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Resonance Raman scattering of β-carotene solution excited by visible laser beams into second singlet state

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ABSTRACT

This study aimed to use self-absorption correction to determine the Raman enhancement of β-carotene. The Raman spectra of β-carotene solutions were measured using 488 nm, 514 nm, 532 nm and 633 nm laser beams, which exhibited significant resonance Raman (RR) enhancement when the laser energy approaches the electronic transition energy from S_0 to S_2 state. The Raman intensity and the actual resonance Raman gain without self-absorption from S_2 state by β-carotene were also obtained to evaluate the effect of self-absorption on RR scattering. Moreover, we observed the Raman intensity strength followed the absorption spectra. Our study found that, although 488 nm and 514 nm pumps seemed better for stronger RR enhancement, 532 nm would be the optimum Raman pump laser with moderate RR enhancement due to reduced fluorescence and self-absorption. The 532 nm excitation will be helpful for applying resonance Raman spectroscopy to investigate biological molecules in tissues.

1. Introduction

Raman spectroscopy is a panoply method in optical biopsy that has been used for decades in detecting human diseases, biochemical and metabolic changes of molecules. It is label free and less invasive without special preparations [1–3], in comparison to histology and with high-pressure liquid chromatography (HPLC), which is the standard technique that adds dyes for measuring antioxidant substances [4]. There are several Raman methods: spontaneous Raman, resonance Raman (RR), Surface Enhance Raman and stimulated Raman (SRS) processes. Spontaneous Raman scattering is approximately 10^{-6} of the incident laser intensity. When the incident laser wavelength is near an electronic absorption, the Raman scattering intensity is enhanced due to the resonant effect from the poles of the Raman scattering cross section probability. The resonance enhancement of the vibrational modes becomes an efficient tool for investigating large molecular components in tissues. Spontaneous Raman and resonance Raman (RR) are important in biomedical applications to investigate native molecules such as flavins, NADH (nicotinamide adenine dinucleotide) and carotenoids [5–12]. SRS uses two laser beams, one at pump and the other at Stokes beams to perform gain or loss imaging of molecules complex in tissue for lipids and protein content.

Carotene is one of carotenoids (N = 11 all trans C=C chain) antioxidant pigment that gives color and protects cells throughout the body from damage [13,14]. It is synthesized by plants and is the main chromophore in carrot, tomatoes and skin. It is involved in numerous physiological processes in humans. The body converts β-carotene into vitamin A which is needed for eye health, the immune system, and skin health. Other carotenoids such as lycopene are also involved in antioxidant processes of the defense mechanism of the body [15]. The optical energy states of β-carotenoid molecules are well known that transition from the ground S_0 ($1\,^1Ag$) state to the first S_1 ($2\,^1Ag$) state is not allowed due to symmetry restrictions [16,17], and the second excited S_2 ($1\,^1Bu$) state absorbs light between 450 and 480 nm and decays rapidly to S_1 via nonradiative relaxation [18]. The fluorescence from S_2 is weak due to the rapid S_2 to S_1 nonradiative relaxation.

Recently, RR using 532 nm light was found to be a magic Raman wavelength for tissues because of small absorption and enhancement of scattering from flavins and carotene while producing small fluorescence for detecting cancer and plaque [3,19–21]. The common excitation lasers, such as 532 nm and 514 nm, are over the edge of absorption curve of the β-carotene S_2 state. Both the incident pump laser and Raman scattering light are absorbed by β-carotene molecules, which will attenuate the actual Raman scattering intensity collected by the detector.

The focus of this paper is to measure the resonance Raman

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scattering of lasers at excitations 488 nm, 514 nm, 532 nm, and spontaneous Raman at 633 nm, to evaluate the strength of Raman follows the absorption spectra, and to correct for self-absorption in order to obtain the original intensities of the resonance Raman scattering efficiencies without self-absorption. One presumption was that 532 nm was the optimum Raman pump laser with RR enhancement for tissues.

