Eur Biophys J (1992) 21, 257–261

European Biophysics Journal

Direct spectroscopic determination of functional sulphydryl groups on intact cell surfaces by surface-enhanced resonance Raman scattering

B. N. Rospendowski, J. M. Campbell, J. Reglinski, and W. E. Smith*

Department of Pure and Applied Chemistry, University of Strathclyde, 295 Cathedral St., Glasgow, Scotland, G1 1XL, UK

Received September 24, 1991/Accepted June 22, 1992

Abstract. Semi-quantitative and direct determination of labelled sulphydryl groups on the surface of intact erythrocytes has been accomplished for the first time with surface-enhanced resonance Raman scattering (SERRS). The method, which involves the use of citrate-reduced silver colloids, is sensitive and selective. A 10^{-8} M effective concentration of picomole quantities of sulphydryl groups was determined in the presence of the normally overwhelming signal from haemoglobin. This seminal study suggests that SERRS may be applied to other in situ, site-directed labelling experiments.

Key words: Surface-enhanced resonance Raman scattering (SERRS) – Intact cells – Erythrocyte – Membrane proteins – Bio-engineering

Introduction

Surface-enhanced resonance Raman scattering (SERRS) provides a method for the elucidation of structural and electronic information on molecularly specific sites of proteins in contact with aqueous solution (Cotton 1988). It has the potential to provide detailed information on the nature of key biological sites such as enzyme active centres. Surface-enhancement increases sensitivity compared to Raman scattering by several orders of magnitude. Amplification of the scattering is achieved by coupling with the surface plasmon resonance on carefully designed metal or dielectric surfaces (Van Duyne 1979; Otto 1983; Moskovits 1985). In addition to the improved sensitivity, surface selection rules provide orientational information (Creighton 1988). Furthermore, by tuning the exciting laser radiation to be resonant with the electronic transition of a chromophore, a significant enhancement in Raman scattering is observed from vibrational modes which involve specific movements of the atoms in the resonant chromophore. This provides selectivity as well as additional sensitivity (Clark and Dines 1986; Spiro 1988; Clark and Hester 1986). The combination of surface enhancement and resonance is SERRS.

The main problem with the technique is the necessity for the use of a metal surface, preferably a silver surface, which may react with biological systems and may cause denaturation. Recently, we reported that on carefully prepared citrate-reduced colloidal silver particles, a coating of citrate appears to protect proteins from direct interaction with the metal and so confers a degree of biocompatibility (Wolf et al. 1988; Rospendowski et al. 1989a, b; Farrens et al. 1989). Using this method is was possible to preserve the heme spin-state of cytochrome P-450 and to detect benzphetamine-induced low-to-high spin-state conversion (Rospendowski et al. 1991). Thus, it seemed of interest to determine whether or not these bio-compatible colloids could be used to study reactive sites at intact cell surfaces.

Sulphydryl groups on the proteins of the exofacial erythrocyte membrane surface are of interest because they are chemically reactive (Reglinski et al. 1988) and are structurally important (Bennett 1985). They are damaged by disease or modified by certain drugs (Chilles et al. 1990; Reglinski et al. 1989). These sites have been studied previously using fluorescent labels (Dufton et al. 1984). However, fluorescence tends to be molecularly ambiguous owing to the broad nature of the signals and it has only been used with red cell ghosts (Devaux and Seigneuret 1985). In contrast, the resolution of resonance Raman scattering and the vibronic structure of resonance Raman excitation profiles would enable direct molecular identification and the assignment of bands to localised bonds or moieties (Spiro and Czernuszewicz 1990) and aid an understanding of electron-nuclear dynamics (Myers 1990). In addition, specific amino-acid residues of proteins may be identified and scrutinised (Fodor et al. 1989). In this work, SERRS is used to selectively target and quantify the sulphydryl groups of erythrocyte membrane-bound proteins. This is the first direct determination of sulphydryl groups on the membrane surface of intact, haemoglobin rich cells. The use of colloidal particles in-

* *Correspondence to:* W. E. Smith

tracellularly to study a drug/DNA interaction has recently been reported (Nabiev et al. 1991).

Experimental

Raman spectra were obtained from samples held in a 1 cm pathlength fluorimeter cell. The instrument used was an Anaspec modified Cary 81 double spectrometer (1200 grooves/mm gratings) equipped with photon counting electronics (Anaspec Series 2000 Quantum Photometer) and a Thorn EMI 9658 photomultiplier tube (S20 photo-cathode). 90° scattering geometry and 5 cm^{-1} slits were used. The samples were not spun as the SERRS signals were stable during the time of each scan (less than 10 minutes). Excitation was with the 457.9 nm line from a Spectra Physics argon ion laser (model 2045).

