Plasmonic Nanobiosensing: from in situ plant monitoring to cancer diagnostics at the point of care

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Abstract

Nucleic acid biosensing technologies have the capability to provide valuable information in applications ranging from medical diagnostics to environmental sensing. The unique properties of plasmonic metallic nanoparticles have been used for sensing purposes and among them, plasmonic sensors based on surface-enhanced Raman scattering (SERS) offer the advantages of sensitive and multiplexed detection owing to the narrow bandwidth of their characteristic Raman spectral features. This paper describes current applications that employ the unique SERS-based inverse molecular sentinel (iMS) nanobiosensors developed in our laboratory. Herein, we demonstrate the use of label-free iMS nanoprobes for detecting specific nucleic acid biomarkers in a wide variety of applications from cancer diagnostics to genetic monitoring for plant biology in renewable biofuel research.

1. Introduction

The development of sensitive and selective biosensing techniques is of great interest for various applications, ranging from medical diagnostics to environmental monitoring. In regards to medical applications, practical and sensitive diagnostic tools with the capability to detect multiple biomarkers simultaneously are highly desirable [1–4]. Nucleic acid biomarkers have been considered valuable diagnostic biomarkers to monitor the presence and progression of various diseases [5–7]. Recently, much effort has been devoted to developing practical detection strategies for nucleic acid biomarkers, such as DNA, mRNA and microRNA, to overcome challenging analytical aspects in currently adopted laboratory-based methods [8–13].

Raman spectroscopy, which provides narrow spectral features characteristic of the probed analytes, is a powerful tool for specific and multiplexed detection. Surface-enhanced Raman scattering (SERS), which increases the number of Raman scattering photons considerably, enables the application of Raman spectroscopy for extremely sensitive detection. The origin of this enhancement arises primarily from the excitation of localized surface plasmons of metallic nanostructures by incident light. In this process, an intense localized electromagnetic field is generated which can interact with molecules adsorbed on or near the metallic surface, potentially increasing the normally weak Raman scattering process by factors of $10^{7–15}$ [14–18]. Due to the sharp, molecularly-specific Raman peaks, SERS allows for discrimination of multiple targets simultaneously, offering significant advantages over methods such as fluorescence and chemiluminescence, which exhibit broader emission bands [19–23].

SERS research has afforded exciting possibilities for a wide range of biosensing applications; however, in order to generate a strong SERS signal, most existing methods require multiple incubations, post-incubation reactions or washing steps. We have developed a variety of SERS plasmonic substrates and nanoplatforms for chemical and biological sensing over the past several years. In particular, we have demonstrated the highly...
sensitive, specific and multiplexed biosensing using the combination of SERS-active metallic nanoparticles and a unique probe, referred to as the ‘molecular sentinel’ (MS) [24, 25]. The MS probe consists of a silver nanoparticle functionalized with DNA strands having a Raman label molecule at the opposite end. The stem-loop structure of the MS probe recognizes the complimentary target DNA. The MS sequence is designed to form a hairpin loop in the absence of target DNA in order to keep the Raman label close to the surface of the nanoparticle. Under such a configuration, an intense SERS signal is induced upon laser excitation. When complementary target DNA is introduced, hybridization of the target to the MS hairpin loop occurs, physically separating the Raman label from the surface of the metallic nanoparticle, thereby decreasing the SERS signal.

More recently, we have reported the development of a novel homogenous plasmonics-based biosensor, referred to as the ‘inverse Molecular Sentinel (iMS)’ nanoprobe, for the detection of nucleic acid targets with an OFF-to-ON detection scheme [26, 27]. The iMS nanobiosensor, which uses a SERS-platform consisting of silver-coated gold nanostars (AuNS@Ag), utilizes a nonenzymatic strand-displacement process to allow for the conformational change of hairpin probes for target identification, as illustrated in figure 1. Similar to the MS nanoprobe, one end of the stem-loop is immobilized onto the surface of the metallic nanoparticle while the other end is functionalized with a Raman label. The unique aspect of the iMS nanobiosensor is the use of an additional ‘placeholder’ strand that is partially complimentary to the hairpin probe and disrupts the stem-loop structure to keep the Raman label physically separated from the nanoparticle surface. In this configuration, the iMS nanoprobe displays low SERS signal intensity (OFF state). Once the target is introduced, it binds to the toehold overhang of the placeholder and begins displacing the stem-loop probe from the placeholder. Following this branch migration process [28, 29], the placeholder is released, which allows the stem-loop structure to close and move the Raman label close to the plasmonics-active nanostar surface, providing a strong SERS signal (ON state). As the iMS homogenous assay requires no target labeling or subsequent washing steps, this positive-readout platform provides a powerful sensing technique for a variety of applications.