2. Materials and Methods

2.1. Solution Preparation and Absorption Measurement

Type I β-carotene powder (2.2 mg, MW 536.87, C9750, Sigma-Aldrich) was dissolved in 10.0 mL pure acetone to make a saturated solution (saturated concentration 0.37×10^{-3} mol/L). Then the solutions were diluted 10 times and 50 times to obtain solutions with concentration 3.7×10^{-5} mol/L and 7.4×10^{-6} mol/L, respectively.

A Cary 500 UV-VIS-NIR spectrophotometer was used to measure the absorbance curve of β-carotene solution in a clean quartz cuvette with 10 mm path length. The concentration c of β-carotene in acetone is obtained by Eq. (1):

$$c = \frac{Abs}{\varepsilon l} \tag{1}$$

where *Abs* is the absorbance, ε is the molar attenuation coefficient of β-carotene in acetone ($= 134 \times 10^3$ L/mol/cm at 454 nm in acetone), *c* is the molar concentration in mol/L, and *l* is the path length (cm). The concentrations calculated by Eq. (1) are 3.4×10^{-5} mol/L([Caro] = 34 μM) and 6.8×10^{-6} mol/L([Caro] = 6.8 μM). The decreased concentration compared with the original solution is possibly due to no addition of antioxidants in the solution.

2.2. Resonance Raman Spectroscopy

Raman scattering is an inelastic scattering of a photon when encountering molecules. As shown in Fig. 1(A), a molecule interacting with a photon at frequency of ω_L is excited from the ground state to a virtual state, and then makes a transition back to the vibrational state by giving off a photon at Stokes frequency ω_S ($\omega_L - \omega_S = \omega_{20}$). The anti-Stokes frequency at ω_{AS} corresponds to the case where the photon gains energy at the expense of the molecular vibrational state. There are two possible resonances in the Stokes nonlinear polarization: one involving the vibrational state and the other for electronic transitions in Raman process. The Stokes polarization is [22]

$$\widehat{P_s} = \sum_j |\widehat{E_L}|^2 \widehat{E_s} \frac{\mu_{0j}\mu_{j2}\mu_{20}}{4h^2(\omega_{j0} - \omega_L)(\widetilde{\omega}_{20} - \omega_L)(\omega_{j0} - \omega_L)} \tag{2}$$

where μ_{0j} is dipole transition from 0 ground state to all *j* states, μ_{j2} is the dipole transition from *j* state to 2 vibrational states, $\widehat{E_L}$ is the laser electric field and $\widehat{E_S}$ is the Stokes field, $\widetilde{\omega}_{20} = \omega_{20} - i\Gamma_2$, where Γ_2 is the linewidth for the given state (one over the lifetime), and ω_q is the vibrational frequency.

The Raman intensity for different pumping wavelengths follows the absorption coefficient $\sim(\omega_s^4/\omega_L)\ \alpha(\omega_L)$ [23,24]. When the laser ω_L enters the absorption states *j*, the Stokes polarization (cross section) increases due to the denominator in Eq. (2) approaching zero. This effect is called resonance Raman (RR). In addition, when ω_{20} approaches the vibrational frequency ω_q, the middle term in the dominator becomes smaller and the polarization increases that can blow up 10 to 1000 times. Resonance Raman spectroscopy can be obtained when the energy of the excitation laser is close to the energy of electronic absorption transition of the molecule (Fig. 1(B)). The occurrence of resonance Raman will result in significant enhancement of Stokes peak intensity up to 10^3 times. Besides the increased RR scattering, chromophores can also emit fluorescence after absorbing laser photons.

The Raman spectra were acquired by using a Raman microscope (IDRaman Micro, Ocean Optics, Inc.) at excitation 532 nm with the