Citrate reduced silver colloids were produced as reported previously (Turkevich et al. 1951; Lee and Meisel 1982). Briefly, 90 mg of silver nitrate was dissolved in 500 ml H_2O to which 10 ml of 1% of sodium citrate was added after the 500 ml H_2O had been brought to boiling. Boiling with continuous mechanical stirring was continued for approximately 1 hr and the stability and monodispersivity of the colloid, as indicated by the position of λ_{max}, was measured. A single band with a λ_{max} of 410 nm (60 nm full width at half maximum) in the extinction spectrum of the colloid indicated that a fairly mono-disperse (30 nm average particle size diameter) silver colloid had been produced with little aggregation and that a uniform citrate layer surrounded the metal particles. Cleanliness of the glassware and high purity reagents are essential for formation of a stable and biocompatible colloid.

10 ml of red blood cells were collected and placed in lithium heparin tubes. In all cases cells from normal (i.e. healthy) human subjects were used. The cells were centrifuged at 1500 r.p.m. for five minutes. The upper plasma layer was removed and discarded. The packed red cells were washed three times with phosphate buffered saline (PBS, 0.125 M Na_2HPO_4/0.154 M NaCl adjusted to pH 7.4 with conc. HCl). By counting, 10^{10} cells per ml were found. Ellman's reagent 5,5′-dithio-bis-(2-nitrobenzoic acid), Sigma Chemical Co. Ltd., Poole, UK, used as supplied) was added at 5×10^{-3}, 2.5×10^{-3}, 1×10^{-3} and 7.5×10^{-4}, 5×10^{-4}, 3×10^{-4} and 1×10^{-4} M final concentration in a PBS solution containing 10^9 cells/ml. The cell suspension was incubated with the different concentrations of Ellman's reagent for 1 hr. at room temperature.

After concentration of the cells by centrifugation, 100 µl (approx. 10^9 cells) aliquots of the Ellman's reagent modified erythrocytes were placed in 5 ml of PBS and were mixed with 5 ml of the citrate-reduced silver colloid (approx. 10^{14} to 10^{15} particles/ml). The colloid/cells were incubated in the PBS for 1 hr. at 25 °C. After centrifugation, the supernatant was removed from the colloid-coated erythrocytes. The coated erythrocytes were washed twice with PBS. Very little lysis of the cells was visible. Optical microscopy of the cells indicated that lysis did not occur with the colloid-coated cells.

For SERRS measurements, 5 µl of washed colloid treated cells were resuspended in 2 ml of PBS (final cell concentrations – 10^6 cell/ml).

Ellman's anion was prepared by a disulphide exchange reaction using cysteine. Precipitation of the cysteine-Ellman's mixed disulphide at pH 5 allowed isolation of the unstable, soluble anion. The concentration of the anion was determined from Beers Law (Riddles et al. 1979). A 3×10^{-3} M solution of the anion was prepared and was used immediately for the Raman measurements.

Results and discussion

The probe used for reaction with membrane sulphydryl groups on the surface of the erythrocyte was 5,5′-dithio-bis-2-(nitrobenzoic acid) Ellmans reagent (ESSE) (Ellman 1959). Its reaction with thiols is shown below. This method has been used previously to measure sulphydryl group concentrations in lysates, on cell membranes (Chilles et al. 1990), and in reactions with plasma proteins (Grimaldi 1980). In each case it is the Ellmans anion (ES^-) released into solution which is measured.

$$\text{Membrane-SH} + \text{ESSE} \rightleftharpoons \text{Membrane-SSE} + \text{ESH}$$
$$\text{ESH} \rightleftharpoons \text{ES}^- + \text{H}^+$$

SERRS can overcome the problem of low analyte concentration. Optical signal enhancement is obtained from a biocompatible, nanoscale silver colloid (Wolf et al. 1988; Rospendowski et al. 1989a) which can be used as a probe of the membrane environment. The citrate coated particles are supranegatively charged (Jolivet et al. 1985). They will preferentially migrate to the most electropositive sites on the membrane since the red cell is negatively charged overall (Absolom and Neumann 1987) as a consequence of the sialic acid groups on the surface. However, channels exist through this carbohydrate matrix which enable anions to reach the anion transport protein on the membrane. It is postulated that large, negatively charged molecules and probably therefore the silver colloidal particles use these channels to reach the membrane. Viewed through an optical microscope, there is no evidence for the presence of the citrate-reduced colloidal particles on the cell surface. This indicates that colloid aggregation does not occur to a great extent over the cell surface.

