Photophysical characterization of hematoporphyrin incorporated within collagen gels

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Abstract

Physical properties of hematoporphyrin-enriched collagen gels relevant to the photodynamic treatment of cancer are characterized. The incorporation of the sensitizer within the gels does not affect either the structure of the gel or the absorption and fluorescence spectra of the sensitizer. The gel-embedded sensitizer photodegrades efficiently with the formation of a product emitting near 635 nm. Differences in the collagen scattering are observed following sensitization *vs.* prolonged irradiation with Ar^+ laser, indicating contrasting structural modifications effected to the biopolymer matrix in the two cases. The results correspond well to *in vivo* observations, suggesting that hematoporphyrin-enriched collagen gels may be appropriate systems for modeling the influence of the semisolid nature of tissues, and in particular of the tumour stroma collagen, on the photodynamic phenomenon.

Key words: Photosensitization; Photobleaching; Hematoporphyrin; Collagen; Gels

1. Introduction

The growing interest in the photodynamic therapy (PDT) of cancer [1–3] has prompted extensive research into the mechanism of the phenomenon on a cellular level. Through these studies, cellular structures involved in the absorption and accumulation of sensitizers in cells, *e.g.* as presented in refs. 4 and 5, and cellular targets of the PDT cytotoxicity have been specified, *e.g.* in refs. 6–8. A general understanding of the mechanisms by which PDT impairs cellular functions and metabolism is evolving.

However, *in vivo* studies [9–11] strongly suggest that direct cell killing is only one of the effects of the PDT phenomenon, with multiple processes contributing to the tumour necrosis. In particular, significant amounts of the sensitizers are generally found to accumulate in the tumour stroma [12, 13], where they mediate extensive photodegradation with significant implications for the PDT mechanism and its effectiveness [9–15]. Despite this accumulating evidence, little work has been directed at studying the role of stroma biocomponents in the photodynamic phenomenon.

In this paper, we report a preliminary study on the role of collagen in the hematoporphyrin (Hp)-mediated photosensitization mechanism. Collagen has been chosen for study because of its high abundance in tissue fibrous matrix, and because of its well-known high affinity for porphyrins [16]. The collagen is employed in the gel form commonly used as substrate for keratinocyte cellular cultures. The collagen constitutes highly purified extract from calf skin and in the gel form it maintains its native state [17]. Scanning electron microscopy (SEM) (Fig. 1) shows the prepared film to consist of networks of randomly oriented interleaved protein fibrils. Its appearance corresponds well to characterizations of skin collagen [18]. Conse-

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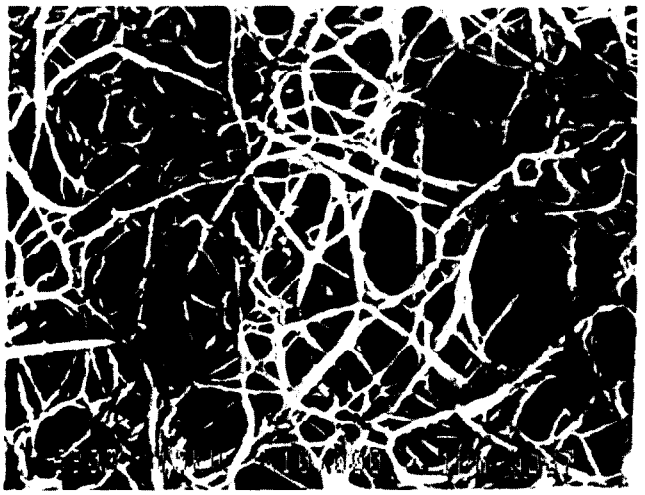

Fig. 1. Scanning electron micrograph of the pure collagen gels employed in this study. An identical structure is obtained for gels enriched in Hp dihydrochloride. (Magnification, 10 000×).

quently, the gel is expected to simulate realistically the semisolid nature of tissues. This is important in view of the demonstrated high sensitivity of the photosensitization process to medium polarity and nature [19].

We find that Hp can be incorporated within collagen gels, wherein it displays interesting photophysical and chemical behavior.

2. Experimental details

Collagen G, from Biochrom KG (Switzerland), is prepared in gel form according to an established procedure [17]. For the preparation of the Hp-enriched gels, Hp IX dihydrochloride, used as received from Sigma GmbH (Germany), is dissolved just prior to use in 0.1 M NaOH, which is subsequently mixed with the collagen stock solution. Absorption spectra of the gels (in cuvettes of 0.1 cm path length) are recorded at room temperature on a Lambda-6 Perkin–Elmer spectrophotometer.

