Remote temperature monitoring in ocular tissue using confocal Raman spectroscopy

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Abstract. We demonstrated the feasibility of Raman spectroscopy for remote temperature monitoring within the aqueous humor of the rabbit eye *in vivo*. Using a confocal Raman spectroscopy system, Raman spectra from 2580 to 3800 cm^{-1} were recorded in HPLC-grade water and in the aqueous humor of the rabbit eye under *in vivo* and *ex vivo* conditions within a temperature range of 14–34 °C. The ratio between the integrated Raman intensities of two temperature dependent OH-vibrational regions (OH2/OH1) in the spectra of water showed high linear dependence on temperature both in pure water [$0.0049(\pm 1.2\%)T + 0.4522(\pm 0.5\%)$, $R^2 = 0.99$, $n = 50$, $p < 0.05$], as well as in the rabbit aqueous humor [$0.0036(\pm 2.8\%)T + 0.4966(\pm 0.6\%)$, $R^2 = 0.98$, $n = 162$, $p < 0.05$] with a high degree of reproducibility and sensitivity (~0.2–0.7 °C). Raman spectroscopy can be used for high resolution and remote monitoring of temperature in the aqueous humor under *in vivo* conditions. [DOI: 10.1117/1.1911901]

Keywords: Raman spectroscopy; temperature; remote sensing; ocular tissue.

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Introduction

Raman spectroscopy (RS) is a powerful optical method for the biochemical characterization of biological media.[1] In particular, temperature effects on various biological systems have been studied *in vitro* using this spectroscopic technique.[2–5] The temperature of samples and specimens can also be determined since Raman scattering is a temperature dependent process. A well-established method for thermometry using RS entails the assessment of the Stokes/anti-Stokes Raman intensity ratio.[6] Because the anti-Stokes lines are usually extremely weak at physiological temperatures, the applicability of this method in living biological tissues is limited. Another previously reported method consists of assessing the temperature-dependent changes in the shape of the Raman spectrum of water[7,8] and has been applied for the remote temperature assessments of subsurface ocean waters[9] and biological tissues.[10] The latter could have significant applications in biology and medicine since most biological tissues are embedded in water.

The feasibility of using RS for remote temperature measurements in the eye was investigated. This is of clinical significance because it could potentially yield the evaluation of the temperature reducing effect of ophthalmic anti-inflammatory drugs[11,12] and the monitoring of therapeutic interventions involving potentially hazardous increases in intraocular temperature. The latter could be an important issue during phaco-emulsification of the ocular lens,[13] laser photorefractive surgery of the cornea,[14] or localized heating of the eye during chemotherapy of intraocular tumors.[15] In addition, currently used steady-state temperature or heat-transfer models of the eye could be validated through the accurate determination of local variations in temperature within the eye.[15,16] However, no suitable technique is available for these kinds of assessments. Invasive methods such as the use of thermocouples are generally too intrusive for application in the eye and are unpractical for spatially resolved temperature measurements. The noninvasive detection of infrared radiation from the eye does not usually permit direct intraocular temperature assessments because of the strong water absorption of this type of radiation. This technique has been used in the past to measure corneal temperature as a predictor for the pharmacological potential of ocular anti-inflammatory drugs.[12]

We have previously studied the use of Raman scattering to detect biological molecules within aqueous humor[17,18] specimens and have also reported on a noncontact confocal Raman spectroscopy (CRS) system specifically designed for the rapid and sensitive characterization of biochemical properties of ocular tissues *in vitro*.[19] Apart from the application of CRS for pharmacokinetic measurements in the eye,[20] the spatial and temporal distribution of corneal hydration in anesthetized rabbits[21,22] was assessed. With this method, the first *in vivo* Raman spectra of human corneas were successfully recorded and changes in corneal hydration due to the application of a topical dehydrating agent[23] were detected.

The noncontact mode of operation of the CRS technique and the ability to assess small volumes of material make this technique applicable for remote temperature measurements within the eye. Thus, with this project, it was sought to deter-

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mine the feasibility of CRS for temperature monitoring in aqueous solutions and the aqueous humor of the rabbit eye under *in vivo* circumstances.

