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Raman Spectroscopic Measurement of Relative Concentrations in Mixtures of Oral Bacteria

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Abstract

Near-infrared Raman spectroscopy has been used for species identification of pure microbial specimens for more than a decade. More recently, this optical method has been extended to the analysis of specimens containing multiple species. In this report, we demonstrate rapid, reagent-free quantitative analysis of a simplified model of oral plaque containing three oral bacteria species, *S. mutans*, *S. sanguis*, and *S. gordonii*, using near-infrared Raman spectroscopy. Raman spectra were acquired from bacterial mixtures in 200 seconds. A prediction model was calibrated by the partial least squares method and validated by additional samples. On a scale from 0 to 1, relative fractions of each species could be predicted with a root mean square error of 0.07. These results suggest that near-infrared Raman spectroscopy is potentially useful in quantification of microbial mixtures in general and oral plaques in particular.

Keywords

Raman spectroscopy; Quantification; Microorganisms; Bacteria; Oral; Partial least squares; PLS

INTRODUCTION

Rapid microbial analysis is important in many fields such as industrial clean room maintenance, food processing, and medical diagnostics. If a pure specimen of a single microbial species is available, for instance, if cultured on an agar plate or if studied at the single-cell level, then an analysis method only needs to identify what microbe is present. More typically, however, a sample contains a mixture of different microbes. In such cases, relative concentrations of different species become relevant parameters. In medical cases, prompt and accurate acquisition of this information can be crucial to delivering proper treatment. In laboratory settings as well, it is desirable to specify the relative bacterial content of biological samples, which often contain similar species.

One important example of polymicrobial mixtures is dental plaque, which accumulates on the surfaces of teeth and is dominated by *Streptococcal* bacteria in the human oral cavity.^{1,2} Among the various supragingival streptococcal species present on tooth surfaces, *Streptococcus mutans* is particularly important because of its unique role associated with tooth decay. Elevated levels of *S. mutans* have been strongly associated with dental caries.^{2–4} The level of *S. mutans* is often compared to that of a competitive species, *Streptococcus sanguis*, which is by far the most prevalent bacterial constituent in healthy plaque.¹ The *S. mutans* to

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S. sanguis concentration ratio can be regarded as an indicator of oral health. Routine quantitative measurement of the relative concentration of *S. mutans* in oral plaque would be valuable for studying animal models of caries as well as for human clinical purposes.

There are many methods of identifying a bacterial colony's species, including conventional biological or biochemical methods,⁵ more recently developed molecular biology assays such as polymerase chain reaction (PCR),^{6,7} and analytical spectroscopic methods, including pyrolysis mass spectrometry (PyMS),^{8,9} Fourier transform infrared spectroscopy (FTIR),^{10,11} surface-enhanced Raman scattering (SERS),¹² and FT-Raman,^{13,14} ultraviolet (UV) resonance Raman,^{15,16} visible Raman,^{17,18} and near-infrared Raman spectroscopy.^{19–25} To date, the PCR methods offer the best discrimination ability while the spectroscopic methods usually take the least time and do not require additional chemicals.

In principle, all of these approaches could be developed into quantitative methods to analyze concentrations in polymicrobial specimens. Examples of existing approaches include “viable counting” (i.e., visually tabulating the number of colonies) after conventional agar culturing²⁶ and quantitative PCR (q-PCR).^{27,28} Viable counting is by far the most widely used in laboratory and clinical applications; however, it usually takes 12 to 48 hours to obtain results, and uncertainties in transferal and growth efficiency limit the accuracy of the measured ratio to order-of-magnitude at best.^{6,29} Q-PCR is currently the gold standard for quantitative analysis of polymicrobial specimens. At present, however, the approach requires many pre- and post-processing steps, multiple chemical agents, and hours of sample incubation. This limits its routine application, particularly in clinical situations.

Recently, we reported an idea for a quantitative approach to polymicrobial analysis based on Raman spectroscopy.³⁰ In an initial test, concentrations of *S. mutans* and *S. sanguis* in prepared mixtures were determined from their Raman spectra using chemometric calibration. The relative fraction of *S. mutans*, on a scale from 0 to 1, was predicted with an error of ± 0.04 . In this study, however, there was complete correlation between the two species' fractions, since an increase in one meant a decrease in the other. This artificial correlation presumably aided the calibration process and generated unreliably low prediction errors. Extrapolation to mixtures of more than two species, where such correlation would not exist, was not straightforward. The next logical step in developing the Raman method for analysis of complex human and animal specimens, therefore, was to study mixtures of more than two microbial species.