Approximately 60% of the surface thiols which react with Ellmans reagent are present on the hexose transport protein. 13% are present on the anion transport protein. The remainder are distributed between other proteins and antibody receptor sites. However, the 60% on the hexose transport protein correspond to two thiol groups which remain hidden except during active sugar transport. In these experiments, it is unlikely that such groups are revealed on the hexose transport protein or that the colloid particles will reach them. Thus, it would appear that the colloidal probe will detect mainly the 13% of the total thiol groups present on the arginine rich, anion transport protein.

SERRS spectra of ESSE and ES^- are shown in Fig. 1. The strongest band observed at approximately

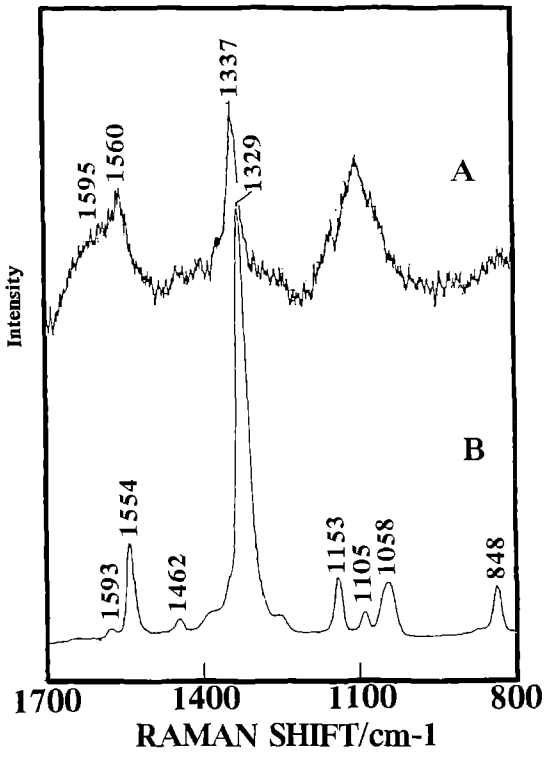

Fig. 1. A SERRS spectrum of 1×10^{-4} M ESSE adsorbed on a citrate-reduced silver colloid in the presence of M/10 PBS. **B** SERRS spectrum of 5×10^{-4} M ES^- adsorbed on a citrate-reduced silver colloid in the presence of M/10 PBS. $\lambda_{ex} = 457.9$ nm. 100 mW power

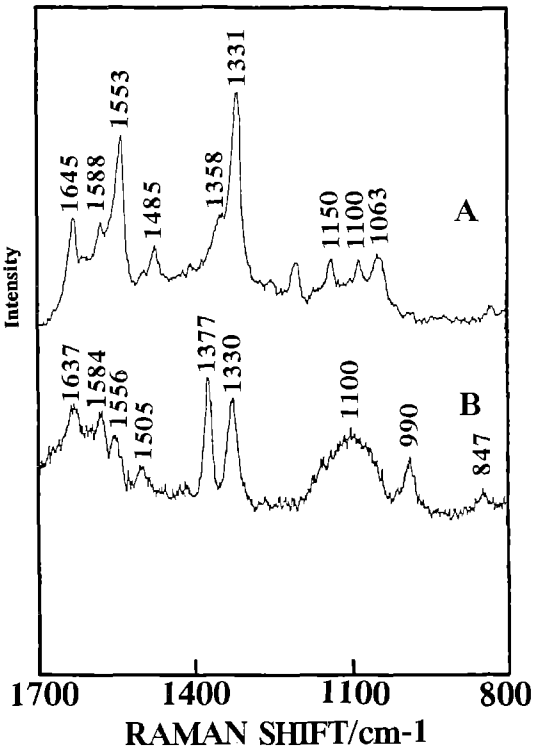

Fig. 3. A SERRS spectrum of 5×10^{-6} Ellmans reagent modified albumin in PBS. **B** SERRS spectra from a red blood cell suspension (10^6 cells/ml) after treatment with 5×10^{-3} ESSE and citrate-reduced silver colloid. $\lambda_{ex} = 457.9$ nm. 100 mW power