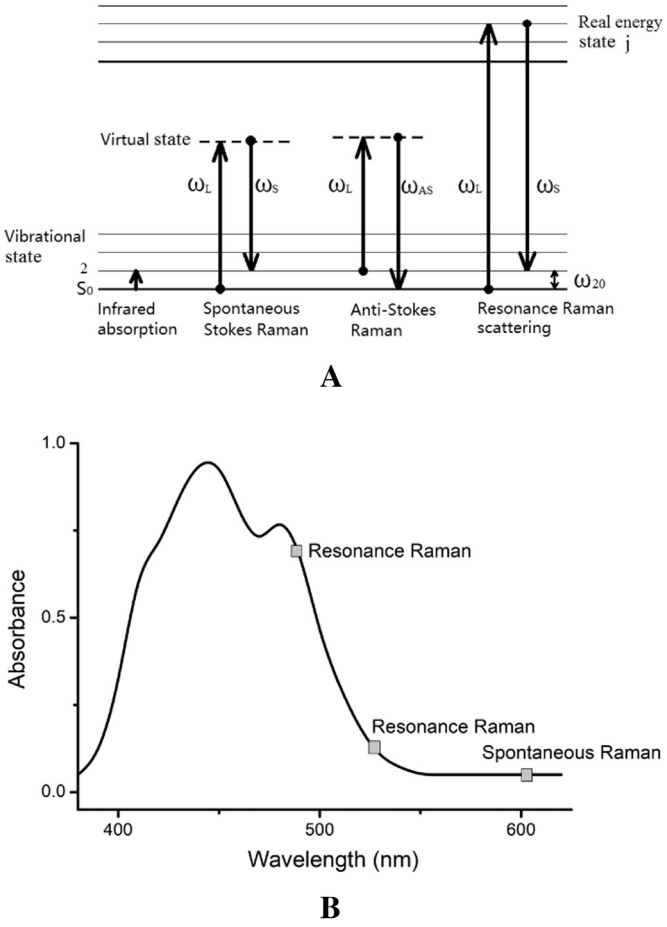

Fig. 1. (A) Theory of resonance Raman spectroscopy, IR absorption, and spontaneous Raman spectroscopy transitions energy level diagram for virtual and real Raman transitions [23]; (B) When the laser enters the absorption states *j* of carotene, the cross section increases since the poles in denominator approach zero and this process is called Resonance Raman.

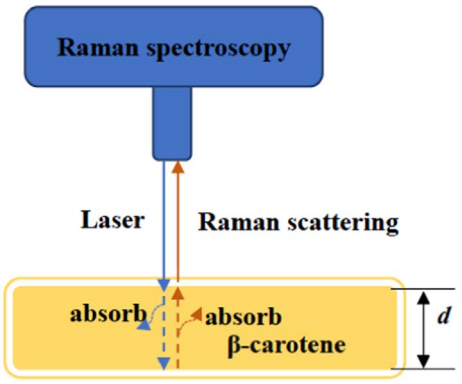

Fig. 2. Diagram for measurement of Raman scattering.

laser power of 1.6 mW, and home-made Raman spectroscopy for the excitations of 488 nm, 514 nm, and 633 nm, respectively. As shown in Fig. 2, the inelastic scattering (Raman scattering) occurs when a laser beam goes through the β-carotene solution. During the process, a part of the incident laser is absorbed by the molecules, so is the Raman scattering light.

2.3. Self-absorption Correction

The intensity of Raman scattering with self-absorption can by

described by Beer's law:

$$I_R(z) = (I_{R0}e^{-\alpha_L z})e^{-\alpha_R z} \quad (3)$$

where I_{R0} is the original intensity of Raman scattering without absorption, and α_L and α_R ($\alpha_i = \varepsilon_i c$, $i = L, R$) are extinction coefficients (cm^{-1}) at the wavelength of incident laser and Raman scattering.

When the thickness of solution layer is d (cm) (Fig. 2), the total intensity of Raman scattering (I_{Rm}) measured is the integral of Eq. (3) as follows:

$$I_{Rm,tot} = \int_0^d (I_{R0})e^{-(\alpha_L+\alpha_R)z}dz, \quad \text{and} \quad (4)$$

$$= \frac{I_{R0}}{-(\alpha_L+\alpha_R)}(e^{-(\alpha_L+\alpha_R)d} - 1) \quad (5)$$

Thus, after self-absorption correction, the original intensity of Raman scattering without absorption can be given as follows:

$$I_{R0} = I_{Rm.tot}\frac{\alpha_L+\alpha_R}{1-e^{-(\alpha_L+\alpha_R)d}} \quad (6)$$