In the experiments we present in this paper, three bacterial species, *S. mutans*, *S. sanguis*, and *S. gordonii* were included in the mixture system. Previously acquired Raman spectra of pure cultures of these three species (Fig. 1a) had shown that they could be unambiguously classified using the area underneath selected peak regions, as shown in Fig. 1b. Below, we present quantitative estimation of relative bacterial concentration in three-species mixture samples. The results demonstrate that the Raman technique, as a general method, is able to obtain quantitative bacterial information in multi-species bacterial mixtures without strong concentration correlations and holds promise specifically for analysis of oral plaque specimens.

METHODS

System Setup

The Raman microscope system used in this study has been described previously^{24,30} and is shown schematically in Fig. 2. Raman scattering was excited by a diode laser at 830 nm (Process Instruments, Salt Lake City, UT), with the near-infrared wavelength chosen to reduce autofluorescence compared to visible excitation. Laser light was delivered to the microscope by a multimode optical fiber (core diameter 63 μm), which was selected for increased power

at the expense of a greater spot size. Although the laser spot size could be reduced for higher spatial resolution, in these experiments a volume-averaged signal was in fact desirable. The laser light was cleaned by a band pass filter, reflected at efficiency >95% by a dichroic beam splitter, and then coupled into a Nikon E400 upright microscope. The objective (50 $\times$, 0.8 NA, Nikon) focused the incident laser power of 100 mW to a 30 μ m spot on the sample. Back-scattered light was collected by the objective and sent back to the beam splitter. Photons of wavelength longer than 860 nm passed through the beam splitter and a long pass filter and were coupled into a spectrometer (HoloSpec *f*/1.8, Kaiser) by a 100 μ m diameter core fiber. A thermoelectrically cooled, back-thinned, deep-depletion charge-coupled device (CCD) (DU420-BR-DD, Andor) was used to record Raman spectra. Spectral resolution of the system was better than 6 cm^{-1} as measured by neon gas emission lines. Depth resolution was about 30 μ m as determined by translation of a glass/air interface through the focal plane, following the method described by Caspers et al.^{31,32} The spectrograph was calibrated for wavelength using neon emission lines and for relative wavenumber using indene.³³ Spectra were recorded between 600 and 1880 cm^{-1} , spanning the so-called “fingerprint region” where biological samples exhibit their most characteristic peaks (c.f. Fig. 1).

Sample Preparation

S. mutans strain UA159, *S. sanguis* strain NCTC10904, and *S. gordonii* strain DL1 were used in this study. Approximately 10 μ L volumes of frozen bacteria were used to streak cultures on separate plates containing Todd-Hewitt (TH) (Difco, Detroit, MI) agar medium. Bacteria were then inoculated in liquid TH medium from agar plates and incubated for approximately 16 hours at 37 $^{\circ}\text{C}$ in an atmosphere of 95% air/5% CO_2 . The final optical density (OD, measured with 600 nm light) of each sample was approximately 0.8, with the bacteria in stationary growth phase. Limiting samples to this phase is consistent with eventual clinical applications, in that bacteria harvested from the oral cavity are predominantly in the stationary phase. In addition, we have observed in a separate study that spectra of *S. mutans* and *S. sanguis* in various growth phases can be grouped robustly according to species (data not shown), suggesting that growth-related spectral variations would not severely alter the results reported here.

Mixture samples were constructed by pipetting as described previously³⁰ except that three species were used. The concentration combinations followed a triangle design as shown in Fig. 3. In each sample the relative fractions of the three species were integral multiples of 0.1, and this set of 66 samples contained all possible such combinations. The consistent OD (of approximately 0.8) for all three single-bacteria stocks ensured that the resulting concentration ratios in the mixtures were similar to the ratios of the pipetted volumes. The resulting bacterial samples were centrifuged in 10 mL Corning tubes at 3000 rpm for 10 minutes at 4 $^{\circ}\text{C}$, washed by deionized water, and centrifuged again, after which the concentrated bacterial infranant was stored in a freezer. Because the mixtures were prepared in a single session but Raman spectral acquisitions were long (requiring multiple days to run all samples), freezing was the best way of ensuring consistent treatment of all samples. No spectral artifacts due to the freezing process were observed.

