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Investigation of the Molecular Nature of Low-molecular-mass Cobalt(II) Ions in Isolated Osteoarthritic Knee-joint Synovial Fluid

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High field ^{1}H NMR spectroscopy demonstrated that addition of Co(II) ions to osteoarthritic knee-joint synovial fluid (SF) resulted in its complexation by a range of biomolecules, the relative efficacies of these complexants/chelators being citrate $\gg$ histidine $\sim$ threonine $\gg$ glycine $\sim$ glutamate $\sim$ glutamine $\sim$ phenylalanine $\sim$ tyrosine $>$ formate $>$ lactate $\gg$ alanine $>$ valine $>$ acetate $>$ pyruvate $>$ creatinine, this order reflecting the ability of these ligands to compete for the available Co(II) in terms of (1) thermodynamic equilibrium constants for the formation of their complexes and (2) their SF concentrations. Since many of these SF Co(II) complexants (e.g. histidinate) serve as powerful $^{\cdot}$OH scavengers, the results acquired indicate that any of this radical generated from the Co(II) source in such complexes via Fenton or pseudo-Fenton reaction systems will be "site-specifically" scavenged. The significance of these observations with regard to cobalt toxicity and the *in vivo* corrosion of cobalt-containing metal alloy joint prostheses (e.g. CoCr alloys) is discussed.

Keywords: Low-molecular-mass cobalt ions; Osteoarthritis; ^{1}H NMR; Speciation; Synovial fluid; CoCr metal alloy joint prostheses

INTRODUCTION

Despite being an essential dietary trace metal ion, being required as an activator for enzymes (e.g. cholinesterases and carboxylases), and an essential constituent of cyanocobalamine (vitamin B_{12}), excessive exposure to cobalt ions can give rise to a range of toxic effects. Indeed, early studies found that cobalt salts are likely to be carcinogenic,[1] and affect α-pancreatic cells,[2] liver cytochrome P-450 levels[3,4] and platelet aggregation.[5] Both cobalt metal particles [Co(0)] and cobalt(II) [Co(II)] ions have recently been found to be cytotoxic towards alveolar macrophages as well as alveolar type II cells,[6] and the hazards facing those employed in refinery industries and the production of appropriate alloys such as hard metal (HM, a mixture of tungsten carbide, cobalt and other metals) are well documented.[1,7] Co-induced interstitial lung diseases (more specifically interstitial alveolitis, fibrosis and asthma) are believed to be ascribable, at least in part, to the adverse generation of reactive oxygen species (ROS) within lung tissue, with the subsequent development of oxidative damage to critical biomolecules, a phenomenon which is likely to be of much significance with regard to the toxicological effects exerted by Co(II).

Adverse effects associated with excessive superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) production are largely attributable to their ability to form the aggressively reactive $^{\cdot}$OH radical via Fenton or pseudo-Fenton reaction systems, and, depending upon critical criteria such as biological

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microenvironment, availability of Co(II)-chelating/complexing biomolecules, redox potentials of the complexes so formed, the nature and concentrations of accessible $^{\cdot}$OH radical scavenging antioxidants, etc. it remains a strong possibility that Co(II) ions can promote the generation of $^{\cdot}$OH radical from H_2O_2 (Eq. 1). This toxicological pathway[8–11] has now been augmented by studies that have demonstrated DNA damage caused by the generation of ROS by Co(II)/(H_2O_2) mixtures,[12–14] an observation consistent with the further hypothesis that endogenous ROS play a role in metal/metal ion-induced carcinogenesis as indeed they do via their indirect generation from organic carcinogens.[15] A GC-MS study has identified 11 hydroxylated purine/pyrimidine bases in renal, hepatic and pulmonary chromatin DNA of rats that had received an i.p. dose of a Co(II) salt,[16] a result similar to that from an earlier study of the reaction products of Co(II), H_2O_2 and chromatin isolated from cultured human cells.[12] This hydroxylation process has been corroborated by a recent *in vitro* study involving the treatment of calf thymus and human diploid fibroblasts with Co(II) and H_2O_2.[17] It has been proposed that these oxidation products arise from a free radical-mediated intra-strand cross-linking reaction.[18]

$$\mathrm{Co(II)} + \mathrm{H_2O_2} \rightarrow \mathrm{Co(III)} + {}^{\cdot}\mathrm{OH} + \mathrm{OH^-} \qquad (1)$$

Furthermore, studies reporting cobalt ion-induced DNA strand-breaks in cultured animal cells[19] have been supported by investigations demonstrating that Co(II) causes mitochondrial DNA damage.[14] Co(II) also interferes with cellular DNA repair processes, a phenomenon which inhibits nucleotide incision and polymerization repair steps in human fibroblasts.[20] Shi,[21] Leonard[22] and Van den Broeke[23] have provided evidence supporting the hypothesis that Co-induced cell injury arises from its ability to generate $^{\cdot}$OH radical *in vivo*.

The reaction between Co(II) and H_2O_2 does not generate hydroxyl radical ($^{\cdot}$OH) to such a great extent as Fe(II),[21] although it is clear that the redox potential (E_0) of the metal ion complex involved is of critical importance. However, electon spin resonance (ESR) studies have suggested that Co(II) does not react with H_2O_2 via a "classical" Fenton reaction system at physiological pH values.[24] Although the E_0 value of the $Co(II)_{(aq.)}/Co(III)_{(aq.)}$ system (+1.8 V[23]) clearly precludes the participation of the lower oxidation state metal ion in Fenton or Fenton-type reaction systems, for its 1:3 glycinato complex it is + 0.20 V [26] and hence the capacity of Co(II) to promote the generation of $^{\cdot}$OH from H_2O_2 is, at least in principle, substantially enhancable by its complexation by ligands available in human biofluids and tissues.

