Grafted Iron(III) Physiological-type Porphyrin Aggregates: Syntheses, Magnetic Measurements and Resonance-Raman Study

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In a modelling effort to mimic the degradation products of haemoglobin, iron(III) proto- and deutero-porphyrin aggregates were synthesised using the corresponding iron porphyrin grafted on agarose gel. It is demonstrated, using resonance-Raman and EPR spectroscopy and magnetic measurements, that the major molecular species of the aggregates is an unexpected monomeric high-spin hydroxoiron(III) porphyrin, whereas the expected μ-oxo-bridged dimer is a minor species.

Haemoglobin is the haem protein of blood that transports oxygen. It contains iron protoporphyrin IX (3,7,12,17-tetramethyl-8,13-divinylporphyrin-2,18-dipropanoic acid, H_2L^1) embedded in a protein matrix. The degradation of haemoglobin by biological and chemical processes generates by-products some of which have been identified previously as iron(III) protoporphyrin derivatives.[1,2] Several types of superstructured iron porphyrins, such as crown,[3] picket-fence,[4] strapped,[5] capped[6] and basket-handle[7] porphyrins, have been designed in order to mimic the microenvironment of the metallic centre.

On oxidation of iron(II) synthetic complexes of porphyrins using dioxygen the corresponding monomeric hydroxo derivatives containing hindered porphyrins or tetraphenylporphyrin with bulky substituents are obtained.[8-12] Similar results are obtained in the metathesis of the corresponding chloroiron(III) complexes with hydroxide.[8-12] Treatment of complexes possessing somewhat relaxed steric constraints has been reported to yield mixtures of hydroxo and μ-oxo-bridged species,[13] whereas the ultimate products isolated in the case of iron porphyrins of physiological interest are the μ-oxo-bridged dimers.[14-17]

With the aim of mimicking the aggregation of the iron(III) protoporphyrin released during haemoglobin degradation, we have undertaken the synthesis and the characterization of iron(III) porphyrin aggregates from aqueous solutions of iron(III) physiological-type porphyrins using the corresponding iron(III) porphyrins grafted on agarose gel. Previously, several kinds of metalloporphyrins have been anchored on inorganic or polymer supports including silica,[18,19] polystyrene and polyphosphazene[20] for oxygen-carrier, catalytic or electrochemical applications. In this study, protoporphyrin IX or deuteroporphyrin (3,7,12,17-tetramethylporphyrin-2,18-dipropanoic acid, H_2L^2) were grafted on agarose gel. The grafted iron(III) porphyrins initiate aggregation of the corresponding porphyrin compound from aqueous solution. We report here the preparation and characterization by diffuse reflectance, UV/VIS, EPR, micro-Raman spectroscopies and magnetic measurements of such grafted iron(III) porphyrin aggregates.

Experimental

Synthesis.—The agarose gel beads (0.1 mm in size) were purchased from Pharmacia; [FeL^1(OH)] (haematin), [FeL^1(Cl)] (haemin), and resorcinol (all from Aldrich) were used without further purification. The complex [FeL^2(Cl)] was obtained as described previously.[21] The corresponding dimethyl esters [FeL^3(Cl)] and [FeL^4(Cl)] were obtained as previously described.[14] The μ-oxo-bridged dimers [$(FeL^1)_2O$] and [$(FeL^2)_2O$] were prepared from [FeL^1(Cl)] and [FeL^2(Cl)], respectively, as reported previously,[14-17] and the corresponding dimethyl esters [$(FeL^3)_2O$] and [$(FeL^4)_2O$] were obtained analogously.

