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Citation for published version:
Rickard, D, Hatton, B, Murphy, DM, Butler, IB, Oldroyd, A & Hann, A 2011, 'FeS-Induced Radical Formation and Its Effect on Plasmid DNA' Aquatic geochemistry, vol. 17, no. 4-5, pp. 545-566. DOI: 10.1007/s10498-010-9116-x

Digital Object Identifier (DOI):
10.1007/s10498-010-9116-x

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Aquatic geochemistry

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Cite As: Rickard, D, Hatton, B, Murphy, DM, Butler, IB, Oldroyd, A & Hann, A 2011, 'FeS-Induced Radical Formation and Its Effect on Plasmid DNA' *Aquatic geochemistry*, vol 17, no. 4-5, pp. 545-566.

FeS-induced radical formation and its effect on plasmid DNA

D. Rickard • B. Hatton •D. M. Murphy• I. Butler• A. Oldroyd • A. Hann

Abstract

Plasmid DNA was incubated at 25°C with aqueous solutions of dissolved Fe(II), S(-II) and nanoparticulate FeS with a mackinawite structure, FeS_m. At ≥ 0.1 mM total dissolved Fe(II) and S(-II) an increase in the proportion of the relaxed plasmid DNA occurs, through scission of the DNA backbone. In solutions where FeS_m was precipitated, nanoparticulate FeS_m binds to the DNA molecules. In solutions with concentrations below the FeS_m solubility product, nicking of supercoiled pDNA occurs. Plasmid DNA appears to be a sensitive proxy for radical reactions. The reactant is proposed to be a sulfur-based radical produced from the iron-catalysed decomposition of bisulfide, in a manner analogous to the Fenton reaction. This is further supported by experiments that suggest that sulfide free radicals are produced during the photolysis of aqueous solutions of polysulfides. Supercoiling of DNA affects nearly all DNA-protein transactions so the observation of relaxation of supercoiled forms through reaction with

D. Rickard · B.Hatton. A. Oldroyd
School of Earth and Ocean Sciences, Cardiff University, Cardiff CF103YE, UK
e-mail: rickard@cardiff.ac.uk
D. M. Murphy
School of Chemistry, Cardiff University, Cardiff CF103YE, UK.
I. Butler
School of Geosciences, University of Edinburgh, Edinburgh EH9 3JW UK
A.Hann
School of Biosciences, Cardiff University, Cardiff CF103YE, UK.

FeS solutions has direct implications to biochemistry. The results of this experimentation suggest that genotoxicity in FeS-rich systems is a further contributory factor to the limited survival of organisms in sulfidic environments. Mutations resulting from the interactions of organisms and mobile elements, such as plasmids, in sediments will also be affected in sulfide-rich environments.

1 Introduction

In natural environments, iron sulfides are usually formed in anoxic, sulfidic systems which are relatively rich in organic matter (1987; Rickard and Morse 2005). This is because the sulfide is mainly produced by the activities of heterotrophic, sulfate-reducing bacteria (Morse et al. 1987) The reactions between iron sulfides and organic compounds are significant but little understood. Free radical reactions, in particular, are common in organic chemistry but their geochemical significance has not been widely investigated. An exception is the generation of hydroxyl radicals, OH●, through the reaction between adsorbed water and Fe(III) in defect sites on pyrite surfaces (Borda et al. 2001). Hydroxyl radicals readily react with DNA causing strand breaks. Cohn et al.(2003; 2006) demonstrated that nucleic acids are destroyed in the presence of pyrite due to this reaction.

There have been a number of published descriptions of HS• radical moieties but they have been reported under a variety of names; for example, mercapto radicals (Norman and Storey 1971),sulfhydral radicals (Mills et al. 1987) and thiyl radicals (Baldridge et al. 1987), although thiyl is also used to refer to any molecule containing a sulfur atom with an unpaired electron (RS•) especially in organic chemistry (Bonini and Augusto 2001). Sulfenyl has been

used as a general term for RS• radicals where R ≠ H. Hydrogen sulfide or HS• radicals (Stachnik and Molina 1987; Wang et al. 1987) are widely used as unambiguous names for the radical monomers. HS• radicals are also reportedly produced during the thermal decomposition of alkane thiols (Baldridge et al. 1987) and by the reaction between H_2S and OH• (Stachnik and Molina 1987). Tapley et al. (1999) produced evidence, using EPR, for the generation of sulfide free radicals when sulfide is oxidised. However, HS• radicals were not specifically identified or trapped by Tapley et al. (1999) and they were suggested to be involved in the production of other radicals. HS• radicals appear to be highly reactive but transient forms.

