Research Article

Determination of the Immunoglobulin G Spectrum by Surface-Enhanced Raman Spectroscopy Using Quasispherical Gold Nanoparticles

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Background. Immunoglobulins (Ig) are glycoprotein molecules produced by plasma cells in response to antigenic stimuli involved in various physiological and pathological conditions. Intravenous immunoglobulin (IVIG) is a compound whose composition corresponds to Ig concentrations in human plasma, predominantly IgG. It is used as a replacement treatment in immunodeficiencies and as an immunomodulator in inflammatory and autoimmune diseases. The determination of IgG concentrations is useful in the diagnosis of these immunodeficiencies. Surface-enhanced Raman spectroscopy (SERS) is a technique that allows protein quantification in a fast and straightforward way. Objective. This study is aimed at determining the Raman spectrum of IVIG at physiological concentrations using quasispherical gold nanoparticles as a SERS substrate. Methods. We initially determined the Raman spectrum of IVIG at 5%. Subsequently, for SERS’ characterization, decreasing dilutions of the protein were made by adding deionized water and an equal volume of the 5 nm gold quasispherical nanoparticle colloid. For each protein concentration, the Raman spectrum was determined using a 10x objective; we focused the 532 and 785 nm laser on the sample surface, in a range of 500-1800 cm⁻¹, with five acquisitions and an acquisition time of 30 seconds. Results. We obtained the IVIG spectrum using SERS up to a concentration of 75 mg/dl. The Raman bands correspond to aromatic amino acid side chains and the characteristic beta-sheet structure of IgG. Conclusion. The use of 5 nm quasispherical gold nanoparticles as a SERS substrate allows for detecting the Raman spectrum of IVIG at physiological concentrations.

1. Introduction

Immunoglobulins (Ig), also known as antibodies, are glycoprotein molecules produced by plasma cells in response to antigenic stimuli involved in various physiological and pathological conditions. The primary function of immunoglobulins corresponds to the adaptive immune response. They are subdivided, depending on the structure of the heavy chains they contain, into several classes: IgM, IgG, IgD, IgA, and IgE. IgG is also subdivided into IgG1, IgG2, IgG3, IgG4 (decreasing order of abundance), and IgA in IgA1 and IgA2. IgG is the most abundant, with a plasma concentration of 700-1600 mg/dl, and constitutes 75 to 80% of all Ig. IgA corresponds to about 15%, with a plasma concentration of 70-400 mg/dl, while IgM’s plasma concentration varies from 40 to 230 mg/dl. The determination of immunoglobulin
concentrations is useful in diagnosing immunodeficiencies and evaluating the response to treatment in these patients [1, 2].

Intravenous immunoglobulin (IVIG) is an immunoglobulin concentrate derived from thousands of healthy donors (no fewer than 3500). IgG plays a fundamental role in adaptive humoral immunity. Therefore, IVIG reflects the donor population’s collective exposure to the environment and is expected to contain a repertoire of multiple specific antibodies against a broad spectrum of infectious agents (bacterial and viral), self-antigens, and anti-idiotype antibodies.

The IVIG composition corresponds to the Ig concentrations in human plasma, mainly IgG, IgA, and traces of other Ig, cytokines, and soluble receptors.

IVIG is prepared using the Cohn-Oncley fractionation procedure, precipitating donor plasma with cold ethanol to enrich the IgG fraction, followed by chromatography purification [3]. Commercial products vary concerning the presence of excipients used to stabilize proteins and prevent the aggregation of IgG (such as glucose, maltose, D-sorbitol, or amino acids such as glycine or proline), as well as the levels of sodium, pH, osmolarity, and the presence of another Ig [4].

IgG comprises more than 90% of the different commercial IVIG preparations and is the main component required to observe this drug’s therapeutic effects [5]. IVIG treatment is aimed at providing sufficient IgG antibodies that passively neutralize or opsonize a broad spectrum of infectious pathogens and trigger the activation of cell-mediated immunity. The indications for IVIG administration can be classified according to the mechanism of action and the underlying disease: replacement therapy in immunodeficiencies, immunomodulatory therapy (in hematological and organ-specific autoimmune diseases), an anti-inflammatory agent (in rheumatic, infectious, inflammatory, and neurological conditions).

