Amide I Band Analysis Applied to Vibrational Micro-Spectroscopies of Gingival Crevicular Fluid Samples for Orthodontic Treatment Monitoring †

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Abstract: Vibrational micro-spectroscopies have been applied to investigate gingival crevicular fluid (GCF) for monitoring the orthodontic treatment with fixed appliances. GCF samples have been investigated using Fourier transform infrared, Raman, and Surface-Enhanced Raman spectroscopies. The GCF spectra collected at different times of orthodontic tooth movement have been used for characterizing biochemical changes occurring during the treatment. We examined the Amide I band region by means of deconvolution analysis using Gaussian-Lorentzian curves for infrared spectra and Lorentzian curves for Raman spectra. This analysis allowed us to evidence the contribution of the different subcomponents of Amide I band and the changes occurring during orthodontic treatment. These changes can be ascribed to modifications in the secondary structure of protein content and can contribute to make vibrational spectroscopies a useful tool for monitoring the individual patient’s response to the orthodontic force application.

Keywords: Amide I; band deconvolution; Raman spectroscopies; infrared spectroscopies; orthodontic tooth movement; gingival crevicular fluid

1. Introduction

The analysis of vibration modes of the Amide I band allows to obtain essential information on the structure of biological tissues and fluids. For this reason, this band has been widely considered to monitor protein molecular changes due to pathology or stress mechanisms [1,2]. Typically, the spectroscopy investigation is performed on dried sample of the fluid to limit the signal from the water. This is a demanding condition for Fourier transform infrared (FT-IR) spectroscopy while a direct spectroscopic assessment is allowed on hydrated samples in the cases of conventional and Surface-Enhanced Raman (SERS) spectroscopies. The Amide I band is mainly related to vibrational modes of C=O bonds. Due to the different mechanisms involved, the intensity and positions of observed modes differ for FT-IR and Raman spectroscopy. This aspect has to be considered when data from these spectroscopies are compared. Our previous work has evidenced that the use of these optical techniques for the investigation of Gingival Crevicular Fluid (GCF) can be a useful tool for investigating changes occurring during orthodontic treatment [3–
In the present work, we focused our attention on the Amide I region of FT-IR, Raman, and SERS spectra to monitor changes induced by the orthodontic treatments. Data were compared and the changes occurring in the Amide I band were discussed in relation to the orthodontic treatment time.

2. Materials and Methods

The GCF samples were collected from two different groups of subjects with dental malocclusion needing orthodontic treatment with fixed appliances: the first was constituted by young subjects ranging between 11 and 18 years, whereas the second group was composed by adults over 18 years of age. Bracket bonding have been applied to the patients and GCF was collected during the process of orthodontic tooth movements (OTM) before the treatment (T0 samples) and after 1 or 2 (T1), 7 (T2), and 14 or 28 (T3) days of OTM treatment, respectively [3–5]. Paper points were inserted into the gingival crevice for about 30 s and stored in an 80 °C refrigerator before analysis. After the adding of 10 μL of distilled water, the samples were vortexed and centrifuged to extract the CGF [3,4].

A Perkin Elmer Spectrum One FT-IR spectrometer was used to record FT-IR spectra. Spectral acquisitions were performed in specular reflection mode with a few microliters drop of sample placed on a metallic IR-reflective surface and left to dry. All spectra were collected using 64 scans in the range from 1500 to 1800 cm⁻¹ with a 4 cm⁻¹ spectral resolution [4].

Raman spectroscopy was performed using a He-Ne laser as excitation source at a nominal power of 17 mW. The Jobin-Yvon 180 monochromator, equipped with a liquid N₂-cooled CCD and a grating of 1800 grooves/mm, allowed a spectral resolution of 4 cm⁻¹. A 100× optical objective (N.A. 0.90) and an excitation spot area of about 5 μm of size were used, with accumulation times ranging from 60 to 300 s. SERS spectra were collected using the same apparatus with a 50× (N.A. 0.75) objective and by adopting an accumulation time of 300 s. A small amount (5 μL) of the GCF sample was dropped on the microscope glass decorated with dried gold nanoparticles and SERS measurements were performed immediately after [3].

