Published in final edited form as:

Nanoscale. 2012 July 7; 4(13): 3917–3925. doi:10.1039/c2nr30747b.

Monitoring Enzymatic Degradation of Pericellular Matrices through SERS Stamping

Bo Yan^a, Yan Hong^a, Tianhong Chen^a, and Björn M. Reinhard^{a,*}

^aDepartment of Chemistry and The Photonics Center, Boston University Boston, MA, 02215, USA

Abstract

We introduce a surface enhanced Raman spectroscopy (SERS) stamping approach for acquiring cell-surface specific vibrational spectra of individual living cells under physiological conditions. The SERS stamping approach utilizes a nanostructured metal surface on top of a lithographically defined piston that can be translated in 3-dimensions with nanometer resolution to contact living cells in solution with a pristine metal surface. We applied this approach to characterize the chemical composition of the cellular surface of living MCF7 breast cancer cells and to monitor its change upon addition of the enzyme hyaluronidase, which degrades major constituents of the pericellular matrix. Although the cell surface spectra show significant cell-to-cell fluctuations, a statistical barcode analysis of the spectra ensembles reveals systematic changes in the cell surface SERS spectra upon addition of hyaluronidase, which are consistent with a thinning of the pericellular matrix.

Introduction

The plasma membrane of mammalian cells defines the boundary between the cell interior and the exterior and, therefore, the fundamental “building blocks” of higher organisms. Since the activities of individual cells needs to be coordinated in higher organisms, efficient mechanisms for cell communication and regulation have evolved. These biochemical regulation mechanisms are aided by surface-specific proteins, transmembrane proteins, glycoproteins and glycans. Together with lipids and other components of the plasma membrane, these cell surface functionalities define the “cell surface chemistry”. Perturbations of this chemistry are often associated with serious diseases, most prominently cancer.¹ Consequently, there is significant interest in analytical methods that can identify disease related abnormalities in the cell surface chemistry and make them amenable as biomarkers in research and diagnostics.^{2–4}

Due to its complex composition the cell surface poses significant analytical challenges.⁵ The conventional approach in cell surface diagnostics is to analyze the individual components one-by-one, for instance by mass spectroscopy.⁶ This method requires the selective extraction of the components of interest, for instance, transmembrane proteins or lipids, which often proves to be difficult and time-consuming. To avoid membrane extraction altogether, special assays have been developed to study individual cell surface moieties, such as glycans,⁷ membrane bound proteins,⁸ and lipids in an intact membrane using optical labels. These assays are useful research tools for specific surface species and provide valuable information about their spatial distribution, but they are limited in scope and do not facilitate a systematic surveying of the entire cell surface chemistry.

*bmr@bu.edu.

[†]Electronic Supplementary Information (ESI) available: [Figure S1–S5 and Table S1 are available online]. See DOI: 10.1039/b000000x/

Label-free analytical approach that can screen for unknown changes in the entire cell surface chemistry would greatly alleviate the identification of new biomarkers and could eventually facilitate the detection of patterns of correlated changes that arise from simultaneous modifications of multiple cell surface components. One potential candidate for this challenging sensing task is surface enhanced Raman spectroscopy (SERS).⁹ SERS is a label-free, fast, and non-destructive vibrational spectroscopy which requires little to no sample preparation.¹⁰ Different from Infrared Spectroscopy (IR) water has a very small Raman cross-section, a characteristic that makes the method suitable for analysis of living cells in solution.¹¹ On top of all of these advantageous performance characteristics, which SERS shares with conventional Raman, SERS has additional unique properties that make it the method of choice for a spectral characterization of the cell surface chemistry.¹² It is well-established that intrinsically low molecular Raman cross-sections can be enhanced by many orders of magnitude ($10^3 - 10^{12}$) in the evanescent E-field in the vicinity of a nanostructured metal surface.¹³ The signal enhancement results from a resonant amplification of the incident and scattered radiation fields through the surface plasmon resonances of the nanoparticles.^{14–16} The SERS signal scales approximately with the fourth power of the E-field,¹⁵ which results in a strong distance dependence of the signal amplification effect. Only the signal of those molecules located within < 5 nm away from the surface are amplified. The high signal enhancement together with the strong distance dependence of the signal amplification suggest SERS as a platform for acquiring surface specific vibrational spectra of cells in direct contact with the SERS substrate.

SERS is, however, not free of complications. The signal amplification through the nanostructured metal surface comes at a price. Previous studies have observed considerable spectral fluctuations even for relatively simple molecules.^{17–20} Molecular interactions between the analyte and the substrate, as well as point-to-point fluctuations in the E-field intensity due to heterogeneities in the nanostructured metal surface are sources of additional variability when compared with conventional Raman.¹⁷ These signal fluctuations can be expected to increase in biological systems. Cells exhibit natural cell-to-cell variations in the cell surface phenotype. Especially for cancer cells, which are known to show a high degree of genetic and epigenetic variability,^{21–23} it is possible that the cell surface structure and composition differs significantly between individual cells and even between different locations on one cell. SERS signal fluctuations due to heterogeneities in the cell surface, the SERS substrate, and the cell-metal surface interactions make robust data acquisition approaches in combination with statistical data analysis strategies indispensable for the identification of common molecular features in an ensemble of cells. We implement in this manuscript a “SERS stamp” and demonstrate that this approach –in connection with a barcode data analysis²⁴ – enables to detect the enzymatic degradation of major components (hyaluronic acid and chondroitin sulfate) of the pericellular coat of living MCF7 breast cancer cells.²⁵