The grafted iron porphyrin aggregates were prepared according to a method previously described:[22,23] the FeL^1 and FeL^2 moieties were covalently bound to Sepharose using CNBr-activated agarose, 1,6-diaminohexane and carbodiimide as described previously.[22,23] Fresh iron porphyrin solution {5 mg of [FeL^1(Cl)] or [FeL^2(Cl)] in 1 cm^3 of 0.01 mol dm^{-3} NaOH} was added to a suspension of dry grafted gel (3 cm^3) in phosphate buffer (20 cm^3), pH 7.65. After gentle agitation the gel was filtered off and washed with the phosphate buffer until the pH remained constant. The dry gel loaded with iron porphyrin was finally suspended in phosphate buffer (30 cm^3) and stored in the dark at 5 °C.

Instrumentation.—The EPR spectra of the frozen, wet loaded and neat agarose beads were recorded at 77 K in the X-band frequency with a Bruker ER 200 D spectrometer.

Magnetic measurements were carried out with a Faraday-type magnetometer equipped with a continuous-flow cryostat operating from room temperature to 4.2 K. Wet beads (*ca.* 30 mg) were introduced in the cryostat, partially dried *in vacuo* and frozen at 260 K before the weight equilibration of the system. No diamagnetic correction was effected. The iron content of each sample was determined by atomic absorption spectroscopy after the magnetic measurements.

All the Raman spectra were recorded at room temperature with the use of a Dilor X-Y instrument equipped with an optical microscope. An Olympus X100 microscope objective was used with a half-acceptance angle value $\theta_m = 64.2°$. The instrument employs a multichannel detection system consisting of an intensified photodiode array of 1024 elements. The resonance-Raman scattering was excited using argon laser lines (457.9, 488.0 and 514.5 nm) with low power at the sample (less than 1 mW). Typical illumination and black times were 5 s. The

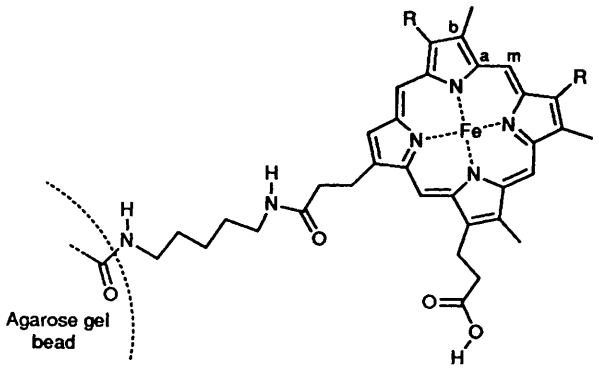

Fig. 1 Schematic representation of the iron porphyrin, with atom labelling, bound on agarose gel. For FeL^2, R = H; for FeL^1, R $CH{=}CH_2$

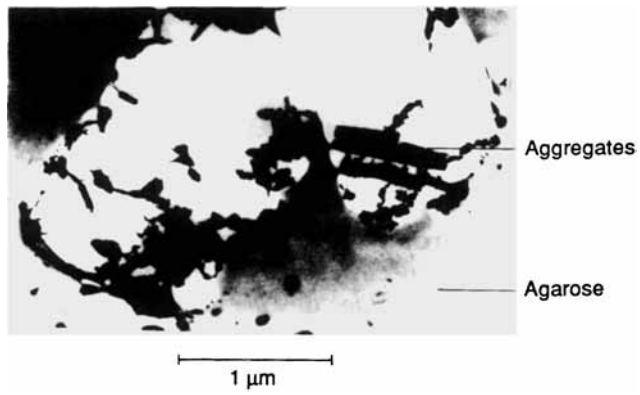

Fig. 2 Electron micrograph of a bead of agarose gel grafted with protoporphyrin iron(III) aggregates (clear, agarose gel; dark, porphyrin aggregates)

spectra were averaged over at least 50 accumulations. The laser beam was focused at the surface of a single wet bead of agarose gel without sampling. The analysed volume was approximately 4 μm^3. Raman spectra of the bulk compounds were also obtained using spinning-cell techniques and $KClO_4$ as solid diluent. Infrared spectra were run using the diffuse reflectance Fourier-transform technique on a Bruker IFS-113V spectrometer. The beads were dried under vacuum before mixing with microcrystalline dry KBr (the reference used). The electronic spectra of the dry beads were obtained after mixing with $BaSO_4$, using the diffuse-reflectance technique on a Varian instrument. Spectra of aqueous solutions were recorded with the same instrument.

