SERS study on myeloperoxidase and its immunocomplex: Identification of binding interactions

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Abstract. Surface Enhanced Raman Spectroscopy (SERS) has demonstrated significant benefit in the identification of biological molecules. In this paper we have examined how to identify and differentiate the 150 kDa protein myeloperoxidase (MPO) from its corresponding antibody (Ab) and their immunocomplex through the use of SERS. The SERS signal of these biological molecules was enabled by 40 nm gold nanoparticles. The SERS spectra for both MPO and the Ab (an IgG molecule) demonstrated results consistent with previous published work on the Raman spectra of MPO and IgG antibodies. The immunocomplex SERS spectra showed peak shifts and intensity variations that could be attributed to conformational changes that occur during immunocomplex formation. Several key spectral areas have been identified which correspond to specific amino acids being shielded from undergoing resonance while new amino acid residues are made visible in the SERS spectrum of the immunocomplex and could be a result of conformational binding. These results indicate that SERS can be used to identify binding events and distinguish an immunocomplex from its individual components.

Keywords: SERS, myeloperoxidase, antibody, IgG, immunocomplex, gold nanoparticles

1. Introduction

Raman scattering, first noted by C.V. Raman in 1928 [27], is the basis for Raman spectroscopy, a widely used molecular material characterization method. A major challenge in Raman spectroscopy is separating the weak inelastic Raman scattering signal from the overwhelmingly dominant elastic Rayleigh scattering. The ratio of photons undergoing Rayleigh scattering to those experiencing Raman scattering is $\sim 10^{16}$ to 1.

Surface-enhanced Raman spectroscopy (SERS) provides a solution to this challenge by enhancing the signal intensity of Raman scattering of molecules adsorbed to rough metal surfaces by as much as $10^{15}$ [22]. Fleischman et al. [11] first noted this enhanced effect in 1973 while observing the adsorption of pyridine on a silver electrode. Later Jeanmarie et al. [16] and Albrecht et al. [2] independently realized this as a unique phenomenon and proposed an electromagnetic mechanism and a chemical mechanism, respectively, as theoretical explanations for the enhanced signal. SERS substrates frequently used include

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gold nanoparticles (GNPs) and silver nanoparticles because of their high sensitivity and stability [15,18, 19,33].

In 1975, Painter et al. [24] observed variations in the Raman signal of an antigen, its corresponding antibody (Ab), and their immunocomplex, and credited these variations to the conformational changes during binding. Using an excitation wavelength of 514.5 nm they examined the Raman spectrum of both lyophilized and solution forms of human IgG, human IgM, and rabbit IgG and found that both IgG and IgM produce similar spectrum in the Amide I and III regions (assigned to Raman wavenumbers regions of 1673 and 1259 cm$^{-1}$, respectively). The strong intensities of Amide I and III bands indicate that these immunoglobulins are predominately composed of $\beta$-sheets. They also compared the Raman signal of the lyophilized forms of rabbit anti-ovalbumin Ab and the immunocomplex of rabbit anti-ovalbumin Ab with the corresponding antigen, ovalbumin. The most significant change in the Raman signal of the immunocomplex is a decrease in the Amide III region (1259 cm$^{-1}$) that is attributed to the binding of ovalbumin and its Ab.

Fagnano et al. [7,8] also characterized the Raman spectrum signals of lyophilized and solution forms of rabbit IgG and anti-IgG and compared them to the lyophilized form of the anti-IgG and rabbit IgG immunocomplex. Similar to Painter et al., using an excitation wavelength of 514 nm, they found IgG to contain mostly $\beta$-sheets. The anti-IgG Ab produced more intense Raman peaks compared to the Raman intensity of the rabbit IgG antigen spectrum. Upon formation of the anti-IgG and rabbit IgG immunocomplex the most noted Raman peak shift occurred between 450–530 cm$^{-1}$, which is attributed to conformational changes through vibrations at the disulfide bonds.

In 1988, Rohr et al. [28] first used SERS to observe Ab-antigen interactions. Using an excitation wavelength of 457.9 nm, they observed a proportional increase in the SERS signal intensity when increasing the concentration of thyroid stimulating hormone (TSH) that was binding to anti-TSH Ab on chemically coated silver surfaces.

