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Raman spectroscopic and low temperature calorimetric investigation on the low energy vibrational dynamics of hen egg-white lysozyme

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ABSTRACT

We have investigated the low frequency vibrational dynamics of chicken hen egg-white lysozyme using Raman spectroscopy and low temperature calorimetry. An amorphous-like behaviour of low frequency dynamics in this protein has been observed. By using inelastic light scattering data and resorting to a fitting procedure, the low temperature specific heat trend has been theoretically reproduced, confirming that, as in disordered systems, the same low energy excitations give rise to the observed anomalies in low frequency vibrational and low temperature thermal properties. A further study of polarized and depolarized Raman spectra has allowed us to infer information about the symmetry of these modes. **The frequency dependence of light-vibrations coupling constant has also been analysed.**

1. INTRODUCTION

The idea that proteins can be described as disordered atomic structures performing specific biological functions is today largely diffused and continues to strengthen thanks to many evidences that they undergo a dynamic transition (usually referred to as the “protein glass transition”) [1] and have anomalous behaviours in their low frequency vibrational dynamics [2] similar to amorphous systems.

As a matter of fact, alike to disordered materials, proteins have shown the presence of low energy modes exceeding the contribution expected by the Debye model in their vibrational density of states $g(\nu)$ (VDOS). More precisely, in inelastic neutron [3,4] and Raman [5] scattering spectra, these excitations merge as a peak which has been traced back to the so-called Boson Peak, a sort of signature of the amorphous state whose origin is still widely debated.

Recently, the amorphous-like behaviour of proteins has been **also** revealed by performing low temperature specific heat measurements [6]. Indeed, **as in glassy systems [7], the low temperature**

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specific heat of globular proteins, when plotted as C_p/T^3 as a function of temperature, shows the presence of a peak whose origin is referred to the excess of modes observed in the low energy vibrational density of states.

In the topics of disordered materials, both low temperature calorimetry and Raman spectroscopy have turned out to be very useful techniques to investigate low frequency VDOS.

In fact, performing specific heat experiment at low temperature allows us to obtain information about vibrational excitations, just in the low energy range where it's possible to neglect any other kind of modes. Thus, low-temperature specific heat, C_p , and $g(\nu)$ are connected by the following equation:

$$C_p = 3N\kappa_B \int_0^{\nu_0} g(\nu) \cdot \left(\frac{h\nu}{\kappa_B T}\right)^2 \cdot \frac{e^{\left(\frac{h\nu}{\kappa_B T}\right)}}{\left[e^{\left(\frac{h\nu}{\kappa_B T}\right)} - 1\right]^2} \cdot d\nu \quad (1)$$

where N is the numerical density, k_B is the Boltzmann constant and ν_0 is the Debye's frequency.

On the other hand, also Raman scattering studies on amorphous materials have been extensively carried out and Shuker and Gammon [8] have deduced a mathematical relation between experimental light scattering intensity $I(\nu)$ for the Stokes side of the spectrum and $g(\nu)$:

$$I(\nu) = C(\nu) \cdot g(\nu) \cdot \frac{[n(\nu) + 1]}{\nu} \quad (2)$$

where $C(\nu)$ represents the light-vibration coupling coefficient, ν is the frequency shift and $[n(\nu)+1]$ is the Bose-Einstein population factor. When normalized by $\frac{[n(\nu)+1]}{\nu}$, the Raman scattering intensity gives a measure of the low frequency vibrational density of states multiplied to $C(\nu)$, a coefficient containing the polarization dependence of the scattering. Different vibrational bands, in fact, can couple to the radiation with varying strength, giving rise to the observed dependence of this coefficient on frequency shift. More precisely, the vibrations couple to light through the displacement dependence of the molecular polarizability of the material, $\bar{\alpha}$, thus, a study of Raman scattering spectra with selective polarization in excitation and detection allows to gain insight into the symmetry of molecular motions.

Therefore, the knowledge of $C(\nu)$ and of its frequency dependence has significant importance for the topic of low frequency vibrations, because it provides a relatively simple method to extract the vibrational density of states from Raman experiment.

In this paper we have focused our attention on the parallel use of two different experimental techniques, low temperature calorimetry and low frequency Raman spectroscopy, gaining a better insight into the low frequency vibrational dynamics of lysozyme.

2. EXPERIMENTAL DETAILS

Lyophilized powder of hen (chicken) egg-white lysozyme (14.3 kDa) has been purchased from Sigma and used without further purification.