Organic-iron sulfide reactions have generated renewed interest over recent years because of the development of the iron-sulfur world hypothesis for the origin and early development of life. Iron sulfide minerals are thought to have adsorbed organic molecules and promoted the reactions between them that lead to the evolution of a mineral bound proto-organism or proto-biont (Russell and Arndt 2005; Russell and Hall 1997, 2002; Russell and Martin 2004; Schoonen et al. 1999; Wächtershäuser 1998). This hypothesis is interesting because various aspects of it can be tested experimentally. The results of these experiments are, of course, not only of interest to understanding Early Earth environments but contribute to our knowledge of geochemistry at the interface between the organic and inorganic worlds.

Most experimentation in this system has involved the reactions with fragments of bulk stable minerals, such as pyrite and pyrrhotite or undefined FeS precipitates or mixtures (e.g. Huber and Wächtershauser 1997; Russell and Hall 1997). By contrast, the initial precipitate from the reaction between Fe solutions and S(-II) at low temperatures (e.g. $<120^{o}C$) is nanoparticulate, stoichiometric FeS with a tetragonal mackinawite structure (Rickard et al. 2006; Wolthers et al. 2003). Hereafter, we refer to this material as FeS_m.

Lower temperature anoxic conditions, under which FeS_m forms, are more amenable to the preservation of large biological molecules. The FeS_m nanoparticles are of the same order of size – and often smaller- than large biological molecules such as nucleic acid polymers. Thus the idea of adsorption of these organic molecules on FeS_m, as has been discussed with respect to pyrite (e.g. Cohn et al. 2004; Zhang et al. 2006) and pyrrhotite (Schoonen et al. 1999), may not be appropriate. Hatton and Rickard (2008) showed that FeS_m nanoparticles attach to nucleic acid polymers in a similar manner to ZnS quantum dots (cf. Jamieson et al. 2007).

In this paper we address the effects of dissolved Fe(II) + S(-II) concentrations below the stability limits of solid FeS_m on plasmid DNA. Hatton and Rickard (2008) used chromosomal or "wild" DNA in their study of the effects of nanoparticulate FeS on nucleic acids and we extend this into plasmid DNA in this study. We use plasmid DNA as a novel indicator of the production of sulfide free radicals during the reaction between DNA and solutions containing Fe(II) and S(-II).

1.1 DNA

We chose DNA as a nucleic acid since it is, necessarily, a robust molecule. Reactions which cause changes in DNA are likely to have an even greater effect on less robust nucleic acid polymers. Hatton and Rickard (2008) showed that nanoparticulate FeS_m not only reacted with wild DNA, for example, but also to other nucleic acids and their derivatives in the order chromosomal DNA > RNA > oligomeric DNA > deoxadenosine monophosphate ≈ deoxyadenosine ≈ adenine.

We used small molecular weight, double stranded DNA plasmids (pDNA) as a reactant since these can be readily isolated as negatively supercoiled molecules. If one or more phosphate bonds in the supercoiled DNA ribose-phosphate backbone are broken, the molecule unravels into a relaxed, open circular form (Fig 1). During electrophoresis, supercoiled forms move more easily through the gel and are separated from the relaxed circular forms (Jezowska-Bojczuk et al. 2002; Rozenberg-Arska et al. 1985; Toyokuni and Sagripanti 1992). Plasmids are therefore widely used as test molecules in studies of nucleic acid chemistry since their behaviour during reactions is readily traceable.

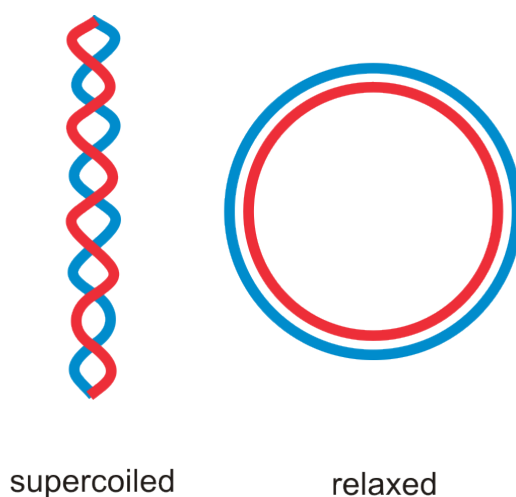

Fig 1. Supercoiled and relaxed, circular plasmid DNA.