Exposure of cobalt-containing metal alloy joint implants to synovial fluid (SF) is an area of much clinical interest since it is well known that particulate alloy matter can leach away from the joint under wear conditions, i.e. the condition of systemic dissemination. In addition to the availability of Co(II) complexants/chelators, the oxidation of cobalt metal [Co(0)] to Co(II) in physiological environments is greatly facilitated by an increase in alloy surface area which is, of course, inversely related to particle size. A study of human subjects fitted with cobalt-chrome (CoCr) prostheses demonstrated that both metals were detectable in local and distant lymph nodes, bone marrow, liver and spleen as ions.[27] Subjects with loose, worn joint prostheses had the highest levels of cobalt and chromium ions, and cases with the greatest wear exhibited necrosis of the lymph nodes. Moreover, the presence of a significant T lymphocyte infiltrate at implant-bone interfaces where CoCr alloy was used has been reported.[28] 8-hydroxy-2′-deoxyguanosine in urine, and substantially elevated blood cobalt levels have been employed as markers of cobalt ion release from metallic implants and the potential onset of cytogenetic modifications in patients.[29] These observations reflect the deleterious surface corrosion of contemporary metal alloy joint prostheses *in vivo*, a complex process likely to involve interdependent dissolution, acid-base, redox and complexation reactions. Indeed, the dissolution of alloy metals (as ions) is aided by phagocytically-generated H_2O_2.[30]

Leonard *et.al.*[22] explored the generation of ROS from Co(0) and suggested that a Co(I)-mediated Fenton-type reaction was responsible for the production of $^{\cdot}$OH monitored by an ESR spin-trapping technique involving DMPO. The reaction of Co(0) with water consumed molecular O_2 and generated Co(II), observations consistent with $O_2^{\cdot-}$ being derived from the single-electron reduction of O_2 by Co(0), and H_2O_2 required for this Fenton-type system from the dismutation of $O_2^{\cdot-}$.

Since to date there has been little or no attempt to clarify the precise molecular nature of implant-derived metal ions in adjacent tissue and joint SF, studies aimed at characterising the precise molecular nature of cobalt ions in appropriate biofluid media can, at least in principle, provide much valuable information regarding the biological activity/toxicity of this metal ion. High resolution nuclear magnetic resonance (NMR) spectroscopy is a technique which is particularly applicable to the "speciation" of metal ions in biological samples via an evaluation of selective resonance broadening ascribable to ligand-exchange processes and/or paramagnetism in the case of transition metal ions,[31–32] as well as complexation-dependent modifications to resonance frequencies and spin-system patterns.[33]

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We report here for the first time Co(II) ion-induced modifications in the ^{1}H NMR properties of low-molecular-mass biomolecules in intact osteoarthritic (OA) SF, an experimental model which provides much valuable information regarding the particular chemical nature of solution-phase Co(II) complexes arising from the potentially deleterious corrosion of cobalt-containing alloy implants during *in vivo* wear episodes.

MATERIALS AND METHODS

Sample Preparation

SF was aspirated for therapeutic reasons from the knee-joints of patients ($n = 7$, mean $\pm$ s.d. age 61 $\pm$ 7 years) attending a rheumatology clinic who had previously been diagnosed as osteoarthritic (OA) according to standard clinical criteria.

Analytical grade $Co(NO_3)_2{\cdot}6H_2O$ was obtained from Sigma-Aldrich Company (Gillingham, Dorset, UK). Aliquots of a stock Co(II) solution in HPLC-grade water (1.00×10^{-2} mol dm^{-3}) were introduced to the SF samples by means of a graduated syringe (Hamilton microliter 900 series, 5 μl capacity, accurate to $\pm$1% of syringe volume). All samples were then equilibrated for a period of 1.0 h at ambient temperature before being stored at -60°C until required for NMR analysis. The pH values of each specimen were determined electrometrically both prior and subsequent to Co(II) addition (Hanna HI 1270 combination pH electrode and meter).

NMR Spectroscopy

^{1}H NMR spectra were recorded at a probe temperature of 25°C on a Bruker Avance 600 spectrometer (departmental facility, Department of Chemistry, Queen Mary, University of London) operating in quadrature detection mode at 600.13 MHz for ^{1}H. Typically, 0.60 ml of each SF sample (control or treated with increasing concentrations of Co(II)) was placed in a 5-mm diameter thin-walled NMR tube (Goss Scientific, Great Baddow, Essex, UK), and 0.07 ml of ^2H_2O was added to provide a field-frequency lock. For each intact biofluid sample, both single-pulse and Carr-Purcell-Meiboom-Gill (CPMG) spectra were recorded for purposes of comparison. Pulsing conditions for single-pulse spectra were: sweep width 8389 Hz, pulse width 8.5 μs (pulse angle 70°), acquisition time 3.9 s, pulse delay 2 s, data points 32,768 (and subsequently zero-filled to 65,536) and 64 transients. An exponential function corresponding to a line-broadening of 0.3 Hz was applied to FIDs before Fourier transformation. Macromolecular resonances (e.g. those arising from proteins such as albumin) present in the single-pulse spectra were suppressed in the CPMG experiments through the use of the pulse sequence $D - 90°_x - [(\tau/2) - 180°_{\pm y} - (\tau/2)]_n -$ acquire, where $D = 2$ s, $\tau = 2$ ms, $n = 64$ and the acquisition time was 3.9 s. Chemical shifts were referenced to external sodium 3-(trimethylsilyl)-[1,1,2,2-d_4]-propionate (TSP, $\delta = 0.00$ ppm). The intense H_2O signal was suppressed by the application of a presaturation pulse at the water resonance frequency. Resonance assignments were routinely made by a consideration of chemical shift values, spin–spin coupling patterns and coupling constants. Component concentrations were determined by electronic integration of the appropriate spectral regions via application of the spectrometer proprietary software (XWIN-NMR), with maintenance of the exact integral regions for each spectrum acquired.

The intensities of OA SF biomolecule ^{1}H NMR signals were determined by electronic integration, and the concentrations of components detectable were estimated by comparison of their resonance areas with that of a 3.40×10^{-2} mol dm^{-3} standard solution of TSP located within a coaxial NMR tube insert. This procedure was employed in order to avoid broadening of the TSP signal which arises from its binding to OA SF proteins or alternative macromolecules. Of course, the levels given reflect only the non-macromolecule-bound (i.e. NMR-visible) fraction of these biomolecules and therefore are expected to be somewhat lower than their total concentrations in this biofluid.

Computer-generated Simulation of the Distribution of Co(II) amongst OA SF Biomolecules

For selected OA SF specimens, simulation of the competitive complexation of Co(II) by a range of low-molecular-mass biomolecules therein at their ^{1}H NMR-estimated concentrations throughout the pH range 2–12 was performed with the SolEq computer program (Academic Software, Otley, Yorkshire, UK). For these computations, the total ionic strength (I) was set at 0.10 mol dm^{-3}.