Experimental section

Fabrication of SERS stamps

The SERS stamps were fabricated using standard photolithography procedures. Briefly, the photoresist (*AZ 1813*) was spin-coated on a 4" silicon wafer to form a ~ 1.5 μm thick layer. The wafer was then exposed to 365 nm UV light through a predesigned mask to form periodic squares on the surface. The squares had dimensions of $100\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$ and a separation of 2 mm from each other. After development, the wafer was etched by $\sim 60\text{ }\mu\text{m}$ through deep reactive ion etching (etching/passivation: $\text{SF}_6/\text{C}_4\text{F}_8$) creating quadratic pistons with $100\text{ }\mu\text{m}$ edge length and $60\text{ }\mu\text{m}$ height. Subsequently, the photoresist on the pistons was removed with acetone. In the last step the wafer was diced into $2\text{ mm} \times 2\text{ mm}$ chips, each containing one piston in their center.

Gold nanorod arrays on the stamp were generated by oblique angle metal deposition.²⁶ The silicon chips were first cleaned in piranha solution (3:1 H₂SO₄:H₂O₂) for 30 min at 70 °C followed by 3 times wash with DI water and blow dried with N₂. Then 5 nm Cr and 50 nm Au film was deposited on the chip surfaces at normal incidence angle. After this base layer had been formed, the chips were fixed on a substrate holder at an angle of 84°. Deposition of more metal at this angle of incidence led to the formation of well aligned gold nanorod arrays in a shadowing growth mechanism.^{26, 27} The deposition of gold was stopped when the length of the nanorods reached about 500 nm as indicated by a thickness monitor. The final SERS stamps were then stored in vacuum and plasma cleaned right before usage. The gold nanorod arrays showed an ensemble averaged enhancement factor for the 1077 cm⁻¹ C-S stretch band in paramercapto aniline (pMA) of $\sim 2 \times 10^5$.

Cell growth and sample preparation

Breast tumor cell lines MCF7 were cultured on quartz cover slides (22 × 22 × 0.25 mm thick) in Eagle's Minimum Essential Medium following the supplier's instructions (ATCC). When the cells had grown to a 70% – 80% confluency (normally four days after cell passage), the slides were washed three times with Hanks' buffer (*Invitrogen*) to remove all of the carbohydrates and proteins in growth medium. Next, 10 mL of bovine testes hyaluronidase (*Sigma H3506*) solution in Hanks' buffer (pH 6) was added to incubate with the cells at 37°C for 5–30 min. The enzyme was used at concentrations of 10 U/mL (corresponds to 17 µg/mL) and 600 U/mL (corresponds to 1 mg/mL). The cells were then washed 3 times with Hanks' buffer and mounted upside-down on a sample holder for SERS measurements. The cells of the controls were incubated in Hanks' buffer (pH 6) for 10 min and then washed with Hanks' buffer before recording SERS spectra.

Immunolabeling HA with functionalized nanoparticles

The 40 nm gold nanoparticles were pre-functionalized with propargyl-PEG-anti-biotin as described in our earlier work.²⁸ MCF7 cells were cultured on silicon chips and treated with hyaluronidase (10 U/mL, 600 U/mL in Hanks' buffer) before fixed with 4% formaldehyde solution. Then the cell chips were washed with 1x PBS and blocked with 1% BSA (in PBS buffer) for 1 h at room temperature. After that the cells were washed again with PBS and incubated with 50 µg/mL anti-HA antibody (*Abcam ab53842*) at 4°C overnight. The excess antibodies were removed with PBS on the next morning. The cells were then incubated with 10 µg/mL biotin-labeled secondary IgG antibody (*Abcam ab97128*) at room temperature for 1h, and subsequently washed three times with PBS. Finally functionalized gold particles were incubated on the chips for 3h at room temperature in a water vapor saturated atmosphere. The chips were cleaned with PBS and water before SEM imaging. For the control measurement, cells were incubated with Hanks' buffer (pH 6) for 10 min, and the HA was then labeled in an identical process as described above. The labeled cell samples were imaged in a Zeiss Supra 40VP SEM. For each condition, 13 cell surface areas (each with an area of $\sim 70 \mu\text{m}^2$) were analyzed to calculate average nanoparticle densities (ρ).

SERS measurements

SERS spectra were collected using an upright microscope (*Olympus BX51WI*) equipped with a 300 mm focal length imaging spectrometer (*Andor Shamrock 303i*) and a back-illuminated CCD camera (*Andor Idus*) optimized for the Near-Infrared (peak quantum efficiency > 90% at 785 nm). A 1200 lines/mm grating with a blazing wavelength of 750 nm was used. The excitation laser was a 785 nm diode laser (*Innovative Photonic Solutions*). After passing through a 785 nm laser line filter (*Semrock, LL01-785-25*), the laser light was injected into the objective using a dichroic (*Semrock, LPD-785RU*) and focused into the sample plane by a 100x water immerse objective (numerical aperture (NA) = 1). The laser power at the sample was 11 mW, which corresponds to a power density of 0.0183 mW/µm².