More recently, Zhang et al. [37] investigated the effects of SERS signal changes during immunocomplex formation. Mouse IgG and goat anti-mouse IgG were each immobilized on silver nanoparticles grown on silicon nanowires; the immunocomplex was formed by adding both antigen and Ab together so they could react on the silver nanoparticle/silicon nanowire substrate. The mouse IgG antigen demonstrated a more intense Raman signal with peak shifts compared to the goat anti-mouse IgG Ab. The immunocomplex exhibited new Raman peaks, distinct from both the antigen and Ab peaks, predominately in the 1100–1700 cm$^{-1}$ wavenumber region.

Subsequent groups used similar techniques with novel variations in order to increase sensitivity of the system, as well as promote more rapid results with fewer steps. Hawi et al. [14] used SERS to detect specific membrane bound enzymes within cells and correlated prostaglandin-H-synthase antigen levels to SERS signal intensity. Dou et al. [6] used near infrared (excitation wavelength of 1064 nm) SERS to detect the conformational binding of anti-mouse IgG (bound to GNPs of 29.7 nm diameter) to mouse IgG antigen. They observed significant increase in the amide I and III bands at 1645 and 1261 cm$^{-1}$, respectively (similar to the work of Painter et al. [24]), when the immunocomplex was formed and an increase in tryptophan residues at 1467, 1112 and 880 cm$^{-1}$.

Myeloperoxidase (MPO) is a lysosomal protein found in neutrophilic granulocytes that has an instrumental role in attacking bacteria and foreign pathogens through chemical reactions. MPO produces both hypochlorous acid and tyrosyl radicals in independent pathways, which are both cytotoxic. Hypochlorous acid is produced from the oxidation of chloride by hydrogen peroxide, while the tyrosyl radical is produced through the oxidation of tyrosine by hydrogen peroxide. It is related to other mammalian per-
oxidases like eosinophil peroxidase, lactoperoxidase, thyroid peroxidase and prostaglandin H synthase [5,17,21].

The crystal structure of human MPO was first reported in 1995 [9] at a 2.3 Å resolution. Later the crystal structure of the native (oxidized) form of MPO, as well as MPO bound to bromide, chloride [10] and cyanide (–CN) [4] have been analyzed at higher resolutions of 1.9 and 1.8 Å. MPO is a 150 kDa dimer composed of two identical halves, each with a covalently bound heme. The two identical halves of MPO are connected by a lone disulfide bond. MPO is composed of two identical sets of polypeptides that are 108 and 466 amino acids long. Their secondary structure is composed predominately of α-helices with very little β-sheets. The heme is bound to MPO by two ester linkages of the carboxyl group of Glu 242 and Asp 94 to the methyl groups on the pyrrole rings A and C (positions 1 and 5, respectively). The terminal β-carbon on the vinyl group of the pyrrole ring A (position 2) connects the heme to a sulfonium ion linkage at the sulfur atom of Met 243 [4]. In addition to the heme group located on each half of the MPO molecule there is a bound calcium ion and three Asn-linked glycosylations (at Asn 189, Asn 225, Asn 317) [36].

Sibbett et al. [30] examined the structure of canine MPO using resonance Raman spectroscopy (RRS). They examined the effects of varying laser excitation wavelength (406 and 454 nm) and temperature (77 and 273 K) on the spectra of MPO. They report that the hemes contain no formyl substituents in conjugation with the macrocyclic ring. Additionally they conclude that MPO contains two equivalent chlorin prosthetic groups.

Babcock et al. [3] purified MPO from human white blood cells and characterized its resonance Raman spectrum. They found native MPO adopts a six-coordinate high-spin configuration. MPO proved stable under 457.9 nm laser excitation and did not photoreduce under illumination. In agreement with Sibbett et al., they did not find any formyl substituents, nor did they find any protonated Schiff’s base groups.

Puppels et al. [26] acquired the Raman microspectroscopic signatures of the nucleic and cytoplasmic regions of human neutrophilic, eosinophilic, and basophilic granulocytes. MPO’s Raman spectrum is so dominant that when compared to the spectrum of a neutrophilic granulocyte there is very little difference between the two. This similarity in spectra occurs despite the fact that MPO accounts for only 5% of the dry weight of a neutrophilic granulocyte [29].