Materials and Methods

A noncontact confocal CRS system, for which a detailed description and overall performance have been described elsewhere,[19] was used for Raman spectroscopic assessments of aqueous solutions and aqueous humor of rabbit eyes under *in vivo* and *in vitro* circumstances at various temperatures. The major instruments of this system are an argon laser (CR-4 model from Coherent Laser Group, Palo Alto, CA) as the excitation source (514.5 nm), a planachromat (25×numerical aperature=0.5; ausJena, Jena, Germany) microscope objective with a 12 mm working distance that focuses the incident laser light and collects the backscattered Raman signal, and a single grating spectrometer (SPEX 500 M; Spex Industries, Edison, NJ) with a nitrogen cooled charge coupled detector array (CCD) consisting of 1024×256 pixels. A 400 μm diameter fiber (2 m long) that transmits the Raman signal to the spectrometer and acts as the pinhole in the confocal configuration, providing a 920 μm integration depth within the aqueous humor.

For calibration purposes, HPLC-grade water was heated to ~50 °C, transferred to a quartz cuvette, and left to cool to ~30 °C while monitoring the temperature with a calibrated thermocouple as the control. Raman spectra in the Raman shift range from 2580 to 3780 cm^{-1} were recorded at regular intervals using 25 mW of argon-laser light and exposure times of 10 s. In addition, tests to determine whether the Raman spectral features of water would change due to the direct exposure of laser radiation were performed. Consequently, to examine potential heating of water by the probing laser beam, Raman spectra were recorded in HPLC-grade water at a constant temperature of ~23 °C at irradiation powers ranging from 5 to 150 mW and exposure times ranging from 1 to 120 s.

Under an Institutional Review Board-approved animal protocol that complied with the ARVO Resolution on the Use of Animals in Research, the animal studies were performed on 16 eyes from a total of nine NZW rabbits (~3.5 kg). Prior to Raman spectroscopic assessments, the rabbits were anesthetized with Ketamine and Xylazine and were restrained in a holder to reduce movement artifacts. For reference temperature measurements, either a rectal thermoprobe was used alone ($n=13$ eyes), or in conjunction with a needle-thermocouple ($n=3$ eyes) which was positioned in the aqueous humor of the eye. In order to obtain a wide range of intraocular temperatures, the aqueous humor of the rabbit eyes was probed *in vivo* at different core temperatures with or without applying additional whole body heating with a heating pad (~40–41 °C), after euthanasia with the eye cooling down *in situ (ex vivo)*, and during warming up of a cold stored (15 °C; 1 h) enucleated eye *in vitro*. In all cases, Raman studies were conducted using the same wavelength of 514.5 nm, along with a laser power of 25 mW and an integration time of 10 s.

Initially, the Raman spectra of HPLC-grade water were analyzed qualitatively in order to find temperature dependent Raman spectral features. Consequently, the most valid, sensitive, and reproducible Raman spectral feature was utilized to predict the temperature in aqueous solutions and the aqueous humor of the rabbit eyes.

Results

Figure 1(a) shows typical Raman spectra in the range from 2580 to 3780 cm^{-1} of HPLC-grade water at two temperatures (30 and 45 °C). It can be seen that the Raman spectral features of water change as a function of temperature, since the regions from ~3000 to 3400 and ~3500 to 3650 cm^{-1} show marked differences in intensity and shape with changing temperature. However, these changes are rather subtle and can be visualized more clearly when plotting the change in Raman intensity per degree change in temperature ($\Delta I/\Delta T$) as a function of the Raman shift, as depicted in Fig. 1(b). With increasing temperature, the region labeled OH1 (2878–3430 cm^{-1}) decreases in intensity while the region OH2 (3430–3729 cm^{-1}) increases in intensity. These changes are in the order of 0.5% of the maximum photon counts at ~3190 and 3550 cm^{-1}/°C change in temperature. The occurrence of an isoskedastic point at ~3430 cm^{-1} corresponds well with reported numbers in the literature.[24] Data taken from the HPLC-water *in vitro* and the rabbit aqueous humor *in vivo* measurements yield comparable results, as can be seen from the dashed and solid curves in Fig. 1(b), respectively. This suggests a consistency in the relationship between the changes in the Raman spectral features of water and the change in temperature.

In order to find the most suitable Raman spectroscopic predictor (RP) for the temperature in an aqueous sample, eight Raman spectroscopic features were tested for their reproducibility, sensitivity, and validity, by evaluating the relationship $RP=A(\pm SE_A)T+B(\pm SE_B)$, where A and B were the slope and the intercept, respectively, of the curve-fit between water temperature (T in °C) and the Raman spectroscopic predictor. The standard errors SE_A and SE_B were used as a measure of reproducibility, while the correlation coefficient R^2 could be used as a measure of accuracy. The results are summarized in Table 1. As can be seen in Table 1, the best predictor for water temperature using Raman spectral information was the integrated Raman intensity ratio IR(3430–3729)/IR(2878–3430) with the lowest SE_A and SE_B and the highest R^2. The relationship between this predictor (OH2/OH1) and the water temperature of HPLC-grade water (T) from 30 to 46 °C is depicted in Fig. 2 yielding the formula: $OH2/OH1=0.0049(\pm 1.2\%)T+0.4522(\pm 0.5\%)$, $R^2=0.99$, $n=50$, $p<0.05$. This particular ratio was used for the remainder of the experiments.