Raman scattering experiments have been performed, at room temperature, by using an Ar⁺-ion laser ($\lambda = 514.5$ nm), with a power of 300mW as excitation source and a double monochromator Yobin Yvon U-1000 spectrometer. The sample has been prepared by sealing the powder between sapphire windows. Sapphire contributes with a negligible intensity to the light-scattering spectra in the frequency range of interest ($\nu < 200$ cm⁻¹), where the Raman spectrum is dominated by lysozyme modes [9]. The light scattering data have been acquired in the 90° scattering geometry, with a resolution of the experimental set-up of 1 cm⁻¹. The scattered intensity of the Stokes Raman spectra has been recorded in the 8-600 cm⁻¹ window, in both polarized (I_{VV}) and depolarized (I_{VH}) experimental conditions. The subscripts V and H correspond to directions that are vertical and horizontal with respect to the scattering plane, defined by the orthogonal initial and final wave vectors of the light.

A weak fluorescence contribution (less than 10% of the scattered intensity) to the spectra has been systematically revealed in the background signal. Its magnitude has been approximated by a second-order polynomial using a fitting procedure and, then, subtracted from raw spectra.

Specific heat measurements have been carried out in the temperature range between 1.5 K and 23 K by means of an automated calorimeter operating with thermal relaxation method [10]. The dry powder of lysozyme has been packed into an aluminium foil cell housing a total amount of about 15 mg of sample.

3. EXPERIMENTAL RESULTS AND DISCUSSION

In figure 1 the scattered intensities of polarized [6] and depolarized Raman spectra of dry lysozyme at T=300 K, corrected for the fluorescence background, are represented as $I_r(\nu) = \frac{I(\nu)}{\nu \cdot [n(\nu) + 1]}$ in the frequency range below 300 cm⁻¹. The spectral region above this frequency value has been excluded from this representation, since it is characterized only by Raman modes of sapphire upon a background contribution.

Both these spectra exhibit a similar spectral shape but the scattered intensity, in the vertical-horizontal configuration, is reduced to 16% of I_{VV} .

The low frequency Raman spectra of proteins are typically composed of two overlapping contributions: a quasi-elastic tail and a vibrational inelastic contribution. The first, observed in the very low frequency regime ($\nu < 15 \text{ cm}^{-1}$), reflecting relaxational motions (local conformational jumps and fluctuations of structural molecular conformations), is usually connected to the flexibility of the protein [5], while, the second one, lying at frequency higher than 20 cm^{-1} , is supposed to arise from collective motions (involving correlated displacements of groups of atoms, residues, secondary structures and domains [11]) which are related to the elasticity of the protein [5] and considered very important for biological functions [12].

The analysis of light scattering data requires the separation of relaxational and vibrational contributions. Unfortunately, the magnitude of the quasi-elastic contribution is a function of temperature and is expected to be relevant at $T=300 \text{ K}$. Furthermore, the low frequency limit ($\nu = 8 \text{ cm}^{-1}$) of the investigated region, strongly reducing the range where relaxational modes are clearly observed, has prevented from correctly subtracting this contribution from the Raman scattered intensity. However, since the quasi-elastic component is usually described by a Lorentzian shape line extending up to 50 cm^{-1} [13], it can be supposed that its occurrence doesn't affect heavily the inelastic part of the Raman spectrum, where its contribution is expected to drop following the bare Lorentzian tail end.

According to Shuker and Gammon [8], the normalized Raman intensity, $I_r(\nu)$, represents the product between the vibrational density of states as $g(\nu)/\nu^2$ and the coupling constant:

$$I_r(\nu) = \frac{I(\nu)}{\nu \cdot [n(\nu) + 1]} = \frac{C(\nu) \cdot g(\nu)}{\nu^2} \quad (3).$$

This representation allows a more detailed investigation of the low frequency Raman spectrum, making more visible the excess of modes in the VDOS respect to the Debye behaviour ($g(\nu) \sim \nu^2$) and giving a more defined representation of the Boson peak.

In Fig. 1, clearly, the Raman signal appears to be characterized by a strong quasi-elastic contribution at frequency below 20 cm^{-1} and by the presence of two bands in the frequency range between $20 < \nu < 300 \text{ cm}^{-1}$, where harmonic vibrations of lysozyme are expected to dominate [9]: the first band is really closed to the quasi-elastic tail, while the second one is placed at higher frequencies (behind 50 cm^{-1}) and appears as a shoulder on the right side of the first bump. A fitting procedure has been performed by using two Lorentzian shapes and the theoretical curves well reproduce both polarized and depolarized Raman scattering spectra (see fig. 1).

The frequency positions and the intensity values of the two bands in the Raman spectra have been obtained as fitting parameters. While the second band is set at about 80 cm^{-1} in both polarized and

depolarized spectra, different values have been observed for the frequency location of the first peak. In fact, this bump, that corresponds to the Boson peak, is centred at about 20 cm^{-1} in the polarized spectrum and at about 22 cm^{-1} in the depolarized spectrum. The location of this band at slightly lower frequency than that observed in previous light scattering study on dry lysozyme [14, 5] ($\nu \approx 25 \text{ cm}^{-1}$) could be due to the quasi-elastic contribution whose intensity, at room temperature, covers the low frequency tail of the Boson Peak and prevents from having a high definition for the profile of this bump. This instance appears more pronounced for the polarized spectrum, where the quasi-elastic contribution is stronger than that observed in depolarized Raman spectrum.