RESULTS

600 MHz ^{1}H NMR CPMG spectra of intact OA SF samples contain resonances ascribable to a wide variety of low-molecular-mass components (together with those attributable to the more mobile portions of macromolecules such as lipoproteins and carbohydrate side-chains of *N*-acetyl glycoproteins which are incompletely suppressed by the CPMG pulse sequence). The CPMG ^{1}H NMR profiles of OA SFs acquired here contained many prominent

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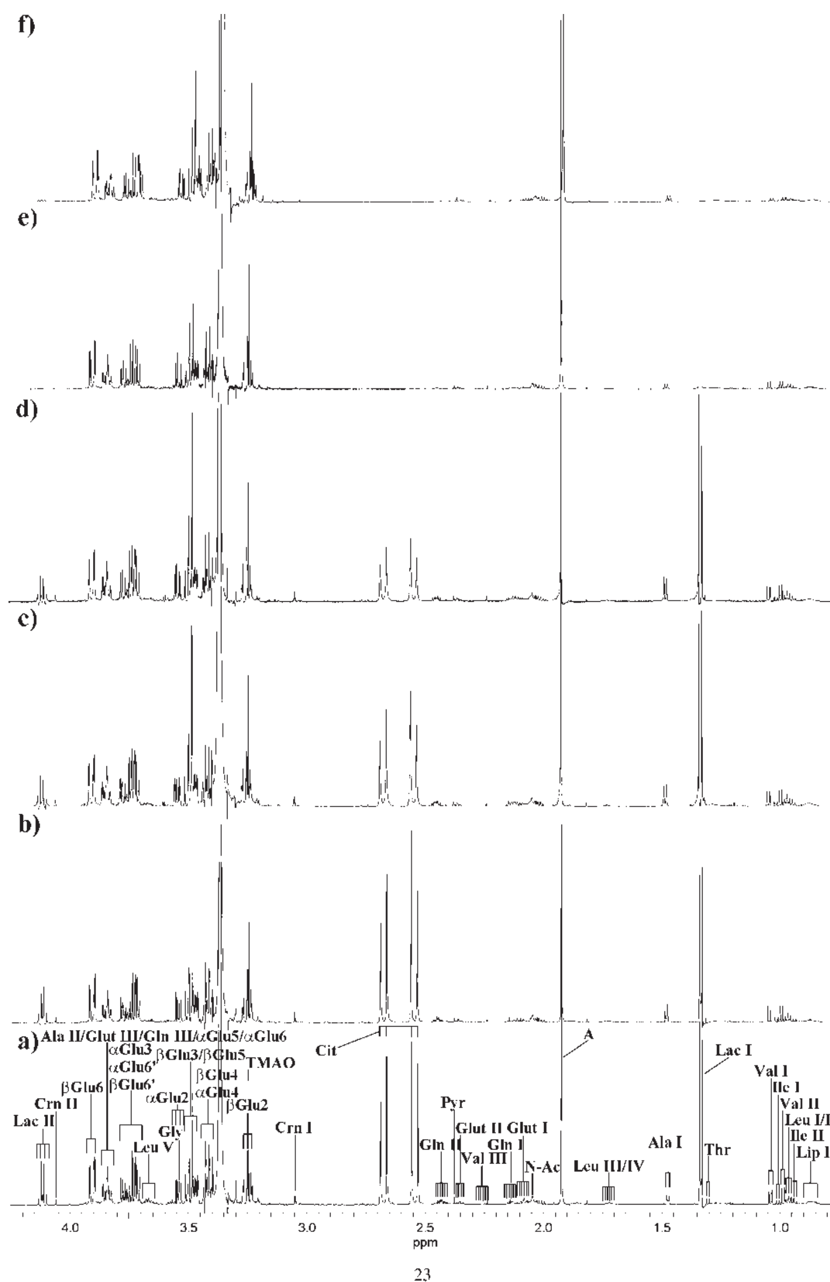

FIGURE 1 Expanded 0.50–4.50 ppm region of the 600.13 MHz CPMG ^{1}H NMR spectra of (a) intact OA SF specimen and the same sample following equilibration with Co(II) at concentrations of (b) 1.20×10^{-5}, (c) 7.10×10^{-5}, (d) 1.36×10^{-4}, (e) 7.48×10^{-4}, and (f) 1.497×10^{-3} mol dm^{-3}. Abbreviations: A, acetate-CH_3; Ala, alanine-CH_3; Cit, citrate *AB* —CH_2CO$_2^-$ protons; Crn I and II, creatinine —CH_3 and —CH_2 protons, respectively; α-Glu 2-6′, α-glucose ring protons; β-Glu 2-6′, β-glucose ring protons, Glut I and II, glutamate *A*BX and A*B*X β protons, respectively; Glut III, glutamate AB*X* α proton, Gln I and II, glutamine *A*BX and A*B*X β protons respectively; Gln III, glutamine AB*X* α proton; Gly, glycine α proton; Lac I and II, lactate —CH_3 and —C*H* protons respectively; Ile I and II, isoleucine δ and β protons respectively; Leu I, II, III and IV, leucine δ, δ, γ, and β protons respectively; Lip I and II, terminal —CH_3 and bulk chain (—CH_2—)$_n$ protons, respectively, of lipoprotein-associated triacylglycerols (i.e. those of chylomicrons, VLDL, LDL and HDL); Met, methionine S-CH_3 proton; N-Ac, spectral region for acetamido methyl groups of *N*-acetylsugars present in the molecularly-mobile carbohydrate side-chains of "acute-phase" glycoproteins; Pyr, pyruvate-CH_3; Thr, γ-CH_3 proton; TMAO, trimethylamine *N*-oxide -N(CH_3)$_3$ protons; Val I, II and III valine γ-CH_3 and β-C*H* protons, respectively.

resonances, e.g. those assignable to valine, threonine, lactate, acetate, glutamate, glutamine, pyruvate, citrate, creatinine, glycine, α- and β-D-glucose, tyrosine, histidine, phenylalanine and formate. The high- and low-field regions of a typical spectrum are shown in Figs. 1(a) and 2(a), respectively. The intensity of relatively broad lipoprotein-associated lipid and "acute-phase" glycoprotein *N*-acetylsugar resonances (the latter predominantly corresponding to those of α_1-acid glycoprotein) are of a much lower intensity than those of serum specimens collected from OA patients, an observation which serves as a potential diagnostic index for this inflammatory joint disease which, in view of an enhanced permeability,