The mass spectrum of commercial haematin (Aldrich) was recorded using a Riber Mag 10-10 spectrometer: $C_{34}H_{33}FeN_4O_4$ gives *m/z* 633 and 616. The metal content of the loaded beads was determined by atomic absorption spectroscopy after decomposition of the samples using concentrated $HClO_4$ and HNO_3 of high purity. The chloride content of the loaded beads was verified using conventional methods.

Results

Synthesis.—When solid [FeL^1(Cl)] or [FeL^2(Cl)] samples were shaken with an aqueous solution of NaOH a dark red colour developed. This was previously attributed to mono- and di-meric water-soluble iron porphyrin species.[24–29] Taking into account the general trend[30] of the kinetics of conversion from a hydroxo monomer to a μ-oxo dimer, the stock solutions of iron porphyrins of the physiological type were used rapidly after complete dissolution of [FeL^1(Cl)] and [FeL^2(Cl)] solids in aqueous 0.01 mol dm^{-3} NaOH solution.

The method of functionalizing the agarose gel is analogous to those previously described:[22,23] first, CNBr-activated agarose was coupled to 1,6-diaminohexane, then the free amino groups were coupled to iron protoporphyrin or deuteroporphyrin with carbodiimide in the non-aqueous medium dimethylformamide. The resulting structure of the iron porphyrin ring covalently bound on agarose gel can be represented as in Fig. 1. The grafted iron porphyrin serves as the base for the aggregation of iron porphyrin moieties contained in the aqueous solution. Thus aggregates were obtained by mixing an iron(III) porphyrin stock solution with the agarose gel beads grafted with the corresponding iron porphyrin and suspended in water. The resulting solution was maintained at pH 7.65 using phosphate buffer. A blank assay was undertaken by mixing the iron(III) porphyrin solution with the ungrafted agarose gel, suspended in water at pH 7.65 using a phosphate buffer. After 1 h the gel was filtered off and washed with phosphate buffer. No significant amount of iron was detected by elemental analysis. In contrast, with the grafted system a significant amount of iron was detected after the same treatment. Pseudo-crystalline particles approximately 0.5 μm in size were observed in electron micrographs of the grafted aggregates (Fig. 2).

The iron(III) porphyrin aggregates anchored on the agarose gel were characterized with wet beads as samples (or as otherwise indicated), using several techniques including elemental analysis, UV/VIS, EPR, IR, resonance-Raman spectroscopies and magnetic measurements. The metal content of the wet beads was approximately 0.15% according to elemental analysis. To identify the molecular species contained in the aggregates it is convenient to compare the spectroscopic properties to those of the bulk solids [FeL^1(OH)] (haematin), [FeL^1(Cl)], [$(FeL^1)_2O$] and the deuteroporphyrin analogues, reinvestigated under our experimental conditions. Solid haematin can be formulated as [FeL^1(OH)]. The elemental analysis, infrared and mass spectra are in good agreement with a major monomeric hydroxo species. In particular, the molecular peak in the mass spectrum is at *m/z* = 633 and a relatively intense peak occurs at *m/z* = 616, which corresponds to the FeL^1 moiety.

UV/VIS Spectroscopy.—The UV/VIS spectra of the dry beads, obtained using the diffuse-reflectance technique, exhibit intense absorptions in the expected wavelength region for iron(III) porphyrin compounds.[31,32] However, optical spectroscopy was unable to distinguish between mono- or di-meric derivatives. It has been noted earlier that while the spectra of the chloro and hydroxo derivatives of iron(III) porphyrins are significantly different, those of the hydroxo and μ-oxo complexes show considerable similarity.[12,13]

EPR Study.—The EPR spectra of the grafted protoporphyrin and deuteroporphyrin iron(III) aggregates are shown in Fig. 3. The spectra of the frozen wet beads (77 K) show characteristic features at *g* 5.8 and 2 as expected for monomeric high-spin iron(III) porphyrin compounds with a $^6A_{1g}$ ground state.[13,30,33] The intensities of the EPR signals are indicative of a significant amount of monomeric high-spin iron(III) porphyrin in the aggregates. Although the presence of an antiferromagnetically coupled μ-oxo dimer cannot be ruled out, the EPR characteristics are very weak[33] and cannot be detected in Fig. 3.

