paper on methodology

Surface enhanced Raman spectroscopic monitor of *P. acnes* lipid hydrolysis in vitro

Millicent K. Weldon,* Michael D. Morris,[1,*] A. B. Harris,[†] and Janice K. Stoll[†]

Department of Chemistry,* University of Michigan, 930 N. University Ave., Ann Arbor, MI 48109-1055, and Department of Biologic and Materials Science,[†] School of Dentistry, University of Michigan, 1011 N. University Ave., Ann Arbor, MI 48109-1078

Abstract Surface enhanced Raman spectroscopy (SERS) at a silver microelectrode was used to monitor bacterial hydrolysis of triglycerides in lipid mixtures that model sebaceous gland secretions. Mixtures of wax esters, squalene, triolein, and triisostearin were used as model skin secretions. The transformation was followed in vitro as changes in the SERS caused by hydrolysis of triglyceride to fatty acid. The fatty acid was adsorbed as its carboxylate, which is readily identified by the characteristic band at ca. 1395 cm^{-1}. Co-adsorption of propionate was also observed. The technique can also confirm the presence of bacteria by detection of short chain carboxylic acids released as products of fermentation during the growth of these cells.—Weldon, M. K., M. D. Morris, A. B. Harris, and J. K. Stoll. **Surface enhanced Raman spectroscopic monitor of *P. acnes* lipid hydrolysis in vitro. *J. Lipid Res.* 1998.** 39: **1896–1899.**

Supplementary key words surface-enhanced • Raman spectroscopy • microelectrode • lipids • fatty acids • triglycerides • bacterial hydrolysis

Propionibacterium acnes (*P. acnes*) is a major microbial inhabitant of human skin. These bacteria colonize sebaceous follicles and skin surface and it is thought that they use the nascent sebum emulsion as a source of nutrition. Skin surface lipids are comprised of approximately 43% triglycerides, 25% wax esters, 16% free fatty acids, 12% squalene, and 2.5% cholesteryl esters (1). Nascent sebum from the sebaceous gland has no free fatty acids. By the time sebum reaches the skin surface it contains small amounts of diglycerides, monoglycerides, free fatty acids, and free sterols (2). Marples, Downing, and Kligman (3) have shown that bacteria located in the follicular lumen produce lipases that hydrolyze triglycerides. The reduction in triglyceride composition as the lipids progress toward the surface of the skin is proportional to the increase in free fatty acid concentration. Ingham et al. (4) purified the lipase and characterized its lipolytic activity for a range of triglycerides. These investigations suggest that the enzymes probably serve a function in the nutrition of the organism. Gribbon, Cunliffe, and Holland (2) suggested an additional role related to the adhesion of the bacteria to the lipid rich skin. However, if overproduced, the free fatty acids may serve as irritants in the skin (5).

Several in vitro studies have been performed to investigate the hydrolysis of lipids by *Propionibacterium acnes* (2, 3, 6–8). Until now, the detection of free fatty acids in sebum and skin surface lipid mixtures has been performed by two methods: thin-layer chromatography with quantification by photodensitometry (6, 7); or by extraction of the long chain fatty acids using a two-phase solvent system followed by titration (2).

We have used surface enhanced Raman scattering (SERS) at silver (Ag)-deposited silicon substrate microprobes to monitor the bacterial hydrolysis of target triglycerides as well as lipid mixtures in situ. We have already shown that these silver SERS surfaces are selective for fatty acids in a mixture of model sebum lipids (9). We also show that we can detect the presence of bacteria by assaying for the short chain carboxylic acids released as products of fermentation during the cycle of these cells.

The probe design is derived from those of electrodes designed for neurophysiological experiments on living rodents. The silicon substrate is strong enough to survive insertion into small openings, such as the canal of a sebaceous pore. Therefore, these or similar probes may find ultimate use in monitoring the biochemistry of the sebaceous follicles.