3. Results

3.1. Raman Spectra and Fluorescence Background at Different Excitation Wavelength

Fig. 3 shows Raman spectra of β-carotene solution at concentration of 34 μM (the spectra curves for [Caro] = 6.8 μM were similar so not shown here) excited at 488 nm (blue curve), 514 nm (olive curve), 532 nm (light green curve), and 633 nm (red curve). Both β-carotene and acetone molecules contributed to the peaks in Raman spectra of carotene solution. The two intense Raman peaks at 1157 cm^{-1} and 1525 cm^{-1} corresponded to symmetric C—C and C═C stretching vibrations of β-carotene, respectively. Another smaller Raman peak at 1006 cm^{-1} was from rocking motions of the methyl groups in β-carotene molecules. The most intense peaks from acetone were at 787 cm^{-1} and 2923 cm^{-1}. When the incident laser was gradually changed from blue (488 nm) to red (633 nm), Raman peaks from β-carotene dropped off significantly compared with peaks (such as 787 cm^{-1}) from acetone. In addition, some small Raman-active modes at 2161 cm^{-1}, 2310 cm^{-1}, 2529 cm^{-1}, and 2672 cm^{-1} almost disappeared when laser wavelength was greater than 532 nm. The

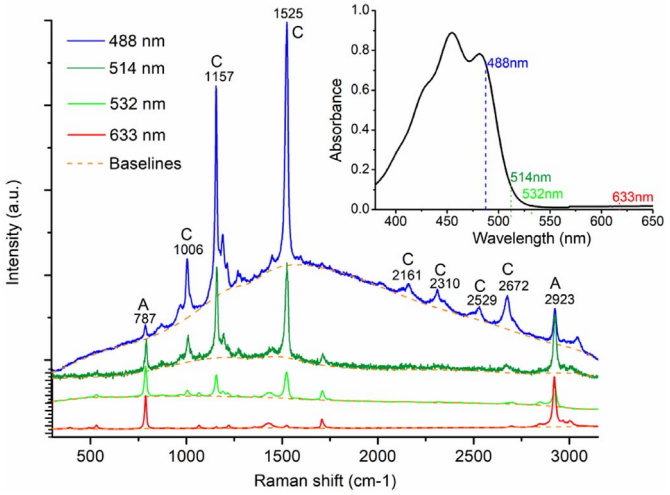

Fig. 3. Raman spectra of β-carotene solution ([Caro] = 34 μM) excited at 488, 514, 532 and 633 nm, respectively. The orange dash curves are the baselines for Raman spectra. The inset graph is the absorbance curve of β-carotene solution in a 2 mm thick cuvette. The numbers with letter 'C' represent Raman peaks from β-carotene, and the numbers with 'A' are peaks from acetone molecules. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

Table 1
Ratios of Raman peak intensity to background intensity ($I_{R/B}$).

Concentration	Excitation			
	488 nm	514 nm	532 nm	633 nm
6.8 μM	1.06	0.246	0.565	0.132
34 μM	1.36	1.17	2.42	0.442

variations among four Raman spectra are most likely due to different locations of excitation laser at absorption spectrum of β-carotene solution (the inset graph in Fig. 3), and thus the attenuation of resonance effect of β-carotene.

The four spectra also displayed different shapes and amplitudes of baselines (orange dash curves in Fig. 3), which were mainly from the fluorescence of β-carotene molecules. Intensity ratios of Raman peak to background ($I_{R/B}$) at 1525 cm^{-1} were calculated and shown in Table 1. At [Caro] = 34 μM, the value of $I_{R/B}$ in the Raman spectrum excited at 532 nm was the highest among the four spectra. While at lower concentration of 6.8 μM, $I_{R/B}$ at 532 nm was larger than those at 514 nm and 633 nm, but smaller than that at 488 nm due to the much higher RR peak excited at 488 nm. The 488 nm shows the RR vibrations marked with C in silent Raman region of 1800 cm^{-1} to 2700 cm^{-1}.