Light scattered off the sample was collected by the same objective and filtered by the dichroic, a 803 nm long pass filter (*Semrock LP02-785-RS*) and a laser blocking notch filter (*Semrock NF01-785U-25*). The active area for recording SERS spectra was horizontally confined by a slit in the entrance port of the spectrometer to a strip with a width of 3.9 μm . The CCD chip was vertically sub-divided using the camera software into eight bins with a height of 3.7 μm . In this way spectra were recorded simultaneously from eight regions of interest, each with an area of 3.9 $\mu\text{m} \times 3.7 \mu\text{m}$, which corresponds to $\sim 1/6$ of the footprint area of the investigated cells (MCF7).²⁹ Ten individual acquisitions with 1s integration time were accumulated for each spectrum.

The SERS stamp was fixed in the center of a petri-dish and immersed in Hanks' buffer (pH 7.4) at 37 °C. The whole dish was mounted on a three-dimensional nanopositioning stage (*Thorlabs*) with minimal step size of 25 nm and a range of motion of 100 μm along x and y and 80 μm along z. The cell culture slide was immersed in the same dish and mounted facing the SERS stamp on a separate mechanical 3-axes stage. We recorded a background spectrum of the SERS stamp when substrate and cells were separated by several tens of microns. Then the SERS stamp was raised to contact the cells and a cell surface SERS spectrum was acquired. To establish efficient contact between cell surfaces and the substrate, the SERS stamp was raised at a minimum speed of 25 nm steps until initial perturbation on cell's morphology was observed.

For the acquisition of SERS spectra of pMA, cholesterol, and mucin the following sample preparation procedures applied. In case of the pMA the SERS stamps were incubated with a 10 mM solution of pMA in ethanol for 3 hours. Then the SERS stamp was washed and transferred into Hanks' buffer and SERS spectra were recorded. A cholesterol solution in chloroform was drop-dried on a SERS stamp. Then the stamp was washed and transferred into Hanks' buffer to acquire a SERS spectrum. The same sample preparation applied to a 0.5 mg/mL solution of mucin in Hanks' buffer.

Data analysis

Data processing was performed using home-written Matlab (version 2007B) analysis codes. First, the background subtracted spectra were baseline corrected by the subtraction of a 3rd order polynomial baseline. Next, we applied a minimum intensity criterion (three times the average signal-to-noise of background spectra) and a high intensity threshold (99% confidence interval of all SERS spectra) to eliminate low and high intensity outliers. The remaining spectra were then smoothed with a FFT filter and second derivatives were calculated. We chose a value of 10% of the minimum slope detected in the 2nd derivative to define spectral peaks (see Figure S1). To transform the original spectra into barcodes, all peaks that passed both the intensity and 2nd derivative selection criteria were assigned a numerical value of one, while all other points were set to zero. In the last step all of the barcodes for each experimental condition were histogrammed using a bin size of 14 cm^{-1} .

Results and discussions

The acquisition of surface specific SERS spectra of living cells involves two major technical challenges. First, it is necessary to establish a very close contact between a nanostructured metal surface and the cell surface of interest. Second, it is important to avoid contamination of the SERS substrate with growth medium, cellular debris, or secreted material, since the SERS spectra of these compounds can have similarities with cellular surfaces. Our experimental approach to fulfill these requirements is illustrated in Figure 1. It is based on a nanostructured gold surface on a fabricated piston, which we refer to as "SERS stamp". The SERS stamp enables a controlled contacting of the cellular surface of one or a few adjacent cells with a pristine nanostructured metallic surface in solution. Similar as in tip-enhanced

Raman spectroscopy (TERS)^{30–32} only the molecular species located in the contact area of the SERS substrate will experience a strong amplification of their Raman cross-sections through the enhanced E-field. Due to this enhancement, the SERS signal from the contact area will dominate the recorded Raman spectrum, resulting in a surface-specific Raman spectrum. We emphasize here that the spatial resolution obtained with the SERS stamp is not comparable to that in TERS, where sharp tips are used to collect vibrational information with nanometer spatial resolution.³³ The SERS stamping approach seeks, instead, to capture spatially averaged surface spectra from the contact area between the stamp and the cells. The ultimate goal is to use optimized SERS stamps for a chemical profiling of cellular surfaces on the single cell level.

The application of SERS for a label-free characterization of the entire cell surface chemistry has more stringent experimental requirements than other already established sensing schemes that apply SERS for the detection of specific surface antigens using functionalized noble metal nanoparticles. These approaches rely on some recognition functionality, usually an antibody, to target the nanoparticles to specific moieties on the cell surface.^{28, 34–36} Antibodies and other potential chemical recognition functionalities will have profound effects on the recorded SERS spectra since they not only act as a spacer between the plasma membrane and the noble metal nanoparticles but also contribute to the recorded SERS spectra.³⁵ In addition, surface bound nanoparticles can be uptaken by the cells through endocytosis, which results in a loss of cell surface specificity of the recorded SERS spectra.³⁷ The SERS stamping approach in Figure 1 was designed to avoid the background problems associated with a cell surface tethering as well as the nanoparticle uptake by contacting the cellular surface with a pristine metal surface immobilized on a silicon support.