EXPERIMENTAL

Reagents

P. acnes 6919 was obtained from ATCC. The lipid mixture included triolein, triisostearin, isostearyl isostearate, oleyl oleate, and squalene. Triisostearin was received as a

Abbreviations: SERS, surface enhanced Raman spectroscopy; *P. acnes*, *Propionibacterium acnes*; SCE, saturated calomel electrode.

[1] To whom correspondence should be addressed.

gift from Unichema Corp. All other reagents were obtained from Sigma (99% purity) and used as received. The bacteria were first streaked out on Schaedler Blood Agar plates and then cultured in liquid broth that consisted of yeast extract (3 g/L), beef extract (10 g/L), peptone (10 g/L), soluble starch (10 g/L), glucose (1 g/L), cysteine hydrochloride (0.5 g/L), sodium chloride (5 g/L), sodium acetate (3 g/L), agar (0.5 g/L), and glycerol as an emulsifier (1%). The cultures were incubated anaerobically at 35°C under an atmosphere of 85% N_2, 5% H_2, 10% CO_2. Silver surfaces on the silicon substrate microprobes were prepared using an AgCN plating solution (10). A 0.1-m KCl solution was used for the activation of the SERS surfaces. A 0.1-m KH_2PO_4 solution was used in experiments involving electrochemical adsorption.

Instrumentation

Lithographically constructed silicon substrate SERS microprobes were used. These consist of electrodeposited and roughened silver on an iridium base layer. The fabrication process has been outlined elsewhere (11). The iridium base layer is a strip 10 μm × 700 μm on the silicon substrate with electrical contact through a macro support and integrated circuit connector. The iridium is overcoated with silver from a silver cyanide solution by electroplating (10) at −1.2 V versus SCE.

The Raman microprobe has been previously described (12). The excitation source was a 633 nm He-Ne laser. Powell lens illumination (K. A. Christensen and M. D. Morris, unpublished results) was used in all experiments. Power at the sample was 100 μW/μm^2. An Olympus 10×/0.3 NA objective was used to focus the laser light onto the microelectrode and to collect the SERS signal. The spectrograph was a Kaiser Optical Systems Holospec f/1.8i fitted with a Photometrics CH250 thermoelectrically cooled CCD detector. The microelectrodes were positioned on an Olympus IMT-2 inverted research microscope using micromanipulators. The silver SERS microprobe was positioned with a hydraulic micromanipulator (Narishige MO-303). In all measurements the long axis of the silver strip was oriented approximately parallel to the beam.

Procedures

For activation, a probe was immersed in a drop of 0.1 m KCl solution spotted on a cover glass. The probe was biased to +200 mV versus Ag/AgCl for 0.5 s and then to −600 mV for 0.5 s. Several oxidation–reduction cycles were used to condition the surface.

Hydrolysis of lipids by bacteria was performed following the protocol of Reisner et al. (6) with slight modifications. Triolein (20 μL) was added to 0.5 mL liquid medium at room temperature and then autoclaved for 15 min at 121°C. Fresh cultures of *P. acnes* grown anaerobically at 35°C on Schaedler Blood Agar plates were used as inocula. Organisms were suspended in liquid medium and standardized to an optical density of 0.61 at 420 nm. Each reaction tube was inoculated with 40 μL of cell suspension. Controls of media with triglyceride and inoculated media without triglycerides were included in each experiment. Tubes were incubated anaerobically at 35°C for 2–10 days under an atmosphere of 85% N_2, 5% H_2, 10% CO_2. The tubes were agitated daily under anaerobic conditions on a Vortex mixer to ensure even dispersion.

After incubation two techniques were used to detect the lipolysis products. First, the lipids and long chain fatty acids were extracted using Dole's reagent (2): 40% (v/v) propan-2-ol, 10% (v/v) heptane, 1% (v/v) sulfuric acid. The solvent was evaporated to approximately 50% of its initial volume under a stream of nitrogen at room temperature. An activated probe was then inserted into the solution and allowed to adsorb for 5 min.