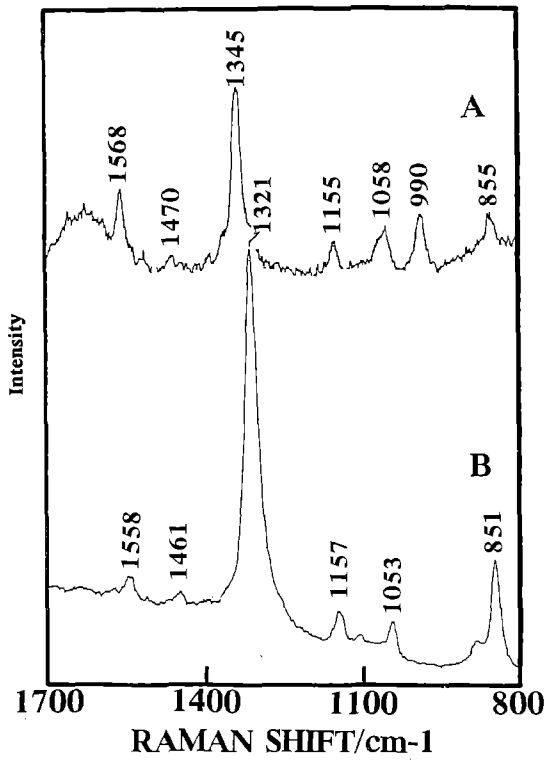

Fig. 2. A Resonance Raman spectrum of 5×10^{-3} M ESSE in PBS. **B** Resonance Raman spectrum of 1×10^{-3} M ES^- in PBS. $\lambda_{ex} =$ 457.9 nm. 100 mW power

1330 cm^{-1} is attributable to the symmetric stretch of the nitro group. The exact wave number position for this band has been shown in resonance Raman scattering to be dependent on the oxidation state of the thiol group. In the case of SERRS, ES^- occurs at 1329 cm^{-1} and ESSE at 1337 cm^{-1}. These values compare with 1321 and 1345 cm^{-1}, respectively for ES^- and ESSE in resonance Raman scattering (Fig. 2). The shifts indicate that the absorption of the reagent to the colloidal silver surface perturbs the electron density of the nitro group. In the SERRS spectrum of albumin in which the one available thiol group on the protein has been labelled by exchange with Ellmans reagent to form a mixed disulphide (Fig. 3), the stretching frequency for the nitro group occurs at 1331 cm^{-1}. A reduction in the relative intensity of this band, as compared to that observed for ES^- and ESSE (Fig. 1), is detected with respect to the bands in the 1600 cm^{-1} and 1000 cm^{-1} regions and the frequency is closer to that of ES^- than that of ESSE. These changes are due in part to the nature of the albumin site and in part to the different electronic and surface selection rules (Creighton 1988) for a SERRS active group held away from the surface by the presence of the protein. The thiol group is held in a pocket of the protein so that the environment round the entire ES^- moiety has a different dielectric constant to water. There will also become steric hindrance. The restricted environment is most likely to cause the lowering of the frequency from that anticipated

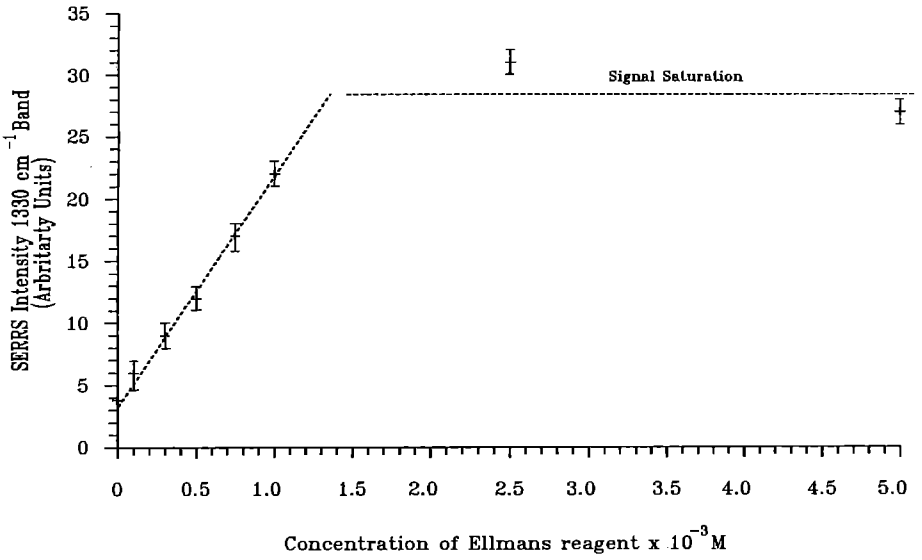

Fig. 4. Graph of SERRS intensity of 1330 cm^{-1} band versus concentration of Ellmans reagent (ESSE) with which the red blood cells were incubated

position close to that of the disulphide ESSE. Both factors will affect intensity.

