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REVIEW

Application of Surface-Enhanced Raman Spectroscopy to Biological Systems

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This review summarizes the literature relating to the application of surface-enhanced Raman scattering (SERS) and surface-enhanced resonance Raman scattering (SERRS) techniques to the study of biological molecules. The emphasis is on publications that have appeared during the period from 1985 to 1991. The review is divided into six major parts. First, a brief overview of the current understanding of the mechanistic aspects of SERS/SERRS is given, with an emphasis on the relationship between theoretical predictions and experimental results. The most common experimental systems (colloids, metal island films, and electrodes) are described. Studies of biological systems are described in the second (small molecules), third (DNA and proteins) and fourth (membranes proteins and membrane preparations) sections. In the fifth or conclusion section, the potential use of SERS or SERRS as a method for obtaining spectra of native biological molecules is evaluated. Finally, the sixth section describes advances in Raman instrumentation in terms of their possible impact on future applications of SERS/SERRS techniques to biological molecules.

INTRODUCTION

The advantages of surface-enhanced Raman scattering (SERS) as a technique for the detection and identification of molecules adsorbed at surface were clearly obvious from the early experimental work in this field. Initial studies by Fleischmann *et al.*,[1] Jeanmaire and Van Duyne[2] and Albrecht and Creighton[3] demonstrated that Raman scattering from pyridine on a roughened silver electrode was enhanced by approximately six orders of magnitude. From these quantitative studies, it became evident that SERS was a new, sufficiently sensitive spectroscopic method for surface science, analytical applications and biophysics. These early results stimulated considerable experimental and theoretical interest in this phenomenon which resulted in a large number of publications (as reviewed in Refs 4–14). In spite of this rapid increase in experimental results and theoretical work, it became clear that a single model, which could explain all of the observed features of the effect and would have predictive power, would not suffice. This fact inhibited the wide application of SERS.

In the early 1980s, three research groups began to study SERS of biological molecules in order to obtain new structural–functional information. Koglin and Séquaris[15] initially focused on nucleic acid components and DNA. Nabiev *et al.*[16] investigated amino acids, water-soluble and membrane proteins and nucleic acids. Cotton[17,18] studied surface-enhanced resonance Raman scattering (SERRS) from a number of proteins containing chromophores. Following these pioneering studies, many other researchers have continued to demonstrate the immense potential of this approach for solving a number of problems in biochemistry, biophysics and molecular biology. In this review, a brief description of the theoretical concepts relating to SERS will be given first, followed by a more detailed discussion of experimental systems relevant to biomolecules. The major focus will be given to the newest examples of SERS of different types of biomolecules (amino acids, nucleic acids, porphyrins, water-soluble, membrane and chromophore-containing proteins and DNA). Both the practical and fundamental aspects of SERS phenomena will be discussed for these molecules. From a practical point of view, the objective is to demonstrate that enhanced Raman scattering offers tremendous potential for obtaining new structural information on extremely small amounts of biomaterials and, at the same time, to provide evidence that the functionality of the biomolecules is preserved on the surface. Because a number of excellent reviews[15–18] have summarized the literature up to *ca.* 1987, no attempt will be made to cite all of the earlier work in this field.

MECHANISTIC ASPECTS OF SERS/SERRS AND THE RELATIONSHIP TO EXPERIMENTAL PROCEDURES

In this section, the purpose is to describe the relationship between theoretical predictions and experimental observations. An understanding of the physical and/or chemical basis of the enhancement process is essential for the design of appropriate experiments and for an interpretation of the resulting data.

Theoretical considerations

The major theoretical concepts which are concerned with the origin of the surface enhancement effect can be classified within two general categories, electromagnetic (EM) and molecular (chemical) mechanisms (reviewed in Refs 4–14). The former invoke an enhancement of the

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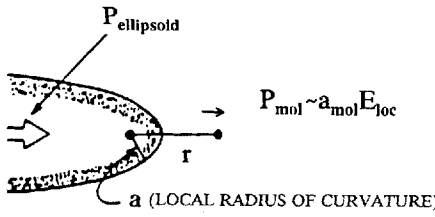

$$I_{RAMAN} \sim \left|\frac{a}{r}\right|^{12} \left(\frac{\epsilon - 1}{\epsilon + \epsilon_R}\right)^2 \left(\frac{\epsilon - 1}{\epsilon + \epsilon_R 1}\right)^2$$

Figure 1. Schematic diagram of a molecule located a distance (r) from the center of the local radius of curvature (a) of the tip of a surface protrusion. When either the incident light or the Raman scattered light is resonant with the collective electron oscillation of the metal particle ($\epsilon = -\epsilon_R$ or $\epsilon = -\epsilon'_R$, respectively), it induces a large enhancement of the local electromagnetic field (E_{loc}). The total Raman scattering intensity (I_{RAMAN}) in this expression does not include the image and chemical enhancement effects (adapted from C. A. Murray in Ref. 14).

electromagnetic field near the metal surface due to plasmon resonances.[10] These resonances occur at the frequencies of the incident and scattered light in small metal particles or protuberances on a rough metal surface. Examples of such systems include metal colloids, metal island films and electrodes. The EM theories predict long-range enhancement. The magnitude and distance dependence of the enhancement are strongly affected by the radius and shape of the metal particles or surface roughness (Fig. 1). In general, EM enhancement is not sensitive to the chemical nature of the molecule and produces spectra that should be similar to the Raman or resonance Raman spectra of the molecules in solution.

Theories which invoke a molecular mechanism consider the molecular interaction between the molecule and the metal as the origin of the enhancement of Raman scattering.[9] This interaction leads to a change in molecular polarizability due to charge transfer, σ- or π-bonding to the metal, interaction with adatoms near the metal surface or even electrostatic interactions. Because the molecular mechanisms are more specific as to the nature of the molecules, not all adsorbates are predicted to give rise to enhanced Raman scattering in these cases. In addition, spectra that result from these mechanisms should be different from solution-phase spectra. Large band shifts, changes in relative band intensities and new bands may be observed.[19]

Experimental systems

Various experimental tests have been developed to validate specific theoretical models. In many cases, a given theoretical model can be proved by certain experiments, but not all experimental observations fit a single model. For example, in the case of metal colloids the existence of plasmon resonances which produce enhancement is obvious. However, in many cases, the SERS spectra of molecules on colloids are completely different from the spectra of these species in solution. Also, the SERS excitation profile does not follow the extinction spectrum of the colloid and a new maximum appears in the profile.[20] These facts indicate that a strong interaction exists between the molecules and the surface which is important for the SERS effect. Many examples of systems that exhibit behavior characteristic of both EM and chemical enhancement may be found in the literature. For this reason, the experimentalist should be familar with theoretical predictions. More than one type of enhancement mechanism may be operative in an experimental system which provides a total enhancement of 10^5–10^6-fold. For application purposes, the main problem is to determine the relative contributions of different types of mechanisms to the total enhancement and how these change in different experimental systems.

The three most common systems that have been employed in SERS studies include electrodes, colloidal metals and vapor-deposited island films from silver, gold and copper. Each has certain advantages as well as limitations. Electrodes provide a fast and convenient monitor of the metal surface following an oxidation–reduction cycle (ORC).[12] The ORC causes changes in surface morphology at the atomic scale (formation of adatoms and defects in crystal structure) and/or the nanometer scale. Large-scale changes have been observed by scanning electron microscopy (SEM) and scanning tunnelling microscopy (STM) following an ORC cycle.[21] Adatoms and defects in crystal structure can function as active centers for the formation of complexes with molecules, resulting in a chemical enhancement. Nanometer-scale protuberances provide plasmon resonances and in this manner produce EM enhancement. The potential of the electrode also affects the redox state of the adsorbate and its orientation, surface coverage and molecular symmetry, which can result in different selection rules for Raman scattering. The main disadvantage to the use of electrodes is the inability to control independently each experimental parameter. It is very difficult to create a roughness which is necessary for EM enhancement by an ORC without also creating active centers which give rise to chemical enhancement. Conversely, an ORC which redeposits only a few monolayers of metal will be just sufficient to create active centers. However, long-range electromagnetic enhancement has also been observed on these electrodes, because there is always some roughness associated with an electrode before an ORC. This fact makes electrodes inconvenient for fundamental investigations of the SERS effect. However, for applied studies electrodes are useful because they offer wide possibilities for changing the experimental conditions.

As mentioned above, EM enhancement of Raman scattering in colloidal metals is obvious. These systems consist of small (10–100 nm), well defined, close to spherical particles which are ideal for producing plasmon resonances.[22,23] The SERS effect usually occurs after aggregation of the colloids, which could be caused by adsorbates. In this case, the aggregation affects an increase in the electromagnetic field between particles and makes it possible to observe enhanced Raman scattering. However, SERS has been reported for non-aggregated or particularly aggregated colloids and the enhancement was comparable, or even greater, for some molecules. Also, it has been reported that different anions can strongly affect SERS spectra and the magnitude of the enhancement, without perceptibly

changing the aggregation state of the colloid.[24] This means that there is also a chemical enhancement, and colloids cannot be considered as SERS systems with only EM enhancement of Raman scattering.

The use of metal colloids for SERS studies is common because they are easy to prepare and easy to investigate by spectroscopic methods, electron microscopy, etc. These systems are also very convenient for theoretical simulations of SERS phenomena. In practical applications, they provide the lowest detection limit for many molecules. A disadvantage of colloidal metals is the poor reproducibility of their properties from one preparation to another. The poor reproducibility makes control of the size (distribution function), shape and surface properties of particles and aggregates extremely difficult.