Alternatively, a probe was inserted into the liquid reaction mixture. After exposure to the mixture, the electrode was biased to −200 mV versus SCE and immersed into a 0.1 m phosphate buffer (pH 6.8) for 5–10 s to allow complete adsorption of carboxylates. After the probe was removed from the buffer, the bias was removed. The conditioned probe was positioned in the microscope optical path and spectra were recorded along the length of the silver strip.

RESULTS AND DISCUSSION

Figures 1A and **1B** show the SERS spectra of neat triolein and of the extracted lipolysis products after 10 days incubation in the anaerobic chamber. The major bands of triolein include CH_2 rocking (700 cm^{-1}–800 cm^{-1}), skeletal C–C stretches (1000 cm^{-1}–1150 cm^{-1}), CH_2 twist (1290 cm^{-1}), CH_2 bend (1440 cm^{-1}) (13, 14). Similar bands are seen in the lipolysis product spectra. Additionally, there is a strong symmetric carboxylate stretch at 1400 cm^{-1}. Earlier work with model sebum mixtures showed that the symmetric carboxylate stretch in the 1390–1400 cm^{-1} region can be used as an indicator of the presence of free fatty acids in a lipid mixture. The 1440 cm^{-1} decreases over time, indicating displacement of tri-

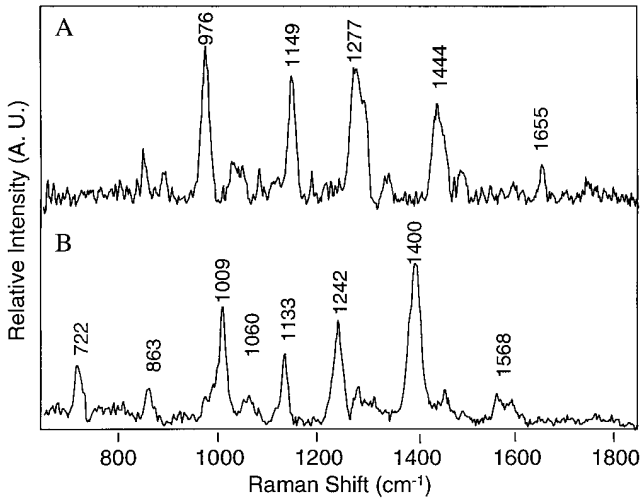

Fig. 1. A: SERS spectrum of triolein in liquid state; B: SERS spectrum of hydrolysis products extracted from inoculated media. Laser power at the sample was 100 μW/μm^2.

olein by oleate produced by the lipase of the bacteria. It is known that these bacteria produce propionic and acetic acids as products of fermentation during their growth (15, 16). These short chain carboxylic acids may be coadsorbed as their carboxylates. After 10 days of incubation of the lipids with the bacteria, as shown here, the spectrum is essentially that of a carboxylate (17).

The experiment was then repeated with a mixture of lipids (triolein, triisostearin, oleyl oleate, isostearyl isostearate, and squalene). The reaction tube remained in the anaerobic chamber for 8 days. The results are shown in **Fig. 2**. The symmetric carboxylate stretch (1395 cm^{-1}) is of comparable intensity to the CH_2 bend (1448 cm^{-1}). The presence of this intense methylene chain marker, which is absent in the carboxylates, indicates co-adsorption of one or more of the lipids. Because *P. acnes* hydrolyzes only triglycerides, we expected to see co-adsorption of other lipids as well as any unhydrolyzed triglycerides. This finding is consistent with previous results from model sebum studies containing 43% fatty acids.

The use of an extraction procedure has drawbacks, however. Extraction may change the relative concentrations of lipids and fatty acids. Adsorption from the extract favors short chain acids. Extraction is not compatible with the use of the probes in confined environments, such as sebaceous follicles.

Because of these problems, the extraction step was eliminated. Adsorption from probes directly inserted into the reaction media was examined. In order to eliminate protein interferences the probe was biased to −200 mV versus SCE. This potential is just positive to the potential of zero charge of silver in 0.1 m phosphate. The positively charged metal surface should facilitate carboxylate adsorption and repel any positively charged species such as proteins.