Magnetic Measurements.—The monomer/dimer ratio of the iron(III) porphyrin mixtures of the grafted aggregates was determined by variable-temperature magnetic susceptibility studies. The iron content of each sample under the magnetic field was determined by elemental analysis after the magnetic measurements. The knowledge of the iron content and the susceptibility permits the determination of the fractional content of the monomeric species in the mixtures, taking into account some approximations. The variations of the magnetic susceptibility of the wet grafted aggregates *versus* $1/T$ are shown

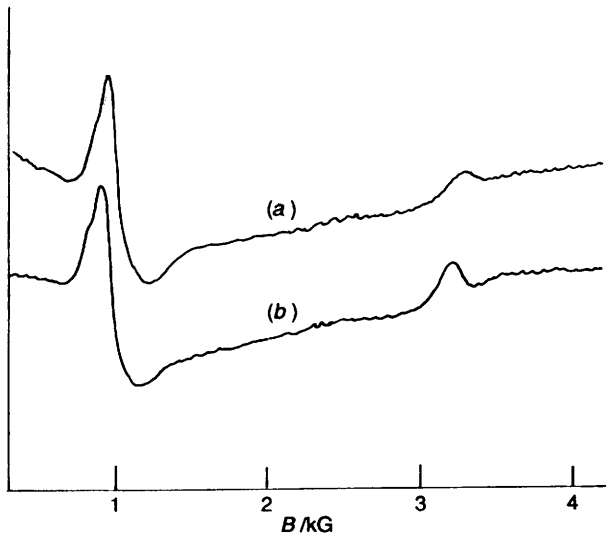

Fig. 3 The EPR spectra, at 77 K, of (*a*) grafted iron(III) protoporphyrin aggregates on agarose gel and (*b*) grafted iron(III) deuteroporphyrin aggregates on agarose gel. G = 10^{-4} T

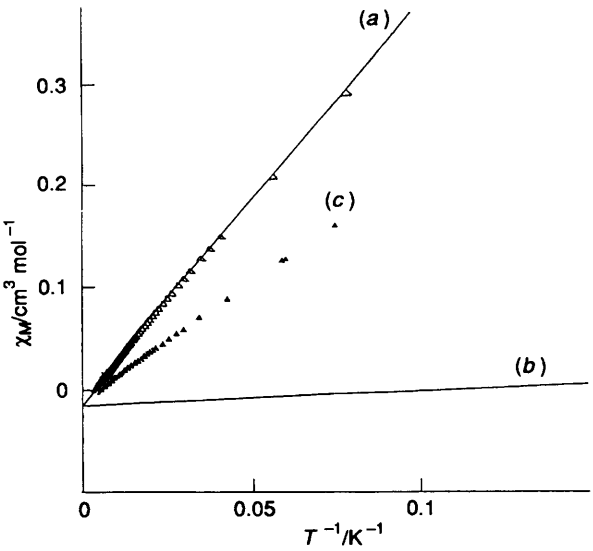

Fig. 4 Plots of magnetic susceptibility χ_M *vs.* $1/T$; (*a*) calculated values for a monomeric high-spin iron(III) moiety [equation (3), $S = \frac{5}{2}$], (*b*) calculated values for an antiferromagnetically coupled high-spin diiron(III) moiety (parameters taken from ref. 34) and (*c*) experimental values obtained from grafted iron(III) protoporphyrin aggregates on agarose gel