The influence of the photothermal interaction of the laser beam with water on the Raman spectral features revealed no appreciable change in the integrated Raman intensity ratio OH2/OH1 due to increasing light fluence. Analyzing similar signal-to-noise ratio spectra of HPLC-grade water at ~23 °C showed a ratio OH2/OH1 of 0.5670±0.0016, corresponding to a calculated temperature of 23.4±0.1 °C and a standard error of ~0.3%.

The results on the noncontact Raman spectroscopic assessment of the aqueous humor temperature (range 14–34 °C) using the intraocular thermocouple as a standard are shown in Fig. 3. A strong linear relationship was found between the

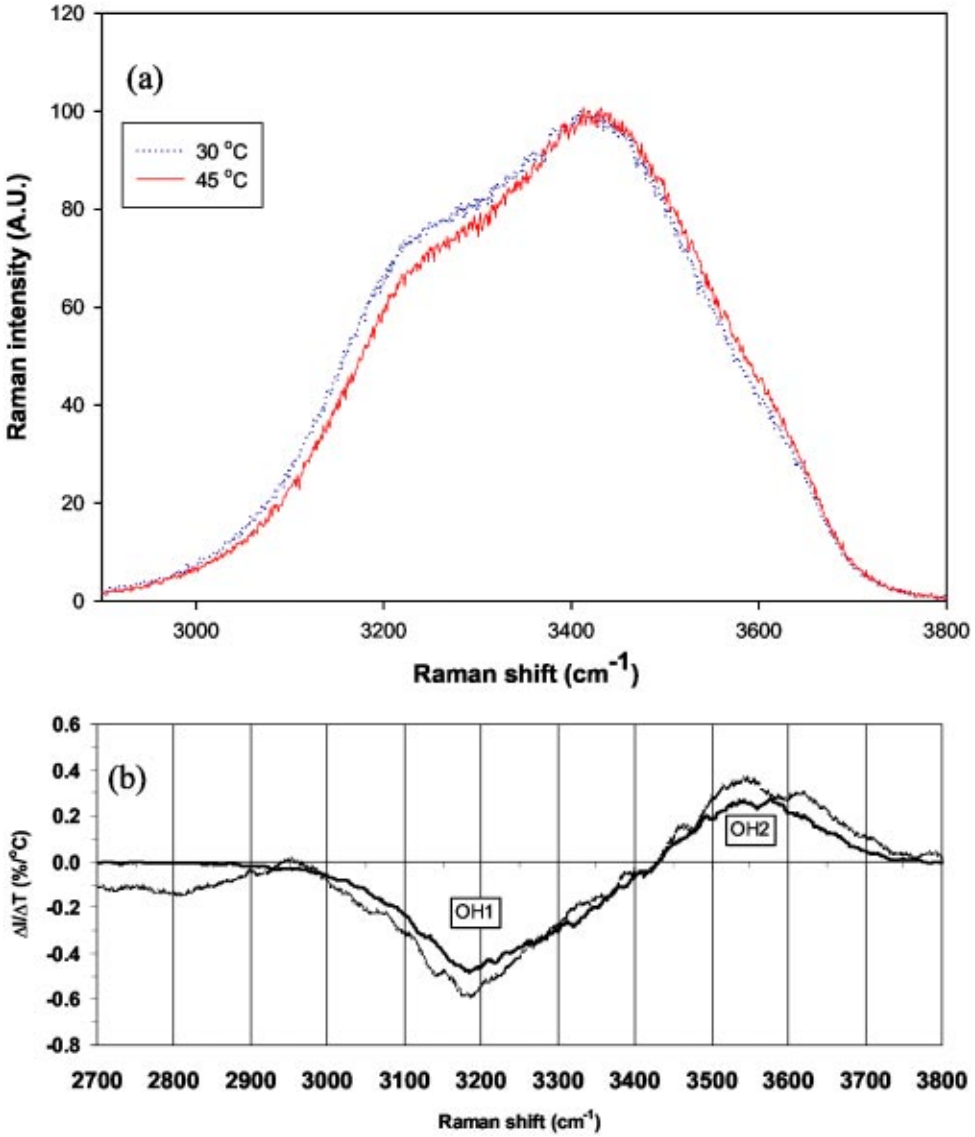

Fig. 1 (a) Raman spectra of HPLC-grade water in a quartz cuvette at two different temperatures (measured via a calibrated thermocouple), (b) change in Raman intensity (ΔI) in the spectrum of water as a function of the change in temperature (ΔT) within the HPLC-grade water (bold line) and the rabbit aqueous humor (dashed line).