Resonance Raman analysis was conducted on neutrophilic granulocytes, MPO-deficient neutrophilic granulocytes, cytochrome b558-deficient neutrophils, and human MPO isolated and purified from leukocytes at laser excitation wavelengths of 413.1 nm [32] and 457.9 nm [31]. Similar to Puppels et al., Sijtsema [31] also recognized the overwhelming impact of MPO on the Raman spectrum of neutrophilic granulocytes. The MPO-deficient neutrophils further exemplify the influence of MPO on the Raman signal by displaying a significantly altered spectrum when compared with normal neutrophilic granulocytes and purified MPO.

Confocal Raman microspectroscopy of MPO by Otto et al. [23] provided another verification of the MPO Raman signal with similar peak positions as previous groups.

Here we report, for the first time, the SERS spectra of MPO, its Ab, and their immunocomplex immobilized on gold nanoparticles. We have identified bands that are unique to the immunocomplex of MPO and its Ab and explore the use of such bands as signatures of binding events.

2. Experimental methods

All chemicals were purchased from Sigma Aldrich unless otherwise mentioned, Ab (polyclonal rabbit anti-human myeloperoxidase) was purchased from ABD-Serotec and MPO was purchased from Lee
Gold nanoparticles of 40 nm diameter were prepared according to Frens [12] with added modifications. Briefly, 500 µl of 1% chloroauric acid were added to 50 ml of distilled H₂O and heated to a boil. 1% citric acid was added to the solution at various volumes to control the particle size. The solution was refluxed until the color changed from dark blue to red. The solution was then cooled to room temperature, dialyzed to remove excess citrate ions, and characterized with UV-vis spectroscopy [13], atomic force microscopy, and scanning electron microscopy.

The GNPs were then immobilized on silane functionalized glass slides that were prepared according to the method of Park et al. [25]. In brief, glass slides were cleaned using piranha solution (1:3 v/v, H₂O₂:H₂SO₄) and dried under nitrogen. Cleaned glass slides were then immersed in 3% 3-aminopropyltrimethoxysilane in methanol for 3 h. Silanized slides were then rinsed thoroughly with methanol followed by DI water and dried with a jet of dry nitrogen. Silanized glass slides were then immersed in the dialyzed GNPs solution for 3 h. Slides with immobilized GNPs were further washed with DI water and air dried. A ∼100 µl well was constructed on top of the glass slide to facilitate Ab immobilization and subsequent SERS data collection.

Antibody was immobilized by letting 100 µl of 100 nM Ab solution interact with the GNP coated slides for 15 min at room temperature followed by thorough washing with 1× PBS buffer (pH 7.4). GNPs coated with Ab were then allowed to interact with 100 µl of 1 µM MPO for 15 min at room temperature followed by washing. A similar procedure was followed to immobilize MPO directly on GNPs. The Ab and MPO physically adsorbed to the surface of the GNPs. SERS data was collected from the GNP-Ab conjugates, GNP-immunocomplex, and GNP-MPO conjugates immediately after preparation using a Renishaw RM1000 confocal Raman microspectrometer with a 50× long focal microscope. The Raman microspectrometer has a spectral resolution of 1 cm⁻¹. A 785 nm diode laser (15 mW) was used to collect the SERS signal. Care was taken to maintain ∼50 µl of PBS buffer to minimize thermal damage and the SERS data was collected from an area of ∼40 × 40 µm square located at the center of the well. SERS signal from glass slides coated with GNPs was also collected and served as the background signal.

An average of 75 spectra per sample were obtained and subjected to a three point baseline correction. The three points for baseline correction were kept the same for all spectra and samples, typically the first and last point corresponding to the beginning and end of the wavenumbers of the spectra and the third point being set at 1369 cm⁻¹. This baseline correction applied to all data sets helped smooth the data and allow an appropriate comparison. Following the baseline correction individual spectra were normalized and averaged. In order to remove the GNP signature from the spectra, the average GNP spectrum set was subtracted from averaged spectra of Ab, MPO and the immunocomplex.