The ratio, A_1/A_2 , of the intensities of the Boson Peak respect to the peak placed at 80 cm^{-1} has been evaluated 1.76 for the polarized spectrum and 1.38 for the depolarized spectrum. The difference observed for the two polarizations is essentially due to a more pronounced second peak in the VH-polarized spectrum respect to the VV-polarized spectrum. This can be more clearly observed in figure 2, where $I_r(\nu)$ normalized to the scattered intensity value at the Boson Peak frequency (I_{BP}) is plotted for both the polarizations. This is a manifestation of the band dependence of the coefficient $C(\nu)$ and evidences a stronger coupling of the second band to the optical radiation field respect to the Boson Peak.

The polarization properties of the Raman spectrum can be used to infer information about the symmetries of vibrational modes. In fact, the symmetry property of a mode having frequency ν , in the Raman spectra of amorphous and biological systems, is quantitatively characterized by a depolarization ratio $\rho(\nu)$ defined as the ratio I_{VH}/I_{VV} [15].

The depolarization ratio can assume values which depend on the type of Raman-active vibration to which it corresponds. More precisely, for a totally symmetric vibration $\rho(\nu)$ is equal to zero while the non-symmetric character of a vibration increases if the depolarization factor assumes values from 0 up to 0.75.

It is worth emphasizing that, in contrast to the case of an ordered single crystal, a disordered material, such as a polycrystal, liquid, gas or biological structure, is composed of molecules which don't exhibit perfect orientational and positional correlation. Therefore, in order to obtain information about the symmetry of modes, it is necessary to consider a proper orientational average to the molecular polarizability tensor $\bar{\alpha}$.

In the case of linearly polarized incident radiation, it is useful to express the average polarizability in terms of two separate quantities, that are invariant to rotation, namely isotropic (α) and anisotropic (β) scattering, which are related to $\rho(\nu)$ as follows:

$$\rho(\nu) = \frac{I_{VH}}{I_{VV}} = \frac{3\beta^2}{45\alpha^2 + 4\beta^2} \quad (4).$$

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3 On the basis of the last equation, the depolarization factor is therefore a measure of the relative
4 contributions of isotropic and anisotropic Raman components, being the latter sensitive to
5 depolarized modes.
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8 Furthermore, since the Raman spectrum of amorphous solids and also of proteins is continuous, the
9 depolarization ratio associated with it is a continuous function of frequency shift and its features
10 reflect the different symmetry properties of vibrational modes.
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13 In the inset of figure 1, the depolarization ratio of dry lysozyme is shown in the frequency range 10
14 - 200 cm^{-1} , where the two bands in the Raman spectra have been observed. It has been found almost
15 frequency independent in the investigated frequency range and to have a value of about 0.2
16 comparable to that previously obtained for amorphous systems [16]. The occurrence, at frequencies
17 below 50 cm^{-1} , of a deviation of $\rho(\nu)$ from its constant value suggests a character of the modes lying
18 in the Boson Peak frequency region slightly more polarized compared to the vibrations contributing
19 to the second band in the Raman spectra. Furthermore, the low value assumed by the depolarization
20 ratio lets us to suppose a predominantly symmetric character of the active Raman modes
21 contributing to the low frequency range, even though, the non-zero value of $\rho(\nu)$ could be indicative
22 of the presence of coupled modes having mixed (symmetric and anti-symmetric) character.
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31 Considering these results, the frequency dependence of polarized inelastic light scattering intensity
32 appears the most representative Raman spectrum of the low frequency modes characterizing dry
33 lysozyme sample. For this reason, in this paper, only the I_{VV} spectrum will be considered from here
34 on in order to get additional information on the coupling between light probe and elastic features of
35 this system.
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40 Concerning the evaluation of the true vibration coupling coefficient, it is not obvious whether all
41 vibrations at one frequency contribute to the Raman spectra with the same $C(\nu)$, or there are
42 different kinds of vibrations and each contributes with its own $C(\nu)$.
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46 The Raman coupling coefficient can be extracted by light scattering data if the vibrational density
47 of states is known. Figure 3 shows the frequency dependence of the coupling coefficient for dry
48 lysozyme in the frequency range 10-80 cm^{-1} obtained by comparing our experimental Raman data
49 and $g(\nu)$ measured in previous inelastic neutron scattering experiments at $T=150\text{K}$ [3]. It is
50 important to remark that, even though neutron and Raman scattering data at different temperatures
51 have been compared, this doesn't greatly affect the evaluation of the coupling constant. In fact, it
52 has been shown that the low energy vibrational excitations of dry proteins in the Boson Peak region
53 are not strongly influenced by temperature [17] and have a prevalent harmonic character [18].
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60 The analysis of $C(\nu)$ has been performed in a restricted frequency range since inelastic neutron data
were not available at energy values higher than 10 meV and the influence of the quasi-elastic