Data Acquisition

At the time of spectrum acquisition, samples were thawed. A droplet, approximately 1 μ L, of bacteria from each tube was transferred to a CaF_2 plate and allowed to dry into a film for approximately 15 minutes. The resulting samples were discs less than 1 mm in diameter and less than 100 μ m thick. Taking the volume of a bacterial cell to be a few μm^3 and considering their folding and packing efficiency, we estimate that each sample contained approximately 10^7 bacteria, an amount that can easily be recovered from the surface of a tooth.^{34,35} The laser excitation spot itself had a much smaller diameter (30 μ m) than the dried disc and illuminated only a few thousand bacteria.

From each sample disc, three spectra were taken at different lateral positions. About 15 minutes of photo-bleaching was performed to reduce the fluorescence background. Each spectrum contained 20 individual 10-second frames, permitting cosmic ray spikes generated within the CCD array to be identified and removed. The total effective spectral acquisition time was 200 seconds.

Data Processing and Analysis

All spectra were corrected for system background and spectral response, and a fourth-order Savitzky–Golay filter^{36,37} removed spatial-frequency noise above the 6 cm^{-1} resolution. Fluorescence background was approximated by a fifth-order polynomial fit and subtracted. To correct for variations in sample density, a step of amplitude normalization was necessary. Spectra were normalized to equal areas under the 1450 cm^{-1} peak, corresponding to CH_2 deformations. The spectra were thus rescaled to the same amount of hydrocarbons, and by extension to approximately the same number of bacteria.

Partial least squares (PLS) regression analysis³⁸ was performed on the total of 198 processed spectra ($66\text{ samples} \times 3\text{ lateral positions}$). Thirty-six (36) samples (108 spectra) were uniformly selected as the calibration group (marked with dots in Fig. 3), while the remaining 30 samples (90 spectra) were designated as the validation group. The spectral range used was from 614 to 1870 cm^{-1} , containing 750 datapoints. PLS models of different rank (number of iterations of the PLS process) were evaluated using a leave-one-sample-out cross-validation. Once the lowest-error cross-validation model was identified, its rank was used to generate a model from the whole calibration set. This PLS prediction model was then applied to the validation set.

RESULTS AND DISCUSSION

Figure 4 plots the PLS predicted *S. mutans* concentration fractions versus the reference values from the pipetting preparation, using a model of rank 11. This rank produced a minimum root mean squared error of cross-validation (RMSECV) for *S. mutans* of 0.068 (with concentration ranging from 0 to 1). When the 11-vector calibration model was applied to the validation set, a similar root mean squared error of prediction (RMSEP) of 0.067 was obtained, which coincidentally was the minimum RMSEP for all prediction model ranks. The fact that it took 11 loading vectors to model the system rather than two (as would be needed in the ideal case) indicates the presence of additional variables (such as system drift) beyond the changes in bacterial concentrations.

Models were also constructed for *S. sanguis* and *S. gordonii*. In the same fashion as for *S. mutans*, calibration sets were chosen to span the variations in the target bacteria's concentration evenly. The RMSECV was 0.065 for *S. sanguis* and 0.071 for *S. gordonii*, optimizing at 11 loading vectors in both cases as well. The RMSEP values for the validation sets were 0.071 and 0.070, respectively.

To test whether spectral shot noise was the limiting factor for the prediction error, we performed additional validations using less than the full 200 seconds' worth of data per spectrum. The improvement of RMSEP was not significant for integration times longer than 100 seconds; increasing from 100 to 200 seconds yielded an improvement of less than 3%. This suggests that our prediction model at 200 seconds is not shot noise limited.

Reference concentration error, i.e., the uncertainty in the mixture preparation process, is not a trivial fraction of the total error. We estimate that the preparation error was 2–3 percent due to pipetting technique. Another factor is the limited sampling volume. As stated earlier, we took Raman spectra only from a few thousand bacteria each time. The bacterial fractions in this small volume could be different from that of the bulk mixture; for a nominal 50/50 mixture

of two species, this sampling error could be as high as $\sqrt{0.5 \times (1 - 0.5)} / \sqrt{1000} \approx 1.6\%$. Differences in the large fluorescent background (which is not fully removed by polynomial fit) could be another major error source.