By exchanging the sulphydryl population of the red cell membrane with Ellmans reagent, we were able to place on the membrane surface a chromophoric, Raman active marker for the sulphydryl group of the membrane proteins. The SERRS signals (Fig. 3) from the Ellmans treated cells show a band assigned to the nitro function at 1330 cm^{-1} and the weaker aromatic vibration at 1556 cm^{-1}. In addition, peaks due to haemoglobin are observed at 1377 cm^{-1}, v_4, 1505 cm^{-1}, v_3, 1584 cm^{-1}, v_2, and 1637 cm^{-1}, v_{10}.

Semiquantitative SERRS detection of the sulphydryl group content of the erythrocyte membrane is shown in Fig. 4 as a graph of the intensity of the 1330 cm^{-1} band vs. concentration of Ellman's reagent used in the incubation with the erythrocytes. The SERRS intensity of the 1330 cm^{-1} band increases as the concentration of Ellmans reagent used in the initial incubation of the red cells at the labelling stage of the experiments is increased up to 5×10^{-3} M. At Ellmans reagent incubation concentrations above 1.5×10^{-3} M the signal level appears to plateau. Presumably, all the exchangeable thiol groups have reacted with reagent concentrations greater than about 1.5×10^{-3} M. Hence no further increase in the signal is observed with increasing concentration. The signal is observed in the presence of relatively large quantities of haemoglobin, which would preclude observation of the signal under resonance Raman scattering conditions alone.

An immediate concern in this study is that the colloidal particles may migrate over the cell surface or that cleavage of the protein mixed disulphide link could occur due to affinity of the Ellmans reagent for the cell surface. Thus, the sol could be acting as a "sink" for the Ellmans reagent/anion and any specific membrane structure information would be lost. However, both Ellmans anion and Ellmans reagent yield SERRS signals which differ in wave number and relative intensity from that observed for mixed disulphides between the cell membrane and the Ellmans probe. Furthermore, the SERRS observed from the albumin labelled suspension is different from that from the cells. Thus, the signal obtained from the cell surface appear to be specific and due to the bound disulphide.

Thus, the development of a new technique for the study of membrane bound constituents of intact whole cells has been accomplished. The SERRS of sulphydryl groups is of particular interest because of the current interest in these groups in disease states, but it may be that with suitable probes other sites on the membrane can be studied. In addition, it is of interest that semi-quantitative behaviour was obtained from these initial measurements indicating that the technique has the potential to detect both the number and activity of the groups.

Acknowledgements. We would like to thank the Science and Engineering Research Council, the Nuffield Foundation and the Arthritis and Rheumatism Council for financial assistance to BNR, JR and JMC respectively. We thank Dr. R. Wilson of Glasgow Royal Infirmary for the provision of blood samples.

References

Absolom DR, Neumann AW (1987) In: Matijevic E (ed) Surface and Colloid Science. Vol. 10, pp. 215–263, Plenum Press, New York

Bennett V (1985) The membrane skeleton of human erythrocytes and its implications for more complex cells. Ann Rev Biochem 54:273–304

Chilles C, Mulheron M, McCrae F, Reglinski J, Smith WE, Brzeski M, Sturrock RD (1990) Concentration and reactivity of the sulphydryl group population on the membrane of intact erythrocytes in patients with rheumatoid arthritis. Ann Rheum Dis 49:668–671

Clark RJH, Dines TJ (1986) Resonance Raman spectroscopy and its application to inorganic chemistry. Angew Chem Int Ed Engl 25:131–158

Clark RJH, Hester RE (eds) (1986) Advances in spectroscopy: spectroscopy of biological systems. Vol 13, Wiley & Sons Ltd, Chichester

Cotton TM (1988) In: Clark RJH, Hester RE (eds) Advances in spectroscopy: spectroscopy of surfaces. Vol 16, pp 91–153, Wiley & Sons Ltd, Chichester