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3 contribution has prevented from considering the Raman spectrum at frequency below 15 cm^{-1} .
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5 However, despite these restrictions, the investigated region shown in fig. 3 completely covers the
6
7 range where the two bumps are observed.

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9 A monotonic increase of $C(\nu)$ with frequency has been revealed. **More precisely it has been found**
10 **that the coupling coefficient follows a linear dependence described by the function $C(\nu) \propto \nu + A$,**
11 **where A represents a non vanishing value assumed by $C(\nu)$ in the limit $\nu \rightarrow 0$.** This result is fully in
12 agreement with the universal linear frequency behaviour of $C(\nu)$ found for a large number of
13 different glasses near the Boson Peak frequency region [19,20,21] and puts into evidence, once
14 again, the existence of a similarity between amorphous and biological systems.

15
16 It is worth to emphasize that, a linear frequency trend of the coupling coefficient for biological
17 systems has long been supposed in analogy to glassy systems [22,9] but, to our knowledge, this is
18 the first time that this assumption has been experimentally confirmed.

19
20 To further test the validity of this result, information about the frequency dependence of the
21 coupling constant can be inferred by comparing Raman scattering and low temperature specific heat
22 data [23]. For this purpose, the spectral dependence of $g(\nu)$ has been calculated from Raman spectra

23
24 by using equation (2) and normalizing the scattered intensity to $\frac{[n(\nu)+1]}{\nu}$. Assuming a specific
25 frequency dependence of the coupling coefficient, $C(\nu) \propto \nu^\alpha + A$, the reduced specific heat, C_p/T^3 ,
26 as a function of temperature, has been reproduced by inserting into equation (1) the VDOS obtained
27 by Raman data. Afterwards the α value has been obtained as fitting parameter, and the frequency
28 dependence of $C(\nu)$ has been derived.

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30 In Fig. 4, **the specific heat** of dry lysozyme [6] and the fitting curve, obtained as described above,
31
32 are **plotted** as a function of temperature.

33
34 The experimental calorimetric data have shown the presence of a broad band extending on all the
35 investigated temperature range and the indication of an increasing contribution at temperature
36 below 3 K. The origin of this second contribution could be ascribed to the existence of tunnelling
37 states in strict analogy with glasses [24]. A deep study of this upturn requires more detailed
38 measurements at lower temperatures and is, therefore, excluded from the topic discussed in this
39 paper. **It's worth to note that, just for this reason, the fitting procedure has been limited to the**
40 **temperature range $T > 3\text{K}$, where the main contribution to the specific heat is due to vibrational**
41 **excitations.**

42
43 The bump, located at about 6 K, represents a strong deviation from the expected Debye contribution
44 to the specific heat and its presence can be considered as a further analogy between protein and
45 disordered materials [7]. In glasses this excess of specific heat is supposed to arise from the same

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3 modes merging into the Boson Peak, taking into account the equivalence between temperature and
4 frequency values due to the dominant phonon approximation ($h\nu \approx 4 \div 5k_B T$). The credibility of
5 this statement is further supported by a complete agreement between the temperature position of the
6 bump observed in the temperature dependence of the reduced specific heat and the Boson Peak
7 frequency in the Raman spectrum as in glasses as in our lysozyme sample. In this regard, the
8 observed asymmetry in the right tail of the bump in the C_p/T^3 of lysozyme could arise from an
9 additional contribution due to the modes which give rise to the shoulder revealed at 80 cm^{-1} in the
10 Raman spectra.
11

12 A good agreement between Raman and low temperature specific heat measurements is also deduced
13 by observing how the theoretical fit reasonably reproduces the temperature dependence of C_p/T^3 of
14 dry lysozyme in terms of intensity, position and shape of the observed bump. The alpha exponent,
15 obtained as fitting parameter, is 0.75. This result is consistent with the observed linear frequency
16 dependence of $C(\nu)$ as can be also pointed out by the overlapping of the linear and quite-linear
17 curves shown in Fig. 3. Furthermore, in fig. 4, the function obtained with $\alpha=0$ (corresponding to
18 $C(\nu) = \text{const}$) and $\alpha=1$ are also reported in order to show the very narrow range of variation allowed
19 for the parameter α in this approach.
20