1.2 FeS

FeS occurs in two main forms at low temperatures : (1) FeS nanoparticles with a mackinawite structure, FeS_m, and (2) aqueous FeS clusters, FeS_{aq}. FeS_m nanoparticles are very reactive. Their propensity to oxidation has been widely described (e.g. Rickard and Luther 2007). In particular,

they undergo a relatively rapid solid state transformation to the more stable thiospinel greigite, Fe_3S_4, and react with O_2 to form Fe(III) oxyhydroxides in water. FeS_m is soluble compared with its more stable iron sulfide counterparts, such as pyrite. In acid to neutral solutions the solubility is described by

$$FeS_m + 2H^+ \rightarrow Fe^{2+} + H_2S \qquad (1)$$

where $\log K_{sp,1} =$ 3:5 ±0.25 (Rickard 2006). In neutral to alkaline solutions, the solubility can be closely described by the intrinsic solubility

$$FeS_m \rightarrow FeS^0 \qquad (2)$$

where FeS^0 represents an aqueous FeS_{aq} cluster monomer and $\log K_{sp,0} = -5.7$ (Rickard 2006).

Theberge and Luther (1997), Rickard (1997), Luther and Rickard (2005) characterised aqueous FeS clusters (FeS_{aq}) and showed that these species are intimately involved in iron sulfide chemistry. Rickard et al.(2001) showed that aldehydic carbonyls inhibit FeS_{aq} formation.

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1.3 Free radical reactions

Biological macromolecules are sensitive to hydrolysis and oxidation reactions. Although reactive oxygen species have received much attention, many different free radical species, generated by the redox reactions of intracellular iron and other metal ions, induce mutations in biochemical systems, due to their capacity to oxidise DNA (Berglin and Carlsson 1986; Imlay et al. 1988; Kawanishi et al. 2001; Toyokuni and Sagripanti 1992). In this context it is important to note that pyrite induces hydrogen peroxide formation in water regardless of the presence of oxygen whilst iron(II) monosulfide does not (Borda et al. 2001). Thus, if iron (II) monosulfide is involved in free radical reactions, it is likely that the free radical species involved are not based

on conventional peroxide generation. By analogy with the damage cause by reactive oxygen species, it is expected sulfur radicals will also lead to disregulation of biological processes and may induce mutations.

2. Materials and methods

2.1 Sample preparation

Sterile, O_2-free deionized, 18MΩ water was used throughout. MilliQ ™ 18 MΩ H_2O was sterilized by autoclaving for 2 h at 120°C and 2 Bars in a Priorclave™ and deoxygenated by bubbling O_2-free N_2 for at least 1 h. Studies of the resultant dissolved O_2 concentration (Butler, et al. 1994) shows that the solution contains measurable O_2 in the 1-10 ppmv range. O_2 level in the anoxic cabinet was monitored and did not exceed 10 ppmv. All containers were sterile on purchase or sterilized through autoclaving as above except plastic bottles for metal sulfate and sodium sulfide stock solutions which were washed with 2% Decon 90® and then rinsed with ethanol.

All reagents were of analytical grade. Stock solutions of 0.5, 5, 50, 500 mM Fe(II) and S(-II) were prepared with analytical grade $(NH_4)_2Fe(SO_4)_2.6H_2O$ (Mohr's salt) and $Na_2S.9H_2O$ (Fisher Chemicals™) dissolved in H_2O. Aliquots of these stock solutions were taken to make up solutions with defined FeS concentrations. In order to make it possible to extend the FeS concentrations across the regions where dissolved and solid species are present, we define the concentration in terms of the FeS_m equivalent, $FeS_{eq.}$ We are able to do this since FeS_m is stoichiometric $Fe_{1.0}S_{1.0}$ (Rickard et al. 2006). Thus, for example, a solution containing 10 mM $(NH_4)_2Fe(SO_4)_2.6H_2O$ and 10mM $Na_2S.9H_2O$ produces 10mM FeS_{eq}. In a Labmaster *130*™

anoxic glove box, filled with O_2-free N_2, sodium sulfide solution was added to Tris buffer (a pH 8 buffer commonly used in DNA experiments) in a 0.2 mL microfuge tube. To this solution, $(NH_4)_2Fe(SO_4)_2.6H_2O$ was added and finally pDNA solution was added to give a final volume of 50 µL.