Creighton JA (1988) In: Clark RJH, Hester RE (eds) Advances in spectroscopy: spectroscopy of surfaces. Vol 6, pp 37–89, John Wiley & Sons, Ltd, Chichester

Devaux PF, Seigneuret M (1985) Specificity of lipid-protein interactions as determined by spectroscopic techniques. Biochim Biophys Acta 822:63–125

Dufton MJ, Hider RC, Cherry RJ (1984) The influence of melittin on the rotation of band 3 protein in the human erythrocyte membrane. Eur Biophys J 11:17–24

Ellman GL (1959) Tissue sulphydryl groups. Arch Biochem Biophys 82:70–77

Farrens DL, Holt RE, Rospendowski BN, Song P-S, Cotton TM (1989) Surface enhanced Raman scattering spectroscopy applied to phytochrome and its model compounds. 2 Phytochrome and Phycocyanin chromophores. J Am Chem Soc 111:9162–9169

Fodor SPA, Copeland RA, Grygon CA, Spiro TG (1989) Deep-ultraviolet Raman excitation profiles and vibronic scattering mechanisms of phenylalanine, tyrosine and tryptophan. J Am Chem Soc 111:5509–5518

Grimaldi MG (1980) Serum sulphydryl concentration and anti-rheumatic drugs in rheumatoid patients. J Pharm Pharmacol 32:876

Jolivet JP, Gzara M, Mazieres J, Lefebvre J (1985) Physiochemical study of aggregation in silver colloids. J Coll Interface Sci 107:429–441

Lee PC, Meisel D (1982) Adsorption and surface-enhanced Raman of dyes on silver and gold sols. J Phys Chem 86:3391–3395

Moskovits M (1985) Surface-enhanced spectroscopy. Rev Mod Phys 57:783–826

Myers A (1990) Femtosecond molecular dynamics probed through resonance Raman intensities. J Opt Soc Am B 7:1665–1672

Nabiev IR, Morjani H, Manfait M (1991) Selective analysis of anti-tumor drug interaction with living cancer cells as probed by surface-enhanced Raman spectroscopy. Eur Biophys J 19:311–316

Otto A (1983) In: Cardonna M, Guntherodt G (eds) Topics in applied physics: Light scattering in solids IV. Vol 54, pp 289–418, Springer, Berlin Heidelberg New York

Reglinski J, Hoey S, Smith WE, Sturrock RD (1988) Cellular response to oxidative stress at sulphydryl group receptor sites on the erythrocyte membrane. J. Biol Chem 263:12360–12366

Reglinski J, Smith WE, Belch JJF (1989) The action of captopril at a cellular level. J Chem Res 316

Riddles PW, Blakeley RL, Zerner B (1979) Ellmans reagent 5,5′-dithiobis-(2-nitrobenzoic acid) – a re-examination. Anal Biochem 94:75–81

Rospendowski BN, Schlegel VL, Holt RE, Cotton TM (1989) In: Allen MJ, Clearly SF, Hawkridge FM (eds) Charge and field effects in biosystems II, pp 43–58, Plenum Press, New York

Rospendowski BN, Farrens DL, Cotton TM, Song P-S (1989) Surface enhanced resonance Raman scattering (SERRS) as a probe of the structural differences between the Pr and Pfr forms of phytochrome. FEBS Lett 258:1–4

Rospendowski BN, Kelly K, Wolf CR, Smith WE (1991) Surface-enhanced resonance Raman scattering from cytochromes P-450 adsorbed on citrate-reduced silver sols. J Am Chem Soc 113:1217–1225

Spiro TG (1988) Biological applications of Raman spectroscopy. Vols I–III, Wiley-Interscience, New Nork

Spiro TG, Czernuszewicz RS, Li X-Y (1990) Metalloporphyrin structure and dynamics from resonance Raman spectroscopy. Coord Chem Rev 100:541–571

Turkevich J, Stevenson PC, Hillier J (1951) A study of the nucleation and growth processes in the synthesis of colloidal gold. Dis Faraday Soc 11:55–75

Van Duyne RP (1979) in: Moore CB (ed) Chemical and biochemical applications of lasers. Vol 4, pp 101–185, Academic Press, New York

Wolf CR, Seilman S, Burke MD, Rospendowski BN, Kelly K, Smith WE (1988) Evidence that the catalytic differences of two structurally homologous forms of cytochrome P-450 relate to their heme environment. Biochemistry 27:1597–1603