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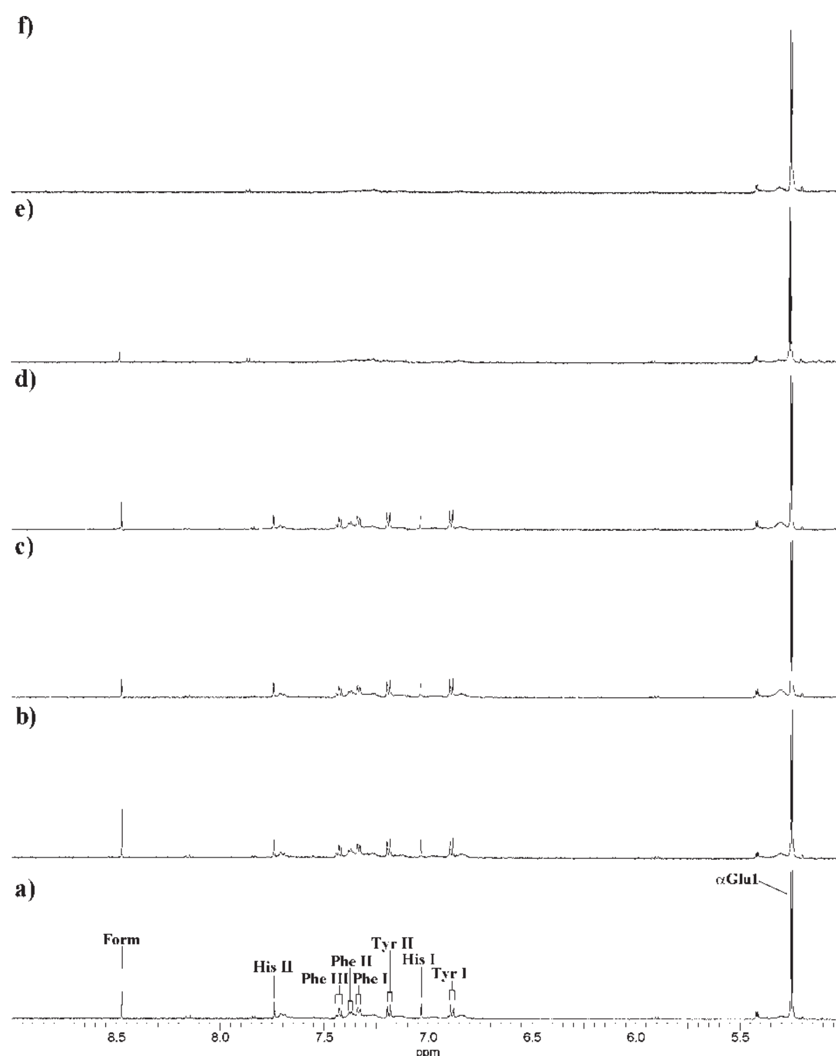

FIGURE 2 Expanded 5.00–9.00 ppm region of the 600.13 MHz CPMG ^{1}H NMR spectra of OA SF shown in Fig. 1(a)–(f) correspond to the added Co(II) concentrations of Fig. 1 (i.e. $0.00–1.497 \times 10^{-3}$ mol dm^{-3}). Abbreviations: Form, Formate-H proton; α-Glu 1, α-glucose ring proton; His I and II, histidine imidazole ring protons; Phe I, II and III, phenylalanine aromatic ring protons; Tyr I and II, tyrosine aromatic ring protons.

allows the filtration of these macromolecules through the damaged synovial membrane.[34] The ketone bodies 3-D-hydroxybutyrate and acetone detectable serve as indicators of the utilization of lipids as a source of energy in view of a reduced local supply of glucose. Moreover, the glycerol detectable by this technique reflects triglyceride hydrolysis.[35]

Equilibration of the OA SF samples with increasing sequential levels of added paramagnetic Co(II) gave rise to concentration-dependent increases in the linewidths of resonances of selected biomolecules therein. The glucose ^{1}H resonances remained Co(II) ion-insensitive throughout the spectroscopic titrations and therefore the α-D-glucose anomeric ^{1}H resonance ($\delta = 5.23$ ppm) served as a convenient reference centre. High- and low-field regions of a typical spectral titration involving the addition of $0–1.50 \times 10^{-3}$ mol dm^{-3} Co(II) are shown in Figs. 1 and 2, respectively. With the exception of the internal α-D-glucose reference, the relative linewidths of all signals affected by the addition of Co(II) are given in Table I. Particularly notable are the line broadenings for citrate, histidine and threonine which were observed at low added Co(II) levels (i.e. 7.10×10^{-5} mol dm^{-3} for citrate and 1.36×10^{-4} mol dm^{-3} for histidine and threonine), data which indicate the involvement of these biomolecules in the complexation of this metal ion at concentrations which may be relevant or similar to those liberated from a cobalt-containing joint prosthesis alloy into a local SF environment *in vivo*, i.e. at the prosthesis–SF interface. At higher levels of added Co(II), SF signals arising from biomolecules with the most powerful Co(II)-chelating capacity disappeared completely

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TABLE I Table. Mean ± s.e. ($n = 4$) relative increases in signal linewidth for OA SF biomolecule signals found to broaden in the Co(II) titration experiments.*

	[Co(II)]					
	Control	0.012†	0.071†	0.136†	0.748†	1.497†
Valine	1.00	1.00	1.00	1.00	1.28 ± 0.03	1.65 ± 0.03
Threonine	1.00	1.00	1.00	1.12 ± 0.02	∞	∞
Lactate	1.00	1.00	1.00	1.00	2.42 ± 0.11	∞
Alanine	1.00	1.00	1.00	1.00	1.66 ± 0.05	2.00 ± 0.04
Acetate	1.00	1.00	1.00	1.00	1.10 ± 0.02	1.74 ± 0.03
Glutamate	1.00	1.00	1.00	1.00	∞	∞
Glutamine	1.00	1.00	1.00	1.00	∞	∞
Pyruvate	1.00	1.00	1.00	1.00	1.00	1.50 ± 0.04
Citrate	1.00	1.00	1.28 ± 0.06	1.65 ± 0.07	∞	∞
Creatinine	1.00	1.00	1.00	1.00	1.00	1.32 ± 0.05
Glycine	1.00	1.00	1.00	1.00	∞	∞
α-D-glucose	1.00	1.00	1.00	1.00	1.00	1.00
Tyrosine	1.00	1.00	1.00	1.00	∞	∞
Histidine	1.00	1.00	1.00	1.13 ± 0.02	∞	∞
Phenylalanine	1.00	1.00	1.00	1.00	∞	∞
Formate	1.00	1.00	1.00	1.00	4.22 ± 0.25	∞

*Where "∞" denotes the complete disappearance of a signal. †$\times 10^{-3}$ mol dm^{-3}.