For all three species, the RMSEP was close to 0.07, whereas the error in analyzing two-species mixtures had been 0.04.³⁰ Because oral plaque can potentially contain many species at the few-percent concentration level, it is important to consider whether this increase in error would continue as samples became still more complex. The increase is due at least in part to the elimination of spurious correlations between pairs of species' concentrations; this was a particular artifact of the two-species study only. In addition, however, the addition of new species also reduces the spectral uniqueness of *S. mutans* (i.e., increases the spectral overlap); this could continue to degrade the RMSEP as the number of species in the mixtures is increased. Further studies will be needed to determine the fundamental limits on the RMSEP and how it continues to change as sample complexity increases.

At present, we can not offer a chemical interpretation on the spectral differences among these bacterial species. It is promising, however, that the 910 cm^{-1} region retains a distinctive *S. mutans* feature previously observed³⁰ even with two other species present (c.f. Fig. 1b). If this region retains its uniqueness as more oral bacteria are included in future mixtures, then a straightforward *S. mutans* assay in clinical plaques could be envisioned.

CONCLUSION

We have demonstrated the ability of Raman spectroscopy to measure the relative concentrations of bacteria in mixtures of three species with uncorrelated concentration pairings. We have shown that *S. mutans*, *S. sanguis*, and *S. gordonii* can be quantitatively measured in mixtures using Raman spectra acquired within 200 seconds, with an error of ± 0.07 on a normalized concentration scale of 0 to 1. The number of cells required is realistic for clinical collection of oral plaque. The Raman spectral region of 890–940 cm^{-1} reveals distinctive spectral features of *S. mutans* that might be retained in more complex mixtures. These results show that the Raman technique can quantitatively measure the concentration fractions of bacteria comprising a few percent or more of a microbial specimen. Efforts are currently underway to apply this technique to more complicated samples in more realistic environments, for example, in biofilms.

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References

1. Socransky SS, Manganiello SD. J. Periodontol 1971;42:484.
2. Marsh PD. Cariology 1999;43:599.
3. de Stoppelaar JD, van Houte J, Dirks OB. Caries Res 1969;3:190. [PubMed: 5267921]
4. Loesche WJ. Microbiol. Rev 1986;50:353. [PubMed: 3540569]
5. Kral TA, Daneo-Moore L. J. Dental Res 1981;60:1713.
6. de Soet JJ, van Dalen PJ, Appelmelk BJ, de Graaff J. J. Clin. Microbiol 1987;25:2285. [PubMed: 3323224]
7. Li Y, Caufield PW, Redmo Emanuelsson I, Thornqvist E. Oral Microbiol. Immunol 2001;16:16. [PubMed: 11169134]