For the SEM examination, the gels are dehydrated by treatment with a series of ethanol baths and critical point drying with CO_2. The samples are subsequently coated with 60Au–40Pd and examined on a JEOL model JSM-840 (Japan) scanning electron microscope at 15 kV.

In the fluorescence and "photobleaching" experiments, the laser beam ($d_{diam} = 3$ mm) (Spectra-Physics 2016 argon ion laser) propagates through the collagen (prepared within Petri dishes) approximately 0.5 cm below the gel surface, and the emission is collected by an optical fibre (600 μm thickness) placed perpendicular to and 0.1–0.5 cm above the gel surface. An x–y precision micrometer ensures accurate positioning of the fibre in measuring the spatial distribution of the fluorescence intensity. The light from the fibre is spectrally analyzed on a 0.5 m monochromator, interfaced to a Princeton optical multichannel OMA III system. A mercury lamp is used for the wavelength calibration of the system. Fluorescence intensities represent integrated areas of the peaks, corrected for the background signal (due to the strong scattering by collagen) and for inner-filter effects, which are generally small.

3. Results and discussion

In accordance with the previous evidence on the high affinity of collagen for porphyrins [16], we find that we can produce collagen gels with Hp concentrations as high as 10^{-1} M. The incorporation of the Hp does not appear to influence the matrix structure, as judged by SEM examination and by specific volume measurements (*i.e.* change in the collagen volume solidified in the presence and absence of the hematoporphyrin). Furthermore, the incorporated Hp is not easily removed by repeated washings of the gel with saline solution. Strong hydrogen bonding and limited diffusivity of the molecule within the dense collagen fibril network probably account for the difficulty in extracting the Hp from the biopolymer substrate. The strong binding of Hp to collagen may relate to the prolonged retention of the sensitizer in the tumour stroma that has been observed in the *in vivo* studies.

Interestingly, the incorporation of Hp within the collagen does not effect noticeable changes to the absorption and fluorescence spectra of the sensitizer. In the range of examined concentrations from 10^{-5} to 10^{-3} M, the Soret and Q bands appear almost at the same wavelengths as for the corresponding aqueous solutions. To ensure that the integrity of the gel does not affect the validity of the conclusion, the absorption measurements for each Hp concentration were repeated several times with different samples. Furthermore, immediately after each spectrum referenced to pure collagen gel, an analogous spectrum was obtained with water as reference. An overall small value for the transmission coefficient and an absorption spectrum typical of the collagen substrate were always obtained, establishing that the interrogated gel was well structured and that its integrity was not compromised during the scan. With these precautions, we feel confident that the Hp ab-

sorption spectrum is not perturbed by the incorporation within the collagen gel. Essentially the same observation is made for the emission spectrum, for which the 615 nm and 675 nm peaks are found blue-shifted by 3 ± 1 nm and 5 ± 1 nm respectively.

This wavelength insensitivity of the spectral features suggests that the polarity of the local Hp environment within the gel is very close to that of aqueous solution. Evidently, significant water concentration is incorporated within the gel during polymerization. In addition to the polarity factor, the observed insensitivity suggests that the polymeric matrix does not disrupt the aggregated species that are present in the aqueous solutions of the sensitizer at concentrations higher than 10^{-6} M [20]. It appears that the collagen matrix has enough flexibility to accommodate the aggregates without perturbing their equilibrium or nature. It should be noted, however, that small quantitative changes exist, especially in the fluorescence quantum yields, but the analysis of these effects relies on the establishment of an accurate actinometer in the collagen gel, measurement of the gel's refractive index etc., to be presented in an subsequent publication.

The incorporated Hp provides via its fluorescence an effective probe for mapping the propagation of the Ar^+ laser beam through the gel. The fluorescence distribution along the beam propagation axis decreases in exponential manner, as illustrated by the full curve in Fig. 1(a), whereas a nearly gaussian distribution is observed (full curve in Fig. 1(b)) for directions perpendicular to the light propagation axis and in the plane of the beam. In principle, analysis of the curves on the basis of well-established theoretical models [21] can yield the absorption and scattering coefficients of the collagen gels. However, the analysis is complicated by the photodegradation of the sensitizer that occurs in parallel during gel irradiation. Thus different curves are indicated depending on the extent of illumination. This is illustrated in Fig. 2 by comparing data obtained for minimum irradiation time (full circles) with corresponding data recorded upon extensive laser irradiation (open circles).