3.2. Resonance Raman Scattering and the Effect of Self-absorption

Different from β-carotene molecules, the absorption peaks of acetone are located in the UV range. When excited by visible light, Raman lines of acetone obtained were from spontaneous Raman scattering, and thus intensities of Raman peak from acetone remained about the same in our experiments. In this paper, measured total relative intensities (I'_{Rm_tot}) from β-carotene molecules were used as the ratios of peak intensities at 1157 cm^{-1} to those at 787 cm^{-1} (from acetone) excited by lasers with different wavelengths. Our results showed that at β-carotene solution concentration of 34 μM, I'_{Rm_tot} decreased from 14.70 to 0.0588 (Table 2A) when excited from 488 nm to 633 nm,

Table 2
Intensity of Raman peaks at 1157 cm^{-1} and resonance Raman gain (RRG) before and after self-absorption correction when (A) [Caro] = 34 μM and (B) [Caro] = 6.8 μM.

Wavelength (nm)	Extinction coefficient α (cm^{-1})	I'_{Rm_tot}	RRG_{Rm_tot}	I'_{R0}	RRG_{R0}
A					
λ_{laser} = 488	3.62126				
$\lambda_{Raman\ shift}$ = 517	0.34085	14.70	250	59.37	918
λ_{laser} = 514	0.47428				
$\lambda_{Raman\ shift}$ = 547	0.05814	3.08	52.4	3.97	61.4
λ_{laser} = 532	0.09532				
$\lambda_{Raman\ shift}$ = 567	0.0535	0.716	12.2	0.771	11.9
λ_{laser} = 633	0.08447				
$\lambda_{Raman\ shift}$ = 683	0.10904	0.0588	1.0	0.0647	1.0
B					
λ_{laser} = 488	0.72046				
$\lambda_{Raman\ shift}$ = 517	0.05931	2.81	183.7	4.05	263
λ_{laser} = 514	0.08627				
$\lambda_{Raman\ shift}$ = 547	0.00139	0.656	42.9	0.685	44.5
λ_{laser} = 532	0.00912				
$\lambda_{Raman\ shift}$ = 567	0.000543	0.138	9.0	0.139	9.0
λ_{laser} = 633	0.00573				
$\lambda_{Raman\ shift}$ = 683	0.01059	0.0153	1.0	0.0154	1.0

respectively; and I'_{Rm_tot} decreased from 2.81 to 0.0153 (Table 2B) at β-carotene concentration of 6.8 μM.

At the different excitation wavelength of 488 nm, 514 nm, 532 nm and 633 nm, the Raman peak at 1157 cm^{-1} shifted to 517 nm, 547 nm, 567 nm and 683 nm, respectively. According to the absorbance curve of β-carotene solution in Fig. 3, extinction coefficients α declined rapidly when the wavelengths of excitation laser (λ_{laser}) and Raman shift ($\lambda_{Raman\ shift}$) were changed from blue to red (Table 2). After self-absorption correction by using Eq. (6), the original relative intensities of Raman scattering (I'_{R0}) decreased from 59.37 to 0.0647 when the incident laser changed from 488 nm to 632 nm (Table 2A). At the lower concentration (6.8 μM), I'_{R0} was reduced from 4.05 to 0.0154 (Table 2B).

Although the fluorescence of β-carotene was excited by blue or green light located in the wing of the absorption peak, Raman peaks were enhanced significantly due to resonance effects compared with spontaneous Raman peaks excited using 633 nm. Resonance Raman gains (RRG), which are equal to the ratio of I'_{Rm_tot} or I'_{R0} at each wavelength compared to that at 633 nm, were used to estimate the resonance effect (Table 2). Before self-absorption correction, RRG_{Rm_tot} of β-carotene solution with concentration of 34 μM were 250, 52.4, and 12.2 at laser wavelengths of 488 nm, 514 nm, and 532 nm, respectively. After self-absorption corrections, RRG_{R0} was upregulated largely to 918 at 488 nm excitation, while RRGs at 514 nm and 532 nm changed slightly after correction, to 61.4 and 11.9, respectively. When β-carotene solution was diluted to a lower concentration, RRG at 488 nm excitation changed from 183.7 to 263 after correction, while RRGs at 514 nm and 532 nm remained almost the same after correction. Both RRGs with and without self-absorption correction were smaller than those at [Caro] = 34 μM (Table 2B).