measured aqueous humor temperature and the Raman intensity ratio OH2/OH1 (solid line in Fig. 3), with OH2/OH1 $=0.0036(\pm 0.0001)T+0.4966(\pm 0.0030)$ ($R^2=0.9763$, $n=162$). The slope of this relationship differed slightly from the results obtained from the experiments performed on water. However, the *in vivo* assessments agreed well with the HPLC-grade experiment (dashed line in Fig. 3). In anesthetized rabbits at a steady-state core temperature of 38.9 ± 0.7 °C, the aqueous humor temperature was 33.5 ± 0.2 °C as determined by the invasive thermocouple measurements. Noncontact Raman spectroscopic assessment of the aqueous humor revealed an integrated Raman intensity ratio OH2/OH1 of 0.613 ± 0.005 ($n=65$ assessments), corresponding to the temperature of 32.8 ± 0.3 °C using the calibrated HPLC-grade water data. No statistical significant difference between the direct and remote measurements of intraocular temperature was observed. In comparison, in anesthetized rabbits without the intraocular thermoprobe at a steady-state core-temperature of 37.9 ± 0.6 °C, an integrated Raman intensity ratio OH2/OH1 of 0.617 ± 0.009 was found ($n=199$ assessments), corresponding to a temperature of 33.6 ± 0.13 °C when utilizing the HPLC-grade water data for calibration.

Discussion

The CRS was used to determine the temperature-dependent changes in the Raman spectra of aqueous solutions and the aqueous humor of the rabbit eye at various temperatures in order to test the feasibility of CRS for assessing the intraocular temperature remotely.

A highly linear relationship was found between the OH2/OH1 Raman intensity ratio and the temperature of the aque-

Table 1 Raman spectroscopic predictors of water temperature. *I*=Raman peak intensity; IR=integrated Raman intensity.

Raman spectroscopic predictor (RP)	RP=$A(\pm SE_a)T+B(SE_b)$; with T=water temperature (°C)				
	A	SE_a(%)	B	SE_b(%)	R^2
I(3184)	–0.47	0.01(2.5)	74	0.45(0.6)	0.9720
I(3558)	0.25	0.01(5.0)	47	0.48(1.0)	0.8916
I(3558)/*I*(3184)	0.014	0.0003(1.9)	0.53	0.01(1.9)	0.9832
IR(2878–3430)	–96	3.0(3.1)	25170	118(0.5)	0.9551
IR(3430–3729)	43	1.7(3.9)	12075	65(0.5)	0.9311
IR(3430–3729)/IR(2878–3430)	0.0049	6E–5(1.2)	0.4522	0.0023(0.5)	0.9928
I(3558)–*I*(3184)	0.72	0.014(1.9)	–25	0.5(2.1)	0.9825
IR(3430–3729)–IR(2878–3430)	140	2.1(1.5)	–13100	84(0.6)	0.9887

ous humor of rabbit eyes over a temperature range from 14 to 34 °C with a high degree of reproducibility as measured by the standard error (~1.2%) of the slope. The sensitivity of the proposed thermometric application of CRS is solely a characteristic of the signal-to-noise ratio of the recorded spectra and is determined by the incident light energy used and the shot noise of the CCD camera. The aforementioned standard error corresponds to a standard deviation of ~8%. Thus, a difference in the integrated Raman intensity ratio of ~16% corresponding to a temperature difference of ~0.2 °C is easily resolved within the experimental conditions used in this study. In the *in vivo* studies, a temperature sensitivity of ~0.7 °C was achieved. As compared to the previously reported temperature measurements,[8] the improvement of the temperature sensitivity in our measurements can be attributed to the high signal-to-noise ratio Raman spectra that can be obtained with our confocal system, and, more importantly, to the fact that the whole Raman spectral characteristics of water can be recorded in single spectral scan.

