaneous Raman spectrum. Similar results have been noted for the SERS spectrum of thiophenol, which is attributed to the formation of a charge transfer state and a resonance Raman

enhancement. While the idea of resonance Raman is not new,³³ it has been revived to explain phenomena not captured by pure electric field models in SERS and TERS.³⁴

Aromatic amino acids have been shown to be selectively enhanced compared to other residues in proteins.²¹ Recently stimulated Raman experiments were demonstrated to gain sensitivity by accessing pre-resonant states of molecules.³⁵ The changes in intensity observed for tryptophan suggest this mechanism is consistent with the enhancements in proteins observed. In the case where the tip may physically contact the protein, strong coupling may result in additional enhancements. Future work will be needed to investigate this mechanism.

4. CONCLUSIONS

In this report we have demonstrated that the enhanced Raman signal obtained from nanoparticles interacting with protein receptors can be used to identify the receptor and also measure the specificity of the functionalizing ligand. Significant variance in the observed SERS signal is indicative of multiple interaction sites on the cell. The data suggests that more detailed analysis of the observed spectra may provide a means to investigate local protein structure.

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Figure 1.

A) The cyclic peptide RGDfC (structure shown) is covalently attached to citrate capped gold nanoparticle. The cysteine (C) residue in the peptide has a thiol moiety that bonds to the gold nanoparticle. B) The peptide functionalized nanoparticles are incubated with cells and particles on the plasma membrane surface are detected by TERS. C) The observed enhanced Raman from the TERS tip – nanoparticle interaction can be correlated to reference spectra of specific membrane receptors, the signatures of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins determined from purified receptor protein are shown.

Figure 2.

A). The average enhanced Raman spectrum determined from purified integrin protein complexing with RGDfC functionalized Au nanoparticles are shown. This signature was used to determine which receptor bound the nanoparticle on the surface of cells. The differences in the spectra are attributed to the subtle, yet significant changes in residue orientation as observed in (B) the reported crystal structures of the extracellular domains of the $\alpha_v\beta_3$ (1LG3) and $\alpha_5\beta_1$ (3VI4) integrin receptors. The structures were plotted using Chimera molecular modeling software.³⁰

Figure 3.

A) shows the PCA scatter plot for the TERS signals obtained from the ligands Cyclic-RGDfC, cyclic-isoDGRFC and CisoDGRC peptides functionalized to gold nanoparticles. The clustering suggests how many different sites the ligand can bind to on the cell membrane. B) The mean spectrum for each cluster is shown, illustrating how much spectral variation is observed from cluster to cluster.

Figure 4.

The Raman spectrum of tryptophan under different conditions are shown: A) powder spectrum, B) solution spectrum, C) SERS spectrum. The SERS spectrum in C was obtained in solution using citrate capped gold nanoparticles. The intensities scale shown are arbitrary. All spectra were collected with a 633 nm laser.