in Fig. 4. These data are typical of mixtures of a paramagnetic monomeric high-spin iron(III) compound ($S = \frac{5}{2}$) and of a dimeric iron(III) compound exhibiting weak magnetic susceptibility values. Indeed, the antiferromagnetic exchange coupling significantly decreases the magnetic susceptibility values. The magnetic susceptibility χ_M per ion for a pair of exchange-coupled $S = \frac{5}{2}$ ions has been given previously, and with a coupling constant near 150 cm^{-1}, as expected for μ-oxoiron(III) porphyrin dimers,[34,35] is very low at low temperature. So, the presence of the high-spin iron(III) porphyrin monomer obscures the magnetic susceptibility of the μ-oxo iron porphyrin dimers, below approximately 200 K. The effect of the μ-oxo dimer on the observed magnetic susceptibility was accounted for by use of expression (1) where p is the proportion of uncoupled iron(III).

$$\chi_{obs} = p\chi_{mono} + (1 - p)(\chi_{dimer}/2) + \Sigma\chi_{dia} \quad (1)$$

If $\chi_{dimer}/2$ is assumed to be negligible, expression (2) for the total magnetic susceptibility results. In equation (3), N, β and k have

$$\chi_{obs} = p\chi_{mono} + \Sigma\chi_{dia} \quad (2)$$

$$\chi_{mono} = Ng^2\beta^2S(S + 1)/3kT \quad (3)$$

their usual meanings;[34,35] g was held at 2.0.[34,35] The plots of χ_{obs} *vs.* $1/T$ in Fig. 4 correspond to the expected straight line; from the slope and the overall iron content it was possible to determine the p value for each sample. The p values were found to be in the range 0.6–0.8. No significant difference was found between the iron(III) deuteroporphyrin and iron(III) protoporphyrin aggregates when obtained under the same experimental conditions. The main parameter for obtaining pure monomeric iron(III) porphyrin aggregates appears to be the rapidity in isolating the beads from the freshly prepared iron(III) porphyrin solutions. Porphyrin aggregates grafted on agarose gel appear stable at −20 °C for several months. Indeed, the proportions of the uncoupled high-spin iron(III) compounds had not changed significantly after 2 months.

Infrared Spectroscopy.—Infrared spectroscopy should be very useful to characterize the molecular structure of the iron(III) porphyrins contained in the grafted aggregates. Attempts were undertaken to obtain meaningful IR spectra using the diffuse reflectance technique after dehydration of the beads. Unfortunately, the intense IR bands of the agarose gel obscured the characteric bands of the iron porphyrin moieties and we were unable to obtain sufficient information to provide evidence for the molecular structure, particularly for the axial ligand.

Resonance-Raman Microspectrometry.—The enhancement of the Raman scattering corresponding to some vibrational modes of the iron porphyrin species when the exciting radiation wavelength coincides with that of the electronic absorption permits characterization of the metalloporphyrin moiety at a low level in non-absorbing media, such as agarose gel. To record resonance-Raman spectra with appropriate signal/noise ratio using beads as the sample it is more convenient to use a Raman microprobe equipped with an immersion high-aperture objective in order to reduce the intense background induced by the agarose gel. In addition, the highly sensitive multichannel detector permits the use of the low power of the exciting laser radiation to avoid degradation of the illuminated sample. To compare carefully the vibrational spectra of the grafted aggregates with those obtained from the bulk solids, the spectra of the [FeL^1(OH)], [FeL^1(Cl)], [(FeL^1)$_2$O] and their deuteroporphyrin analogues were recorded under the same experimental conditions, without any solid diluent. These spectra are analogous to those recorded using $KClO_4$ as solid diluent and spinning-cell techniques, except for some broadening of the bands due to local heating effects. The spectra are shown in Figs. 5 and 6; they are compared with those obtained from the aggregates.