Metal island films are prepared by vacuum deposition of metal (50–150 Å average mass thickness) on solid substrates. The island structures have been well characterized by SEM (Fig. 2) and scanning transmission electron microscopy (STEM).[25–27] The surface structure and the temperature of the substrate as well as the deposition rate affect the particle size, shape and interparticle spacing[27] and, as a result, the frequency of the plasmon resonance. Two kinds of plasmon resonances could be present in island films: local resonances within a single island and surface electromagnetic waves. The first makes island films similar to colloids. Surface electromagnetic waves are the dominant mechanism for enhancement of EM fields near the surface of periodical structures (e.g. gratings).[7] However, chemical enhancement of Raman scattering could also be important for island films. In so-called 'coldly deposited' island films, strong SERS was observed only at low temperatures. After raising the temperature above 200 K, the spectra disappeared.[28] This has been attributed to annealing of surface defects which may function as active sites for chemical enhancement.

For applications, especially for biological systems, island films are not a very attractive alternative because the enhancement of Raman scattering from adsorbed molecules is usually less in comparison with colloids and electrodes. On the other hand, island films provide a unique opportunity for performing 'chemically pure' studies of SERS phenomena, especially under ultra-high vacuum conditions.

Numerous other substrates have also been used to elicit SERS. Examples include surfaces prepared by microlithography or ion bombardment, diffraction gratings, spheres coated with silver and filter-paper sprayed with colloidal silver.[11,28,29] The search for an ideal substrate exhibiting maximum enhancement, stability and reproducibility is still in progress.[30,31]

SERS/SERRS STUDIES OF LOW MOLECULAR WEIGHT BIOMOLECULES

SERS has been used to study many different biomolecules of low molecular weight. Recent reviews have summarized this literature.[15–18,32] These include amino acids,[15–18,32–36] peptides,[32,37,38] purines,[15–18,32,39–41] pyrimidines,[15–18,32,41,42] NAD^+/NADH[43,44] and catecholamines.[45,46]

Amino acids and peptides

SERS studies of various types of amino acids and peptides on silver surfaces have been summarized in a number of reviews.[15–18,32] In most cases strong interactions between specific portions of the molecule (e.g. carboxylate groups or aromatic side-chains in amino acids) have provided information about the mode of bonding between these small molecules and the surface. Differences in the amino acid spectra reported by the various groups can be explained in terms of variation in experimental procedures (e.g., colloid preparation procedure, analyte concentration). Chumanov et al.[33] have shown that the SERS response for various amino acids on silver colloids can be divided into three categories. Aliphatic amino acids (Gly, Ala, Val, Cys and Leu) interact with the surface via the carboxyl and an amino group. A second group, Asp and Glu, interact through two carboxyls and one amino group. The third group, aromatic amino acids, produce the most intense spectra and in this case bands attributed to the π-electron system are strongly enhanced. The SERS excitation profiles show that both EM and chemical mechanisms are operative in these systems. The short-range enhancement results from a chemical interaction between the amino acid and the silver surface. These authors also showed that optical activity was induced in the aromatic amino acid–silver complexes.

In an interesting application of SERS to electrochemical processes, Watanabe and Maeda[36] showed that the disulfide bond of cystine was reduced to a thiol to form cysteine at negative potentials. The process was quasi-reversible and the disulfide was reformed at positive potentials. On the other hand, adsorption of the reduced cysteine did not lead to oxidation of the disulfide. The lack of redox activity in the latter case was attributed to steric hindrance.

Earlier studies of dipeptides indicate that adsorption occurs primarily through the carboxylate group and accordingly the spectrum of the residue at the carboxylate terminus was observed.[32] Recently, Herne *et al.*[38] obtained SERS evidence for the interaction of the amino group at the surface. A number of dipeptides were examined and the *N*-terminal residue consistently produced the strongest SERS spectrum with only very weak scattering from the *C*-terminal residue. The only exception to this behavior were the Gly–Tyr and Gly–Tyr–Gly peptides, which exhibited strong scattering from the aromatic side-chain. These authors also examined several enkephalins and observed interactions between the amine groups and aromatic side-chains. In another study of L-tryptophan-containing peptides, Lee *et al.*[37] found that both the carboxylate and the amino groups interact with a silver colloid surface. Once again, the differences between the results of Herne *et al.*[38] and previous workers are probably a result of the experimental conditions used. In particular, the sol preparation procedure may play a very important role, especially with respect to the surface charge density and aggregation state. Citrate sols contain adsorbed citrate ions, whereas borohydride sols should be expected to contain borate ions.

Curley and Siiman[34] examined a number of different 2,4-dinitrophenyl (DNP) amino acids on colloidal silver. These are frequently used as haptens. The data showed

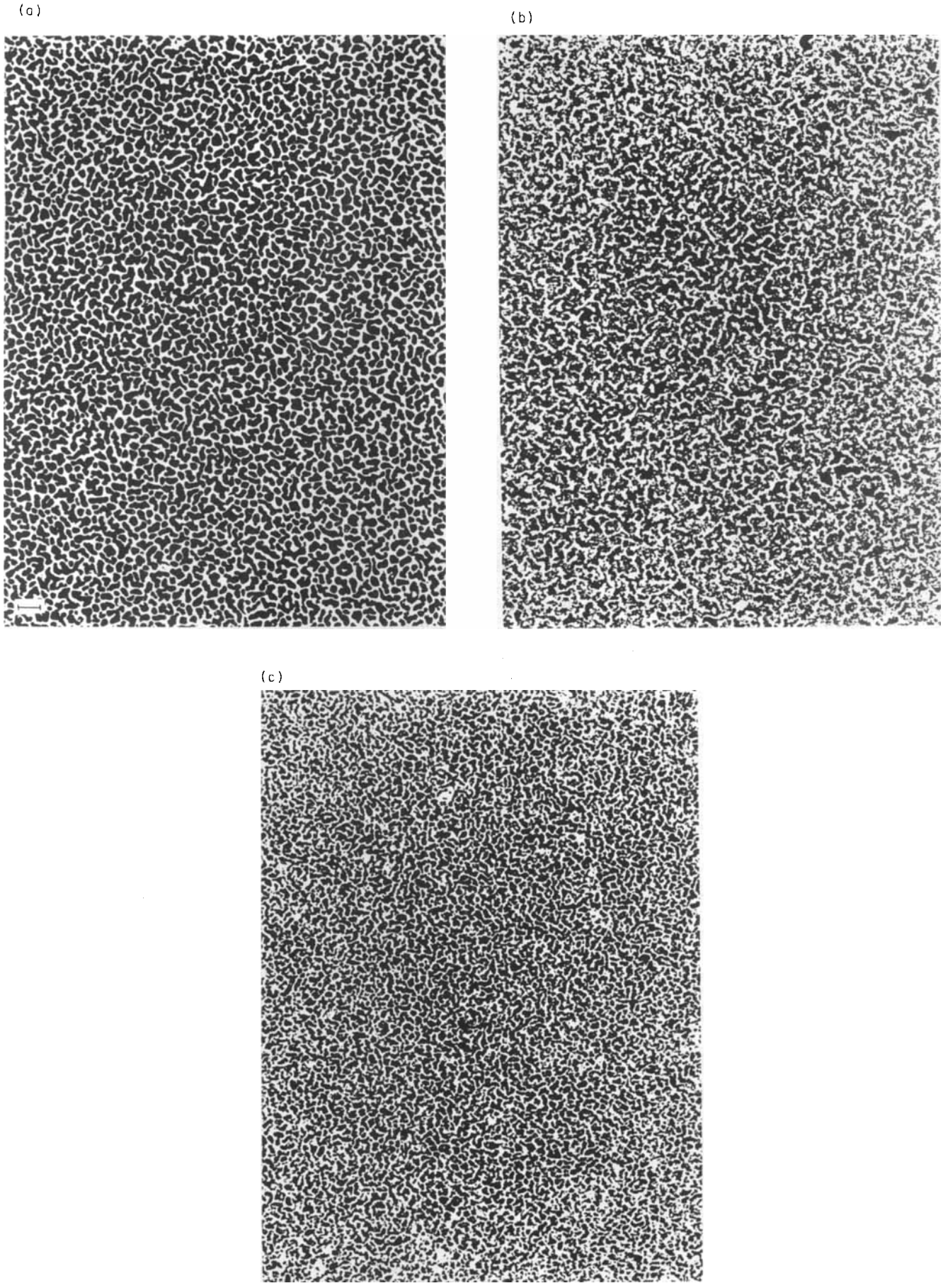

Figure 2. *TEM photomicrograph of silver island films (50 Å average thickness) deposited on glass slides at three different deposition rates:* (a) 0.03; (b) 0.33; (c) 5.00 Å s^{-1}. The slides were maintained at room temperature. The bar in (A) indicates 1000 Å. All of the images were taken at the same magnification.[27]

strong signals from the nitrophenyl group, probably as a result of preresonance enhancement with the electronic absorption band at 360 nm.

Most SERS studies of the small molecules cited above have supported a short-range mechanism of enhancement. Similarly, the NAD^+ results of Austin and Hester[44] suggest that direct contact between the cofactor and the silver surface is required for observation of SERRS. They were not able to observe the cofactor SERRS spectrum when bound to the enzyme glyceraldehyde-3-phosphate dehydrogenase (GADPH).