Figure 3A shows the adsorption spectra from a microprobe inserted directly into a hydrolysis mixture that had been incubating for 8 days. The presence of fatty acids is

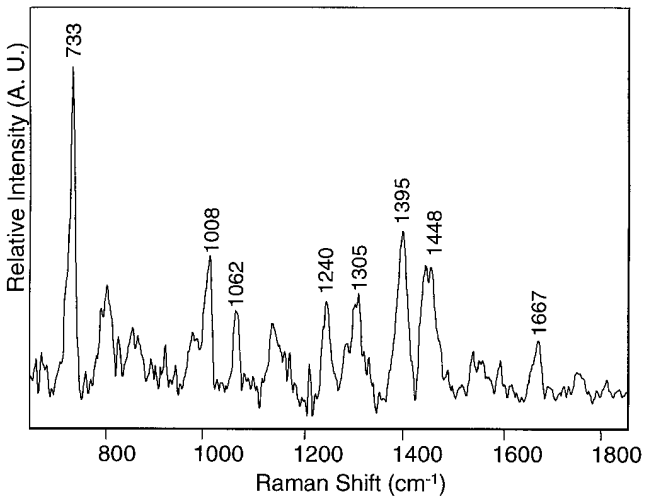

Fig. 2. SERS spectrum of extracted hydrolysis products of lipid mixture (triolein, triisostearin, isostearyl isostearate, oleyl oleate, and squalene). Laser power at the sample was 100 $\mu W/\mu m^2$.

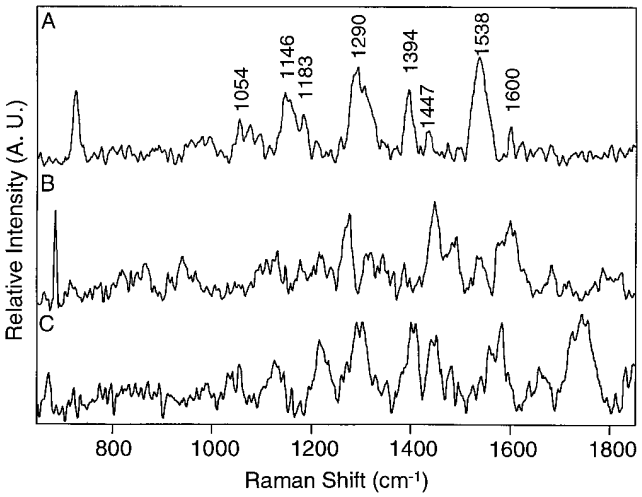

Fig. 3. A: SERS spectrum of hydrolysis products of lipid mixture (triolein, triisostearin, isostearyl isostearate, oleyl oleate, and squalene) monitored in situ; B: SERS spectrum of lipid mixture control (lipid mixture in media, no bacteria) monitored in situ; C: SERS spectrum of *P. acnes* control (*P. acnes* in media, no triglycerides) monitored in situ. Laser power at the sample was 100 $\mu W/\mu m^2$.

indicated by the symmetric carboxylate stretch at 1394 cm^{-1} as well as the asymmetric carboxylate stretches at 1540 and 1600 cm^{-1}. The signal/noise ratio is not as good as is obtained using samples that had been extracted, but it is adequate for detecting carboxylates. Some coadsorption of lipids is indicated by the weak 1447 cm^{-1} band. It should be emphasized that this procedure is rapid. About a minute is required for positioning of the microprobe in the reaction mixture, adjusting the electrode bias, and removing the probe. Spectroscopic measurement requires an additional minute or two for placement of the probe on the microscope stage, focus and other pre-measurement operations. The spectrum acquisition time is 30–60 sec.