It should be noted that the spectra of the aggregates are obtained from one individual bead, however all measurements made were analogous for all the beads from the same preparation. The frequencies of the marker bands ν_2, ν_3, ν_4 and ν_{10} observed for the aggregates (Table 1) are analogous to those reported for the bulk compounds and confirm the presence of Fe^{III} in the high-spin state inserted into a protoporphyrin or deuteroporphyrin ring, in a five-co-ordinated environment.[36] However, because of the lack of enhancement, resonance-Raman spectroscopy does not provide clear evidence for the characteristic vibrations of the axial ligand.

If the wavenumbers of the intense bands of the in-plane porphyrin modes are analogous for all the compounds studied (Table 1), the relative intensities were found to be very different according to the nature of the axial ligand, OH or μ-oxo bridge. It should be noted that the resonance-Raman enhancement is

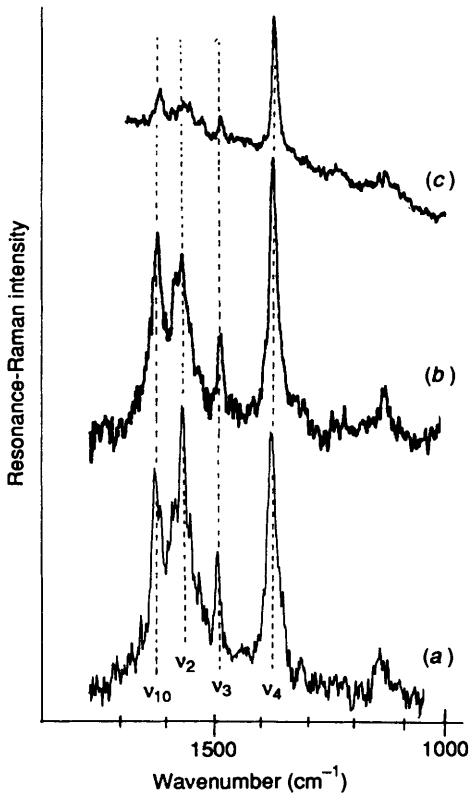

Fig. 5 Resonance-Raman spectra recorded using the multichannel microprobe technique (exciting radiation: 457.9 nm) of (*a*) solid hematin [$FeL^1(OH)$], (*b*) a bead of agarose gel grafted with FeL^1 aggregates, and (*c*) the solid μ-oxo dimer [$(FeL^1)_2O$]

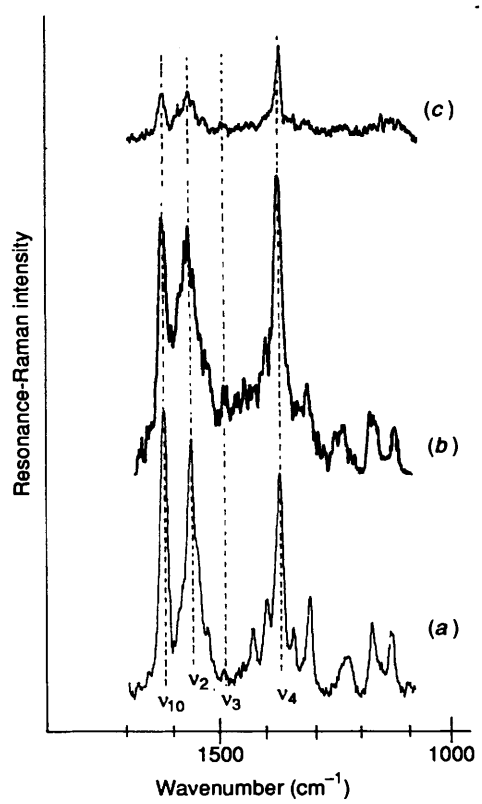

Fig. 6 Resonance-Raman spectra recorded using the multichannel microprobe technique (exciting radiation: 514.5 nm). Details as in Fig. 5