Different doses are administered according to the medical condition being treated. In general, low doses are used in replacement therapies and higher doses when an immunomodulatory or anti-inflammatory effect is required [6].

Raman spectroscopy is a nanocharacterization technique based on the inelastic dispersion of molecular systems, which are illuminated with monochromatic radiation; it changes frequency due to the energy exchange that exists with the matter [7, 8]. Raman spectroscopy provides information on the primary, secondary, and tertiary structure of proteins by identifying associated characteristic bands. By allowing the structural characterization of proteins, it can detect pathological changes in them [9]. The application of the Raman characterization technique in biomedicine is an advance in the detection of biomarkers through noninvasive methods; however, it shows limitations because the signal from various proteins is weak. Due to this, the technique of surface-enhanced Raman spectroscopy (SERS) uses nanostructured surfaces of noble metals such as gold and silver. This technique allows the standard Raman scattering signal to be amplified in a factor from $10^4$ to $10^{16}$ [10]. It has been used to diagnose some diseases and identify contaminants and pathogens, among other applications [8, 10, 11]. It is a non-destructive characterization technique that can be used in aqueous media [12]. SERS could be an alternative to the ELISA test, an immunological assay in which an enzyme is used as a biomarker [13]. The first protein spectra reported were the spectra of hemoglobin and cytochrome C in 1972 [14]; later, spectra of other biological molecules were reported [15].

This study is aimed at determining the Raman spectrum of IgG (IVIG) using 5 nm quasispherical gold nanoparticles as a SERS substrate.

2. Material and Methods

We purchased intravenous normal human immunoglobulin 5% (Octagam® 5%, Octapharma Pharmazeutika Produktionsgesellschaft, m.b.H. Vienna, Austria). It contains 6 g of total protein, 95% unmodified IgG, 12 g of maltose, 600 μg of octoxynol (Triton X-100), 120 μg of tri-N butyl phosphate (TNBP), and 120 ml of injectable water. The antibody content is 0.5 IU per g of immunoglobulin. We kept it at a temperature of 4°C until use. The experiments were carried out at a temperature of 24°C, maintaining a pH in the colloidal suspension of 5.5 to keep it stable and avoid the protein’s aggregation. We used deionized water (AE), with a resistivity ≥ 18.2 MΩcm² at 25°C; and quasispherical gold nanoparticles (AuNPs), 5 nm in diameter, with a concentration of $5 \times 10^{13}$ particles/ml, 0.052 mg/ml; and citrate coating (nanoComposix, Inc., San Diego, CA). Characterization of AuNPs are shown in Supplemental Material Section A.

Raman experiments were conducted in a Horiba Jobin Yvon XploRA ONE Raman spectrometer coupled to an Olympus BX41 optical microscope, using a laser source at 532 nm (green) and 785 nm (red).

We initially determined the Raman spectrum of IVIG at 5 g/100 ml (5%). Subsequently, for SERS’ characterization, dilutions of the protein were made in the mentioned concentration, adding deionized water and an equal volume of the nanoparticle colloid, as discussed below. When preparing the dilutions in this way, all samples have a final concentration of AuNPs of 0.026 mg/ml, and a total concentration of IVIG decreasing from 2.5% to 0.075%.

(1) $20 \mu l$ IVIG 5% + $20 \mu l$ AE
(2) $20 \mu l$ IVIG 5% + $20 \mu l$ AuNP 50 nm
(3) $20 \mu l$ IVIG 5% + $20 \mu l$ AuNP 5 nm
(4) $20 \mu l$ IVIG 2.5% + $20 \mu l$ AuNP 5 nm
(5) $20 \mu l$ IVIG 1.25% + $20 \mu l$ AuNP 5 nm
(6) $20 \mu l$ IVIG 0.62% + $20 \mu l$ AuNP 5 nm
(7) $20 \mu l$ IVIG 0.31% + $20 \mu l$ AuNP 5 nm
(8) $20 \mu l$ IVIG 0.15% + $20 \mu l$ AuNP 5 nm