The SERS stamps used in this work were fabricated by photolithography and subsequent oblique angle deposition as shown in Figure 2. Details of the SERS stamp fabrication are summarized in the Experimental Section. Before we applied the SERS stamp for cell surface measurements, we first characterized its performance with a test panel of molecules of varying molecular complexity. Our panel included para-mercaptoaniline (pMA), the steroid cholesterol, which is contained in biological membranes at high concentrations, and the highly glycosylated protein mucin (Mw = 1–10×10⁶ Da). All spectra were measured in Hanks' buffer (pH 7.4). The small molecule pMA is commonly used to benchmark SERS substrates.^{38, 39} Due to its thiol group the molecule readily chemisorbs onto gold surfaces and assembles into a brush around the substrate. With pMA as test molecule, we obtained a linear SERS signal vs. laser power response in the power range between 3.9–11.56 mW (measured in the sample plane), confirming that photo damage did not occur in this power range (Figure S2). All subsequent SERS measurements were performed with a laser power of ~11 mW, corresponding to a power density of 0.0183 mW/μm².

Figure 3 contains 24 different SERS spectra of pMA recorded on three SERS stamps (A), 63 spectra of cholesterol recorded on four stamps (B), 56 spectra of mucin from six stamps (C). The relatively simple molecule pMA exhibits a clear defined spectral fingerprint comprising a well defined number of vibrational transitions. However, even for this small molecule the relative intensities of the bands show observable fluctuations. For the considerably more complex biomolecules cholesterol and especially mucin, the total signal intensity drops and the signal fluctuations increase. The signal intensity depends on the number of molecules on the SERS substrate, their intrinsic Raman cross sections and the average SERS signal enhancement provided by the substrate. Furthermore, the exact shape of SERS spectra also depend on the orientation of the molecules on the surface and, especially for larger molecules, on structural changes that result from interactions between the molecule and the surface. The observed signal fluctuations in the recorded SERS spectra result from a

convolution of the chemical complexity of the molecules, their interactions with the metal surface, and fluctuations in the $|E|$ -field enhancement due to heterogeneities in the SERS stamp. While the effect of the substrate is comparable for all three investigated molecules, the chemical complexity of the molecules increases going from (A) to (C) in Figure 3. Furthermore - unlike pMA - cholesterol and mucin will not self-assemble with a preferential orientation onto the substrate surface. Instead, these molecules will sample random orientations which increases the number of different interactions between the molecule and the SERS substrate.

Despite the observed signal fluctuations, many of the spectra share common features for both cholesterol and mucin in Figure 3(B) and (C). From an analytical point of view, the vibrational bands that show the largest representation in all of the recorded spectra are most valuable for the identification of the molecule. These bands represent the characteristic spectral features of the investigated samples. One possible approach for their identification is to first detect the most prominent spectral features in each individual spectrum and then to determine their relative probabilities in the entire ensemble of recorded spectra. We implemented this analysis approach through an algorithm that converts each individual spectrum into a “barcode” of only those peaks (as identified by their second derivative) that lie above a defined threshold.²⁴ The individual barcodes were then histogrammed to generate the ensemble barcode that visualizes the most representative SERS peaks of the ensemble. The details of this data analysis strategy are summarized in the Experimental Section.

In Figure 4, we show the obtained ensemble barcodes for the three test molecules. The ensemble barcode of pMA (Figure 4A) contains C-C and C-C-C bending modes at 1010 cm^{-1} , the C-S stretching mode at 1080 cm^{-1} , the C-H bending mode at 1180 cm^{-1} , a C-C stretching/C-H bending combination mode at 1480 cm^{-1} and the C-C stretching mode at 1590 cm^{-1} .⁴⁰ For reasons discussed above, the ensemble barcode of cholesterol (Figure 4B) shows a broader spread of the spectral features when compared with pMA. Nevertheless, most of the observed spectral features can be assigned. The band at 1000 cm^{-1} is assigned to the steroid ring (ring breathing) and the 1040 cm^{-1} band indicates the C-C stretching mode. The bands distributed at around 1200 cm^{-1} arise from C-H in-plane bending modes. The broad features between 1270 cm^{-1} to 1400 cm^{-1} are assigned to bending modes of CH_2 and $=\text{CH}$. The bands at $\sim 1430\text{ cm}^{-1}$ result from CH_3 bending modes and at 1600 cm^{-1} lie the C=C stretching modes.⁴¹

It is interesting to note that our SERS spectra differ somewhat from the published Raman spectra of cholesterol.^{41, 42} Unlike the Raman spectrum, which contains a prominent peak at 1438 cm^{-1} , the SERS spectra are dominated by the steroid ring features in the $1000\text{--}1050\text{ cm}^{-1}$ range. The strong signal enhancement of the molecular features associated with the steroid ring and could be the result of a preferential attachment of the steroid ring onto the SERS substrate. A similar effect was observed before in the Raman/SERS spectra of warfarin.⁴³