2. iMS nanoprobes for detection of miRNA in complex media

To demonstrate the development of a homogenous assay for the detection of miRNAs, our group previously reported the design of the iMS nanoprobe labeled with Cy5 dye to detect the ubiquitously expressed miR-21. MiR-21 has been well established as an oncogenic miRNA due to its aberrant over-expression in various cancers [30, 31]. For example, miR-21 has been reported to be one of the most significantly upregulated microRNAs for breast cancer and its expression has been associated with tumor progression and poor prognosis [32, 33]. As shown in figure 2, in the absence of miR-21 target, the SERS signal of the iMS-placeholder conjugates was low, indicating that the placeholder strands can effectively turn the signal OFF by keeping the Raman labels away from the nanostar surface (figure 2, spectrum a). The SERS signal remained low in the presence of non-complementary sequences (figure 2, spectrum b), indicating the specificity of the detection method. In contrast, the presence of miR-21 targets (figure 2, spectrum c) provides a significantly increased SERS signal, indicating that the Raman labels were brought close to the gold nanostar surface and thus experiencing the enhanced electromagnetic field. Additionally, previous calibration studies using serial dilutions of total small RNA extracted from MCF-7 cancer cells, which highly
express miR-21, demonstrated the quantitative capability of the analytical approach as the signal response assay was linear in the range of 10–500 ng total small RNA sample with good reproducibility (8.6\% variation) [26]. It was demonstrated that miR-21 could be detected in as little as 10 ng of small RNA [26]. In our recent publication, we demonstrated that our IMS technique is capable of detecting miRNA in RNA extracted from GI tissue samples. Tumor and healthy tissues showed a statistical significance difference in SERS signal, which was also shown to be in agreement with the qRT-PCR performed on the same samples. These results clearly demonstrated that our method can quantify miRNA at concentrations required to detect cancer in tissue biopsies [34].

To investigate the potential of the IMS technique as a point-of-care screening test, the effect of complex solutions, such as serum, on the operation of IMS nanoprobes was investigated. In this study, 1 μm of synthetic target was incubated with miR-21 IMS nanoprobes in a PBS buffer solution containing increasing ratios of PBS:fetal bovine serum (FBS). It was found that increasing the content of FBS caused a decrease in the SERS signal of the IMS in the ON condition (hairpin closed). When incubating the IMS nanoprobes in buffers containing more than 50\% FBS, a 72.4\% decrease in SERS signal was observed as compared to that achieved in PBS, indicating that the sensing capability of the IMS nanoprobes is affected when in the presence of serum. Through this study, it was determined that either the viscosity of the solution or the presence of biomolecules within blood and serum have the potential to affect the IMS sensing mechanism. Development of a porous, gel-based protective system would allow for nucleic acid analysis within complex samples. Hydrogels are highly tunable cross-linked hydrophilic polymers with remarkable features including stability as well as facile synthesis and modification. For these reasons, the naturally occurring polymer, agarose, was selected for protection of the nucleic acid based IMS nanoprobe. It was determined that 5\% w/v low temperature gelling agarose allowed for the most homogenously dispersed particles as well as retained the sensing function of the IMS nanoprobes. Higher temperature gelling agarose caused the dissociation of the placeholder from the stem-loop, requiring addition of placeholder following gel formation with several subsequent washes to remove excess placeholder.

The IMS nanoparticle solution was embedded in a gel matrix of 5\% w/v agarose and evaluated the response in PBS containing miR-21 target DNA strands. As shown in figure 3, when comparing the response of IMS nanoprobes in solution in the presence of 1 μm synthetic miR-21 targets with that of nanoprobes embedded in agarose at the same particle concentration (0.5 nm) in the presence of targets, the Raman peaks of the Cy5 label are maintained and a slightly greater SERS intensity increase is observed.