Purines and pyrimidines and related compounds

The SERS spectra of purines and pyrimidines have been extensively studied and the results have been summarized in a number of reviews.[15–18,32] The review by Paisley and Morris[32] provides an excellent and concise summary of publications up to 1987. In most of these early studies, an attempt was made to determine the orientation of the compound at the surface using analysis of band intensities. As with amino acids, some disagreement exists between the data reported by different research groups. Adenine gives intense SERS spectra and at high concentrations it is oriented perpendicular to the silver surface and bound through the amino group. At low concentrations (below 10^{-5} M), the ring may be planar to the surface. Guanine also produces strong SERS, but the nature of the surface interaction and orientation is not clear from existing data. Finally, cytosine and thymine SERS suggest that these molecules are oriented perpendicular to the surface. Of course, there are generalizations and the orientations of all of these molecules may be strongly affected by pH, the presence of counter ions and the concentration. For example, Lee *et al.*[35] investigated cytosine and its derivatives and found that cytosine and 5′-rCMP changed their orientations with changing concentration. The latter compound also changed its orientation with a decrease in pH. Itoh *et al.*[39] showed that the orientation of 9-methyladenine at silver electrodes is very sensitive to pH and electrode potential.

Nabiev[41] showed that the method of preparation of the silver sol can produce marked differences in the SERS spectra of nucleotides, as well as for DNA. When silver sols were activated by the addition of NaCl, only dAMP gave a strong spectrum when an equimolar mixture of the nucleotides was added to the sol. Nonactivated sols, on the other hand, produced spectra containing contributions from all of the nucleotides. The authors postulated that activation of the sol produced sites that have greater specificity for adenine ring interaction.

The differences between SERS spectra of uracil from silver sols and from electrodes were investigated and found to result from changes in orientation of the pyrimidine with variation of surface potential.[42] For adenine and its derivatives, Otto *et al.*[40] found that the external amino group plays an important role in its adsorption behavior. No changes in orientation were observed with changing electrode potential for adenine or any of its derivatives. If, however, the amino groups were methylated, two different orientations were present in the range between -0.2 and -0.7 V *vs.* SCE. At the more positive potential, the ring plane is oriented in a near vertical position, whereas at -0.7 V the plane is parallel to the surface.

The addition of the ribose moiety to the nucleic acid bases results in significant differences in their mode of interaction with electrodes. The potential and concentration affect the adsorption behavior, as summarized in a recent review.[32]

Other compounds related to nucleic acid bases, nicotinamide adenine dinucleotide (NAD^+) and dinucleotides have also been studied by SERS and these results have been reviewed.[15–18,32,43,44] In a more recent study, Siiman et al.[43] examined the orientation and conformation of NAD and NADH on colloidal silver. Their results suggest that NADH is bound with a side-on orientation, through the N-7 and amino sidegroup of adenine, at high concentrations. In contrast, NAD is bound through both the adenine and nicotinamide rings and these are oriented flat on the surface. At low concentrations, both molecules adsorb with the adenine in a flat orientation and the nicotinamide in a stacked conformation.

Catecholamines

The potential of SERS for determining catecholamine neurotransmitters was investigated by Lee *et al.*[45] Dopamine, norepinephrine, 3-methoxytyramine and catechol gave strong spectra on a silver electrode at -0.9 V *vs.* SCE. Epinephrine and isoproterenol, on the other hand, gave comparatively weak spectra. These compounds contain methyl groups in the side-chain and this may cause weaker SERS because of a steric effect. However, epinine, which also contains a methyl group in the side-chain, gave a strong SERS spectrum. The potential of SERS for detecting these neurotransmitters in intact systems was discussed and possible interferences were cited. In a later paper, McGlashen and Morris[46] showed that the simulation of a more realistic biological matrix could be achieved by adding bovine serum albumin (BSA) to dopamine solutions. The SERS spectrum was almost completely obscured when 0.3% BSA was added to the solution. Protein interference was overcome by coating the electrodes with partially hydrolyzed cellulose acetate. The polymer prevented protein adsorption on the electrode surface, but allowed diffusion of dopamine to the surface. Removal of the dopamine from the membrane was also possible, but the response was slow, requiring about 20 min of vigorous stirring in dopamine-free buffer solution. Nevertheless, optimization of the polymer coating thickness suggests this as a promising approach for identifying the catecholamines and their metabolites in biological systens.

Miscellaneous chromophoric species

SERRS studies of small biomolecules have included porphyrins,[15–18,32,47,48] chlorophylls and related compounds,[49–52] flavins,[18,32,53–55] retinals,[56] bile pigments[57,58] and eye lens pigments.[59]

Porphyrins and chlorophylls. In the case of porphyrins and chlorophylls, the spectra excited near the strong Soret

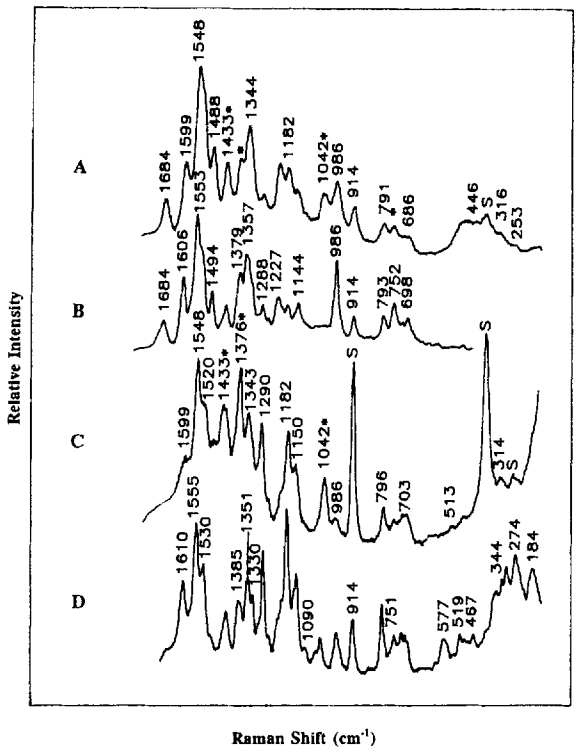

Figure 3. (A and C) RR and (B and D) SERRS spectra of chlorophyll *a* adsorbed on a silver electrode in degassed water at 298 K. Laser power, 25 mW; 16 scans (1 scan s^{-1}); excitation wavelength, (A and B) 406.7 nm and (C and D) 457.9 nm.[50]

absorption band (e.g. at 406.4 nm) were very similar to the solution RR spectra (Fig. 3). At other excitation wavelengths, further to the red (e.g. 457.9 nm), spectral differences between the SERRS and RR spectra arise from the interaction between the adsorbate and the surface. For example, in the case of chlorophylls, the major spectroscopic differences are shifts in the C=O mode and bands between 1500 and 1600 cm^{-1}, as compared with the solution spectra (Fig. 3).[49,50] Other changes, especially including relative intensity differences in many of the bands, may be related to the orientation of the macrocycle relative to the surface. Differences between the SERRS spectra of chlorophyll *a* on silver and gold electrodes reflect differences in the orientation of the macrocycle at these surfaces.[51] The fluorescence quencing that accompanies the adsorption of highly fluorescent molecules on metal surfaces provides a significant advantage for excitation of chlorophyll spectra in the lowest energy (Q_y) transition.[50] Hildebrandt and Spiro[52] also utilized the fluorescence quenching present in SERRX to obtain chlorophyllin spectra on silver colloids using 647.1 nm excitation.

Flavins. The SERRS studies of flavins has been reviewed.[15,32] Early studies of glucose oxidase and other flavoproteins were complicated by the presence of free flavin or the release of flavin in the presence of the silver surface. Moreover, the generation of H_2O_2 in the enzymatic reaction of adsorbed glucose oxidase with glucose led to the formation of a silver–flavin complex at the electrode surface. So far it has not been possible to obtain SERRS spectra of the intact protein. This may be due to the large size of the protein and the fact that the chromophore is buried within the protein.

Retinal. Monolayers containing all-*trans*-retinal produced SERRS on silver island films. The spectra were interpreted to indicate the interaction of the C=O group with the surface.[56] Most perturbations in the SERRS spectrum were associated with the C=O or other groups near this end of the molecule. In addition, it was determined that the enhancement factor decreased from *ca.* 3×10^5 at 514.5 nm excitation to 3.7×10^4 at 457.9 nm excitation, or as the laser wavelength approached resonance with the absorption maximum of retinal. A decrease in the enhancement has been observed previously in the case of resonantly enhanced molecules and this is generally attributed to the shortened lifetime and broadened absorbance of the molecule on the surface.[7,8]

Bile pigments. Several bile pigments have also been examined by SERRS.[32,57,58] These include biliverdin (as reviewed in Ref. 32), bilirubin[57] and the photoisomers of biliverdin.[58] An analysis of the spectrum showed that bilirubin adsorbed as the dicarboxylate ion and maintained its internal hydrogen bonding.[57] When bilirubin was complexed with cyclodextrin, the SERS spectrum was still observed. However, on complexation with albumin the SERS signals were quenched. It was concluded that the bilirubin was not able to interact directly with the silver surface in the albumin case, whereas one end of it was free to bind to silver in the cyclodextrin complex. Although this result suggests that direct contact of bilirubin with the silver surface is required for SERS, it should be noted that SERS is expected to decrease with distance and surface coverage. Decreased SERS intensity is a result of both the distance of the bilirubin from the surface, which is surrounded by the protein, and decreased surface coverage, due to the large size of the complex. Some long-range EM enhancement may still be present, but it is insufficient to produce detectable SERS.

The SERRS spectra of biliverdin dimethyl ester (BVDE) photoisomers were obtained from the compounds adsorbed on a silver electrode at 77 K.[58] The three isomers *Z,Z,Z*, *Z,Z,E* and *E,Z,Z* gave distinct SERS spectra. Moreover, the dissimilarity of the *E* isomer spectra from the *Z,Z,Z* isomer suggests that BVDE is stabilized on the surface. In solution it is not possible to detect spectra of the *E* isomers because on irradiation with white light these are converted to the more stable *Z,Z,Z* isomer. These spectra provided a basis for interpretation of the chromophore conformation in phytochrome, which will be discussed in a subsequent section.