The Raman spectroscopy and SERS data were numerically treated to remove background signal by using a wavelet-based algorithm described in Ref. [7]. The spectra were then analyzed in terms of convoluted peak functions to determine the basic vibrational modes that contribute to the signal using a best-fit peak fitting routine of the GRAMS program, based on the Levenberg–Marquardt nonlinear least-square method. Peaks constituting the spectrum were manually selected in order to define the starting conditions for the best-fit procedure. The best-fit was then performed to determine the optimized intensity, position, and width of the peaks using the χ² parameter to evaluate the performance of the procedure. Lorentzian functions were used to fit Raman and SESR data, while Gaussian/Lorentzian mix functions were used for FT-IR data.

3. Results and Discussion

The Amide I band of the FT-IR spectra of GCF samples collected at OTM increasing times from one of the subjects considered in the study are reported in Figure 1a. The reported signals have been normalized assuming unitary the band area and the spectra have been deconvoluted in terms of Gaussian/Lorentzian mix functions to outline the component modes. The red curve modes were assigned to α-helix secondary structure vibration, while the components at about 1620 cm⁻¹ was assigned to β-sheet secondary and 3₁₀-helix [6], and the modes in the 1660–1690 cm⁻¹ range were ascribed to β-turn and β-sheet contributions as indicated in Figure 1b-i [3,8]. The broad mode at about 1600 cm⁻¹ was assigned to C≡C stretching of amino acids [6]. We have already observed that the relative intensity of the Amide I band with respect to Amide II band intensity decreases with the OTM times indicating a protein rearrangement [4]. The center of the Amide I band moves to higher wavenumber for OTM increasing times, especially for T2 and T3 spectra. The
FT-IR spectrum of the T0 sample was compared with the spectrum resulting from Raman spectroscopy in Figure 1b-ii. The Raman spectrum was deconvoluted in terms of Lorentzian functions and the main secondary structure modes were individuated and assigned to β-sheet (at about 1627 cm\(^{-1}\)), α-helix (at about 1652 cm\(^{-1}\)), β-sheet (at about 1677 cm\(^{-1}\)), and β-turn (at about 1699 cm\(^{-1}\)). The dependence of the wavenumber positions of protein secondary structure on the OTM time are reported in Table 1 for data concerning the FT-IR measurements and in Table 2 for Raman spectroscopy data. The maximum intensity of the Amide I band of the FT-IR signal occurs at a wavenumber value lower than the one of the Raman spectrum, in agreement with data reported in Ref. [9].

![Figure 1](image_url)

**Figure 1.** (a) Amide I region of the FT-IR spectra of CGF sample before orthodontic treatment (T0, reference sample) and after 2 (T1), 7 (T2) and 14 days (T3) of OTM. The deconvolution of the spectra in term of Gaussian/Lorentzian mix functions is reported. The red curves refer to α-helix of the amide I band. (b-i) Comparison of Amide I region of the FT-IR and Raman spectra of CGF sample before orthodontic treatment (T0). (b-ii) SERS spectrum of GCF (T0 sample).

**Table 1.** Assignment of the protein secondary structure modes (in cm\(^{-1}\)) for Amide I band of FT-IR spectra.

<table>
<thead>
<tr>
<th>Mode</th>
<th>T0 (Reference)</th>
<th>T1 (2 Days)</th>
<th>T2 (7 Days)</th>
<th>T3 (14 Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-sheet; 3(_{10})-helix</td>
<td>1630</td>
<td>1619</td>
<td>1617</td>
<td>-</td>
</tr>
<tr>
<td>α-helix</td>
<td>1647</td>
<td>1647</td>
<td>1643</td>
<td>1642</td>
</tr>
<tr>
<td>β-sheet; β-turn</td>
<td>1669</td>
<td>1672</td>
<td>1687</td>
<td>1667/1690</td>
</tr>
</tbody>
</table>