When studying the potential for gel-embedding to retain the IMS sensing mechanism in complex media, it was found that the signal change of the gel-embedded IMS in the presence of 1 μm synthetic miR-21 targets was only slightly lower (5.7\%) when incubated in FBS as compared to PBS. The gel-embedding allowed for retained IMS functionality in FBS as compared to colloidal IMS, which showed a 72.4\% decrease in SERS signal in FBS compared to PBS. This illustrates the excellent protection capability of a gel matrix, suggesting the potential for a robust system for use in serum samples.

The gel-encapsulated method described herein not only allows for protection of the IMS nanoprobes, but also allowed for in vitro SERS sensing with ultra-high sensitivity, as demonstrated by the capability to detect...
Figure 3. (A) SERS intensity (arbitrary units) of OFF miR-21 iMS nanoprobes in solution (spectrum a) and agarose-embedded miR-21 iMS nanoprobes (spectrum b) at the same particle concentration (0.5 nm). (B) SERS intensity (arbitrary units) of the miR-21 iMS nanoprobes in solution (spectrum a) and agarose-embedded (spectrum b) turned ON by the addition of 1 μM target.

Figure 4. Gel-embedded miR-21 iMS nanoprobes in agarose gel successfully detected sub-picomolar (0.9 pM) synthetic miR-21 target in PBS solution.

sub-picomolar concentration of miRNA. As shown in figure 4, the SERS intensity was significantly increased when incubating the iMS-embedded agarose gel with 0.9 pM synthetic miR-21 target in a PBS solution. Based on the standard deviation of the noise, the limit of detection (LOD) is estimated to be 0.3 pM. The gel not only serves as a protecting platform but also as a concentrator, gathering the targets and confining them to a small space for interacting with the embedded iMS nanoprobes. Additionally, the stability of the gel-encapsulated nanoprobes was investigated and it was found that the functionality was maintained after 3 months of storage at 4 °C.

3. Monitoring genomic targets within plants using iMS nanoprobes

3.1. In vitro detection of RGA within RNA extracts from transgenic Arabidopsis thaliana

We have developed iMS nanoprobes to detect the mRNA of the Arabidopsis RGA gene, which plays a critical role in controlling plant biomass and production. The RGA gene, belonging to a 5-gene DELLA family, encodes an important transcriptional regulator repressing the gibberellin (GA) signal transduction pathway in Arabidopsis. The phytohormone GA promotes vegetative and reproductive growth in plants by triggering rapid degradation of nuclear transcription regulators DELLA via the ubiquitin-proteasome pathway [35–40]. Recent studies further indicate that DELLA functions as master growth repressors by integrating GA signaling with multiple other signaling pathways in response to internal developmental cues and external biotic and abiotic signals [41, 42]. Therefore, the development of DELLA-specific nanoprobes can provide useful tools to monitor spatial and temporal regulation of DELLA expression during plant growth.

The detection of the RGA mRNAs using iMS nanoprobes was demonstrated by examining total RNA samples isolated from a transgenic Arabidopsis line, which overexpresses RGA after 2 h steroid hormone dexamethasone (DEX) treatment. RNA samples extracted from wild-type and mock-treated plants were used as controls. The iMS nanoprobes were incubated with 5 μg of total RNA samples for 2 h at room temperature followed directly by SERS measurements. Figure 5 shows that the SERS intensity from the transgenic
Figure 5. *In vitro* detection of RGA mRNA within total RNA extracted from wild-type (WT-RNA), transgenic control with no DEX treatment (Mock-RNA), and DEX-induced (DEX-RNA) Arabidopsis.

Figure 6. (A) Raman map (background subtracted, 524 cm\(^{-1}\)) superimposed over the transmission image of the leaf infiltrated with ‘OFF’ miR-156 iMS nanoprobe. The figures on the right contain spectra from two different pixels within the Raman intensity map: one from an area of the leaf that was not infiltrated with particles (B), and one from a region infiltrated with ‘OFF’ miR-156 iMS nanoprobe (C).

Figure 7. (A) Raman map (background subtracted, 524 cm\(^{-1}\)) superimposed over the transmission image of the leaf infiltrated with ‘ON’ miR-156 iMS nanoprobe. The figures on the right contain spectra from two different pixels within the Raman intensity map: one from an area of the leaf that was not infiltrated with particles (B), and one from a region infiltrated with ‘ON’ miR-156 iMS nanoprobe (C).