Eye lens pigments. The analytical utility of SERRS was demonstrated in an investigation of eye lens pigments.[59] The lens tissue was obtained from a number of different animals (grey squirrels, ground squirrels and chipmunks) and was homogenized in water. Following centrifugation, the supernatant which contained low molecular weight components was mixed with silver colloid preaggregated with $NaClO_4$. A comparison of the spectra obtained from the colloids with those of model compounds under identical conditions provided unambiguous identification of the pigments. The significance of the data was discussed relative to the metabolic/photochemical generation of lens pigments.

SERS/SERRS STUDIES OF DNA AND PROTEINS

Many SERS/SERRS studies of large biomolecules have been reported and the literature prior to 1988 has been reviewed.[15–18] Rather than describing results that have been reviewed previously, this section will focus on examples of recent publications relating to the use of SERS/SERRS for examining protein and complex biomolecules on surfaces. In particular, emphasis will be placed on those papers which are concerned with the structure of the biomolecules on the SERS/SERRS surfaces.

Application of SERS to large biomolecules

Early SERS studies of non-chromophoric biomolecules have included proteins,[15–18] antibodies[60–62] polynucleotides[15–18,32] and DNA.[15–18,41,63] An examination of the mechanism(s) responsible for the enhancement process in these studies indicates that strong scattering from those groups of atoms closest to the surface is expected for a chemical type enhancement. In general, this is what has been observed.

Proteins. The aromatic amino acids on the surface of the proteins produce the strongest SERS signals[15–18,33,61,62] (Fig. 4). In the case of immunoglobulin G, additional groups were also detected.[61] From an analysis of the spectrum, it was concluded that cystine was bound through one of the sulfur atoms of the disulfide bridge, except at high ionic strength, in which case SH groups were also observed. The COO^- groups of aspartic and glutamate residues were also bound. None of the reported protein spectra in the literature show enhancement of the amide I and III bands, apparently because of screening by the aromatic groups on the protein surface.[33] In most cases, long-range enhancement was not present.

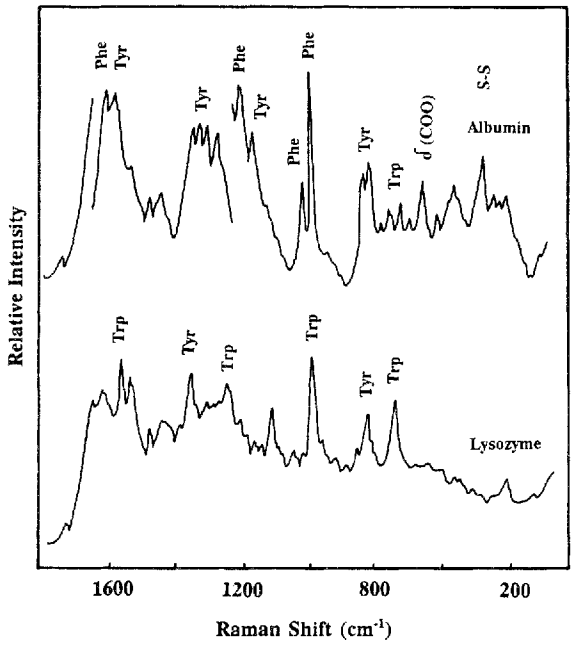

Figure 4. SERS spectra of two water-soluble proteins, serum albumin and lysozyme, on a silver electrode. Experimental conditions: laser excitation wavelength, 514.5 nm; power, 30 mW; electrode potential, −0.65 V *vs.* AgCl; protein concentration, 10^{-6} M; electrolyte, 0.1 M KCl.

DNA. The behavior of DNA at silver colloids was studied by Kneipp and Flemming.[63] Their results are in disagreement with previous reports of the SERS spectrum of DNA, which indicated destabilization of the DNA at the silver surface. These authors found that the SERS spectrum of native DNA is very similar to the solution spectrum if sufficient time (hours) is given for the macromolecule to reach a stationary adsorption state. The initial spectrum (after 8 min) is markedly different from that obtained after 18 h. It was suggested that the results indicate less destabilization of the double helix on the sol used and, based on good agreement between theoretical and experimental excitation profiles for the SERS spectrum, the conclusion was that an electromagnetic enhancement mechanism is operative in this experimental system. Differences between these results and those reported previously are attributed to differences in the surface potential of the sol preparations. Thus, if previous experiments were performed on a more positively charged surface, this would reduce the stability of the DNA. Additional experimentation is needed to determine if this is indeed the case, or if the results are due to the long time period between adding the sample and recording the spectrum. It may be that active sites on the surface of the colloid, which are responsible for chemical enhancement, are quenched with time. Only long-range EM enhancement would be expected if this occurred.

In an interesting study by Nabiev,[41] it was shown that greater chemical specificity of silver colloids could be achieved with DNA by varying the experimental procedure used to detect SERS. This was achieved by preparing two types of sols, non-activated or normal sols and activated sols. Non-activated silver sols produced spectra of all of the nucleotides at the 10 pg level. Activated sols (NaCl was added to reach a concentration of 0.05 M), on the other hand, produced a strong spectrum of adenine only. This chemical specificity was utilized to determine regions of destabilization in DNA double helix. A tenfold increase in the adenine SERS signal was observed for destabilized regions of DNA that were enriched in adenine nucleotides. Other nucleotides produced no SERS signals.

Applications of SERRS to chromophore-containing systems

The combination of conventional resonance enhancement with EM surface enhancement (SERRS) can be used to detect chromophores that are buried within a protein matrix. Resonance enhancement provides an additional two to three orders of magnitude in scattering intensity over the surface enhancement. In addition to the improved sensitivity, the selectivity of SERRS provides another advantage. The resonance Raman effect allows detection of scattering from a limited portion of a complex macromolecule. Moreover, the chromophoric portion is frequently the most interesting because it is the active site for enzymatic catalysis

(e.g. FAD in glucose oxidase), redox reactions (e.g. the heme groups in cytochromes) or for photochemical processes (e.g. the chlorophylls in reaction centers or light-harvesting proteins).

Protein–dye and DNA–drug systems. In addition to the naturally containing chromophore systems, SERRS has also been used in the study of dye-containing macromolecules[60,64] and drug binding to DNA.[65–67] The complex of avidin with the dye 2-(4′-hydroxyphenylazo)benzoic acid (HABA) was used as a model for chromophore-containing proteins.[64] The SERRS spectrum of the complex was markedly different from that of the free dye, indicating that the interaction of the protein with the surface did not disrupt the dye–protein interaction. This same dye was used in another study to develop an immunoassay based on the SERRS effect.[60] The dye was captured by avidin-coated silver island films and gave rise to a logarithmic relationship between dye concentration and SERRS signal over the concentration range 10^{-8}–10^{-5} M. Another dye, *p*-dimethylaminoazobenzene (DAB), was covalently attached to an antibody against the human thyroid stimulating hormone (TSH). The labelled antibody was then used in a sandwich assay to detect the antigen. These results were encouraging as regards the development of a SERRS-based, no-wash immunoassay system.

The interaction of drugs with DNA has been probed by SERS[65] and SERRS.[66,67] Manfait *et al.*[65] were able to incorporate silver colloids into erythroleukemic cells. The cells were then treated with an anti-cancer drug, doxorubicin (DOX) or adriamycin. The SERS spectrum within the cellular compartments of a single cell was recorded by microspectroscopy. The spectra showed that the DOX interacted with the DNA in the nucleus. The interaction of the drug with cytoplasmic components was different than in the case of DNA. In another study, DNA complexes with adriamycin and 11-deoxycarminomycin and their model chromophores were found to produce detailed SERRS spectra when intercalated into DNA.[66] An analysis of the spectral changes on binding to DNA indicated the mode of interaction. The fluorescence quenching aspects of SERRS were particularly advantageous in this study. Resonance Raman spectra were difficult to obtain because of the high fluorescence exhibited by the compounds. Other anthracyclines were also examined by SERRS.[67] Excitation profiles of the free molecules on silver colloids provided evidence for a charge-transfer transition between the silver surface and the chromophore. The authors showed that the data could be rationalized either by an electromagnetic or a charge-transfer mechanism.

Heme-containing proteins. One of the long-term goals of our research is to develop SERRS methodology for the study of chromophores within membrane preparations, especially photosynthetic systems. In order to determine whether this is feasible, we first examined two well characterized proteins, cytochrome *c* and myoglobin.[68] The results showed that it is indeed possible to detect SERRS from the heme group in these proteins. However, it was not clear from these early results whether the SERRS signal was from heme groups buried within the protein matrix, as in the native

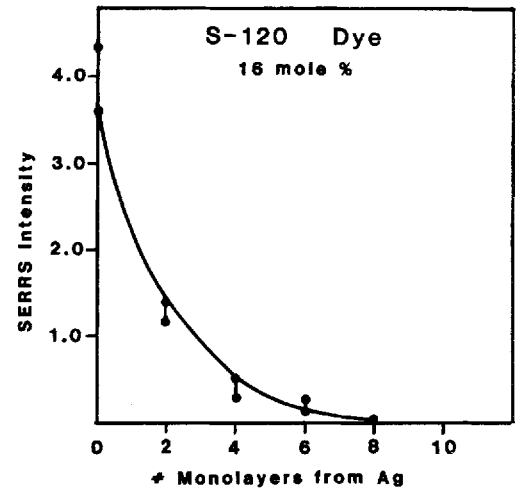

Figure 5. SERRS intensity *vs.* the number of monolayer spacers between an S-120 dye monolayer and a silver island film on a glass slide. Experimental conditions: excitation wavelength, 514.5 nm; laser power, 2 mW; 1 scan (0.5 s integration time).

protein structure, or from heme groups that were in direct contact with the electrode surface. To verify that EM enhancement occurs at distances greater than a few ångstroms from the electrode surface and to determine the relationship between EM enhancement and distance of the chromophore, we utilized Langmuir–Blodgett techniques to prepare a series of samples containing lipid–dye mixtures which were spaced at defined distances from the silver surface.[69] Thin silver films (silver island films) were vapor deposited on glass slides. Monolayers containing long-chain fatty acids or esters were deposited on the slide and used as transparent (at the laser excitation wavelengths) spacers. The dye–lipid monolayer was deposited on top of the spacer layer(s). The results of this study showed that the intensity of the SERRS signals from the dye followed the relationship predicted from EM theory (Fig. 5). Similar experimental evidence for long-range enhancement was obtained from studies by other groups.[70] The coverage dependence of the SERRS signal was also examined by the Langmuir–Blodgett technique. The excellent sensitivity of SERRS was emphasized in this work. It was possible to detect SERRS from dye concentrations less than 1% of a monolayer.[71] These results provided support for utilizing the tremendous sensitivity of SERRS to study chromophoric species contained with a biological matrix.