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from the spectra acquired. Further resonances showed only small increases in linewidth at the more elevated added Co(II) concentrations [e.g. a 10% increase for the $-CH_3$ group singlet of acetate at an added Co(II) level of 7.48×10^{-4} mol dm^{-3}]. An overall appraisal of linewidth increases and, at the higher added Co(II) concentrations, resonance areas established that the relative affinities of OA SF biomolecules was in the order citrate $\gg$ histidine $\sim$ threonine $\gg$ glycine $\sim$ glutamate $\sim$ glutamine $\sim$ phenylalanine $\sim$ tyrosine $>$ formate $>$ lactate $\gg$ alanine $>$ valine $>$ acetate $>$ pyruvate $>$ creatinine. In the case of threonine and glycine, their γ-CH_3 and α-CH_2 resonances, respectively, shifted to lower field at the lower Co(II) levels added [that this chemical shift behaviour was restricted to only two complexants excluded the involvement of any pH chemical shift dependence; pH measurements made on OA SF samples both prior and subsequent to the addition of increasing levels of Co(II) confirmed that their H^+ ion concentrations remained constant throughout, a consequence of the strong buffering capacity of this biofluid]. Such shifts are attributable to the fast exchange of these biomolecules at the Co(II) centre.

Up to the point of their complete, Co(II) concentration-dependent removal from spectra, the resonance broadenings observed were accompanied by decreases in their overall, α-glucose-normalised intensities. For example, in a typical sample, the ^{1}H NMR signals of citrate were reduced by 42%, histidine by 48%, and acetate by 32% on raising the added Co(II) level from 1.20×10^{-5} to 1.36×10^{-4} mol dm^{-3}, 7.10×10^{-5} to 1.36×10^{-4} mol dm^{-3}, and 1.36×10^{-4} to 1.50×10^{-3} mol dm^{-3}, respectively.

For two of the OA SF specimens examined, certain biomolecule signals (e.g. those of formate and alanine) increased in intensity at low concentrations of added Co(II), although the exchange broadening process predominated at higher Co(II) levels. This observation presumably arises from their displacement from protein (or alternative macromolecule) binding-sites by charged Co(II)-bioligand complexes formed immediately after addition of $Co(II)_{(aq.)}$ to the biofluid, i.e. their mobilization from the NMR-invisible "pool" of macromolecule-bound low-molecular-mass biomolecules.[36] For example, complexation of added $Co(II)_{(aq.)}$ by OA SF citrate, forming the 1:2 $[Co^{II}(Cit.)_2]^{4-}$ anion complex[37] (as might be expected at low added and physiologically-relevant concentrations of this metal ion) at sufficient levels may displace electrostatically-bound formate anion from a protein binding-site. Hence, for selected SF samples, this molecular mobilisation of formate and alanine (i.e. increases in their NMR-visible levels) observed at low added Co(II) concentrations precedes the observed line-broadening ability of this metal ion.

The relative Co(II) ion complexing efficacies of the low-molecular-mass NMR-detectable components undoubtedly reflects a combination of OA SF concentration and metal-ligand stoichiometric equilibrium constant (of course, at low added Co(II) concentrations, stoichiometries greater than 1:1 are probable). Thermodynamic stability constant data available for Co(II) (measured potentiometrically) include values for lactate, $\log_{10} \beta_{110} = 1.4$, $\log_{10} \beta_{120} = 2.4$, $\log_{10} \beta_{130} = 2.7$ mol^{-1} dm^3 ($I = 1.00$ mol dm^{-3}); threonine, $\log_{10} \beta_{110} = 4.4$, $\log_{10} \beta_{120} = 8.0$ mol^{-1} dm^3 ($I = 0.10$ mol dm^{-3}); alanine, $\log_{10} \beta_{110} = 4.3$, $\log_{10} \beta_{120} = 7.8$, $\log_{10} \beta_{130} = 9.5$ mol^{-1} dm^3 ($I = 0.10$ mol dm^{-3}); acetate, $\log_{10} \beta_{110} = 1.1$ mol^{-1} dm^3 ($I = 0.16$ mol dm^{-3}); glutamate, $\log_{10} \beta_{110} = 4.6$, $\log_{10} \beta_{120} = 7.9$ mol^{-1} dm^3 ($I = 0.10$ mol dm^{-3}); glutamine, $\log_{10} \beta_{110} = 4.0$, $\log_{10} \beta_{120} = 7.3$ mol^{-1} dm^3 ($I = 0.10$ mol dm^{-3}); citrate, $\log_{10} \beta_{110} = 5.0$ mol^{-1}

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dm^3 ($I = 0.10\,\mathrm{mol\,dm^{-3}}$); glycine, $\log_{10}\beta_{110} = 4.6$, $\log_{10}\beta_{120} = 8.5$, $\log_{10}\beta_{130} = 10.8\,\mathrm{mol^{-1}\,dm^3}$ ($I = 0.10\,\mathrm{mol\,dm^{-3}}$); histidine, $\log_{10}\beta_{110} = 6.9$, $\log_{10}\ \beta_{120} = 12.3\,\mathrm{mol^{-1}\,dm^3}$ ($I = 0.10\,\mathrm{mol\,dm^{-3}}$); tyrosine, $\log_{10}\beta_{111} = 3.9$, $\log_{10}\beta_{122} = 7.5\,\mathrm{mol^{-1}\,dm^3}$ ($I = 0.10\,\mathrm{mol\,dm^{-3}}$); phenylalanine, $\log_{10}\beta_{110} = 4.1$, $\log_{10}\beta_{120} = 7.6\,\mathrm{mol^{-1}\,dm^3}$ ($I = 0.05\,\mathrm{mol\,dm^{-3}}$), formate, $\log_{10}\beta_{110} = 0.7$, $\log_{10}\beta_{120} = 1.2\,\mathrm{mol^{-1}\,dm^3}$ ($I = 2.0\,\mathrm{mol\,dm^{-3}}$).[38]