A slight difference in the OH2/OH1 Raman intensity ratio versus temperature relationship was observed between the animal and the HPLC-grade water experiments, although both relationships exhibited a high linear dependence on temperature. Since the *in vivo* animal results agreed favorably with the HPLC-grade water experiments, additional variables in the *ex vivo* situation besides the temperature could have influenced the changes in the shape of the Raman spectrum of water. This difference could be partly due to the interaction of the applied euthanasia agent with the aqueous humor and the fact that the blood-aqueous barrier breaks down after euthanasia of the rabbits with a consequent leakage of proteins into the aqueous humor. This could possibly result in the occurrence of interfering fluorescence or otherwise alter the background properties of the spectra. It is also possible that the concentrations of anion (Cl^-,HCO_3^-) and cation (Na^+,K^+,Mg^{2+}) electrolytes could cause possible perturbations in the dissociation equilibrium constants of the water molecule clusters, although these concentrations within the aqueous humor are quite small (e.g., Na^+: 142 mmol per Kg of H_2O; Cl^-: 131 mmol per Kg of H_2O). Finally, the curvature of the cornea may also cause slight changes in the collection of the Raman scattered light in the *in vivo* and *ex vivo* measurements. Nevertheless, it was shown that the proposed method was easily applicable in the *in vivo* situation and dem-

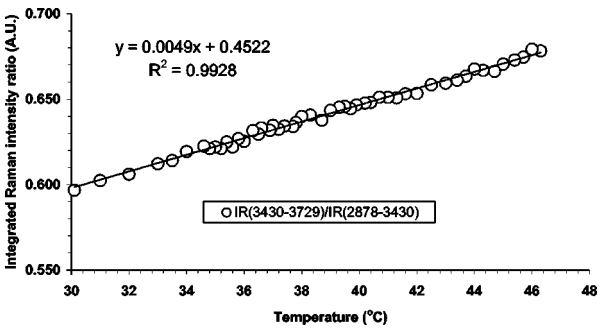

Fig. 2 Integrated Raman intensity ratio of the OH-peak regions 2878–3430 and 3430–3729 cm^{-1} as a function of water temperature.

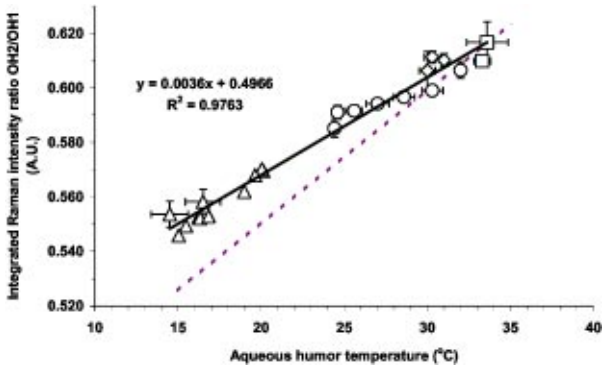

Fig. 3 Relationship between the Raman intensity ratio OH2/OH1 (n =162) and the aqueous humor temperature of rabbit eye *A* (squares: *in vivo*, circles: *ex vivo*) and eye *B* (diamonds: *in vivo*, triangles: *ex vivo*). Dashed line is the fitted linear curve from Fig. 2 extended to lower temperatures.

onstrated that the intraocular aqueous humor temperature of anesthetized rabbits can be reliably monitored remotely using noncontact confocal Raman spectroscopy.

The use of the experimental parameters of light exposure (250 mJ) at a wavelength of 514.5 nm is clearly above the retina safety levels for human applications.[25] In order to address this, we have recently obtained a CCD detecting system, which has twice the quantum efficiency of the detector used in this present study. We are also investigating the use of a longer laser excitation wavelength (632.8 or 647 nm) to improve retinal safety.[25] Changes in the optical setup are also being conducted, such as utilizing a larger diameter collecting fiber, to increase the recorded Raman scattered signal. With these changes, the ability to use this system for human studies is possible (see Ref. 23). It should also be noted that the physical properties of the confocal probe, such as the strong divergence of the excitation beam after focusing on the region of interest and the ability to precisely control positioning of the optical probe at different depths within the eye, makes it virtually impossible to accidentally focus on the retina.

In conclusion, the Raman spectrum of water is subject to change as a result of changing temperature. This indicates that care should be taken when comparing Raman spectra of biological tissues containing water (i.e., either the temperature is kept constant or the Raman spectra are corrected for the temperature dependent changes). More importantly, since water is the major component of most biological specimens, confocal Raman spectroscopy might possibly be used to noninvasively monitor the temperature of these tissues. This pilot study showed that confocal Raman spectroscopy can be applied for remote temperature measurements within the aqueous humor of the rabbit eye under *in vivo* conditions with a high degree of sensitivity, specificity, and reproducibility.

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