The pH of the iron sulfide + DNA solutions was measured using a Jenway 2030 ™ digital pH meter. A pH equivalent to modern seawater (8.1 ± 0.1) was maintained for all but the most concentrated experiments. At 100mM FeS_{eq} the pH was 7.5 and at 150mM FeS_{eq} the pH was 6.8. The drop in pH in these high FeS_{eq} systems reflects the inevitable release of relatively large amounts of H^+ in the FeS_m precipitation reaction. Under all these experimental conditions the solubility of FeS_m is controlled by reaction (2) and the dominant dissolved Fe form is aqueous FeS clusters and the dissolved Fe(II) concentration is equivalent to about 1µM (Rickard 2006).

2.2 Plasmid DNA preparation

pUC18 plasmid DNA (pDNA) was extracted from cultures of *E.coli* grown in LB broth with 100 mg/L ampicillin using Wizard® Plus SV Minipreps DNA Purification System. High purity pUC18 plasmid DNA (Bayou Biolabs™) was used as a standard since this is free of RNA, protein, nucleotide, RNase, and DNase contamination. pUC plasmids are virtually pure supercoiled forms, containing minimal nicked plasmid and chromosomal DNA contamination. pDNA was diluted with H_2O and stored in nuclease free water at -20ºC until required.

2.3. Electrophoresis

All samples were prepared and incubated for 1 h in a Labmaster 130™ anoxic glove box filled with O_2 free N_2. The samples were removed from the glove box for electrophoretic analysis. A 5 μL aliquot of the samples was mixed with 1μL of 5*loading buffer and loaded on a 0.7% agarose gel with ethidium® bromide added to a concentration of 10 μg/ml. 80 V was applied across the gel until the dye had moved sufficiently down the gel. The gels were then photographed with ultraviolet light in a Gene Genius Bio Imaging System™. The amount of pDNA on the gel was quantified using GeneTools™ densitometry from SynGene ™ which computes the quantity of DNA in a band from its fluorescent intensity relative to a standard marker (Bioline HyperLadder 1™) which produces a series of bands containing different quantities of DNA. To increase accuracy, HyperLadder 1™ was added to a minimum of two wells on each gel (5 μL) and quantification was performed using at least 4 different bands from both markers and the mean concentration was taken. Initial pDNA concentrations refer to the total amount of pDNA measured on the gel in both the super-coiled band and the relaxed band in the FeS-free positive control for that experiment. Note that when pDNA is broken into smaller fragments, these travel through the gel at faster rates than both the super-coiled and relaxed forms and are effectively lost to the analysis.

2.4. FeS_{aq} clusters

Aldehydic carbonyls have been shown to react with FeS_m to prevent the formation of FeS_{aq} clusters (Rickard et al. 2001). It is therefore possible to test if these highly reactive clusters are

involved in the reaction with DNA by adding small amounts of formaldehyde to the experimental system. Aqueous formaldehyde solution (Sigma-Aldrich™), was added to four 1 mM FeS_{eq} + pDNA solutions to a final concentration of 2 mM formaldehyde and run on the same gel along with four 1 mM FeS_{eq} + pDNA solutions without formaldehyde.

2.5. Molecular O_2

A problem with the standard methodology described above is that although the reagents and reaction were prepared and carried out under anoxic conditions, electrophoresis was routinely carried out, for obvious practical reasons, in a system exposed to the atmosphere. In order to determine if this had any effect on the reaction, the electrophoresis tank was placed in the anoxic cabinet and the samples loaded directly on to it, without being exposed to atmospheric oxygen at any point. All solvents, including electrophoresis buffer, were thoroughly deoxygenated with oxygen-free nitrogen and the anoxic cabinet was monitored at <10 ppmv O_2.