very sensitive to the wavelength of the exciting radiation (Figs. 5 and 6). The intensities of these bands are clearly distinguished from those of the chloro precursor in the cases of both protoporphyrin and deuteroporphyrin. This result is in good agreement with the elemental analysis, and we can readily assume that OH or O is the axial ligand of the iron porphyrin complexes. It is obvious from the spectra in Figs. 5 and 6 that the relative intensities of the marker bands of the aggregates are the result of the combination of bands from μ-oxo and hydroxo compounds. So, the fractional content of the hydroxo species can be estimated from the relative intensities of the marker bands (*e.g.* ν_2 and ν_4) taking as standard the corresponding intensities of the bulk hydroxo and μ-oxo compounds. The hydroxo/μ-oxo ratio was found to be the same for each bead and each preparation. The value depends only on the rapidity of preparing the starting porphyrin solution, being in the range 0.6–0.8 and in good agreement with the average values obtained by magnetic measurements for the same preparation.

Discussion

When solid [$FeL^1(Cl)$] or [$FeL^2(Cl)$] samples were dissolved in aqueous solutions of NaOH the chloride ligand was readily displaced by a small amount of hydroxide. The subsequent dimerization reaction through the μ-oxo bridge depends on the time, the pH value and the nature of the porphyrin. Mono- and di-meric species were found using EPR, UV/VIS and Mössbauer spectroscopies.[24–29]

The UV/VIS and infrared results are unable to provide any clear evidence for the nature of the molecular species of the grafted aggregates, whereas the EPR and magnetic measurements are in good agreement with a monomeric high-spin iron(III) porphyrin as the major species. The identification of the iron(III) porphyrin chromophores is straightforward by simple comparison between the resonance-Raman spectra of the aggregates and the chloro, hydroxo and μ-oxo parent compounds in the bulk state which are well documented.

Resonance-Raman spectroscopy is currently being applied not only as a probe of size, oxidation and spin state of the metalloporphyrin core, but also to obtain information about the nature of the bonding between the iron and the axial ligand.[36] It is well known that in resonance-Raman scattering the wavenumber is a property only of the ground state whereas the intensity involves both the ground and excited electronic states. Many correlations have been established between the wavenumber of some lines (marker bands) corresponding to the porphyrin core modes and the oxidation state, spin state and core size of metalloporphyrin derivatives.[36–42] The assignment proposed earlier[14–17,43,44] for the bulk mono- and di-meric metalloporphyrins of the physiological type, protoporphyrin and deuteroporphyrin dimethyl esters, can be transferred to the resonance-Raman bands of the non-esterified porphyrin aggregates studied here (Table 1). Indeed, similar wavenumbers were observed for the corresponding lines of the porphyrin core of the bulk esterified and non-esterified compounds.[15–17] In addition, for each porphyrin type the frequencies obtained in the spectra of monomeric and μ-oxo dimeric complexes are all within 4 cm^{-1} of one another.

For the compounds in the bulk state the hydroxide axial ligation or oxo bridge can be differentiated by the use of the low-frequency range of the Raman spectra. Indeed, significant bands are observed at 419 and 410 cm^{-1} for deutero- and protoporphyrin μ-oxo-bridged iron complexes, respectively (Table 1). These bands are readily assigned to the symmetric stretching mode ν_{sym}(Fe–O–Fe) by comparison with previous work concerning ^{18}O-labelled esterified μ-oxo-bridged iron porphyrin complexes.[14] The antisymmetric modes ν_{asym}(Fe–O–Fe) were easily detected in the IR spectra at *ca.* 850 cm^{-1}. According to earlier studies[45] on sterically hindered hydroxoiron(III) porphyrins, the band corresponding to the ν(Fe–OH) mode appears only as a broad and weak feature near 550 cm^{-1}, even when appropriate exciting laser lines are used. No tractable feature can be detected in the low-frequency spectra of both

Table 1 Some characteristic resonance-Raman wavenumbers (cm^{-1}) of iron(III) protoporphyrin and deuteroporphyrin aggregates grafted on agarose gel and of the corresponding non-esterified bulk chloro and μ-oxo-iron(III) compounds (C_a, C_b and C_m are defined in Fig. 1)