In view of the marked variation in the concentrations of citrate in OA SF samples,[39] the Co(II)-complexing ability of a specimen with no ^{1}H NMR-detectable levels of this powerful metal ion chelator was also examined in detail (Fig. 3). As expected, the Co(II)-dependent broadening of resonances was in virtually the same order as that deduced from samples containing higher levels of citrate but with histidine and threonine representing the most powerful complexants.

The computer modeling system employed incorporated a range of potential Co(II)-complexing, low-molecular-mass ligands at concentrations equivalent to those of typical OA SF specimens (i.e. those determined by ^{1}H NMR spectroscopy as outlined in the "Materials and Methods" section), together with the thermodynamic stability constants for their 1:1,

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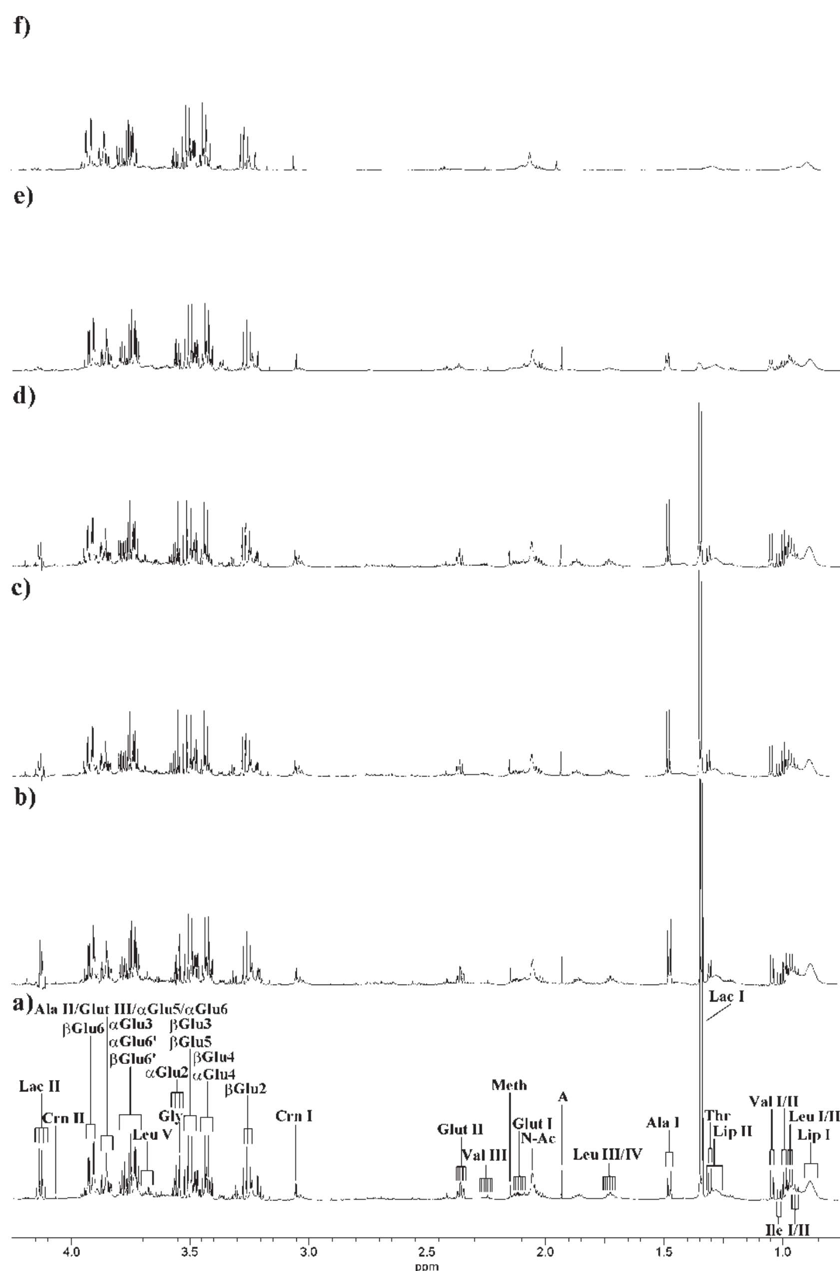

FIGURE 3 Expanded 0.50–4.50 ppm region of the 600.13 MHz CPMG ^{1}H NMR spectrum of (a) an OA SF specimen containing no NMR-detectable citrate, and after equilibration with (b) 1.20×10^{-5}, (c) 7.10×10^{-5}, (d) 1.36×10^{-4}, (e) 7.48×10^{-4}, and (f) $1.497 \times 10^{-3}\,\mathrm{mol\,dm^{-3}}$ added Co(II). Abbreviations: as for Figure 1.

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1:2 and, where appropriate, 1:3 complexes. This modeling system confirmed that at a biofluid-conditional pH value of 7.00, the major Co(II) complexants in this matrix were citrate and histidinate, respectively. Indeed, results acquired for the OA SF sample with its ^{1}H NMR spectrum exhibited in Fig. 1 (i.e. that with a relatively high citrate level) revealed that at an added Co(II) level of 1.36×10^{-4} mol dm^{-3}, the distribution of this metal ion was citrate, 97.7% (as ML only); histidinate, 1.41% (1.34% as ML, 0.072% as ML_2); glycinate, 0.055% (as ML only); threoninate, 0.052% (as ML only); glutamate, 0.003% (as ML only): the ^{1}H NMR-determined concentrations of citrate, histidine, glycine, threonine and glutamate in this specimen were 1.32×10^{-3}, 2.81×10^{-5}, 7.50×10^{-5}, 2.38×10^{-5} and 3.75×10^{-5} mol dm^{-3}, respectively. For the sample giving rise to the spectrum displayed in Fig. 3 (i.e. that with no ^{1}H NMR-detectable citrate), however, the distribution of Co(II) was histidinate, 79.32% (67.71% as ML, 11.61% as ML_2); threoninate, 3.62% (3.48% as ML, 0.14% as ML_2); glutamate, 1.76% (as ML only); glycinate, 0.85% (as ML only); alaninate, 0.38% (as ML only); phenylalaninate, 0.32% (as ML only): the ^{1}H NMR-estimated concentrations of histidine, threonine, glutamate, glycine, alanine and phenylalanine were 2.07×10^{-4}, 9.64×10^{-4}, 1.40×10^{-3}, 5.24×10^{-4}, 6.52×10^{-4} and 2.72×10^{-4} mol dm^{-3}, respectively.