The ensemble barcode of the largest and chemically most complex molecule investigated in this study, mucin, is shown in Figure 4C. Both the C-C stretching mode at 980 cm^{-1} and the phenyl ring breathing (phenylalanine) at 1005 cm^{-1} contribute to the spectral features in the $980\text{--}1010\text{ cm}^{-1}$ window. The C- C_6H_6 stretching mode (phenylalanine, tryptophan and tyrosine) and the amide III band contribute to the broad bars between 1200 and 1350 cm^{-1} . The bars in the range between $1550\text{--}1620\text{ cm}^{-1}$ can be assigned to the amide II band and the C=C stretching modes in tyrosine and tryptophan.^{44, 45}

Our analyses of the spectra in Figure 4 highlight the major advantage of the barcoding approach. It enables to abstract complex spectral information of SERS spectra recorded on various SERS substrates and makes them amenable to a systematic comparison. In the next step, we want to apply this data analysis strategy to verify whether the SERS stamping approach is capable of providing cell surface specific information. Our experimental strategy is to induce controlled changes in the cell surface chemistry and then to test whether the SERS stamping detects these modifications. We introduced such changes to MCF7 cancer cells by treating the cells with hyaluronidase. The latter is an endoglycosidase that degrades hyaluronic acid (HA) and chondroitin sulfate (CS), which are major components of the pericellular matrix.^{46, 47} HA is a uniformly repetitive, linear glycosaminoglycan (GAG) composed of disaccharides of glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc). It has a molecular weight of around 10^6 – 10^7 Da and is preferentially located in the extra- and peri-cellular space of most animal tissues, where it noncovalently interacts with CS and other types of aggrecans through link proteins to form a coat surrounding the cells.^{48, 49}

The synthesis and spatial distribution of HA has been studied in the breast cancer cell line MCF7 in detail,^{48,50, 51} and it is also well known that HA can be enzymatically removed from cellular surfaces through hyaluronidases.^{52–55} In this work we aim to test whether SERS can be used to detect changes in the HA cell coat upon hyaluronidase addition.

We first tested the activity of the hyaluronidase in Hanks' buffer (pH 6) at 37°C using synthetic HA in a conventional gel assay (Figure S3). These experiments confirmed that already an incubation of HA with 10 U/mL enzyme for 10 min led to a measurable degradation. After incubation with 600 U/mL for 30 min, the HA sample was completely digested. In a second control experiment we validated that the hyaluronidase also degrades cell surface bound HA under our experimental conditions. To that end, we incubated MCF7 cells in Hanks' buffer (pH 6) containing 10 U/mL hyaluronidase for 10 min or 600 U/mL hyaluronidase for 30 min at 37°C. After the incubation the cells were fixed and stained for HA with 40 nm Au immunolabels using a recently described multivalent labeling procedure (see Experimental section).²⁸ After immunolabeling the samples were transferred to the scanning electron microscope (SEM) for inspection. Figure 5 contains representative SEM images of controls (A, cells incubated in Hanks' buffer for 10 min without enzyme) and of cells treated with 10 U/mL (B) or 600 U/mL (C) hyaluronidase. Figure 5D contains a histogram of the average NP densities (ρ) obtained from 13 surface areas (each with an area of $\sim 70 \mu\text{m}^2$) for each investigated condition. We also included the standard deviation of ρ as error bars in the histogram. Although the HA surface concentration significantly fluctuates between individual cells (which was confirmed by conventional fluorescence staining (Figure S4) and previous reports⁵⁶), the average NP density overall decreases in the presence of the hyaluronidase. The latter confirms an enzymatic removal of cell-surface HA.

In the next step, we recorded SERS spectra of MCF7 cells incubated with 10 U/mL and 600 U/mL hyaluronidase for 10 and 30 min, respectively, as well as of controls (no enzyme) using SERS stamps. We acquired spectra from all the cells contained within an active area of $3.9 \mu\text{m} \times 29.6 \mu\text{m}$ on the SERS stamp. Every SERS stamp was used only for a single experiment to avoid spurious signals from cellular debris attached to the metallic surface after its initial contact with the cell surface. Overall, we recorded at least 65 spectra for each condition (corresponds to more than 30 cells). For details regarding the data acquisition and analysis, please refer to the Experimental Section.

The resulting SERS spectra and calculated ensemble barcodes are shown in Figure 6A and 6B, respectively. As anticipated, the original cell surface spectra in Figure 6A show an even higher degree of variability than observed for mucin. While the intensity fluctuation among individual SERS spectra (Figure 6A1–A3) complicate a comparison, the corresponding

ensemble barcodes (Figure 6B1–B3) prior and after enzymatic treatment show systematic differences, most prominently in the spectral range of 1300 – 1450 cm^{-1} . Prior to enzyme addition a large number of spectral features were observed in this spectral range (Figure 6B1), but after 10 min of enzyme treatment (10 U/mL) their number is significantly diminished (Figure 6B2). After 30 min in 600 U/ml enzyme solution, this spectral range is almost completely void of any features (Figure 6B3).