21 It is worth emphasizing that, a close α value for the coupling coefficient has been found in a silica-
22 xerogel [23] characterized by a low network connectivity. In this study the existence of a correlation
23 between the fast increase of $C(\nu)$ with frequency and the presence of elastic local inhomogeneities
24 in the glassy matrix has been hypothesized. A similar interpretation can be put forward also for the
25 frequency behaviour of the coupling coefficient in proteins which are characterized by a highly
26 heterogeneous structure.
27

28 4. CONCLUSION

29 Low frequency light scattering measurements on dry lysozyme, performed in polarized and
30 depolarized configuration, have shown the existence of two contributions to the Raman intensity:
31 the Boson Peak placed at about 20 cm^{-1} and a second bump set at 80 cm^{-1} . A stronger coupling of
32 the second band to the optical radiation field respect to the Boson Peak has been observed.
33

34 The depolarization ratio of the Raman scattering at room temperature has been found almost
35 frequency independent in the investigated frequency range and has a value of about 0.2, comparable
36 to that previously obtained for amorphous systems.
37

In agreement with the universal behaviour found for the coupling coefficient in many amorphous systems near the Boson Peak frequency region, a nearly linear frequency dependence of $C(v)$ ($C(v) \propto v^{0.75} + A$) has been found by comparing Raman to neutron scattering and specific heat data.

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FIGURE CAPTIONS

Figure 1: Low frequency normalized Raman spectra of dry lysozyme in the polarized ($-\nabla-$) and depolarized ($-\circ-$) configuration as a function of the frequency shift. The solid lines represent the fitting curves of the spectral shape in the 20-300 cm^{-1} range. In the inset the depolarization spectrum $\rho(\nu)$ as a function of the frequency with the relative error bars.

Figure 2: Comparison of the I_r Raman intensity normalized to the Boson Peak intensity for the polarized ($-\nabla-$) and depolarized ($-\circ-$) spectrum of dry lysozyme.

Figure 3: Frequency dependence of the coupling coefficient $C(\nu)$ for dry lysozyme (\circ). The dashed line is descriptive of a linear frequency dependence while the solid line represents the curve: $C(\nu) \propto \nu^{0.75} + A$.

Figure 4: Temperature dependence of the reduced specific heat of dry lysozyme (\circ). Solid, dotted and dashed lines reproduce the theoretical curves obtained by the fitting procedure described in the test, with $\alpha=0.75$, $\alpha=0$ and $\alpha=1$ respectively.