Fig. 4(A) and (B) show the relationships between the absorption curve and measured total Raman intensity I'_{Rm_tot} (black square) and the original intensity I'_{R0} (red triangle) at concentrations of 34 μM (A) and 6.8 μM (B). At both concentrations, I'_{R0} matched the absorption curve better than I'_{Rm_tot}, indicating the effect of self-absorption on resonance Raman scattering.

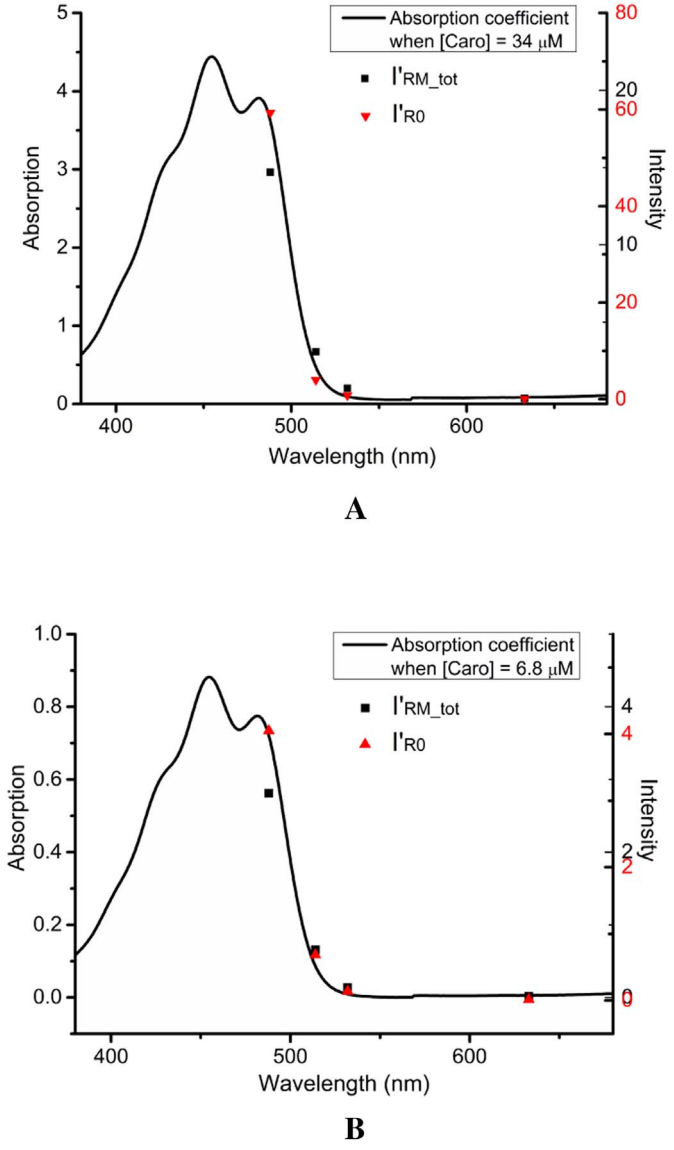

Fig. 4. Relationship between absorption coefficient and Raman intensities of β-carotene solution before and after self-absorption correction. (A) [Caro] = 34 μM. (B) [Caro] = 6.8 μM. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

4. Discussion

Resonance Raman spectroscopy has been shown to be an important tool for studying biological materials and tissues. When the incident laser frequency is close to an electronic transition of the molecule of interest, a subset of Raman-active modes is enhanced greatly, which would facilitate the study of molecules at low concentration and might provide more detailed information. In our study, different excitation laser wavelengths were chosen at the wing of absorption peaks of β-carotene. Raman peak intensity of β-carotene solution was indeed amplified greatly due to absorption transitions. However, under resonance conditions, incident laser and Raman scattering were absorbed by β-carotene molecules, and the fluorescence was unavoidably excited, both of which influence measured Raman spectra. Fluorescence emission contributed a very broad peak to Raman spectra to form the background. Re-absorption of laser light and Raman scattering also attenuated the Raman peak intensities.