The differences in the SERS spectra before and after hyaluronidase addition become even clearer by subtracting the control barcode from the barcode obtained after incubation with 600 U/mL enzyme for 30 min (Figure 7). The difference spectrum confirms a systematic decrease in intensity for spectral features located at around 1040 cm^{-1} , 1100 cm^{-1} , 1150 cm^{-1} , 1320–1375 cm^{-1} and 1430–1470 cm^{-1} respectively, in the presence of hyaluronidase. These spectral features are consistent with recorded Raman spectra of HA in the literature and can be assigned to the C-OH stretching mode (1045 cm^{-1}), acetyl group C-CH₃ stretching mode (1090 cm^{-1}), C₍₄₎-OH and C₍₄₎-H deformation modes (1130–1150 cm^{-1}), the amide III mode (1330 cm^{-1}) and the CH₃ symmetric deformation mode (1370 cm^{-1})^{52–55}. The features at 1430 cm^{-1} and 1470 cm^{-1} are assigned to CH₂ bending modes. Our spectrum assignment was further corroborated by SERS spectra acquired from chemically synthesized HA (*Sigma S9825*) on a SERS stamp (Figure S5), which reproduced the spectral features at 1045 cm^{-1} , 1130–1150 cm^{-1} , 1330 cm^{-1} , and 1430 cm^{-1} . We emphasize that the SERS spectrum of the synthesized HA is not identical to that of the HA in the cell coat since the synthetic molecule misses protein components. For a complete list of tentative assignments of all observed spectral features, please refer to Table S1.^{44–47, 52–54, 57–59}

Overall, the loss in intensity of the HA associated barcodes in response to hyaluronidase addition is consistent with an enzymatic removal of the HA coat. The enzyme does, however, not lead to a global decrease in signal intensity; some spectral features become more intense after addition of the enzyme. These features have positive values in the difference spectrum in Figure 7. After incubation with the enzyme at 600 U/mL for 30 min, SERS features at around 1026 cm^{-1} , 1180 cm^{-1} , 1215 cm^{-1} , 1245 cm^{-1} , 1560 cm^{-1} and 1590 cm^{-1} all increased in intensity. These bands can be assigned to the amide modes of proteins and C=C stretching modes of lipids associated with the plasma membrane.^{44–47, 57–59}

One potential interpretation for the increase in intensity of these bands is that with increasing removal of the HA coat on the cell surface the SERS stamp can approach closer to the plasma membrane. Since the HA associated extra- and peri-cellular coat acts as spacers between the SERS stamp and the plasma membrane, the removal of HA facilitates an enhancement of the signal of chemical features that are otherwise hidden under the coat. This model is consistent with the findings from previous studies in which we compared the SERS spectra of tumor and non-tumor cell lines dried on a nanostructured metal surface. The previous studies indicated that the lipid bands are more pronounced in the SERS spectra of the non-tumor cells than in the spectra of tumor cells, which commonly over-express glycans.⁶⁰

Conclusions

The cell surface is a complex hybrid material whose chemical characterization still poses significant challenges for conventional analytical method. In response to the need for new tools that can provide molecular information about the cell surface composition, we introduced and validated in this study a “SERS stamping” approach. This method enables to contact cellular surfaces in solution with a pristine nanostructured noble metal surface and

thus facilitates the acquisition of SERS spectra from individual living cells in solution. Although the cell-to-cell fluctuations in the recorded SERS spectra are large, we show that a statistical analysis of the spectra recorded before and after hyaluronidase treatment through barcoding nevertheless identifies systematic differences in the spectra ensembles that are consistent with a thinning of the pericellular matrix. Given the significant functions of the pericellular coat, and HA in particular, in cell migration, wound healing, cell growth and cancerogenesis,^{61, 62} the ability to detect changes in the pericellular matrix on living cells is significant and underlines that already the non-optimized SERS stamps applied in this work are an enabling sensor platform. The advantage of the SERS stamping method is its compatibility with rational fabrication approaches for nanostructured metal surfaces.^{63, 64} We anticipate that the use of engineered nanostructures with higher structural conformity as SERS active surface can reduce the substrate related signal fluctuations and will, thus, result in an additional performance improvement of the SERS stamping approach in the future. When combined with statistical spectra analysis methods, SERS stamping represents a promising new approach for the chemical profiling of cellular surfaces and provides new opportunities for a label-free monitoring of cell surface biomarkers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work was partially supported by the National Institutes of Health through grant 5R01CA138509-03 and the National Science Foundation through grants CBET-0853798 and CBET-0953121.