FIGURES

FIGURE 1

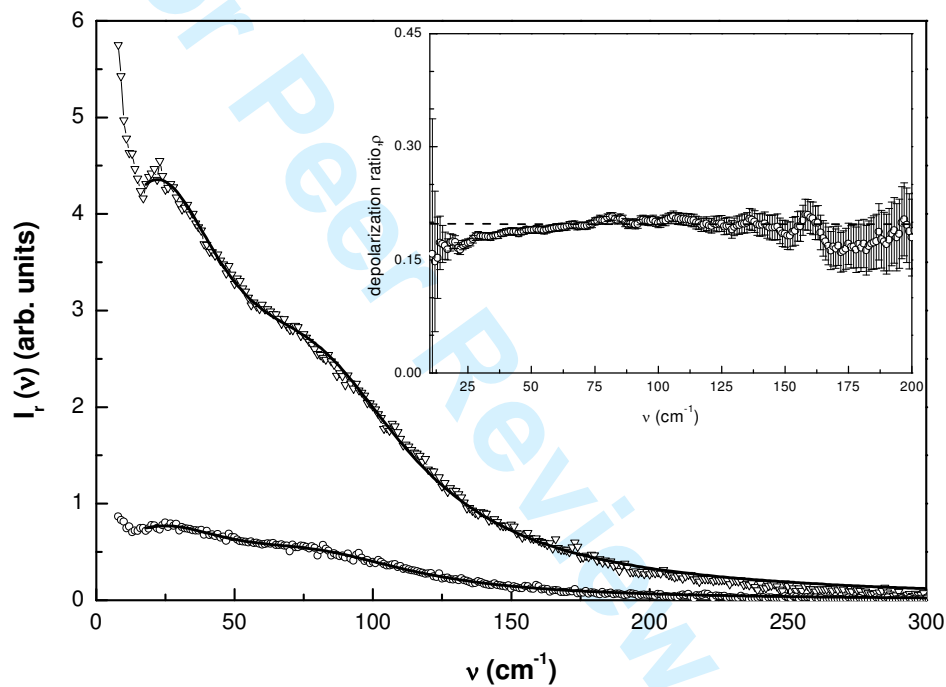


FIGURE 2

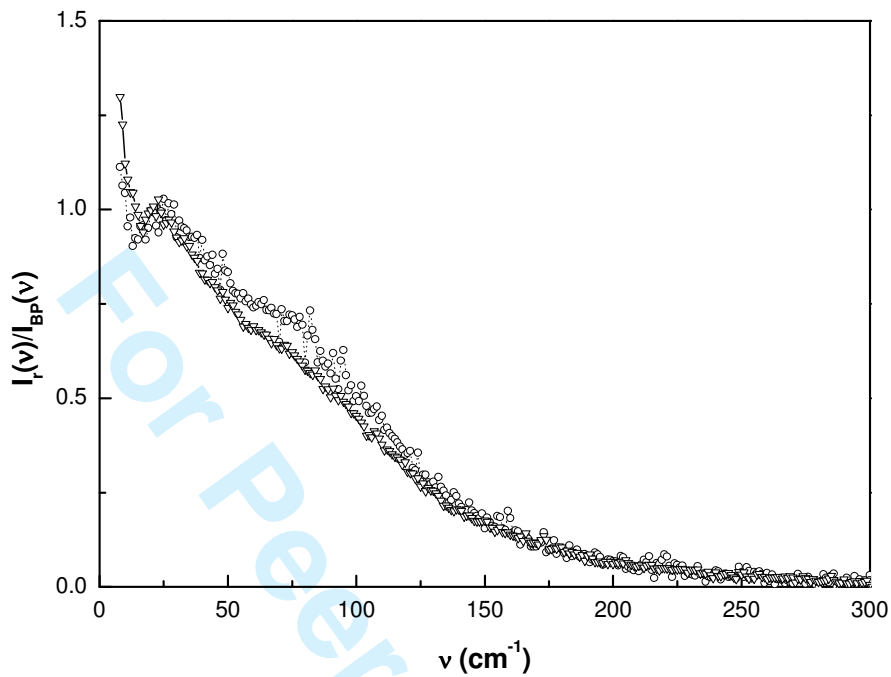