We prepared each of the dilutions and immediately carried out the measurements without requiring additional incubation time. We placed the mixtures in an aluminum cell and obtained the SERS spectrum using a 785 nm laser, in a range of 500-1800 cm⁻¹, with five acquisitions and an
Table 1: Characteristic Raman bands of IVIG.

| Raman shift (cm\(^{-1}\)) | Proposed band assignment                                                                 | Reference |
|---------------------------|-----------------------------------------------------------------------------------------|-----------|
| 710                       | Tyrosine (642, 640-650)                                                                  | [17]      |
|                           | Tryptophan (707)-in IgG-                                                                  | [18]      |
|                           | Carbon backbone v (Ca-C, Ca-C\(\beta\) \(\gamma\) Ca-N) (870-1150)                       | [19]      |
|                           | Glycine, alanine v (CNC) (850-900) assigned to the symmetric CNC stretch mode             | [20]      |
| 852                       | Tyrosine (852)-in IgG-                                                                  | [21]      |
|                           | Tyrosine (843) v (ring)-in IgG-                                                          | [18]      |
|                           | Tyrosine out of plane ring bending mode at 853                                            | [22]      |
|                           | Hydrogen bonding state of tyrosine                                                       | [23]      |
|                           | Phenylalanine (1003, 1000-1010)                                                         |           |
| 1001                      | Symmetric breathing mode of phenylalanine (1003)                                         | [22]      |
|                           | Cysteine (CH bend) (1142)                                                                | [20]      |
| 1130                      | Glutamine (1122) (NH\(_3\) bend and rock modes)                                         | [20]      |
|                           | Amide III region (1230-1340) \(\delta\) (N-H, Ca-H), v (Ca-N)                          | [19]      |
|                           | \(\beta\)-Sheet structure (1239)                                                        | [24]      |
| 1237                      | \(\beta\)-sheet structure, amide III (1230-1240)                                         | [23]      |
|                           | Glutamine (1225) (CH2 bend and twist)                                                    | [20]      |
|                           | Tryptophan, tyrosine \(\delta\) (ring) (1225)-in IgG-                                    | [18]      |
|                           | Amide III, \(\beta\)-sheet and random coils (1242)                                      | [25]      |
|                           | Histidine (1400-1420)                                                                   | [17]      |
| 1396                      | Tryptophan v ring stretching-in IgG-(1366)                                               | [21]      |
|                           | Tyrosine v (ring) (1385)-in IgG-                                                         | [18]      |
|                           | Glycine (1411) CH\(_2\) scissor mode                                                    | [20]      |
|                           | Tryptophan CH\(_2\) scissors-in IgG-                                                     | [21]      |
|                           | Tryptophan or \(\delta\) (CH\(_3\)) (1455)-in IgG-                                     | [18]      |
| 1449                      | Glutamine (CH\(_2\) bend and scissors modes) (1462)                                     | [20]      |
|                           | C-H vibration (1449); CH functional groups in amino acid side chains of proteins        | [25]      |
| 1497                      | Glycine (CH\(_2\) and OH bending modes) (1495)                                          | [20]      |
|                           | Tryptophan, tyrosine v (ring) (1487)-in IgG-                                            | [18]      |

\(\delta\): deformation; v: stretching.
acquisition time of 30 seconds. We focus the laser on the surface of the sample with a 10x objective.

We calculated the SERS enhancement factor in MatLab software using the estimation of surface-enhanced Raman spectroscopy (SERS) enhancement factor [16].

3. Results

It was possible to determine the IVIG spectrum by SERS up to a protein concentration of 0.075%, using quasispherical gold nanoparticles at 0.026 mg/ml and 785 nm laser. The characteristic Raman bands of the IVIG spectrum obtained are shown in Figure 1 and described in Table 1.

Signals from different molecules can constitute Raman bands [26]. In the spectrum, these bands may appear broadened, and therefore, the contribution of various components cannot be easily recognized, causing this to be misinterpreted as noise. In SERS, some signals that conform to the band may be more evident due to the amplification [27].

We perform a spectral truncate in the region of 980 to 1020 cm\(^{-1}\). The band at 1001 cm\(^{-1}\), corresponding to the phenylalanine ring, is shown in red.