8. Irwin, WJ. Analytical Pyrolysis: A Comprehensive Guide. Marcel Dekker; New York: 1982.
9. Goodacre R, Kell DB. Curr. Opin. Biotechnol 1996;7:20. [PubMed: 8791308]
10. Helm D, Labischinski H, Schallehn G, Naumann D. J. Gen. Microbiol 1991;137:69. [PubMed: 1710644]
11. Timmins EM, Howell SA, Alsberg BK, Noble WC, Goodacre R. J. Clin. Microbiol 1998;36:367. [PubMed: 9466743]
12. Grow AE, Wood LL, Claycomb JL, Thompson PA. J. Microbiol. Methods 2003;53:221. [PubMed: 12654493]
13. Williams AC, Edwards HGM. J. Raman Spectrosc 1994;25:673.
14. Edwards HGM, Russell NC, Weinstein R, Wynn-Williams DD. J. Raman Spectrosc 1995;26:911.
15. Nelson WH, Manoharan R, Sperry JF. Appl. Spectrosc. Rev 1992;27:67.
16. Manoharan R, Ghiamati E, Chadha S, Nelson WH, Sperry JF. Appl. Spectrosc 1993;47:2145.
17. Schuster KC, Urlaub E, Gapes JR. J. Microbiol. Methods 2000;42:29. [PubMed: 11000428]
18. Huang WE, Griffiths RI, Thompson IP, Bailey MJ, Whiteley AS. Anal. Chem 2004;76:4452. [PubMed: 15283587]
19. Puppels GJ, de Mul FFM, Otto C, Greve J, Robert-Nicoud M, Arndt-Jovin DJ, Jovin TM. Nature (London) 1990;347:301. [PubMed: 2205805]
20. Maquelin K, Choo-Smith LP, van Vreeswijk T, Endtz HP, Smith B, Bennett R, Bruining HA, Puppels GJ. Anal. Chem 2000;72:12. [PubMed: 10655628]
21. Maquelin K, Choo-Smith LP, Endtz HP, Bruining HA, Puppels GJ. J. Clin. Microbiol 2002;40:594. [PubMed: 11825976]
22. Maquelin K, Kirschner C, Choo-Smith LP, van den Braak N, Endtz HP, Naumann D, Puppels GJ. J. Microbiol. Methods 2002;51:255. [PubMed: 12223286]
23. Kirschner C, Maquelin K, Pina P, Thi NAN, Choo-Smith LP, Sockalingum GD, Sandt C, Ami D, Orsini F, Doglia SM, Allouch P, Mainfait M, Puppels GJ, Naumann D. J. Clin. Microbiol 2001;39:1763. [PubMed: 11325987]
24. Berger AJ, Zhu Q. J. Mod. Opt 2003;50:2375.
25. Hutsebaut D, Maquelin K, de Vos P, Vandenabeele P, Moens L, Puppels GJ. Anal. Chem 2004;76:6274. [PubMed: 15516118]
26. McKee AS, McDermid AS, Ellwood DC, Marsh PD. J. Appl. Bacteriol 1985;59:263. [PubMed: 3932293]
27. Rupf S, Kneist S, Merte K, Eschrich K. Eur. J. Oral Sci 1999;107:75. [PubMed: 10232454]
28. Rudney JD, Pan Y, Chen R. Archives Oral Biol 2003;48:475.
29. Babaahmady KG, Challacombe SJ, Marsh PD, Newman HN. Caries Res 1998;32:51. [PubMed: 9438572]
30. Zhu Q, Quivey RG, Berger AJ. J. Biomed. Opt 2004;9:1182. [PubMed: 15568938]
31. Caspers PJ, Lucassen GW, Puppels GJ. Biophys. J 2003;85:572. [PubMed: 12829511]
32. Rajadhyaksha M, Gonzalez S, Zavislan JM, Anderson RR, Webb R. J. Invest. Dermatol 1999;113:293. [PubMed: 10469324]
33. Wollman ST, Bohn PW. Appl. Spectrosc 1993;47:125.
34. Beckers HJA, van der Hoeven JS. Infect. Immun 1982;35:583. [PubMed: 7056576]
35. Beckers HJA, van der Hoeven JS. Archives Oral Biol 1984;29:231.
36. Savitzky A, Golay M. Anal. Chem 1964;36:1627.
37. Steinier J, Termonia Y, Deltour J. Anal. Chem 1972;44:1906.
38. Haaland DM, Thomas EV. Anal. Chem 1988;60:1193.

Fig.1.

(a) Typical Raman spectra of *S. mutans*, *S. sanguis*, and *S. gordonii* cultured in liquid Todd-Hewitt medium. See the Methods section for system details and culture/acquisition protocols. (b) Identification of pure *S. mutans*, *S. sanguis*, and *S. gordonii* specimens (circles, stars, and squares, respectively) based upon the integrated area under Raman peaks centered at 910 and 820 cm^{-1} . Lines are easily drawn to group the datapoints by species. The Raman peak at 910 cm^{-1} distinguishes *S. mutans* from the other two oral bacteria, while *S. sanguis* and *S. gordonii* can be separated with assistance from a peak at 820 cm^{-1} .

Fig. 2.
Schematic plot of the Raman microspectroscopy system; see text for details.

Fig. 3. Triangle design of percent concentrations for mixture samples used in the three-species study. Each of the 66 clusters represents the composition of one mixture sample, while each of the three circles in a cluster represents a bacterial species (clockwise from the top: *S. mutans*, *S. sanguis*, and *S. gordonii*). The darkness of each circle is proportional to the concentration of the corresponding species. 36 of the 66 samples, identified by a black dot in the middle, were used as a calibration set, while the rest (30 samples) were the validation set.

Fig.4. *S. mutans* concentrations generated via PLS analysis using 11 loading vectors. (○) The cross-validation of the calibration set, and (×) the predictions from the validation set. The dashes indicate the line of perfect prediction and have been added to guide the eye.