Arabidopsis treated with DEX was significantly higher than that from wild type and transgenic control without DEX treatment, indicating that more mRNA targets were detected in the DEX treated sample. The results demonstrated that the washing-free and amplification-free iMS nanoprobe has potential to be used as selective, non-perturbative probes to measure the spatial and temporal expression of signaling molecules in the investigation of bioenergy-relevant plant systems.

3.2. SERS-based imaging of iMS nanoprobe within plant tissues

In order to develop SERS-based functional imaging, the iMS sensing modality was integrated with an imaging system [43]. The eventual goal of this work is to monitor specific nucleic acid biomarkers within living plants with the temporal and spatial resolution required to understand the mechanisms behind the
regulatory role of targets of interest. Specifically, a better understanding of the regulatory role of miRNA would enhance the bioengineering of plants as better bioenergy sources.

To demonstrate the in vivo feasibility of SERS imaging in plants, iMS nanoprobes designed for miR-156, a critical miRNA that regulates plant flowering time, were tagged with Cy7 labels and infiltrated into an Arabidopsis leaf using needleless injection. One leaf was infiltrated with iMS nanoprobes in the open configuration (iMS 'OFF') while another leaf was infiltrated with iMS nanoprobes that had been turned ‘ON’ by incubating with the synthetic target before washing and infiltration. A Raman microscope was built in house which comprised of an inverted microscope coupled to a 785 nm diode laser (300 mW) focused on the image plane of the microscope objective (laser spot = 500 µm; resolution 600 µm). Raman maps of the infiltrated leaves were measured using this lab-built Raman microscope.

Figure 6 provides the results following infiltration of ‘OFF’ miR-156 iMS nanoprobes into an Arabidopsis leaf. Following infiltration, the iMS nanoprobes spread from the infiltration spot. The infiltrated area is visible in the inset of figure 6(A) as the lighter (more translucent) region. Figure 6(A) also displays the Raman map of the 524 cm\(^{-1}\) background-subtracted peak superimposed over the transmission image of the leaf. Figure 6(B) provides the spectra (background subtracted) from a single pixel of a region of the leaf where there were no particles while figure 6(C) shows the Raman signal of an infiltrated region of the leaf. While the spectrum of the infiltrated area shows some characteristic peaks of Cy7, those visible in a region infiltrated with iMS ‘ON’ nanoprobes exhibit a much stronger Raman signal, as seen in figure 7. These results suggest the potential to obtain a SERS map of a leaf which reveals the locations of iMS nanoprobes within the leaf.

4. Conclusion

The SERS-based iMS detection mechanism with positive-readout (OFF-to-ON) does not require target labeling and any subsequent washing steps, providing a versatile and powerful tool for a wide variety of applications. By embedding iMS nanoprobes in a gel matrix, we have demonstrated protection of the nanoprobes from components of complex biological fluids. The sensing mechanism of colloidal iMS nanoprobes was maintained within the gel matrix and increased detection sensitivity was observed. Future studies will investigate the clinical applicability of gel-embedded iMS for POC diagnostics by comparing this method with qRT-PCR. As illustrated herein, the iMS nanoprobe has demonstrated applicability towards cancer diagnostics as well as genetic monitoring in plants. The SERS imaging capability of iMS nanoprobes within leaves lays the foundation of functional imaging of nucleic acid targets in plants for use in research related to biofuel production.

5. Experimental section

5.1. Materials

1 N hydrochloric acid solution (HCl), L(+)-ascorbic acid (AA), trisodium citrate dihydrate, Gold(III) chloride trihydrate (HAuCl\(_4\)· 3H2O), Silver nitrate (AgNO\(_3\), 99.995%), Tween-20, Dulbecco’s phosphate buffered saline (PBS), 6-mercapto-1-hexanol (MCH), and sodium borohydride (NaBH\(_4\)) and agarose were purchased from Sigma-Aldrich (St. Louis, MO). NHS-Cy7 was purchased from Lumiprobe (Hunt Valley, MD). Ammonium hydroxide (NH\(_4\)OH, 29.5%), 1 ml disposablesyringes, 27G × 1/2 in. needles, and carbon-coated copper TEM gridswere obtained through VWR (Radnor, PA). All glassware and stir bars were cleaned using aqua regia. Ultrapure water (18 MΩ · cm) was used in all preparations. All oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

5.2. Oligonucleotide sequences

5.3. Silver-coated gold nanostar synthesis

Gold Nanostars (AuNS) were prepared as previously described \[44\]. Gold seed (12 nm) solution was prepared using a modified Turkevich method. To prepare AuNS, 10 ml of 0.25 mm HAuCl\(_4\) containing 10 µl of the gold seed solution was put under stirring at room temperature. To the stirring solution 50 µl of 2 mm AgNO\(_3\) was added quickly followed by 50 µl of 0.1 m ascorbic acid. A color change from a light yellow to dark blue within 5 s was observed, indicating formation of AuNS. Nanoparticle tracking analysis (NTA) found the stock concentration of AuNS to be approximately 0.1 nm.