Using MatLab, we perform the calculation of the enhancement factor (EF). The values used are listed as follows: wavenumber (Raman Shift), Raman spectrum, SERS spectrum, characteristic Raman band (1001 cm\(^{-1}\)), the wavelength of the incident laser (785 nm), molecular weight of the molecule under test (150 g/mol), the density of the molecule (0.05 g/cm\(^3\)), the numerical aperture of the objective (0.471), and surface area of the molecule under test (27.7 nm). This calculation results in an EF = 2.3726 \(\times\) 10\(^5\) (Supplemental Material Section B).

4. Discussion

The vibrations of the peptide structure in proteins are generally associated with three main regions in the Raman spectrum: (1) the region of carbon backbone (870-1150 cm\(^{-1}\)), comprising the narrowing corresponding to Ca-C, Ca-Cβ, and Ca-N, (2) the extended amide III region (1230-1340 cm\(^{-1}\)), which mainly involves the interface combination of the deformation in the NH plane and the narrowing of Ca-N, as well as a mixture between the deformations of NH and Ca-H, and (3) the amide I region (1630-1700 cm\(^{-1}\)) causes the C=O narrowing [20]. Aromatic amino acids are the dominant characteristics in the Raman spectrum of peptides and...
proteins [22]. In fact, in previous work [18, 21], the spectrum of immunoglobulin G on silver surfaces was represented only by the side chains of aromatic amino acids; this is probably because, in the absence of carboxylate bonds, aromatic amino acids have a higher affinity for the metal surface than nonaromatic amino acids. In contrast, under our experimental conditions and using AuNPs as SERS substrate, it was possible to obtain Raman bands corresponding to the β-sheet conformation (1237 cm⁻¹), characteristics of the IgG protein secondary structure. Using Raman spectroscopy and amplifying the signal with 5 nm gold quasispherical nanoparticles, it was possible to determine the IVIG spectrum at a concentration of 0.075%, 75 mg/dl (normal range in healthy subjects 700-1600 mg/dl) [28]. So, it is possible to propose the use of SERS in the diagnosis of certain immunodeficiencies, for example, severe hypogammaglobulinemia (a condition in which IgG levels are less than or equal to 150 mg/dl and there is a lack of antibody response to vaccination), as well as in the evaluation of treatment after IVIG administration [29].

5. Limitations

This study has several limitations. We are determining the spectrum of purified IgG in an aqueous solution. For the SERS technique to be used in clinical laboratories, it will be necessary for the spectrum to be determined in complex mixtures, such as those found in human serum. Another limitation is that when found in serum, IgG could be associated with other molecules. These interactions may modify the spectrum of the protein when trying to obtain it in experimental models. This mixture with other molecules could also interfere with IgG interaction and the metallic nanoparticles used to amplify the Raman spectrum. Modifying the protein corona on the nanoparticles will indeed affect the spectrum obtained. Further research on this topic is required to consider in the future the application of the SERS technique in the clinical setting.

6. Conclusion

The use of 5 nm quasispherical gold nanoparticles as a SERS substrate allows the Raman spectrum of IVIG to be detected at a concentration ten times lower than normal levels.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

All authors declare that they have no conflicts of interest.

Authors’ Contributions

AOD, ELG, and ESKM conceived of the presented idea. AOD, ELG, JMNLI, LCOD, and AGAC wrote the first version of the manuscript with support from ESKM. JMNLI and LCOD developed the theory. ELG, ESKM, and AOD designed and performed the experiments. LCOD and AGAC verified the analytical methods. AGAC, ESKM, and LCOD analyzed the data. All authors discussed the results and contributed to the final manuscript.

Supplementary Materials

Figure 1: TEM image of AuNPs at a 50 nm scale. Figure 2: TEM image of AuNPs at a 20 nm scale. Figure 3: TEM image of AuNPs at a 10 nm scale. Figure 4: optical spectra of the colloidal AuNPs. Figure 5: the size distribution of the obtained AuNPs through the DLS technique. Figure 6: IVIG SERS enhancement factor. (Supplementary Materials)

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