Following the initial report of cytochrome *c* SERRS spectra,[68] a number of additional studies were undertaken on cytochrome *c* and hemoglobin.[15–18,72–76] Hildebrandt and Stockburger[72] showed that cytochrome *c* on silver sols exists in a temperature-dependent, reversible spin state equilibrium. At low temperatures, their SERRS spectra resembled RR spectra of the protein in solution, whereas at room temperature a significant fraction of the heme existed in the high-spin form. Smulevich and Spiro[75] also used silver sols in their study of hemoglobin. Based on spectral changes on the sol, they proposed that the heme was dissociated from the protein and formed μ-oxo dimers. Cleavage of the heme—protein bond probably resulted from the presence of Ag^+ ions in the sol. Also, borohydride was used as the reducing agent and such sols are consider-

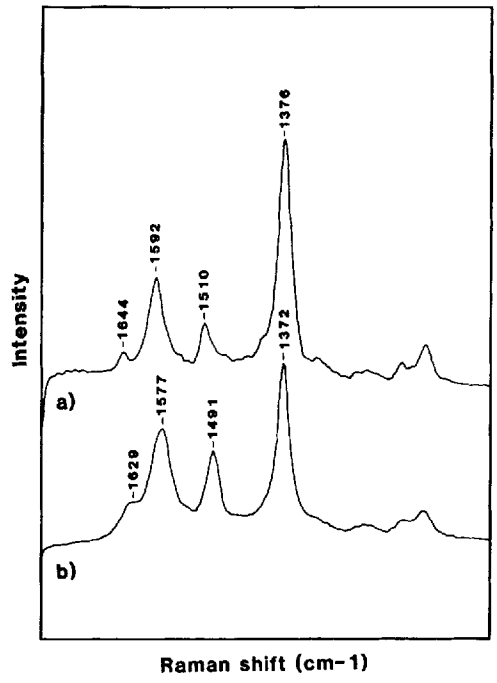

Figure 6. SERRS spectra of cytochrome *c* at (a) 77 K and (b) room temperature adsorbed on a silver electrode. Experimental conditions: excitation wavelength, 406.7 nm; laser power, 15 mW; 1 scan (5.0 s per scan).

ably more hostile to proteins. In a subsequent study by de Groot *et al.*,[76] it was shown that the hemoglobin SERRS spectrum was native when citrate-reduced silver sols were used. Cytochrome *c* is also native if citrate is used to prepare the silver colloid.[77] Finally, the native structure of cytochrome *c* can be preserved if SERRS spectra are recorded at liquid nitrogen temperature (Fig. 6).[78] By monitoring the spectrum as a function of time with a photodiode array, it was demonstrated that photodegradation was responsible for the change in the spectrum from low to high spin. This effect was eliminated when the sample was immersed in liquid nitrogen.

Other cytochromes have also been studied by SERRS, including cytochrome cd_1,[79] c_3[80–82] and P-450.[82–87] Evidence for the native structure of cytochrome c_3 adsorbed on silver surfaces was obtained in two separate studies. Niki *et al.*[80] used voltammetric methods to determine formal redox potentials of the hemes in cytochrome c_3 (*Desulfovibrio vulgaris*, Miyazaki and Hildenborough strains, and *Desulfovibrio desulfuricans*, Norway strain) adsorbed on a silver electrode and found that these were very close to the values measured for the protein in solution. The SERRS data, however, showed the spectrum of only the most positive heme in the protein, indicating that this heme is closest to the surface. Verma *et al.*[81] demonstrated that cytochrome c_3 (*D. vulgaris*, Miyazaki) could be reduced by hydrogen in the presence of hydrogenase, its physiological redox partner. This shows that the enzymatic activity is preserved when the protein is adsorbed on a citrate-reduced silver colloid. A partial change in the heme spin state was observed in the SERRS spectrum, but this was reversible and did not affect the redox properties of the protein. Cotton *et al.*[82] examined the SERRS behavior cytochrome c_3 from *Desulfovibrio desulfuricans* (NCIMB 8372) by adsorbing the protein on citrate-reduced silver colloids. In this study, no change in spin state was observed for the adsorbed protein. The major difference between the SERRS and RR spectra of the protein was in changes in the relative band intensities.

Cytochrome P-450 from a number of different sources has been studied by SERRS. Kelly *et al.*[83] found that spectra of the drug-induced rat liver cytochromes P-450 were native if the appropriate conditions were used for sol preparation, including pH and the sequence of addition of components to the sol. In another study, Hildebrandt *et al.*[84] monitored two different features in the SERRS spectrum to prove the structural and functional integrity of rabbit liver cytochrome P-450 LM2 on silver colloids. These were the substrate-induced spin state changes in the oxidized P-450 and the effect of the thiolate ligand on the oxidation state marker band. The structurally similar cytochrome P-450 PB_{3a} and PB_{3b} (97% sequence homology) were examined by SERRS and found to have different heme environments.[85] This could explain the marked differences in enzymatic activity exhibited by the two proteins. The high sensitivity of SERRS was emphasized in a recent study of four different mammalian liver microsomal P-450 enzymes.[86] Only a few nanograms of sample were required to obtain spectra of methylcholanthrene-induced rat liver cytochrome P-450 IA2, phenobarbital-induced rat liver cytochrome P-450 IIB1 and P-450 IIB2 and rabbit liver cytochrome P-450 IIB4. All were shown to be biological active when adsorbed on a 'biocompatible' citrate sol by demonstrating the low- to high-spin state conversion on addition of benzphetamine.

Other heme proteins that have been examined by SERRS include myoglobin[68,82] and hog thyroid peroxidase (HTP).[87] Myoglobin was found to be particularly sensitive to the nature of the silver surface.[82] The SERRS spectra showed evidence of denaturation following adsorption of the protein on silver electrodes. In contrast, the SERRS spectrum of the protein on a citrate-reduced silver colloid was nearly identical with the RR spectrum (Fig. 7).

Flavoproteins. Flavoproteins are strongly fluorescent and SERRS offers the potential for observing RR scattering directly. Unfortunately, these proteins appear to be destabilized at silver surfaces.[15–18,32,88–91] Initial studies were also plagued by the presence of a small amount of free flavin in the commercial preparations of glucose oxidase.[88] This gave rise to very strong scattering from the unbound flavin. When purified, very weak spectra were observed from the intact protein. This is believed to result from the large distance between the flavin and the surface, in addition to the low surface density because of the larger size of the protein.[91] A number of other flavoproteins (riboflavin-binding protein, glucose oxidase, lactate oxidase, *p*-hydroxybenzoate hydroxylase, Old Yellow Enzyme and flavodoxin (*M. elsdenii*) were also found to produce SERRS from free flavin on silver colloids, suggesting that these flavoproteins are denatured at silver surfaces.[89] The flavin tends to form a complex with Ag^+, and this may be a factor leading to the loss of the prosthetic group. In the case of glucose oxidase, catalytic

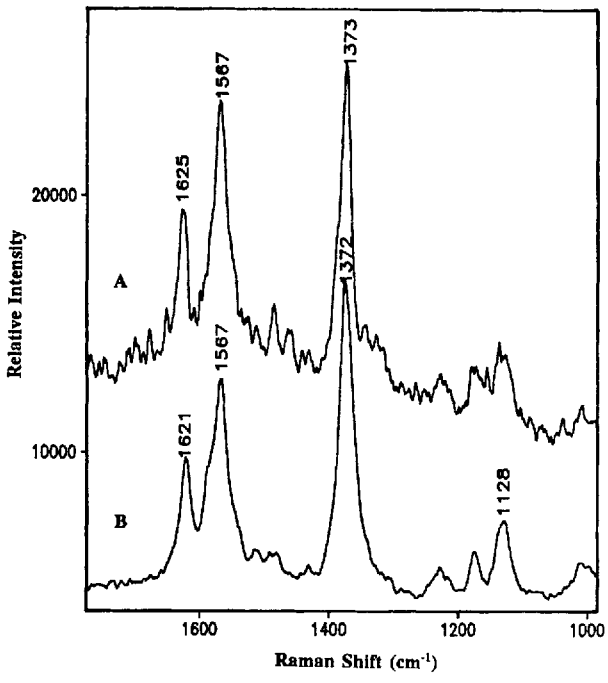

Figure 7. (A) RR and (B) SERRS spectra of myoglobin adsorbed on a silver electrode. Experimental conditions: excitation wavelength, 413.1 nm; laser power, 25 mW; 25 scans (1 scan s^{-1}).

activity leads to the dissociation of the flavin from the protein and formation of the flavin–silver complex.[90]

In summary, the potential for utilizing SERRS for the study of flavoproteins has not been demonstrated so far. It may be that gold surfaces will provide a more suitable substrate. However, because red excitation must be used to evoke enhancement on gold, it may not be possible to utilize the combined RR and surface enhancements in this case.