**Table 2.** Assignment of protein secondary structure modes (in cm\(^{-1}\)) for Amide I band of Raman spectra.

<table>
<thead>
<tr>
<th>Mode</th>
<th>T0 (Reference)</th>
<th>T1 (2 Days)</th>
<th>T2 (7 Days)</th>
<th>T3 (14 Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-sheet</td>
<td>1622</td>
<td>1619</td>
<td>1618</td>
<td>1621</td>
</tr>
<tr>
<td>α-helix</td>
<td>1652</td>
<td>1643</td>
<td>1641</td>
<td>1642</td>
</tr>
<tr>
<td>β-sheet</td>
<td>1677</td>
<td>1662</td>
<td>1661</td>
<td>1666</td>
</tr>
<tr>
<td>β-turn</td>
<td>1699</td>
<td>1687</td>
<td>1690</td>
<td>1695</td>
</tr>
</tbody>
</table>
Figure 2. FT-IR and SERS spectra of GCF (T0) and at increasing OTM times, namely 2 days (T1), 7 days (T2), and 14 days (T3), respectively.

SERS signal is reported in Figure 1b-ii. In this case, the α-helix mode is the prevalent component in the Amide I band region. This indicates that the peptide bonds are on the proximity of the gold nanoparticle surface. Otherwise, the presence of bulky side chain groups orients the peptide bonds too far from the evanescent field of the surface plasmon resulting in a suppression of the Amide I band in the spectra [10]. A comparison of the effect of OTM time on the FT-IR and SERS response in the Amide I spectral region is shown in Figure 2. Differently from FT-IR behavior, the Amide I band center of SERS spectra shifts to lower wavenumbers when OTM time increases, from 1641 cm\(^{-1}\) to 1611 cm\(^{-1}\). This feature can be correlated to a change of both the relative amount and quality of helix secondary structure components. The helix protein secondary structure is typically of type α-helix (3.6 residues/turn), but the 3\(_{10}\)-helix form (3 residues/turn) can also occur. 3\(_{10}\)-helices are typically shorter than α-helices [11] and it is presumed to serve as intermediary steps in the formation and folding of α-helices due to a low reciprocal barrier energy [12]. The energy of the 3\(_{10}\)-helix mode in the SERS spectrum is lower than the one of α-helix [13,14] thus we argued that the 1611 cm\(^{-1}\) observed SERS peak could be assigned to this mode.

4. Conclusions

Changes occurring in the protein secondary structure of GCF during orthodontic processes were assessed in young adults by a compared analysis of the Amide I region of FT-IR, Raman scattering and SERS spectra. This complementary approach allowed a deep insight in the molecular response of CGF component proteins to the OTM process.
stresses, exploiting complementary information that the considered spectroscopy methods offer. The center of the Amide I band of FT-IR spectra moves to higher wavenumber for OTM increasing times indicating a decrease of the α-helix secondary structure component content compared to an increase of unordered and β-sheet components due to protein unfolding processes. Differently from the FT-IR behavior, the Amide I band center of SERS spectra shifts to lower wavenumbers when OTM time increases, from 1641 cm⁻¹ to 1611 cm⁻¹. The SERS response is directly correlated to the protein helix configurations and the observed features can be accounted by the formation of 3i0-helix structures when the OTM time increases, indicating the occurrence of molecular restoring mechanisms of CGF and the alveolar environment after an initial molecular unfolding and disorder increase due to OTM stress.

In the case of FT-IR analysis, a partial overlap of the 3i0-helix and β-sheet spectral ranges do not permit to unequivocally sense the presence 3i0-helix, differently from SERS. These studies demonstrated that the vibrational spectroscopies could be a potential useful tool for an immediate monitoring of the individual patient’s response to the orthodontic tooth movement, aiming to more personalized treatment reducing any side effects.

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**References**