Qualitatively, the differences between the curves obtained in the two cases can be easily accounted for. Thus the decrease in the absorbing molecules effected by the photobleaching minimizes the attenuation (per unit length) of the beam and permits its propagation over a longer distance in the gel (Fig. 2(a)). Likewise, at a given point along the propagation axis, photobleaching permits a wider

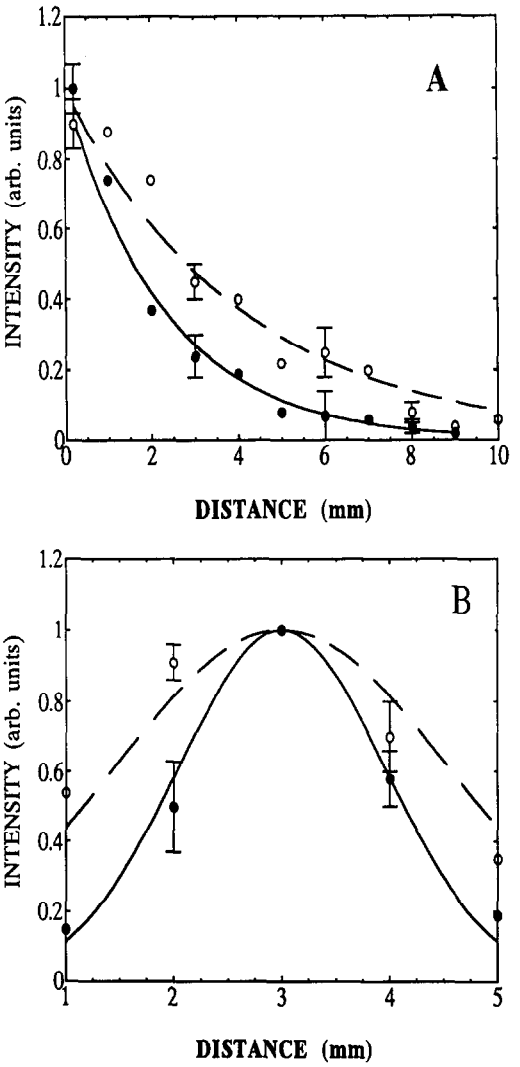

Fig. 2. Distribution of fluorescence intensity of Hp in the collagen gel scales (a) along the Ar^+ ion laser propagation axis and (b) perpendicular to the laser beam and 9.5 mm away from the point of incidence of the laser beam on the collagen surface (here, the fluorescence intensity at the center of the beam — position of +3 mm — is taken arbitrarily as unity and the data points are scaled accordingly): ●, data obtained in the minimum irradiation time (approximately 2 s per point); ○, data obtained upon longer illumination (15 min); error bars represent 2σ, where σ is the standard deviation from five experiments; —, fittings to the full circles (exponential in (a) and gaussian in (b)); – – –, corresponding fittings to the open circles. The sensitizer concentration is 2×10^{-5} M, and the optical fibre (600 μm) is placed 0.2 cm above the collagen surface, with the laser beam ($I_{laser} = 0.15$ W; $\lambda_{laser} = 514$ nm) propagation about 0.5 cm below the collagen surface and perpendicular to the detection axis.

light spread, as evidenced by the larger full width at half-maximum of the broken curve (prolonged irradiation) *vs.* the solid curve (minimal illumination) in Fig. 2(b). Although the quantitative evaluation of the differences requires first assessment of the photodegradation process within gels, it is nevertheless clear that the Hp–collagen

system provides a most realistic system in which to model the propagation of laser light through tissues during PDT.