Such Co(II)-induced spectroscopic changes should, in principle, be reversible, and this was demonstrated by the addition of an excess amount of EDTA (final concentration 1.20×10^{-3} mol dm^{-3}) to a SF sample containing a relatively high level of Co(II) ions (ca. 7.5×10^{-5} mol dm^{-3}) in which the resonances, e.g. for citrate and lactate, had extensively broadened. As expected, these signals re-appeared immediately (i.e. within 10 min.) after EDTA addition. However, these regenerated resonances still exhibited a degree of broadness and were shifted to lower field, an observation putatively ascribable to the retention of a small level of Co(II) by the ligands giving rise to them, a process reflected by a now fast exchange of these complexants between their free and Co(II)-bound forms. These results clearly indicate that the level of EDTA added did not completely remove Co(II) from these complexants (Ca^{2+}, Mg^{2+} and further metal ions in knee-joint SF also compete for this added chelator).

DISCUSSION

Multicomponent analytical data acquired by ^{1}H NMR spectroscopy in this study shows that citrate, histidinate and threoninate are major Co(II)-complexing components present in OA SFs, agents which, either individually or co-operatively, may play a significant role in determining the class and extent of toxicity of this redox-active metal ion released from CoCr and CoCrMo metal alloy joint prostheses. In the presence of added Co(II), the behaviour of individual biomolecule ^{1}H NMR signals can be linked to the complex relationship between through-space paramagnetic-induced line broadening, through-bond isotropic contact and through-space anisotropic pseudo-contact paramagnetic shift effects for each ligand, the latter being known to be particularly prevalent for complexes of this metal ion.[40] Such effects are, of course, weighted by the degree of saturation of the ligand molecule with Co(II), together with the shielding effects reflecting the lifetime spent in the primary co-ordination sphere of the metal ion. The observed spectroscopic parameters will also reflect the precise relationship between the rate of exchange between ligand sites and the energies describing them. In an early pioneering investigation, McDonald and Phillips[41] found that (1) Co(II)-induced ^{1}H NMR contact shifts of histidine are complex and very large (up to 1.2×10^4 Hz), (2) the NMR data acquired throughout the 1 to >11 pH range was ascribable to the formation of two 1:1 and two 2:1 complexes, one of the latter being tetrahedral rather than octahedral, and (3) broadening of both the free and Co(II)-bound histidine resonances observed in the presence of excess concentrations of the ligand was attributable to ligand-exchange processes (as expected, these linewidth increases increased markedly on elevating the pH value from 7 to 10). In the study reported here, at an added Co(II) concentration of ca. 7.00×10^{-5} mol dm^{-3}, complexation by histidine gives rise to broadening and decreases in the overall intensities of its C_2H and C_4H imidazole ring ^{1}H resonances when expressed relative to those of the aromatic ring protons of tyrosine and phenylalanine (Fig. 2), i.e. exchange-induced broadening is being observed.

Although extremely low levels of non-transferrin-bound iron as Fe(II) or Fe(III) ($1-8 \times 10^{-6}$ mol dm^{-3} [42]), and non-caeruloplasmin-bound copper(II) ions (mean concentration 2.59×10^{-7} mol dm^{-3} [32]), which are predominantly complexed by citrate[43] and histidinate/alaninate[32] respectively, are detectable in inflammatory SF and may interfere with the experiments conducted here, these levels are much lower than the minimum added concentration of Co(II) required to exert an effect on the linewidth of OA SF biomolecule resonances and therefore are not expected to influence the results acquired. Furthermore, displacement of Fe(II) or Fe(III) from citrate, and Cu(II) from histidinate and/or alaninate is thermodynamically feasible in view of the higher concentrations of added Co(II) and also their respective stability constant values

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[e.g. log β_{110} for Fe(II)- and Fe(III)-citrate = 4.4 and 11.5[38], values not dissimilar to that for Co(II)].

To the best of our knowledge, the E_0 values of the Co(II)/Co(III) couple has not been measured in aqueous media containing the majority of biological complexants reported here. However, the value of +0.20 V reported for the cobalt-*tris*-glycinato system ($[Co^{II}(gly)_3]^-/[Co^{III}(gly)_3]$, 1.00 mol dm^{-3} KCl, 25°C)[26] is much lower than that of +1.8 V measured for the $Co(II)_{(aq.)}/Co(III)_{(aq.)}$ couple,[25] an observation supporting the role of glycine and further low-molecular-mass SF chelators in promoting the adverse production of $^{\cdot}$OH radical from Fenton or pseudo-Fenton reaction systems involving phagocytically-generated H_2O_2 and ingested or prosthesis-derived Co(II). Notwithstanding, complexation of Co(II) by the endogenous thiol glutathione has been reported to alter the E_0 value of the Co(II)/Co(III) couple and hence facilitate the involvement of this metal ion in Fenton-like reaction systems[22] (Eq. 1). Appropriate spectroscopic and chromatographic experiments aimed at identifying the relative efficacies of such biomolecules to catalyse the production of $^{\cdot}$OH radical from Co(II)/H_2O_2 mixtures are currently in progress in our laboratory.

In contrast to $Co(III)_{(aq.)}$, which rapidly oxidizes water, anionic oxygen-donor ligands can effectively stabilize this higher oxidation state in this solvent system, e.g. *bis*- and *tris*-oxalato Co(III) complexes are stable in aqueous solution. Moreover, chelating ligands with a combination of oxygen- and nitrogen-donor atoms such as EDTA further stabilize Co(III) (the E_0 value for the $[Co^{II}(EDTA)]^{2-}/[Co^{III}(EDTA)]^-$ couple is +0.37 V[44]), and a higher level of stabilization of this metal ion is achieved via occupation of all of its co-ordination sites with nitrogen donor atoms (e.g. E_0 for the $[Co^{II}(NH_3)_6]^{2+}/[Co^{III}(NH_3)_6]^{3+}$ couple is only +0.11 V[44]).