FIGURE 3

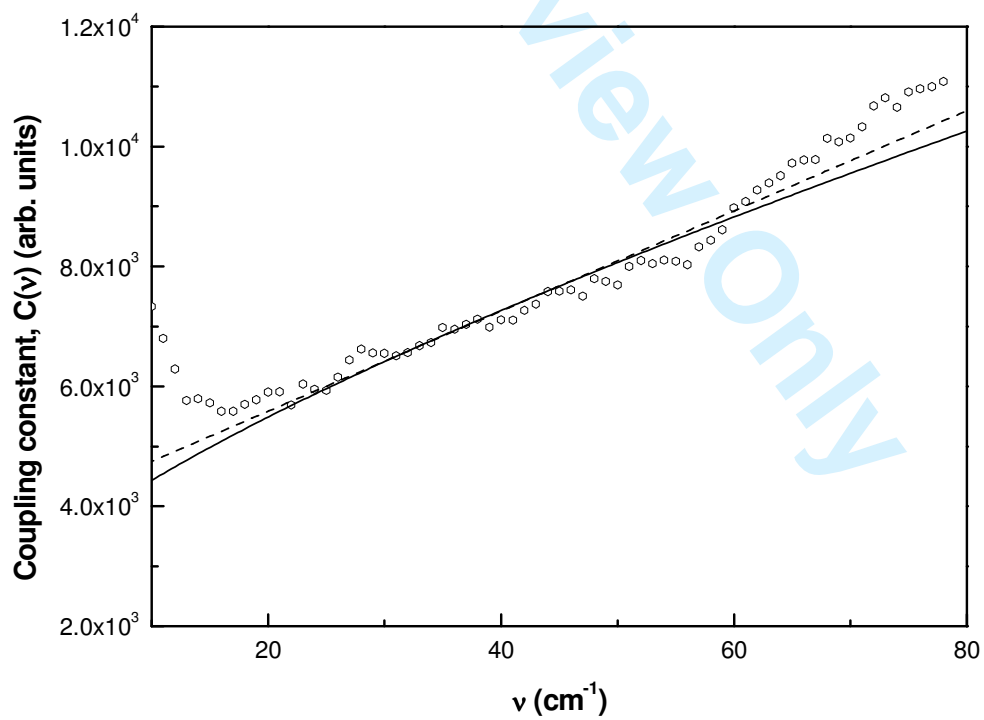
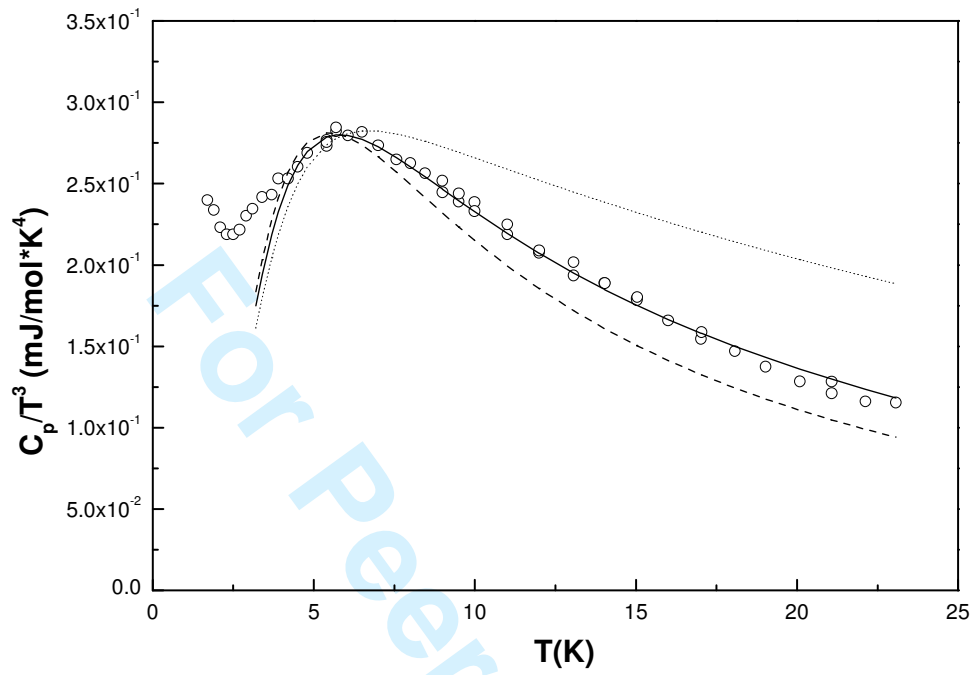
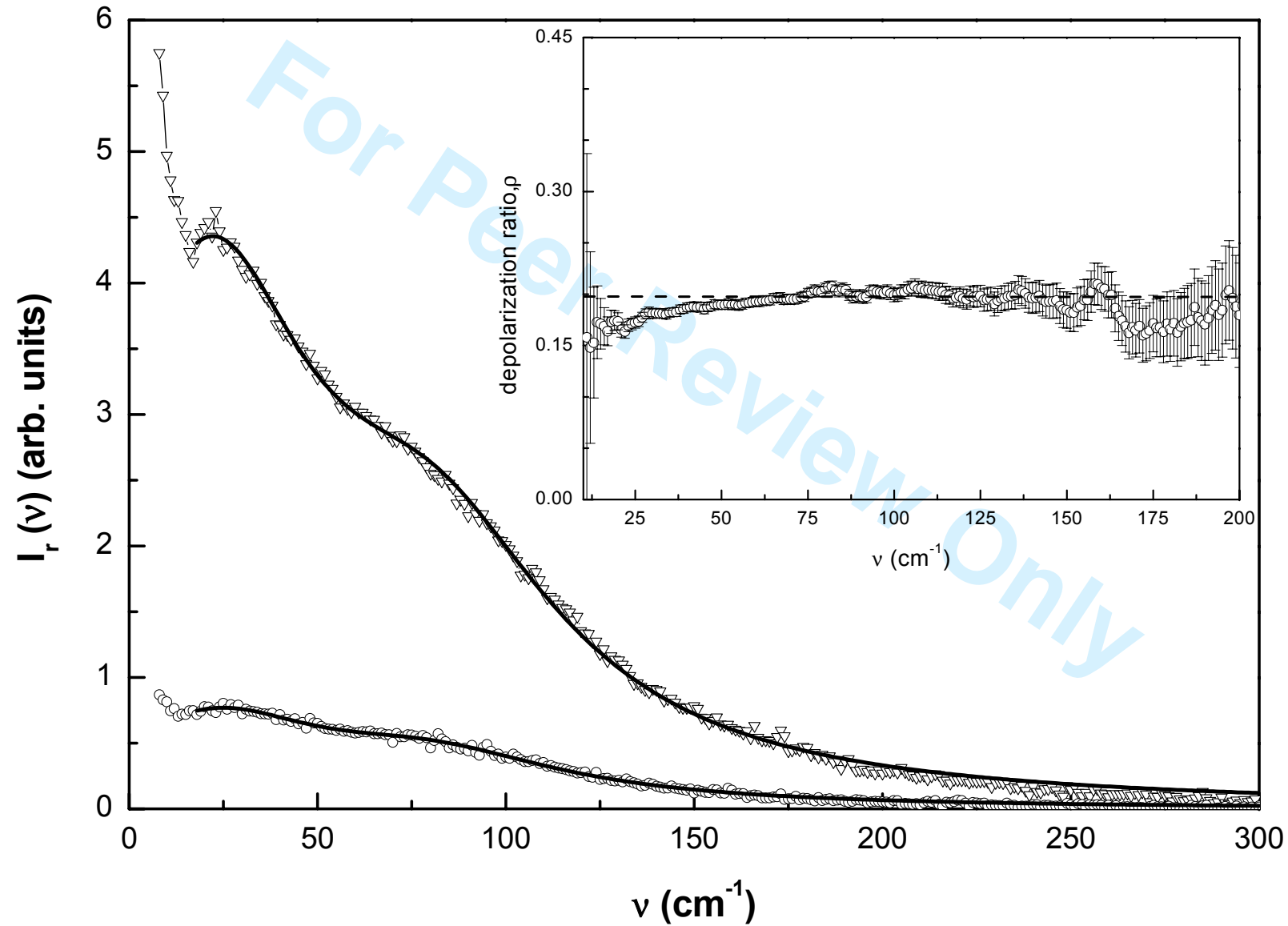