The Raman spectra of β-carotene in acetone were measured with excitation at 488 nm, 514 nm, 532 nm and 633 nm, and the influence of fluorescence on Raman peaks by intensity ratios of Raman peak to background ($I_{R/B}$) was evaluated using simple Beers law method for self-absorption correction. The closer the excitation laser to the absorption peak was, the larger the Raman peak was and with more fluorescence. The Raman peak at 1525 cm^{-1} excited at 532 nm exhibited higher relative fluorescence intensity compared with those at 514 nm and 633 nm. The laser wavelength of 488 nm indeed would excite very high Raman peaks, but meanwhile large fluorescence was excited, especially at a higher concentration (34 μM). Our results also showed that a series of original Raman peak intensities after self-absorption correction excited at different laser wavelength could match the absorption spectra better than that of measured total Raman intensities. The consistency indicated self-absorption effect of β-carotene on laser and Raman light was obvious when the laser wavelength was close to an electronic absorption peak.

Another result showed the effect of self-absorption on resonance Raman gain (RRG). Both the incident lasers and corresponding Raman scatterings were absorbed in different proximity to absorption peak. When excited at 488 nm, resonance Raman gains after self-absorption correction were much larger than that without correction. But RRGs changed slightly when exited at 514 nm and 532 nm due to the reduced edge absorption at Raman frequency of carotene.

Due to the faster transition from S_2 to S_1 in a β-carotene molecule there is not much fluorescence from S_2. On the other hand, flavins have allowed transition from S_0 to S_1 and larger fluorescence from S_1 to S_0. So 532 nm may be a better choice for RR in tissues due to less fluorescence background and weaker self-absorption than 514 nm and 488 nm excitations.

Besides β-carotene, lycopene is another important carotenoid in

human bodies that has different absorption curve from β-carotene. The excitation wavelength at 514 nm, which is close to the peak of the absorption of lycopene, excited more lycopene than other carotenoids. There would be a strong self-absorption by lycopene and apparent influence on the Raman scattering from β-carotene when 514 nm is used to investigate tissues like the skin. In addition, a significant red shift of absorption peak of β-carotene in the skin has been reported [25]. Due to closer proximity to the absorption peak, excitation at 488 nm and 514 nm will result in stronger self-absorption and larger fluorescence background in the skin. Those evidences also support that 532 nm is a better laser beam for RR in biological tissues. In a study of laser wavelength's effect on the enhancement of the 1524 cm^{-1} mode, it was found that 532 nm lies at the onset of the Raman enhancement due to the electronic transition [26]. In contrast to a previous study that using a wavelength with maximum resonance enhancement of the carotenoid [27], we show here that being slightly off resonance can provide better signal to noise ratio when considering self-absorption effects.

This study used β-carotene to examine the self-absorption effect in resonance Raman spectroscopy. In biological tissues, measurement of the Raman spectrum of β-carotene will also be influenced by other factors or other molecules as well. In addition, other carotenoids have different absorption curves from β-carotene, therefore, future studies may use other carotenoids to investigate the effect of self-absorption on resonance Raman. In summary, the Raman spectra of β-carotene solutions were excited by different visible laser wavelengths. Significant RR enhancement occurred when the laser energy became closer to the electronic transition energy from S_0 to S_j for upper $j = 2$. Using the method of self-absorption correction on laser and Raman wavelengths, we obtained original Raman intensity and actual resonance Raman gain without effect of absorption, and evaluated effect of self-absorption on Raman scattering. These results are helpful for applying resonance Raman spectroscopy to investigate biological molecules in tissues for disease detection. The wavelengths of 488 nm and 514 nm seem better for stronger RR enhancement than 532 nm for Raman scattering for β-carotene. Due to the fact that the excited state S_1 is not dipole allowed, the fluorescence from S_2 is weak due to the rapid transfer to S_1 and its reduced nondipole emission. This results in the advantages of less fluorescence and weaker self-absorption effect for 532 nm excitation. The fluorescence peak of flavins is at about 520 nm, therefore the pump wavelength for tissue at 532 nm may be favorable for RR from the flavins because of less resonance, in out resonance, and weaker fluorescence. The work is underway for flavins to validate if the 532 nm is better for tissues and for resonant stimulated Raman scattering (RSRS) processes.

Acknowledgments

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