Irradiation of fresh samples with the Ar^+ ion laser ($\lambda = 514$ nm) results in the appearance of a new fluorescence peak near $\lambda = 640$ nm, with concomitant decrease in the porphyrin emission intensity. These changes are irreversible, establishing that they concern chemical modification of the primary sensitizer. To characterize the degradation kinetics, spectra are recorded every 15 or 30 s during the first 5 min of the process, when the change occurs most rapidly, and every 1 min afterwards. In a logarithmic plot of the integrated peaks *vs.* time (insert in Fig. 3), the data points are found to be described by a straight line to an excellent degree of accuracy (χ^2 being generally above 0.95, except for measurements on 10^{-5} M samples at low laser intensities, for which the comparatively strong scattering by collagen compromises the accuracy of Hp fluorescence measurement and the corresponding certainty of the linear regression). The accuracy of the linear regression establishes that the Hp degradation within gels is well described by first-order kinetics. The initial examination of the photobleaching indicates that its kinetics is weakly dependent on the Hp concentration and that it saturates at higher laser intensities (at about 0.2 W).

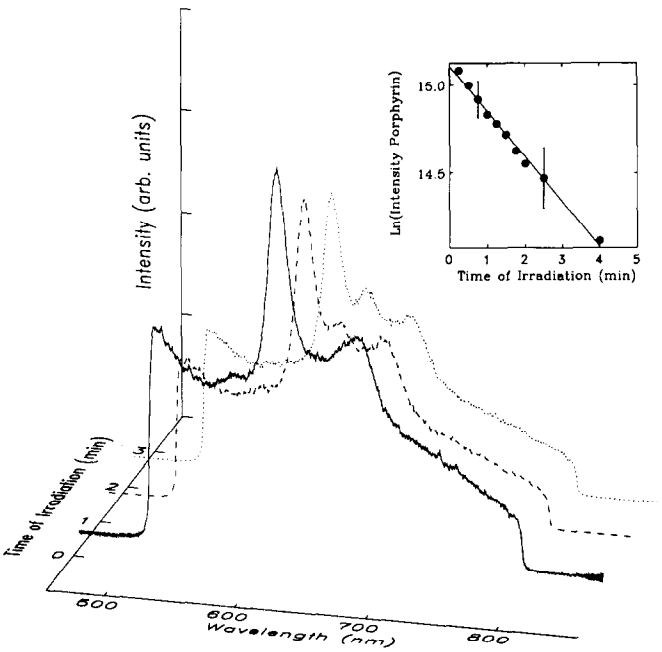

Fig. 3. The first 5 min evolution of the fluorescence spectrum recorded from 2×10^{-5} M Hp-enriched collagen gel under irradiation with a continuous-wave Ar^+ laser ($I_{laser} = 0.15$ W; fiber positioned 0.5 mm away from front surface). The spectrum at $t = 0$ s (——) represents the fluorescence spectrum of pure Hp collagen gel. In the later spectra (· · · · ·, 1 min; – – –, 2.5 min), the intensity of the Hp emission has decreased and a new peak near $\lambda = 640$ nm has emerged. This peak is ascribed to a new species formed by photo-oxidation of the Hp. The inset illustrates that, to a very good approximation, the Hp degradation follows first-order kinetics. The error bars represent 2σ from three runs. The depicted kinetics are followed for approximately the first 5 min of irradiation, after which the degradation slows down.

The product of the Hp degradation is revealed by its emission near $\lambda = 640$ nm ($\lambda_{max} = 635 \pm 1$ nm). Importantly, in control experiments on aqueous Hp solutions (concentration, 10^{-5} M), under otherwise identical conditions, the sensitizer degrades as established by the diminution of its fluorescence, but no new emission peak is detectable ($\Delta I_{640}/I_{640} < 0.2$). This demonstrates the importance of the collagen medium in the formation of the emitting species.

The product itself undergoes further degradation, but its kinetics cannot be characterized accurately because of extensive interference by the much stronger Hp signal. Nevertheless, approximate analysis of the experimental curves suggests also first-order kinetics for its degradation.

These results are in overall good agreement with the data obtained for the "photobleaching" of Hp derivative within cells [22]. This correspondence of observations arises probably because proteins constitute the primary targets of Hp within cells. In any case, the observation of "photobleaching" within collagen gels provides a simple system where the product can be isolated and studied in detail. In particular, it provides for the examination of a previous suggestion on the importance of amino-acid involvement in the photooxidation of porphyrins [23]. The "photobleaching" is currently being studied in experiments with scavengers, so as to establish the exact mechanisms involved in the photodegradation of the molecule.