The results obtained with alanine and histidine are of much significance since the equilibration of Co(II) ions with β-alanyl-3-methyl-L-histidine (anserine) and H_2O_2 has been demonstrated to promote the generation of $^{\cdot}$OH radical, the omission of any of these three components being found to markedly reduce the extent of radical generation.[13] This observation clearly indicates that prior chelation with amino acids or peptides exerts a powerful influence on the redox potential of the Co(II)/Co(III) system.[13,21] ESR studies have demonstrated the generation of $^{\cdot}$OH radical from a mixture of Co(II), H_2O_2 and histidine in phosphate-buffered saline solutions at physiological pH,[23] studies consistent with earlier ones which demonstrated the promotion of $^{\cdot}$OH radical production when Co(II) was bound to EDTA.[42] The replacement of anserine with formate[13] resulted in a moderate decrease in relative $^{\cdot}$OH radical formation, an observation that is likely to be ascribable to the scavenging of this radical by the ligand (second-order rate constant, $k_2 = 3.2 \times 10^9$ mol^{-1} dm^{-3} s^{-1}[45]), and consistent with the hypothesis that its generation by Co(II) is site-specific, as suggested by Moorhouse *et al.*[46] However, histidine is itself an effective $^{\cdot}$OH scavenger ($k_2 = 3.0 \times 10^9$ mol^{-1} dm^{-3} s^{-1}[47]), as indeed are the aromatic amino acids tyrosine and phenylalanine ($k_2 = 3.7$ and 3.5×10^9 mol^{-1} dm^{-3} s^{-1}, respectively [47]) which also displayed significant ^{1}H signal broadenings on addition of Co(II).

However, it should be noted that the relative concentrations of $Co^{II}L_n$ complexes do not necessarily reflect reactive sources of $^{\cdot}$OH radical since the latter is critically dependent on the conditional (and not thermodynamic) E_0 value of the couple (i.e. E_0', representing E_0 at an estimated mean OA SF pH value of 7.0), in addition to the $^{\cdot}$OH scavenging capacity of the bound ligand.

The X-ray crystal structure of the $[Co^{II}(Cit.)_2]^{4-}$ complex anion consists of a distorted octahedron with all co-ordination sites occupied by citrate oxygen donor atoms,[37] whilst that of Co(II)'s complex with formate (as $[Co^{II}(HCO_2^-)_2]\cdot 2H_2O$) has two independent Co(II) sites, the first co-ordinated by oxygen donor atoms from six formate anions, the second by four water molecules and an oxygen donor from each of two formate units (the two classes of octahedra are bridged via one of the formate anions and by H-bonding).[48]

Since the thermodynamic redox potential of the $Co(II)_{(aq.)}/Co(III)_{(aq.)}$ couple can be reduced to values as low as (or lower than) +0.20 V on complexation with bioavailable ligands (as noted above for the glycinato complex), it is conceivable that biofluid O_2 can effect the oxidation of Co(II) to Co(III) either *in vivo*, or *in vitro* during sample preparation, a reaction generating superoxide anion (Eq. 2). The facile autoxidation of Co(II)-amino acid complexes in the presence of atmospheric O_2 has been known for many years. Indeed, Hearon *et. al.*[49] found that aqueous Co(II)-histidinate complexes react reversibly with molecular O_2, generating a diamagnetic, amber-coloured Co(III) complex, which is, of course, expected to have ^{1}H NMR signals in the diamagnetic spectral range (0–10 ppm).[41] However, OA SF electron-donors such as the cysteine-34 residue of albumin, ascorbate or urate may offer sufficient protection against this potentially deleterious reaction system and experiments to determine whether this particular biofluid contains sufficient, Co(II)-stabilising levels of such reductants are currently underway.

$$[Co^{II}(Gly)_3]^- + O_2 \rightarrow [Co^{III}(Gly)_3] + O_2^{\cdot -} \quad (2)$$

Moreover, although glutathione (GSH), glycylglycylhistidine and anserine have the capacity to

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generate a range of ROS putatively responsible for Co-induced cell and tissue damage,[22] excess levels of these peptides or their amino acid components [with respect to those of Co(II) as is indeed the case in physiological environments], can, of course, scavenge $^{\cdot}$OH radical.

Interestingly, Sakurai and Ishizu[50] found that $O_2^{\cdot-}$ could be generated from a Co(II)-tetraphenylporphyrin (TPP)-thiolate-O_2 system, results providing a valuable insight into the mechanism of haem protein-induced oxygen activation in biological systems. Similarly, Sakurai *et. al*.[51] characterised a series of Co(II)-TPP-thiolate complexes and their oxygen adducts. Spectrophotometric studies conducted in our laboratory have revealed the presence of haem proteins in both OA and rheumatoid SFs (Grootveld, M. and Patel, I.Y., unpublished observations) which, dependent on the ability of Co(II) to displace Fe(II) [or Fe(III)] from the porphyrin co-ordination centre, and the availability of free cysteine, could offer a sustainable, local source for this reaction system *in vivo*.

CONCLUSIONS

The studies conducted here provide much useful information concerning the nature of the complexation of Co(II) by SF biomolecules, which advances our understanding of the molecular nature of this metal ion derived from the corrosion of cobalt-containing metal alloy joint implants *in vivo* and potentially gives rise to the development of toxicological testing systems involving specific Co(II) complexes. Comparatively low concentrations of added Co(II) were required to selectively influence the line-width and/or chemical shift values of resonances, an observation supporting the facile application of high resolution ^{1}H NMR spectroscopy to the "speciation" of prostheses-derived metal ions in biofluids and tissues. Such information is also of much value regarding the potential roles for these biomolecular chelators as promoters of the Co(II)-catalysed generation of $^{\cdot}$OH radical and/or "site-specific" scavengers of this highly reactive oxidant, competing phenomena likely to represent major determinants of the nature and level of Co-induced toxicity *in vivo*.

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