Phytochrome.[92,93] A comparison of the SERRS spectra of phytochrome at 77 K and the various isomers of biliverdin dimethyl ester suggested that the chromophore undergoes a $Z \rightarrow E$ photoisomerization during the Pr → Pfr phototransformation. When an electrode was used as a SERRS substrate, the Pfr form underwent substantial dark reversion to the Pr species. This may have been induced by dehydration of the phytochrome by liquid nitrogen, since these spectra were recorded with the electrode immersed in liquid nitrogen. Dark reversion was not a problem with colloidal silver, however, since the protein remained in contact with an aqueous environment. Under these conditions the SERRS spectra recorded with 406.7 nm excitation showed substantial differences between the Pr and Pfr forms that were indicative of a $Z \rightarrow E$ phototransformation.

SERRS STUDIES OF MEMBRANE PROTEINS AND MEMBRANE PREPARATIONS

Many of the active components of membranes contain chromophores. These include, for example, the chlorophylls and carotenoids of photosynthetic membranes, the rhodopsins and bacteriorhodopsin of retinal photoreceptor disks and purple membranes, respectively, as well as electron transport proteins involved in energy transduction in mitochondrial membranes. The possibility of using SERRS to monitor membrane processes has been evaluated on two systems and recent results are summarized here.

Photosynthetic membranes

The chlorophylls and carotenoids in photosynthetic membranes are readily observed by SERRS.[15–18,94–98] Data obtained from a number of different preparations have verified the integrity of these preparations on the silver surface.[98] The chromophores provide ideal probes of membrane structure. For example, the location of spirilloxanthin associated with the B800 antenna complex in the membrane of *Rhodospirillum rubrum* was determined by comparing SERRS results for 'right-side-out' and 'inside-out' membrane preparations. The SERRS signal could be observed only in the latter, indicating that spirilloxanthin is closer to the inner surface of the membrane. The orientation of spheroidenone was determined from SERRS spectra of chromatophores from *Rhodobacter sphaeroides* 2.4.1. membranes.[97]

Photosynthetic reaction centers have also been examined by SERRS.[98,99] In the case of bacterial reaction centers, the bacteriochlorophyll spectrum was observed when the preparation was adsorbed on the electrode surface at negative potentials, whereas the bacteriopheophytin spectrum was observed at positive potentials.[98] This difference may reflect the nature of the protein and/or lipid charged groups near the surface of the preparation.

Photosystem II preparations have also recently been examined at low temperatures.[99] These give strong spectra of the chlorophylls, identical with RR spectra observed in solution. In addition, a spectrum of cytochrome is also apparent. It is especially strong in reduced preparations. The data suggest that the reaction center is adsorbed with the cytochrome oriented close to the electrode surface.

Bacteriorhodopsin and rhodopsin membranes

Nabiev and co-workers have examined membranes from *Halobacterium halobium*[15–18,100] and bovine rod outer segment disks.[15–18,101,102] Several categories of information were gleaned from this work. First, as with photosynthetic membranes, it was possible to determine the location of the chromophore with respect to the membrane. In the case of the purple membrane (PM), bacteriorhodopsin (BRh) was found to be closest to the external side of the membrane surface.[100] In contrast, rhodopsin (Rh) was determined to be closest to the cytoplasmic membrane.[101,102] Second, from reconstitution experiments using apo-purple membranes and arylpolyene aldehydes or 'aromatic' analogues of retinal, the distance between the retinal Schiff base and the external side of the purple membrane was determined to be 6–9 Å.[100] Third, long- and short-range enhancement effects were demonstrated by preparing different colloids (Fig. 8). By using monoclonal antibodies to the C-end of

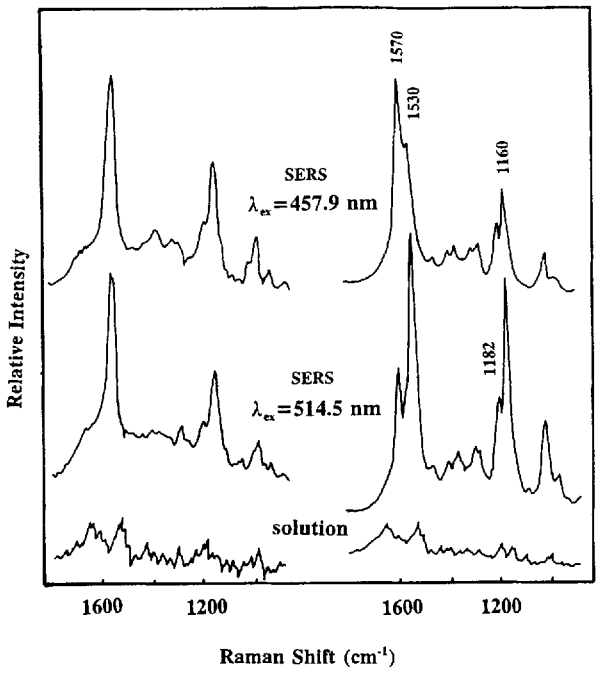

Figure 8. SERS and solution spectra of purple membrane from *Halobacterium halobium* on silver colloids with different excitation wavelengths. The SERS spectra on the right were obtained on aggregated colloids and those on the left from non-aggregated colloids. Experimental conditions; laser power, 10 mW; membrane concentration, 10^{-7} M. The sensitivity was 2.5 times higher for the solution spectra.

retinal, it was shown that the SERS signal could be suppressed, indicating short-range enhancement for non-aggregated silver sols.[101,103] Silver electrodes subjected to a roughening cycle also exhibited primarily short-range enhancement. On the other hand, partially aggregated sols or 'smooth' silver electrodes displayed long-range enhancement. The spectra in this latter case were closer to RR spectra of the preparations in solution. Moreover, the samples were capable of undergoing the normal photocycle on the silver surface, whereas on those substrates which exhibited only short-range enhancement, the photocycle was inhibited.[103] The authors suggested that the use of the short-range type of SERS could provide a means of stabilizing the intermediates. This might be a method preferred over low temperatures; this necessitates the use of organic solvents and could lead to sample denaturation. This work also demonstrates the advantages of short- *vs.* long-range enhancement and the possibility for selecting either of these for a given problem. Short-range enhancement provides information about the topography of the system, whereas long-range enhancement can be used to obtain information that is more relevant to the functional system.

Membrane model systems

Several different types of preparations have been used as models for the lipid bilayer structure of membranes. These include Langmuir–Blodgett monolayers, vesicles and micelles. Each of these model systems has been employed to a limited extent in SERRS studies.

The original studies of Langmuir–Blodgett films as membrane models were undertaken by Cotton *et al.*[69–71] as discussed above. Recently, an SERRS study of isolated retinal in Langmuir–Blodgett films was reported.[56] From an analysis of the data it was concluded that the retinal was interacting with the silver surface near the C=O end of the molecule. Curiously, no C=O vibration was observed. Based on the observation of C–H bending modes at C-14 and C-15, it was concluded that the C=O group was bonded with a specific tilt with respect to the surface.

Vesicles were used to examine the redox properties of a number of viologens.[19] From these experiments, the orientation of the viologen with respect to the surface was determined. In addition, counter-ion effects were observed. Weakly bound anions (sulfate) produced SERS/SERRS spectra of the viologens differing substantially from the solution spectra, whereas strongly bound anions (chloride) produced spectra that were very similar to the solution spectra. The large wavenumber shifts in the case of weakly bound anions were interpreted in terms of strong interaction of the viologen with the surface in addition to a chemical enhancement mechanism. In the case of strongly bound anions, the viologen interacts electrostatically with the surface and an EM mechanism is responsible for the enhancement.

Micelles have not yet been extensively studied. There is one report of the use of surfactants to improve the properties (stability and enhancement factor) of silver colloids.[104] Sun *et al.*[105] have also characterized a number of surfactants.

CONCLUSION

One of the important questions concerning SERS/SERRS studies of biological molecules is whether the molecular structure and function is preserved after interaction with the surface. This is a crucial question if SERS/SERRS is to become a general method for the spectroscopic analysis of biomolecules. Although initial experimental results relating to this question were ambiguous, it now appears that significant structural changes (denaturation) may occur for some proteins under certain conditions, but these are not necessarily due to the interaction of the protein with the surface. For example, it is apparent from SERRS of cytochrome *c* on electrodes that photodegradation can be a serious problem.[78] It was found that after only 25 s of irradiation a significant conversion of the protein from its native low-spin state to a denatured high-spin state occurred. The use of a diode-array detector minimizes the time of sample exposure and also allows the detection of changes in the spectrum which are due to photodegradation.

Many of the earlier criticisms of SERS/SERRS as a technique for the study of proteins were concerned with the possibility of denaturation produced by the interaction of the protein with the surface. It is commonly felt that hydrophobic, water-soluble proteins unravel and spread to form a monolayer when adsorbed on many types of surfaces. This idea was especially prevalent among electrochemists.[106] However, there is evidence to the contrary. In the field of immobilized enzymes, there are ample data that indicate that many

different adsorbed enzymes retain their activity.[107] The fact that some adsorbed redox-active proteins exhibit reversible electrochemical behavior at the same potential, as determined from redox potentiometry of the protein in solution, also lends credence to the probable preservation of native protein structure on the surface. Hildebrandt and Stockburger[72] have shown from SERRS studies of cytochrome *c* that its redox potential is identical with that in solution when the protein is adsorbed at negative potentials. The adsorption behavior of the protein is complex and strongly dependent on electrode potential.