3. Results and discussion

The Ab, MPO, and immunocomplex spectra shown in Fig. 1a–c, respectively, are baseline corrected by subtracting the spectra of the GNP, which exhibit the typical fluorescence of GNPs under experimental conditions [15,18,19,33]. The spectra are averaged from the raw data (not shown) that demonstrated consistency of observed peak positions and peak widths.

The SERS spectrum of MPO (Fig. 1b) exhibits strong similarities to the Raman signals reported in previous studies [3,23,26,30,31]. It is relatively weak below 1100 cm⁻¹, besides three small peaks between 646 (ν₄/ν₂₅) and 684 cm⁻¹ (ν₇), at 835 cm⁻¹ and between 992 (γ(CH)) and 1007 cm⁻¹ (ν₄₅). The minor peak assignments in our study are in excellent agreement with Zbylut et al., including
the vibrational modes at $\nu_{45}$ (1007 cm$^{-1}$), $\gamma$(CH) (992 cm$^{-1}$), $\nu_{46}$ (925 cm$^{-1}$), $\gamma_{10}$ (853 cm$^{-1}$), $\gamma_{15}$ (710 cm$^{-1}$), $\nu_{7}$ (684 cm$^{-1}$) and $\nu_{48}/\nu_{25}$ (646 cm$^{-1}$) [35]. The MPO spectrum from 1120 to 1260 cm$^{-1}$ is also similar to previously published resonance Raman spectra. The major significant peaks at 1137 ($\nu_{44}$), 1214 ($\nu_{13}$) and 1251 cm$^{-1}$ ($\nu_{42}$) are consistent with previous literature on resonance Raman spectra of MPO [3,23,26,30,31]. Our work using a 785 nm excitation wavelength produced results similar to Raman studies of previous groups that used different excitation wavelengths (ranging from 406.7 to 660 nm) [3,23,26,30,31]. This suggests that the key identifying MPO peaks are independent of the excitation wavelength, or the Raman method (RRS or SERS) that was used to collect the data. The SERS spectrum of the Ab has characteristic peaks similar to previously obtained Raman signals of an IgG [7,8], as expected given the structural similarities of an IgG molecule.

In Fig. 1, beginning at 500 cm$^{-1}$ and working towards 1650 cm$^{-1}$ we observe distinct peak shifts, altered intensities, and unique peaks when comparing MPO, Ab and their immunocomplex. Signal intensity alone may not be sufficient to differentiate between biomolecules especially in the SERS mode (amplification factors and specific binding may alter the signal intensity significantly) [1]. It may rather
be a combination of signal intensity and peak position that can provide a more reliable means for identifying a given sample. The differences shown in Fig. 1 suggest that the immunocomplex formation could result in changes of conformation, orientation of bonds, and shifts in the functional groups within the plasmon resonance distance of the GNP. New peaks at 871, 1109 and 1465 cm\(^{-1}\) present in the immunocomplex and not found in either MPO or Ab alone, indicate a tryptophan moiety now made visible in the SERS spectrum, possibly a result of conformational changes after binding. Furthermore, new peaks at 969, 984, 1146 and 1500 cm\(^{-1}\) which are not found in the MPO or Ab signal, indicate the immunocomplex formation. In the Tyr and Amide III region, a peak shift in the Ab spectra from 1276 to 1266 cm\(^{-1}\) in the immunocomplex may also correspond to conformational changes.

Recently Yu et al. [34] employed SERS for studying the effect of charge on the orientation of cytochrome c and concluded that the molecules have random orientation on a bare gold nanohole surface. It is also well known that the amplification factor of the SERS substrate largely depends on the crystal facets to which the molecules are absorbed. In the present study, it is highly probable that orientation and the crystal facet of the GNPs were totally random. Since the SERS data was collected over a large surface area compared to the size of a cluster of particles it would be important to identify the influence of the crystal orientation on the obtained SERS data, especially on the new peaks. We believe it is possible to minimize these variations by improving the homogeneity of the SERS substrate using the methods proposed by Liu et al. [20].

4. Conclusions

In summary, we investigated the potential application of SERS in differentiating the bound immunocomplex of an antigen and its Ab from the unbound complex and its components using MPO as the model antigen. Obtained results indicate that the SERS spectrum of the immunocomplex is different from that of its parent antigen or Ab, and it is possible to identify conformational changes due to immunocomplex formation.

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