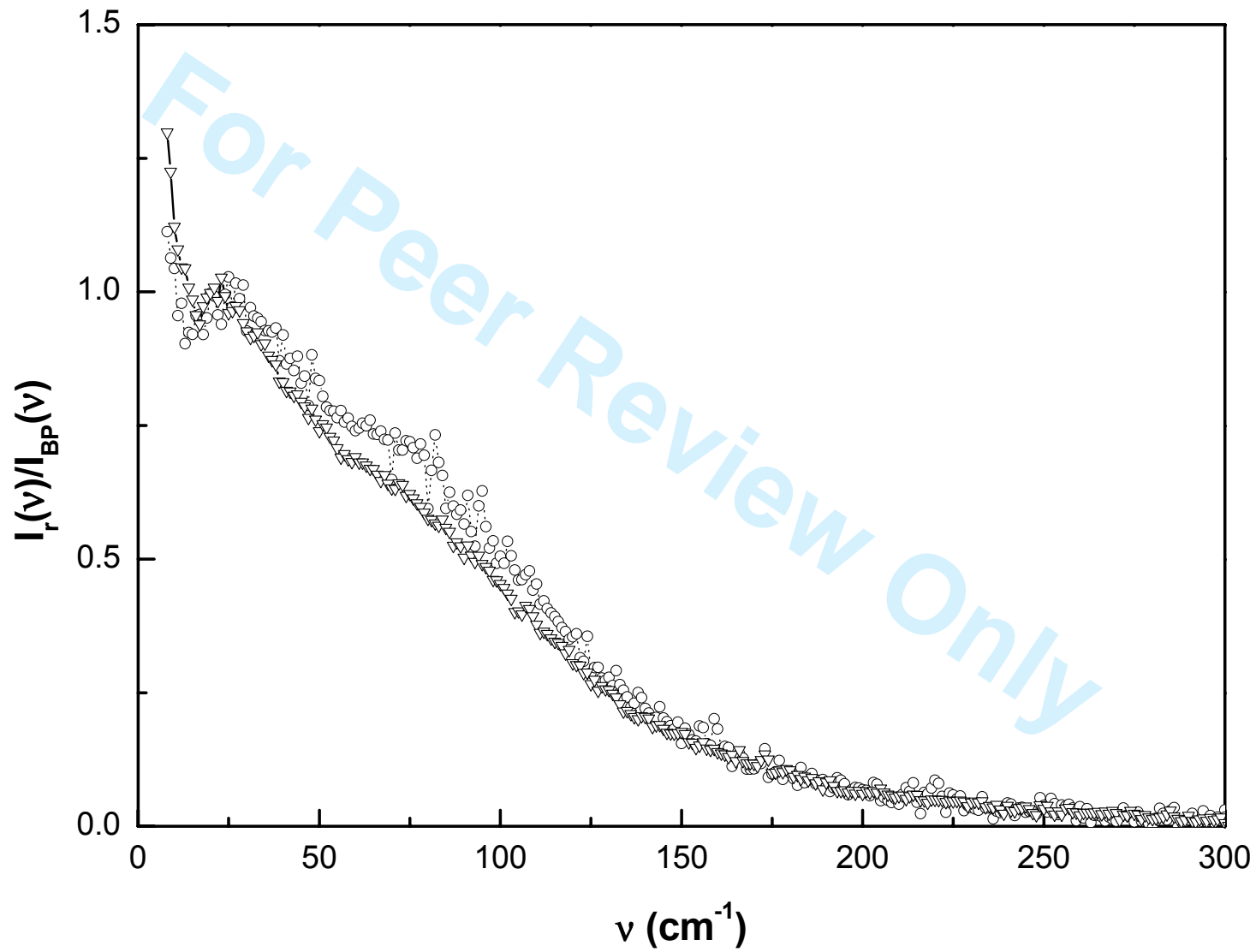


FIGURE 4







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