SERS was also used to examine the lipid controls. As shown in Fig. 3B there is lipid adsorption at −0.2 V versus SCE. The spectrum of the lipid mixture after 8 days in an anaerobic chamber is identical to that obtained when the lipid mixture is first put into the chamber. There is no evidence of carboxylate formation over the 8-day duration of the experiment, as evidenced by the lack of a band at 1395 cm^{-1}–1400 cm^{-1}. Carboxylate formation in our system is caused by bacterial action only.

The SERS spectrum of the *P. acnes* control is shown in Fig. 3C. The striking feature of this weak spectrum is the presence of the symmetric carboxylate stretch at 1395 cm^{-1}. As discussed above, carboxylates are expected as byproducts of bacterial metabolism. We have not attempted to identify the other bands, which may result from adsorption of one or more proteins in the growth medium.

Lipolysis was monitored over the 8-day reaction period. **Figures 4A–B** show spectra from the time course of lipolysis, at day 2, day 6, and day 8, respectively. *P. acnes* are a relatively slow growing bacteria and take at least 24 h to enter

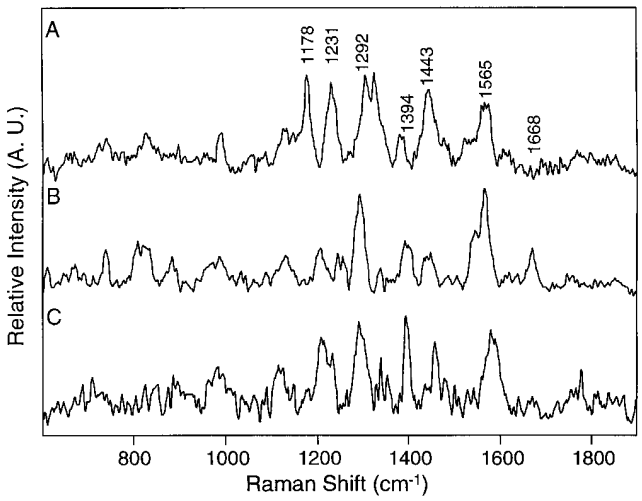

Fig. 4. SERS spectra of temporal lipolysis study monitored in situ. SERS spectrum of lipolysis of lipid mixture (triolein, triisostearin, isostearyl isostearate, oleyl oleate, and squalene); A: after 2 days incubation in anaerobic chamber; B: after 6 days incubation in anaerobic chamber; C: after 8 days incubation in anaerobic chamber. Laser power at the sample was 100 $\mu W/\mu m^2$.

the exponential phase of growth in liquid media. Figure 4A shows that carboxylic acids are detectable as early as 2 days after inoculation. As expected, as the reaction progresses (6 days, Fig. 4B and 8 days, Fig. 4C), the intensity of the carboxylate band increases and the ratio of 1395 cm^{-1}:1443 cm^{-1} changes.

CONCLUSION

SERS at silver microprobes can be used to study the biochemistry of *P. acnes* hydrolysis of triglycerides. The probes are selective for carboxylates. Electrochemical control of adsorption can be used to minimize interference from coadsorbed proteins.

The probes work by adsorption and are monitored after removal from the reaction mixture. There is no impediment to use of the probe as a direct monitor of chemistry in a highly turbid medium such as the oil/salt emulsion that flows through sebaceous follicles, where direct spectroscopic measurements would be difficult. Probes can be constructed with both working and reference electrode on the silicon substrate. Direct deposition of silver is feasible and would eliminate the intermediate silver electrodeposition step. With these further refinements, which are ongoing in our laboratories, SERS may become a convenient probe of this important bacterial/human skin interaction.

We have shown the identifying power of this technique in optically obscure environments. Quantitative data can be obtained as well. With the use of multivariate calibration techniques to extract maximum information from weak Raman spectra (18), these probes can be used to quantitatively assess sebaceous gland chemistry in micron scale pore simulators and eventually in excised skin.

This work was supported in part by Unilever Research Port Sunlight, U.K. MKW would like to thank Dr. Walter Loesche for the use of the laboratory equipment and valuable advice. Dr. Jeremy Andrew of Unilever provided gifts of lipids.