Notes and references

- Hollingsworth MA, Swanson BJ. *Nat Rev Cancer*. 2004; 4:45–60. [PubMed: 14681689]
- Bresalier RS, Rockwell RW, Dahiya R, Duh QY, Kim YS. *Cancer Res*. 1990; 50:1299–1307. [PubMed: 2297775]
- Sawyers CL. *Nature*. 2008; 452:548–552. [PubMed: 18385728]
- Wilson FJ. *Ann Intern Med*. 2006; 144:945–948. [PubMed: 16785487]
- Kellam B, De Bank PA, Shakesheff KM. *Chem Soc Rev*. 2003; 32:327–337. [PubMed: 14671788]
- Rujoi M, Estrada R, Yappert MC. *Anal Chem*. 2004; 76:1657–1663. [PubMed: 15018564]
- Prescher JA, Dube DH, Bertozzi CR. *Nature*. 2004; 430:873–877. [PubMed: 15318217]
- Koehorst RBM, Spruijt RB, Hemminga MA. *Biophys J*. 2008; 94:3945–3955. [PubMed: 18234831]
- Jeanmaire DL, Van Duyne RP. *J Electroanal Chem*. 1977; 84:1–20.
- Jarvis RM, Goodacre R. *Anal Chem*. 2004; 76:40–47. [PubMed: 14697030]
- Petry R, Schmitt M, Popp J. *Chem Phys Chem*. 2003; 4:14–30. [PubMed: 12596463]
- Hering K, Cialla D, Ackermann K, Dorfer T, Moller R, Schneidewind H, Mattheis R, Fritzsche W, Rosch P, Popp J. *Anal Bioanal Chem*. 2008; 390:113–124. [PubMed: 18000657]
- Moskovits M. *J Raman Spectrosc*. 2005; 36:485–496.
- Le Ru, EC.; Etchegoin, P. *Principles of Surface Enhanced Raman Spectroscopy and Related Plasmonic Effects*. Elsevier; Amsterdam: 2009.
- Le Ru EC, Etchegoin PG. *Chem Phys Lett*. 2006; 423:63–66.
- Nie SM, Emery SR. *Science*. 1997; 275:1102–1106. [PubMed: 9027306]
- Etchegoin PG, Le Ru EC. *Anal Chem*. 2010; 82:2888–2892. [PubMed: 20210327]
- Etchegoin PG, Le Ru EC, Meyer M. *J Am Chem Soc*. 2009; 131:2713–2716. [PubMed: 19166340]
- Luo Z, Luo Y, Li J, Liu K, Fu H, Ma Y, Yao J. *Chem Commun*. 2009:1342–1344.
- Schleifenbaum F, Blum C, Subramaniam V, Meixner AJ. *Mol Phys*. 2009; 107:1923–1942.
- Bertucci F, Birnbaum D. *J Biol*. 2008; 7:6. [PubMed: 18304379]

22. Keller P, Lin A, Arendt L, Klebba I, Jones A, Rudnick J, DiMeo T, Gilmore H, Jefferson D, Graham R, Naber S, Schnitt S, Kuperwasser C. Breast Cancer Res. 2010; 12:R87. [PubMed: 20964822]
23. Marusyk A, Polyak K. Biochim Biophys Acta Rev Cancer. 2010; 1805:105–117.
24. Patel IS, Premasiri WR, Moir DT, Ziegler LD. J Raman Spectrosc. 2008; 39:1660–1672. [PubMed: 19714262]
25. Toole BP. Nat Rev Cancer. 2004; 4:528–539. [PubMed: 15229478]
26. Chaney SB, Shanmukh S, Dluhy RA, Zhao YP. Appl Phys Lett. 2005; 87:031908.
27. Karabacak T, Singh JP, Zhao YP, Wang GC, Lu TM. Phys Rev B. 2003; 68:125408.
28. Wang J, Boriskina SV, Wang H, Reinhard BM. ACS Nano. 2011; 5:6619–6628. [PubMed: 21761914]
29. Mendez MG, Kojima SI, Goldman RD. FASEB J. 2010; 24:1838–1851. [PubMed: 20097873]
30. Richter M, Hedegaard M, Deckert-Gaudig T, Lampen P, Deckert V. Small. 2011; 7:209–214. [PubMed: 21213383]
31. Vannier C, Yeo BS, Melanson J, Zenobi R. Rev. Sci. Instrum. 2006; 77: 023104.
32. Yeo BS, Stadler J, Schmid T, Zenobi R, Zhang W. Chem Phys Lett. 2009; 472:1–13.
33. Vitol EA, Orynbayeva Z, Bouchard MJ, Azizkhan-Clifford J, Friedman G, Gogotsi Y. ACS Nano. 2009; 3:3529–3536. [PubMed: 19891490]
34. Hodges MD, Kelly JG, Bentley AJ, Fogarty S, Patel II, Martin FL, Fullwood NJ. ACS Nano. 2011; 5:9535–9541. [PubMed: 22067076]
35. Huang X, El-Sayed IH, Qian W, El-Sayed MA. Nano Lett. 2007; 7:1591–1597. [PubMed: 17474783]
36. Nam JM, Thaxton CS, Mirkin CA. Science. 2003; 301:1884–1886. [PubMed: 14512622]
37. Kneipp J, Kneipp H, McLaughlin M, Brown D, Kneipp K. Nano Lett. 2006; 6:2225–2231. [PubMed: 17034088]
38. Jackson JB, Halas NJ. Proc Nat Acad Sci USA. 2004; 101:17930–17935. [PubMed: 15608058]
39. Yan B, Thubagere A, Premasiri WR, Ziegler LD, Dal Negro L, Reinhard BM. ACS Nano. 2009; 3:1190–1202. [PubMed: 19354266]
40. Osawa M, Matsuda N, Yoshii K, Uchida I. J Phys Chem. 1994; 98:12702–12707.
41. Hanlon EB, Manoharan R, Koo TW, Shafer KE, Motz JT, Fitzmaurice M, Kramer JR, Itzkan I, Dasari RR, Feld MS. Phys Med Biol. 2000; 45:R1. [PubMed: 10701500]
42. Krafft C, Neudert L, Simat T, Salzer R. Spectrochim Acta A. 2005; 61:1529–1535.
43. Renishaw. Application note from Renishaw diagnostic. 2010; 1:RD/Klarite/001.
44. Matthews Q, Jirasek A, Lum J, Duan X, Brolo AG. Appl Spectrosc. 2010; 64:871–887. [PubMed: 20719050]
45. Swain RJ, Kemp SJ, Goldstraw P, Tetley TD, Steyens MM. Biophys J. 2010; 98:1703–1711. [PubMed: 20409492]
46. Bartusik D, Tomanek B, Lattova E, Perreault H, Fallone G. J Pharmaceut Biomed. 2009; 51:192–201.
47. Knudson W. Am J Pathol. 1996; 148:1721–1726. [PubMed: 8669457]
48. Kultti A, Pasonen-Seppanen S, Jauhiainen M, Rilla KJ, Karna R, Pyoria E, Tammi RH, Tammi MI. Exp Cell Res. 2009; 315:1914–1923. [PubMed: 19285976]
49. Toole BP, Yu Q, Underhill CB. Hyaluronan and hyaluronan-binding proteins. Probes for specific detection. Humana Press; New Jersey: 2001.
50. Kultti A, Karna R, Rilla K, Nurminen P, Koli E, Makkonen KM, Si J, Tammi MI, Tammi RH. J Biol Chem. 2010; 285:22901–22910. [PubMed: 20501660]
51. Rilla K, Tiihonen R, Kultti A, Tammi M, Tammi R. J Histochem Cytochem. 2008; 56:901–910. [PubMed: 18574248]
52. Alkrad JA, Mrestani Y, Stroehl D, Wartewig S, Neubert R. J Pharmaceut Biomed. 2003; 31:545–550.
53. Bansil R, Yannas IV, Stanley HE. Biochim et Biophys Acta Gen Subj. 1978; 541:535–542.
54. Cao Y, Shen D, Lu Y, Huang Y. Ann Bot- London. 2006; 97:1091–1094.