Assignment[27]	$[FeL^1]_{agg}$	$[FeL^1(Cl)]$	$[(FeL^1)_2O]$	$[FeL^2]_{agg}$	$[FeL^2(Cl)]$	$[(FeL^2)_2O]$
ν_{10} $\nu(C_a–C_m)$	1630	1626	1625	1630	1630	1628
ν_{37} $\nu(C_b–C_b)$	1591	1590	1590	1586	1586	1585
ν_2 $\nu(C_b–C_b)$	1574	1572	1572	1575	1575	1577
ν_{11} $\nu(C_a–C_m)$	1555	1550	1554	1561	1561	1558
ν_3 $\nu(C_a–C_m)$	1490	1491	1490	1491	1491	1490
ν_4 $\nu(C_a–N)$	1373	1373	1373	1370	1233	1372
ν_{asym}(Fe–O–Fe)			895			833
ν_{16} $\delta(C_a–N–C_a)$	754	754	753	750	750	751
ν_{sym}(Fe–O–Fe)			410			419

solid haematin and aggregates, despite the fact that appropriate exciting radiation wavelengths were used (457.9, 488.0, 514.5 nm) for selective enhancement of the ν(Fe–OH) mode. The enhancement factors of the resonance-Raman scattering corresponding to vibrational modes of the porphyrin core are very sensitive to the nature of the axial ligand.[46] The spectra of the aggregates exhibit intense bands in the wavenumber region corresponding to the ν_2, ν_3, ν_4 and ν_{10} marker bands (Figs. 5 and 6).

It should be noted that the non-esterified propionic acid functions are expected to be deprotonated in the aggregates under study. Indeed, taking into account the pK values ($\approx$4) of these functions the grafted porphyrin aggregates obtained at pH 7.65 are fully deprotonated. Since the peripheral substituents of the porphyrins studied are not themselves chromophoric, at least in the visible–near UV region, resonance-Raman enhancement of their internal modes depends on coupling with the porphyrin core, either electronically or kinematically.[36,46] The internal modes of the vinyl substituents of the protoporphyrin were detected as weak bands in the resonance-Raman spectra of the compounds in the bulk state[36,39,40,47] and in micellar arrangements[31,48] of iron protoporphyrin. These weak bands were not clearly observed in the spectra of the aggregates and no information concerning the conformational changes of the side chains in the molecular packing of the aggregates can be obtained.[49]

All the experimental results (EPR, magnetic measurements and resonance-Raman spectroscopy) of the present work are in good agreement with a monomeric high-spin hydroxoiron(III) porphyrin as the major species in the aggregates. Analogous results were obtained with both the porphyrins of physiological interest: protoporphyrin IX and deuteroporphyrin. The minor species were found to be the expected μ-oxo-bridged derivatives. The monomeric hydroxo complexes of the unhindered iron(III) porphyrins of the physiological type appear to be stabilized through rapid isolation from aqueous solutions using iron porphyrin grafted on agarose gel without any hindered synthetic porphyrin. It is probable that the aggregation is mediated by hydrophobic interactions between the porphyrin cores.[23,50]

From the present experimental results it is difficult to give a realistic geometrical arrangement of the porphyrin moieties in the packing of the aggregates. However, it is tempting to propose a face-to-face stacking of bimolecular entities held together by hydrogen bonding and stabilized in the packing by electrostatic forces and hydrogen networks.

Earlier studies[11,51] of the autoxidation mechanism of iron(II) porphyrins point out that the μ-oxo-bridged dimer is the ultimate product. However, hydrogen abstraction from solvent molecules of oxoferryl (Fe^{IV}=O) intermediates[51] generates hydroxo species which can be stabilized by steric effects. From the biological point of view, it is probable that the ultimate product that could be isolated after the degradation of haemoglobin, and the release of the haem from its pocket, is an unexpected monomeric species stabilized by biological residues of the globin.

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Received 23rd *September* 1992; *Paper* 2/05106K