In parallel with the porphyrin degradation, the background of the spectrum, which is ascribed to light inelastically scattered by the collagen, generally changes in intensity during the irradiation, but the effect is small (amounting, at most, to 10% of the initial signal). Only in few cases (three out of 28 measurements in total) is a different trend observed, namely the scattering by the collagen to increase continuously with irradiation time. We believe that in these cases the collagen integrity is locally compromised and irradiation promotes further disruption of its structure. This interpretation is supported by the fact that the trend is mainly observed or can be reproduced with older (3–4 days old) collagen samples.

Except for these cases, the change in the collagen scattering intensity is always small and partially

reversible. Examination of the irradiated spots by SEM (ten samples after irradiation for 5–10 min) finds the gel morphology essentially identical with that of the control (unirradiated) samples. Thus, within the probing capabilities of the employed techniques, the photosensitization process does not appear to influence the gel structure.

The small change in the collagen scattering is attributed to combined thermal and photodynamic effects, resulting plausibly in limited denaturation and/or subtle modification of the collagen fibrils. Interestingly, Spikes [24] has reported that, following PDT, collagen (in rat tail tendons) has somewhat altered load–strain and stress relaxation properties and increased breaking strength under thermal stress. He proposes that these changes are due to photoinduced cross-linking of the collagen molecules in the tail tendons. Cross-linking would change the number of effective scatterers in the gel, thereby affecting the intensity of the scattering by the collagen. Polymerization and cross-linking is known to modify the light scattering properties in other polymeric systems (see for example refs. 25 and 27), and a similar effect may well be observed for collagen gels.

In sharp contrast with what we found above, upon prolonged (approximately 1 h, depending on laser power) irradiation of either pure or Hp-enriched films at room temperature, the background spectrum is observed to undergo a sudden and abrupt change. The spectrum afterwards is identical with that recorded from optically clear solutions. This indicates that, upon extensive irradiation of the film, the collagen undergoes a thermally induced local degradation. Although the chemical nature of the degradation has not been ascertained at this moment, it is interesting that the changes observed herein parallel the findings of Barr *et al.* [27], who noted contrasting effects following PDT and thermal treatment of colon cancer. In the former case, the collagen fibrils were found to retain their periodicity whereas, in the latter, the architecture and configuration of the normal submucosa were completely lost, suggesting that colonic collagen treated at thermal laser powers is severely damaged.

The preliminary results reported herein indicate that the Hp exhibits rich photophysical and chemical behavior within collagen gels. Further study of this system is expected to enable a better understanding of the influence of solid-like media on the photodynamic phenomenon. Furthermore, the good reproduction of *in vivo* observations in collagen gel suggests that this system may provide a simple and convenient chemical model with which to evaluate the photodynamic potential of "second-generation" sensitizers currently under development.

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References

1 T.J. Dougherty, Photodynamic therapy: status and potential, *Oncology, 3* (1990) 67–73.
2 C.J. Gomer, N. Rucker, A. Ferrario and S. Wong, Properties and applications of photodynamic therapy, *Radiat. Res., 120* (1989) 1–18.
3 B.W. Henderson, Photodynamic therapy – coming of age, *Photodermatology, 61* (1989) 200–211.
4 F. Richelli, G. Jori, G. Moreno, F. Vinzens and C. Salet, Factors influencing the distribution pattern of porphyrins in cell membranes, *J. Photochem. Photobiol. B: Biol., 6* (1990) 69–77.
5 D. Kessel, Porphyrin–lipoprotein association as a factor in porphyrin localization, *Cancer Lett., 33* (1986) 183–188.
6 C. Salet and G. Moreno, Photosensitization of Mitochondria. Molecular and cellular aspects, *J. Photochem. Photobiol. B: Biol., 5* (1990) 133–150.
7 J. Moan, S.E. Rognan, J.F. Evensen and Z. Malik, Cell photosensitization by porphyrins, *Photochem. Photobiophys., Suppl.* (1987) 385–395.
8 C. Candide, J.C. Maziere, R. Santus, C. Maziere, P. Moliere, J.P. Reyftmann, S. Goldstein and L. Dubertret, Photosensitization of Wi26-VA4 transformed human fibroblasts by low density lipoprotein loaded with the anticancer porphyrin mixture Photofrin II: evidence for endoplasmic reticulum alteration, *Cancer Lett., 44* (1989) 157–161.
9 M. Korbelik, Photosensitizers in photodynamic therapy, *Period. Biol., 93* (1991) 563–574.
10 C.J. Gomer, Preclinical examination of first and second generation photosensitizers used in photodynamic therapy, *Photochem. Photobiol., 54* (1991) 1093–1107.
11 B.W. Henderson, S.M. Waldow, T.S. Mang, W.R. Potter, P.B. Malone and T.J. Dougherty, Tumor destruction and kinetics of tumor death in two experimental mouse tumors following photodynamic therapy, *Cancer Res., 45* (1985) 572–576.
12 D. Kessel, P. Thompson, K. Saatio and K.D. Nantwi, Tumor localization and photosensitization by sulfonated derivatives of tetraphenylporphyrin, *Photochem. Photobiol., 45* (1987) 787–790.
13 Q. Peng, J. Moan, G.W. Farrants, H.E. Danielsen and C. Rimington, Location of P-II and AlPCS4 in human tumor LOX *in vitro* and *in vivo* by means of computer-enhanced video fluorescence microscopy, *Cancer Lett., 58* (1991) 37–47.