The development of new methods for minimizing denaturing must be continued. It may be that a general method will be developed. One method that appears promising is to record the spectra at low temperatures.[38] A second approach that appears to hold considerable potential is the use of electrode modifiers. These may be small molecules or lipid monolayers.[77,108] As noted above, de Groot and Hester[76] reported that hemoglobin spectra were native when citrate was used as the reductant. Studies by Rospendowski and co-workers[77,86] indicated that citrate or citrate oxidation products that are present after reduction of $AgNO_3$ are adsorbed to the surface of the sol. This in turn prevents the protein from contacting the metal directly and thereby prevents denaturation. It is apparent from the many studies of Hill and co-workers[108] that certain small molecules when adsorbed on electrode surfaces function as effective promoters for electron transfer between proteins and the electrode. Although the exact mechanism regarding the interactions of the promoter with the electrode and protein is not known, SERS studies show that the promoter is adsorbed.[109] Hence it is likely that it prevents the protein from denaturing and adsorbing irreversibly to the metal surface.

FUTURE DIRECTIONS

The continued development of SERS and SERRS techniques for the study of biological samples appears very promising. As noted above, the three major advantages include the high sensitivity, a unique technique providing new information and significant quenching of fluorescence. Substrates may be developed that serve only as amplifiers of Raman scattering, independent of the chemical nature of the molecules. These will provide EM enhancement. Other types of SERS substrates could be prepared for specific adsorption of molecules and chemical enhancement of Raman scattering. Together, these substrates will lead to a wide range of new analytical applications. To be useful, they should be stable, easy to use and provide reproducible results. Other possibilities for improved methodologies might include powders and gels.

Although silver has been the most common metal for SERS, gold will be used more often in the future. Gold offers two important advantages over silver. First, it is more stable with respect to the formation of surface oxides. Second, its oxidation potential is higher than that of silver and it provides a wider potential window for redox studies at electrodes. The interaction of proteins with gold may also be different as compared with silver. It is possible that fewer problems with denaturation will be encountered.

Surface-enhanced hyper-Raman scattering (SEHRS) has already been applied to several types of biological molecules.[110] New information will result from SEHRS. The hyper-Raman technique also provides the possibility for the combination of the surface enhancement phenomenon, which is strongest in the visible region, with UV resonance Raman spectroscopy, which provides rich information for biological molecules.

Advances in instrumentation will, of course, play a pivotal role in future applications of SERS to biological molecules.

FT-Raman spectroscopy: FT-SERS from gold should be very useful for chromophore-containing biomolecules which are in resonance in the visible region. In this case, near-IR excitation is far removed from resonance and will provide non-resonance SERS. Obviously, near-IR excitation is also less destructive to biomaterials.

Micro-Raman spectroscopy: extremely small amounts of materials may be examined by micro-Raman spectroscopy. Molecular imaging is also possible. However, the tightly focused laser beam may damage the sample in some cases. An alternative approach which also offers high spatial resolution is Hadamard spectroscopy. Treado and Morris[111] have shown that this method can be combined with SERS.

Lasers and detectors: new lasers, such as the Ti–sapphire, provide a simple tunable excitation source in the red region (690–1000 nm). In addition, this laser is relatively inexpensive. When used in conjunction with a charge-coupled device (CCD) detector, the Ti–sapphire laser should prove extremely vaulable for SERS/SERRS studies on gold substrates.

REFERENCES

1. M. Fleischmann, P. J. Hendra and A. J. McQuillan, *J. Chem. Soc., Chem. Commun.* 80, (1973); *J. Chem. Phys. Lett.* **26**, 163 (1974).
2. D. L. Jeanmaire and R. P. Van Duyne, *J. Electroanl. Chem.* **84**, 1 (1977).
3. M. G. Albrecht and J. A. Creighton, *J. Am. Chem. Soc.* **99**, 5215 (1977).
4. R. P. Van Duyne, in *Chemical and Biochemical Applications of Lasers*, edited by C. B. Moore, Vol. 4, p. 101. Academic Press, New York (1979).
5. R. L. Birke and J. R. Lombardi, in *Spectroelectrochemistry, Theory and Practice*, edited by R. J. Gale, pp. 263–348. Plenum Press, New York (1988).
6. A. Wokaun, *Solid State Phys.* **38**, 223 (1984).
7. H. Metiu and P. Das, *Annu. Rev. Phys. Chem.* **35**, 507 (1984).
8. H. Metiu, *Prog. Surf. Sci.* **17**, 153 (1984).
9. A. Otto, in *Light Scattering in Solids IV*, edited by M. Cardona and G. Güntherodt, pp. 289–418. Springer-Verlag, New York (1984).