Silver-coated gold nanostars (AuNS@Ag) were synthesized using a previously described protocol \[45\]. To synthesize AuNS@Ag, unfunctionalized AuNS were kept stirring and 50 µL of 0.1 m AgNO\(_3\) was added to the solution followed by 10 µL of NH\(_4\)OH. The color of the solution changed from blue to dark orange. No purification was required after obtaining AuNS@Ag. The AuNS@Ag were functionalized 2 h after synthesis.
Table 1. iMS oligonucleotide sequences used for the indicated target nucleotide.

| miR-21                  | miR-156                  |
|-------------------------|--------------------------|
| Stem-loop               | Stem-loop               |
| thiol-AAAAAGTCTGTAATAAATAGCTTA | thiol—AAAAAAGCTACCAAAAAAAATACTATGGAG |
| TCA GAC—Cy5             | AAG AGA—amine           |
| Placeholder             | CTCACTCTCTTCTGTCATTTTT   |
| DNA target              | TGACAGAAGAGAGTGAG        |
| RNA target              | 5.4. Preparation of SERS iMS nanoparticles  |
|                         |                         |
| iMS nanoparticles were designed as detailed in Table 1. To prepare the miR-156 iMS nanoprobe, the 3'-amine-terminated stem-loop strand was bound to Cy7 NHS ester following the manufacturer suggested procedure. The NHS-Cy7 was incubated with the amine-terminated MS sequence in a pH 8 bicarbonate buffer under constant stirring for 4 h. A desalting column (NAP-5; GE Healthcare, Little Chalfont UK) was used to purify this solution. This solution can be stored for up to 30 d at −4 °C.  |
|                         |                         |
| The iMS nanoparticles were synthesized as previously described. Briefly, 0.9 ml of 0.1 nm AuNS@Ag solution was placed into a new centrifuge tube followed by the addition of 10  µL of 20  µm stem-loop DNA probe solution and 0.1 ml of 2.5 mm MgCl2 solution. This solution was then incubated overnight at room temperature. O-(2-[3-Mercaptopropionylamino]ethyl)-O’-methylpolyethylene glycol (mPEG-SH, 5000) was added to the solution at final concentration of 1  µm for 30 min to stabilize the iMS nanoparticles, before centrifugal wash (3500 rcf, 10 min) and resuspension in 1 ml of Tris-HCl buffer (10 mm, pH 8.0) containing 0.01% Tween-20. To passivate the nanoparticle surface, 0.1 mm 6-mercapto-1-hexanol (MCH) was added to the solution for 10 min at 37 °C. The nanoparticles were then washed in Tris/Tween-20 buffer using repeated centrifugation (three times, 3500 rcf, 10 min). The purified nanoparticles were finally re-dispersed in Tris-HCl-Tween-20 buffer.  |
|                         |                         |
| 5.5. SERS iMS assay procedure  |
| The iMS nanoparticles were placed in PBS buffer solution containing 0.01% Tween-20 (0.1 nm particle concentration) prior to addition of 0.2  µm placeholder strands. The solution was incubated for 1 h at 37 °C to turn the iMS signal off. The excess placeholders were removed using repeated centrifugation (3500 rcf, 10 min), and re-dispersed in PBS/Tween-20 buffer. The ‘OFF’ iMS nanoparticles (0.05 nm) was then incubated with target analytes at 37 °C for 1 hr. SERS measurements were completed using a Renishaw InVia confocal Raman microscope equipped with a 632.8 nm HeNe laser. The light from the laser was passed through a laser line filter, and focused into the sample solution with a 10x microscope objective.  |
| For tests using total small RNA extracted from patient tissue biopsies, 2  µL of the iMS solution (10 pM) was incubated with 200 ng of total small RNA samples in PBS/Tween-20 buffer at room temperature for 1 h. Mineral oil was added to the mixture to prevent evaporation. After the reaction, the mixture was transferred to a glass capillary tube for SERS measurement. Three SERS measurements were performed per iMS assay and averaged into a single spectrum. The SERS iMS assay was completed three times for each sample (nine total measurements). All SERS spectra reported here were background subtracted and smoothed using a Savitsky-Golay filter.  |
| 5.6. Agarose gel-encapsulation of iMS nanoparticles  |
| To protect the nanoparticles, we designed a strategy to embed the iMS nanoparticles within an agarose gel matrix (5% agarose w/v in PBS). The duplex formed between the miR-21 iMS stem-loop probe and the placeholder has a melting temperature of 44.7 °C. Using 2-Hydroxyethyl agarose with a gelling temperature
of 26 °C–30 °C, iMS in the open configuration (iMS OFF) could be mixed with agarose in liquid form without the possibility of the placeholder dissociating from the stem-loop iMS probe. Upon cooling below 26 °C, a gel forms that contains OFF miR-21 iMS nanoprobe.