14 J. Winther and J. Overgard, Photodynamic therapy of experimental intraocular retinoblastoma-dose–response relationships to light energy and Photofrin II, *Acta Ophthalmol., 67* (1989) 44–50.

15 W.M. Star, H.P.A. Marijnissen, A.E. Berg-Block, J.A.C. Versteeg, K.A.P. Franken and H.S. Reinhold, Destruction of rat mammary tumor and normal tissue microcirculation by hematoporphyrin derivative photoradiation observed *in vivo* in sandwich observation chambers, *Cancer Res., 46* (1986) 2532–2540.

16 D.A. Musser, J.M. Wagner and N. Datta-Gupta, The interaction of tumor localizing porphyrins with collagen and elastin, *Res. Commun. Chem. Pathol. Pharmacol., 36* (1982) 251–259.

17 Von R. Riemschneider, M.Z. Abedin and F. Heisler, Langzeiteinwirkung von äther auf kollagen-haltige materialen im rahmen der gewinnung löslicher kollagene, *Pharm. Ind., 41* (1979) 1077–1081.
Biochrom KG Brochure.

18 C.J. Doilon, C.F. Whyne, R.A. Berg, R.M. Oson and F.H. Silver, Fibroblast–collagen sponge interactions and the spatial deposition of newly synthesized collagen fibres *in vitro* and *in vivo, Scanning Electron Microsc., 3* (1984) 1313–1320.

19 E. Reddi, M.A.J. Rodgers, J.D. Spikes and G. Jori, The effect of medium polarity on the hematoporphyrin-sensitized photooxidation of L-tryptophan, *Photochem. Photobiol., 40* (1984) 415–421.

20 E. Reddi and G. Jori, Steady-state and time-resolved spectroscopic studies of photodynamic sensitizers: porphyrins and phthalocyanines, *Rev. Chem. Intermed., 10* (1988) 241–268, and references cited therein.

21 B.C. Wilson and M.S. Patterson, The physics of photodynamic therapy, *Phys. Med. Biol., 31* (1986) 327–360, and references cited therein.

22 J. Moan, Effect of bleaching of porphyrin sensitizers during photodynamic therapy, *Cancer Lett., 33* (1986) 45–53.

23 M. Krieg and D.G. Whitten, Self-sensitized photooxidation of protoporphyrin IX and related free-base porphyrins in natural and model membrane systems. Evidence for novel photooxidation pathways involving aminoacids, *J. Am. Chem. Soc., 106* (1984) 2477–2479.

24 J.D. Spikes, Effects of photodynamic treatment on the thermal-mechanical properties of collagen (rat tail tendon), *Proc. 21st Annu. Meet., Chicago, IL, 1993,* American Society for Photobiology, Chicago, IL, 1993, p. 317.

25 B. Chu, G. Fytas and G. Zalczer, Study of thermal polymerization of styrene by Raman scattering, *Macromolecules, 14* (1981) 395.

26 N.T. Yu and B.H. Jo, Comparison of protein structure in crystals and in solution by laser Raman scattering II, ribonuclease A and carboxypeptidase A, *J. Am. Chem. Soc., 95* (1973) 5033–5037.

27 H. Barr, A.J. MacRobert, C.J. Tralau, P.B. Boulos and S.G. Brown, The significance of the nature of the photosensitizer for photodynamic therapy: quantitative and biological studies in the colon, *Br. J. Cancer, 62* (1990) 730–735.