Manuscript received 13 February 1998 and in revised form 19 May 1998.

REFERENCES

1. Schurer, N. Y., and P. M. Elias. 1991. The biochemistry and function of stratum corneum lipids. *Adv. Lipid Res.* **24:** 27–56.
2. Gribbon, E. M., W. J. Cunliffe, and K. T. Holland. 1993. Interaction of *Propionibacterium acnes* with skin lipids in vitro. *J. Gen. Microbiol.* **139:** 1745–1751.
3. Marples, R. R., D. T. Downing, and A. M. Kligman. 1971. Control of free fatty acids in human surface lipids by *Corynebacterium acnes*. *J. Invest. Dermatol.* **56:** 127–131.
4. Ingham, E., K. T. Holland, G. Gowland, and W. J. Cunliffe. 1981. Partial purification and characterization of lipase (EC 3.1.1.3) from *Propionibacterium acnes*. *J. Gen. Microbiol.* **124:** 393–401.
5. Puhvel, S. M., and M. Sakamoto. 1977. A reevaluation of fatty acids as inflammatory agents in acne. *J. Invest. Dermatol.* **68:** 93–97.
6. Reisner, R. M., D. Z. Silver, M. Puhvel, and T. H. Sternberg. 1968. Lipolytic activity of *Corynebacterium acnes*. *J. Invest. Dermatol.* **51:** 190–196.
7. Kellum, R. E., and K. Strangfeld. 1969. Triglyceride hydrolysis by *Corynebacterium acnes* in vitro. *J. Invest. Dermatol.* **52:** 255–258.
8. Puhvel, S. M., R. M. Reisner, and M. Sakamoto. 1975. Analysis of lipid composition of isolated human sebaceous gland homogenates after incubations with cutaneous bacteria. Thin-layer chromatography. *J. Invest. Dermatol.* **64:** 406–411.
9. Weldon, M. K., V. R. Zhelyaskov, and M. D. Morris. 1998. Surface enhanced Raman spectroscopy of lipids on silver microprobes. *Appl. Spectrosc.* **52:** 265–269.
10. Page, J. A. 1963. Electrogravimetry. *In* Handbook of Analytical Chemistry. L. Meites, editor. McGraw Hill, New York. 171.
11. Zhelyaskov, V. R., E. T. Milne, J. F. Hetke, and M. D. Morris. 1995. Silicon substrate microelectrode array for surface-enhanced Raman spectroscopy. *Appl. Spectrosc.* **49:** 1793–1795.
12. Todd, E. A., and M. D. Morris. 1994. Micron surface-enhanced Raman spectroscopy of intact biological organisms and model systems. *Appl. Spectrosc.* **48:** 545–548.
13. Gaber, B. P., and W. L. Peticolas. 1977. On the quantitative interpretation of biomembrane structure by Raman spectroscopy. *Biochim. Biophys. Acta.* **465:** 260–274.
14. Bryant, M. A., and J. E. Pemberton. 1991. Surface Raman scattering of self-assembled monolayers formed from 1-alkanethiols: behavior of films at Au and comparison to films at Ag. *J. Am. Chem. Soc.* **113:** 8284–8293.
15. Moore, W. E., and E. P. Cato. 1963. Validity of *Propionibacterium acnes*. *J. Bacteriol.* **85:** 870–874.
16. Cove, J. H., K. T. Holland, and W. J. Cunliffe. 1987. The effects of oxygen on cutaneous propionibacteria grown in continuous culture. *FEMS Microbiol. Lett.* **43:** 61–65.
17. Moskovits, M., and J. S. Suh. 1985. Conformations of mono- and dicarboxylic acids adsorbed on silver surfaces. *J. Am. Chem. Soc.* **107:** 6826–6829.
18. Walker, P. A., III, J. M. Shaver, and M. D. Morris. 1997. Identification of cationic herbicides in deionized water, municipal tap water, and river water by capillary isotachophoresis/on-line Raman spectroscopy. *Appl. Spectrosc.* **51:** 1394–1399.