5.7. Plant material and growth conditions
Arabidopsis thaliana ecotype Ler (wild type) as well as rosette leaves from 4 week old Arabidopsis thaliana ecotype Columbia (Col-0) were used. Seeds were sown directly on soil (Pro-Mix B Biofungicide, Premier Horticulture) in growing trays with 10 cell inserts. Plants were grown at 22 °C under long day conditions (16 h light, 8 h darkness). The transgenic line DEX-RGA was generated by the floral-dipping method using Agrobacterium tumefaciens strain GV3101 pMP90 harboring plasmid pRG218 [46].

5.8. Plasmid construction
To generate the DEX-inducible RGA construct (pRG218), the complete coding sequence of the Arabidopsis RGA gene was cloned into the binary vector pTA7001 as previously described [47, 48].

5.9. DEX treatment, RNA purification
Seeds of wild type or the transgenic line DEX-RGA were germinated on half-strength MS plates supplemented with 1% sucrose in a growth chamber at 22 °C under continuous light (85 µmol m–2 s–1). Eight-day old of seedlings were sprayed until runoff with either 0.01% Tween-20 (Sigma-Aldrich, St. Louis, MO) (mock treatment) or with 10 µm dexamethasone (Sigma-Aldrich, St. Louis, MO) plus 0.01% Tween-20 (DEX treatment). The excess solution was removed and all plates returned to the growth chamber. Seedlings were harvested 2 h after treatment, blotted with paper towels and flash-frozen in liquid nitrogen. Samples were ground to fine powder in liquid nitrogen and stored at −80 °C. Total RNA was extracted from 100 mg of seedling powder with Quick-RNA MiniPrep kit (Zymo Research, Irvine, CA), according to the manufacturer’s instructions.

5.10. Arabidopsis leaf infiltration
Arabidopsis leaves were infiltrated on the abaxial side with ‘OFF’ or ‘ON’ iMS nanoparticles using a needleless syringe. The wax cuticle was gently removed to reduce the pressure required for infiltration, further reducing the potential for damage. Surface particles were washed off of the leaf with deionized water. Leaves were kept in a humid environment prior to imaging.

5.11. Raman measurements and maps
A lab-built Raman setup, comprised of a 300 mW, 785 nm diode laser (Optoeengine Midvale, UT) coupled to an inverted microscope (Ti-U; Nikon Instruments Inc. Melville, NY) focused into the image plane of the microscope objective (laser spot = 500 µm; 2X NA = 0.1; Thorlabs, Newton NJ). The laser was passed and reflected through a filter cube which contains a laser line filter, dichroic mirror and notch filter (Semrock, Rochester, NY). The Raman spectra were collected using a spectrometer (1200 grooves mm–1 grating) connected to a CCD camera (LS785 and Pixis100; Princeton Instruments, Trenton, NJ). The Raman images (600 µm spatial resolution) were obtained through critical illumination of the laser collimated from a fiber optic. To correlate the Raman and transmission images, a bright field image was taken before each map using a CCD camera (ProEM 512B; Princeton Instruments) connected to the microscope. The Raman maps and spectra were performed using 10 accumulations of 100 ms. Raman spectra were background subtracted and smoothed using a Savitsky-Golay filter (Matlab, five-point window and first-order polynomial).

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