10. M. Kerker, *Acc. Chem. Res.* **17**, 271 (1984).
11. M. Moskovits, *Rev. Mod. Phys.* **57**, 783 (1985).
12. R. K. Chang and B. L. Laube, *CRC Crit. Rev. Solid State Mater. Sci.* **12**, 1 (1984).
13. R. K. Chang, *Ber. Bunsenges. Phys. Chem.* **91**, 296 (1987).
14. R. K. Chang and T. E. Furtak (Eds), *Surface Enhanced Raman Scattering*. Plenum Press, New York (1982).
15. E. Koglin and J.-M. Séquaris, *Top. Curr. Chem.* **134**, 1 (1986).
16. I. R. Nabiev, R. G. Efremov and G. D. Chumanov, *Sov. Phys. Usp.* **31**, 241 (1988).
17. T. M. Cotton, in *Surface and Interfacial Aspects of Biomedical Polymers*, edited by J. Andrade, Vol. 2, pp. 161–187. Plenum Press, New York (1985).
18. T. M. Cotton, in *Spectroscopy of Surfaces*, edited by R. J. H. Clark and R. E. Hester, pp. 91–153. Wiley, New York (1988).
19. T. M. Cotton, J.-H. Kim, and R. A. Uphaus, *Microchem. J.* **42**, 44 (1990), and references cited therein.
20. O. Siiman, L. A. Bumm, R. Callaghan, C. G. Blatchford and M. Kerker, *J. Phys. Chem.* **87**, 1014 (1983).
21. I. Otsuka and T. Iwaskai, *J. Vac. Sci. Technol. A* **8**, 530 (1990).
22. J. A. Creighton, C. G. Blatchford and M. G. Albrecht, *J. Chem. Soc., Faraday Trans. 2* **75**, 790 (1979).
23. P. C. Lee and D. Meisel, *J. Phys. Chem.* **86**, 3391 (1982).
24. S. M. Heard, F. Grieser, C. G. Barraclough and J. V. Sanders, *J. Phys. Chem.* **89**, 389 (1985).
25. G. Ritchie and C. Y. Chen, in *Surface Enhanced Raman Scattering*, edited by R. K. Chang and T. E. Furtak, pp. 361–378. Plenum Press, New York (1982).
26. D. A. Weitz, S. Garoff, J. I. Gersten and A. Nitzan, *J. Chem. Phys.* **78**, 5324 (1983).
27. V. L. Schlegel, and T. M. Cotton, *Anal. Chem.* **63**, 241 (1991).
28. I. Pockrand and A. Otto, *Solid State Commun.* **35**, 861 (1980).
29. T. Vo-Dinh, A. Alak and R. L. Moody, *Spectrochim. Acta, Part B* **43**, 605 (1988).
30. C. D. Tran, *J. Chromatogr.* **292**, 432 (1984).
31. J. J. Laserna, W. S. Sutherland and J. D. Winefordner, *Anal. Chim. Acta* **237**, 439 (1990).
32. R. F. Paisley and M. D. Morris, *Prog. Anal. Spectrosc.* **11**, 111 (1988).
33. G. D. Chumanov, R. G. Efremov and I. R. Nabiev, *J. Raman Spectrosc.* **21**, 43 (1990).
34. D. Curley and O. Siiman, *Langmuir* **4**, 1021 (1988).
35. H. Lee, S. W. Suh and M. S. Kim, *J. Raman Spectrosc.* **21**, 237 (1990).
36. T. Watanabe and H. Maeda, *J. Phys. Chem.* **93**, 3258 (1989).
37. H. Lee, S. W. Suh and M. S. Kim, *J. Raman Spectrosc.* **19**, 491 (1988).
38. T. M. Herne, A. M. Ahern and R. L. Garrell, *J. Am. Chem. Soc.* **113**, 846 (1991).
39. K. Itoh, K. Minami, T. Tsujino and M. Kim, *J. Phys. Chem.* **95**, 1339 (1991).
40. C. Otto, F. F. M. de Mul, A. Huizinga and J. Greve, *J. Phys. Chem.* **92**, 1239 (1988).
41. I. R. Nabiev, K. V. Sokolov and O. N. Voloshin, *J. Raman Spectrosc.* **21**, 333 (1990).
42. W. S. Oh, S. W. Suh and M. S. Kim, *J. Raman Spectrosc.* **19**, 261 (1988).
43. O. Siiman, R. Rivellini and R. Patel, *Inorg. Chem.* **27**, 3940 (1988).
44. J. C. Austin and R. E. Hester, *J. Chem. Soc., Faraday Trans. I* **85**, 1159 (1989).
45. N.-S. Lee, Y.-Z. Hsieh, R. E. Paisley and M. D. Morris, *Anal. Chem.* **60**, 442 (1988).
46. M. L. McGlashen, K. L. Davis and M. D. Morris, *Anal. Chem.* **62**, 846 (1990).
47. O.-K. Song, M.-J. Yoon and D. Kim, *J. Raman Spectrosc.* **20**, 739 (1989).
48. J.-S. Ha, O.-K. Song, M. Yoon and D. Kim, *J. Raman Spectrosc.* **21**, 667 (1990).
49. L. L. Thomas, J.-H. Kim and T. M. Cotton, in *XII International Conference on Raman Spectroscopy*, edited by J. R. Durig and J. F. Sullivan, pp. 588–589. Wiley, New York (1990).
50. L. L. Thomas, J.-H. Kim and T. M. Cotton, *J. Am. Chem. Soc.* **112**, 9378 (1990).
51. J. L. Wynn, L. L. Thomas and T. M. Cotton, in *XII International Conference on Raman Spectroscopy*, edited by J. R. Durig and J. F. Sullivan, pp. 348–349. Wiley, New York (1990).
52. P. Hildebrandt and T. G. Spiro, *J. Phys. Chem.* **92**, 3355 (1988).
53. R. E. Holt and T. M. Cotton, in *Redox Chemistry and Interfacial Behavior of Biological Molecules*, edited by G. Dryhurst and K. Niki, pp. 217–228. Plenum Press, New York (1989).
54. R. E. Holt and T. M. Cotton, *J. Am. Chem. Soc.* **111**, 2815 (1989).
55. V. Brabec and K. Niki, *Chem. Lett.* 1445 (1988).
56. J. Y. Huang, A. Lewis and L. Loew, *Spectrochim. Acta*, **44A** (8), 793 (1988).
57. Y.-Z. Hsieh, N.-S. Lee, T.-S. Sheng and M. D. Morris, *Langmuir* **3**, 1141 (1987).
58. R. E. Holt, D. L. Farrens, P.-S. Song and T. M. Cotton, *J. Am. Chem. Soc.* **111**, 9156 (1989).
59. S. Nie, C. G. Castillo, K. L. Bergbauer, J. F. R. Kuck, I. R. Nabiev and N.-T. Yu, *Appl. Spectrosc.* **44**, 571 (1990).
60. T. E. Rohr, T. M. Cotton, N. Fan and P. J. Tarcha, *Anal. Biochem.* **182**, 388 (1989).
61. E. Grabbe and R. P. Buck, *J. Am. Chem. Soc.* **111**, 8362 (1989).
62. A. M. Ahern and R. L. Garrell, *Langmuir* **7**, 254 (1991).
63. K. Kneipp and J. Flemming, *J. Mol. Structure* **145**, 173 (1986).
64. F. Ni and T. M. Cotton, *J. Raman Spectrosc.* **19**, 429 (1988).
65. M. Manfait, H. Morjani, J.-M. Millot, V. Debal, J.-F. Angiboust and I. Nabiev, in *Laser Applications in Life Sciences*, edited by N. I. Koroteev and B. N. Toleutaev, *Proc. SPIE* **1403**, 695 (1991).
66. G. Smulevich and A. Feis, *J. Phys. Chem.* **90**, 6388 (1986).
67. G. Smulevich, A. R. Mantini and M. P. Marzocchi, *J. Phys. Chem.* **94**, 2540 (1990).
68. T. M. Cotton, S. G. Schultz and R. P. Van Duyne, *J. Am. Chem. Soc.* **102**, 7960 (1980).
69. T. M. Cotton, R. A. Uphaus and D. Möbius, *J. Phys. Chem.* **90**, 6071 (1986).
70. G. J. Kovacs, R. O. Loutfy, P. Vincett, C. Jennings and R. Aroca, *Langmuir* **2**, 689 (1986).
71. J.-H. Kim, T. M. Cotton, R. A. Uphaus and D. Möbius, *J. Phys. Chem.* **93**, 3713 (1989).
72. P. Hildebrandt and M. Stockburger, in *Raman Spectroscopy: Sixty Years on Vibrational Spectra and Structure*, edited by H. D. Bist, J. R. Durig and J. F. Sullivan, Vol. 17A, pp. 443–466. Elsevier Sci. Pub, Amsterdam (1989).
73. P. Hildebrandt and M. Stockburger, *Biochemistry* **28**, 6710 (1989).
74. P. Hildebrandt and M. Stockburger, *Biochemistry* **28**, 6722 (1989).
75. G. Smulevich and T. G. Spiro, *J. Phys. Chem.* **89**, 5168 (1985).
76. J. de Groot, R. E. Hester, S. Kaminaka and T. Kitagawa, *J. Phys. Chem.* **92**, 2044 (1988).
77. B. N. Rospendowski, V. L. Schlegel, R. E. Holt and T. M. Cotton, in *Charge and Field Effects in Biosystems—2*, edited by M. J. Allen, S. F. Cleary and F. M. Hawkridge, pp. 43–58. Plenum Press, New York (1989).
78. T. M. Cotton, V. Schlegel, R. E. Holt, B. Swanson and P. Ortiz de Montellano, *SPIE*, Vol. 1055, *Raman Scattering, Luminescence, and Spectroscopic Instrumentation in Technology*, 263 (1989).
79. T. M. Cotton, R. Timkovich and M. S. Cork, *FEBS Lett.* **133**, 39 (1981).
80. K. Niki, Y. Kawasaki, Y. Kimura, Y. Higuchi and N. Yasuoka, *Langmuir* **3**, 982 (1987).
81. A. L. Verma, K. Kimura, T. Yagi, A. Nakamura, H. Inokuchi and T. Kitagawa, *Chem. Phys. Lett.* **159**, 189 (1989).
82. T. M. Cotton, B. Rospendowski, V. Schlegel, R. A. Uphaus, D. L. Wang, L. H. Eng and M. T. Stankovich, in *Laser Applications in Life Sciences, Proc. SPIE*, **1403**, 93 (1991).
83. K. Kelly, B. N. Rospendowski, W. E. Smith and C. R. Wolf, *FEBS Lett.* **222**, 120 (1987).
84. P. Hildebrandt, R. Greinert, A. Stier, M. Stockburger and H. Taniguchi, *FEBS Lett.* **227**, 76 (1988).
85. C. R. Wolf, J. S. Miles, S. Seilman, M. D. Burke, B. N. Rospendowski, K. Kelly and W. E. Smith, *Biochemistry* **27**,

1597 (1988).
86. B. N. Rospendownski, K. Kelly, C. R. Wolf and W. E. Smith, *J. Am. Chem. Soc.* **113**, 1217 (1991).
87. S. Hashimoto, R. Nakajima, I. Yamazaki, T. Kotani, S. Ohtaki and T. Kitagawa, *FEBS Lett.* **248**, 205 (1989).
88. R. E. Holt and T. M. Cotton, *J. Am. Chem. Soc.* **109**, 1841 (1987).
89. N.-S. Lee, Y.-Z. Hsieh, M. D. Morris and L. M. Schopfer, *J. Am. Chem. Soc.* **109**, 1358 (1987).
90. R. E. Holt and T. M. Cotton, *J. Am. Chem. Soc.* **111**, 2815 (1989).
91. R. E. Holt and T. M. Cotton, in *Redox Chemistry and Interfacial Behavior of Biological Molecules*, edited by G. Dryhurst and K. Niki, p. 217. Plenum Press, New York (1988).
92. D. L. Farrens, R. E. Holt, B. N. Rospendowski, P.-S. Song and T. M. Cotton, *J. Am. Chem. Soc.* **111**, 9162 (1989).
93. B. N. Rospendowski, D. L. Farrens, T. M. Cotton and P.-S. Song, *FEBS Lett.* **258**, 1 (1989).
94. R. Picorel, R. E. Holt, T. M. Cotton and M. Seibert, in *Progress in Photosynthesis Research*, edited by J. Biggins, pp. 1.4 423–426. Martinus Nijhoff, Dordrecht (1987).
95. M. Seibert, T. M. Cotton and J. G. Metz, *Biochem. Biophys. Acta* **934**, 235 (1988).
96. R. Picorel, R. E. Holt, T. M. Cotton and M. Seibert, *J. Biol. Chem.* **263**, 4374 (1988).
97. R. Picorel, T. Lu, R. E. Holt, T. M. Cotton and M. Seibert, *Biochemistry* **29**, 707 (1990).
98. R. Picorel, R. E. Holt, R. Heald, T. M. Cotton and M. Seibert, *J. Am. Chem. Soc.* **113**, 2839 (1991).
99. T. M. Cotton and R. P. Van Duyne, *FEBS Lett.* **147**, 81 (1982).
100. I. R. Nabiev, R. G. Efremov, G. D. Chumanov and A. B. Kuryatov, *Biol. Mem.* **2**, 1797 (1989).
101. N. G. Abdulaev, I. R. Nabiev, R. G. Efremov and G. D. Chumanov, *FEBS Lett.* **213**, 113 (1987).
102. N. G. Abdulaev, I. R. Nabiev, R. G. Efremov and G. D. Chumanov, *Biol. Mem.* **3**, 23 (1989).
103. I. R. Nabiev, G. D. Chumanov and R. G. Efremov, *J. Raman Spectrosc.* **21**, 49 (1990).
104. Y. Xu and Y. Zheng, *Anal. Chim. Acta* **225**, 227 (1989).
105. S. Sun, R. L. Birke and J. R. Lombardi, *J. Phys. Chem.* **94**, 2005 (1990).
106. B. A. Kuznetsov, *Bioelectrochem. Bioenerg.* **8**, 681 (1981).
107. W. Hartmeier, *Immobilized Biocatalysts*. Springer, New York (1986); Eng. transl., translated by J. Wieser (1988).
108. F. A. Armstrong, H. A. O. Hill and N. J. Walton, *Acc. Chem. Res.* **21**, 407 (1988).
109. T. M. Cotton, D. Kaddi and D. Iorga, *J. Am. Chem. Soc.* **105**, 7462 (1983).
110. N.-T. Yu and S. Nie, in *Laser Applications in Life Sciences*, *Proc. SPIE*, **1403**, 112 (1990).
111. P. J. Treado and M. D. Morris, *Anal. Chem.* **61**, 723A (1989).