55. Esmonde-White KA, Le Clair SV, Roessler BJ, Morris MD. Appl Spectrosc. 2008; 62:503–511. [PubMed: 18498691]
56. Auvinen P, Tammi R, Tammi M, Johansson R, Kosma VM. Histopathology. 2005; 47:420–428. [PubMed: 16178897]
57. Notingher I. Sensors. 2007; 7:1343–1358.
58. Notingher I, Verrier S, Haque S, Polak JM, Hench LL. Biopolymers. 2003; 72:230–240. [PubMed: 12833477]
59. Ochsenkuhn MA, Jess PRT, Stoquert H, Dholakia K, Campbell CJ. ACS Nano. 2009; 3:3613–3621. [PubMed: 19807067]
60. Yan B, Reinhard BM. J Phys Chem Lett. 2010; 1:1595–1598.
61. Girish KS, Kemparaju K. Life Sci. 2007; 80:1921–1943. [PubMed: 17408700]
62. Varki, A.; Cummings, RD.; Esko, JD.; Freeze, HH.; Stanley, P.; Bertozzi, CR.; Hart, GW.; Etzler, ME. Essentials of Glycobiology. 2. Cold Spring Harbor Laboratory Press; New York: 2009.
63. Yan B, Boriskina SV, Reinhard BM. J Phys Chem C. 2011; 115:24437–24453.
64. Yu Q, Guan P, Qin D, Golden G, Wallace PM. Nano Lett. 2008; 8:1923–1928. [PubMed: 18563939]

Figure 1. Schematic overview of the experimental setup for SERS stamping. The SERS stamp can be raised to contact the cellular surface (inset).

Figure 2. (A) Process flow of the SERS stamp fabrication, (B–E) SEM images of a SERS stamp. Scale bars denote 500 μm in (B), 50 μm in (C), 500 nm in (D) and 200 nm in (E).

Figure 3.
SERS spectra of pMA (A), cholesterol (B), mucin (C)

Figure 4. Ensemble SERS barcodes of pMA (A), cholesterol (B) and mucin (C) (top panel). The bottom panel schematically indicates differences in the size and arrangement of the investigated molecules on the Au surface.

Figure 5.

SEM images of MCF7 cells labeled for HA with 40 nm Au immunolabels obtained after (A) incubation in Hanks' buffer (pH 6) for 10 min (control), (B) incubation with 10 U/mL hyaluronidase for 10 min, and (C) incubation with 600 U/mL enzyme for 30 min. Insets are magnified SEM images on the cell surfaces in each condition. Scale bars denote 2 μm in overview images and 500 nm in magnified images. (D) Average Au NP density (ρ) on the cell surface for the investigated hyaluronidase conditions. Overall, ρ decreases upon addition of hyaluronidase, indicating a successful removal of HA on the cell surface.

Figure 6. SERS spectra from MCF7 cells without hyaluronidase in Hanks' buffer (pH 6) (A1) and after incubation with 10 U/mL hyaluronidase for 10 min (A2) or with 600 U/mL for 30 min (A3). The corresponding barcodes are shown in (B1–B3). The gray areas in (B1–B3) highlight the spectral ranges that show a prominent loss in vibrational transitions.

Figure 7. SERS difference barcode generated by subtracting the control ensemble barcode (no enzyme) from the ensemble barcode obtained after cells treatment with 600 U/mL hyaluronidase for 30 min at 37°C. Grey bars (red fit) indicate spectral features that decrease in intensity due to enzyme treatment, while black bars (blue fit) increase in intensity. Green arrows denote SERS bands that can be assigned to HA. Inset is the chemical structure of HA.