Ti(III) citrate has been used extensively in iron sulfide chemistry as a strong reducing agent (Rickard et al. 2001; e.g. Cornwell and Morse 1987). The measured Eh of an aqueous Ti(III) citrate solution at neutral pH approaches - 400 mV. The addition of Ti(III) citrate to the experimental systems thus removes the possibility of the formation of Fe(III) and the consequent involvement of Fenton reactions in the process. Ti (III) citrate was prepared from 50 mL 0.2 M Na citrate and 5 mL 1.8 M $Ti(III)Cl_3$ brought to a pH of 7 with $NaCO_3$. Ti(III) citrate was added after the precipitation of FeS_m and before the addition of pDNA. The final Ti(III) citrate concentration in the reaction vessel was 3.6 mM. Ti (III) citrate was added to a series of FeS_m + DNA solutions, including the FeS - free control, and run on a gel as described above.

2.6. Free radicals

Two groups of preliminary experiments were performed. In one group, we tested whether we could detect free radicals in our sulfur systems by repeating known reactions and investigated free radical production in polysulfide solutions. In the second, we investigated the potential role of free radicals in the reaction between sulfide and pDNA. This reaction not only has intrinsic interest but, because of the sensitivity of the conformation of pDNA to free radical reactions, can be used to suggest free radical production.

Reagents were of analytical grade or finer and were used without further purification. Sodium tetrasulfide was synthesised from elemental sulfur and anhydrous sodium sulfide using the method of Rosen and Tegman (1971) as modified by Chadwell et al. (2001). For these preliminary investigations of free radical generation in sulfur systems we utilised 5,5-dimethyl-1-pyrroline N-oxide (DMPO) as a spin trap because it is readily soluble in organic solvents and water. All reagents were prepared and handled under nitrogen in an anoxic chamber (MBraun Labmaster 130) at <1ppmv O_2. Organic samples were introduced into the ESR cavity using standard 4mm OD quartz silica tubes in early experiments to test for RS• and OH• trapping. In later experiments both organic and aqueous samples were contained in glass Pasteur pipettes which were sealed at the tip in a Bunsen flame and capped with a butyl rubber seal. The resulting smaller sample volumes made it easier to tune the instrument, especially for aqueous samples.

We trapped hydroxyl, sulfenyl (i.e. R-S R≠H) and, possibly, sulfide radicals in aqueous and organic solvents respectively using a Fenton's reaction via the addition of H_2O_2 to Mohr's

salt solution, FeS and FeS_2 respectively and through photolysis of diphenyl disulfide (C_6H_5-S-S-C_6H_5) in hexane and in ethanol. This approach permitted us to confirm our ability to trap and measure the presence of radicals as spin adducts and to determine the hyperfine coupling constant for the OH• spin adduct with DMPO. Subsequently we examined the behaviour of sodium polysulfide in dimethyl formamide (DMF) and in water; in the latter case using photolysis to attempt to induce radical formation both in the presence and absence of DMPO.

In the plasmid experimentation we used DMSO (Fisher Chemicals™) to test whether free radicals are involved in the reaction of FeS with DNA. It was added to FeS and pDNA solutions to a final concentration of 2.8 M DMSO. One gel was run with 100 and 150 mM FeS_{eq} with and without 2.8 M DMSO and one was run with 4 repetitions of each of 1 mM FeS_{eq}, 2.8 M DMSO and 2.8 M DMSO + 1mM FeS_{eq}.

Catalase is an enzyme which decomposes hydrogen peroxide (H_2O_2) to H_2O and O_2 preventing OH• formation. Catalase from Sigma-Aldrich™ was added to four solutions of pDNA + 0.1 mM FeS_{eq} to a final concentration of 1000 units/mL catalase. These solutions were run simultaneously on a gel as above, quantified with Genetools™ and the percentage pDNA relaxation was measured and compared against a 0.1 mM FeS_{eq} + pDNA control without catalase.

Ultraviolet light enhances Fenton reaction-induced DNA damage. To test if the reaction in this study is similarly enhanced 4 solutions of 1 mM FeS_{eq} + pDNA were incubated under ultraviolet light. The light source was a 4W Hg UV lamp (Mineralight, UVSL-25, giving 80 /tW/cm^2 at 45 cm height, UV Products Inc.). For 30 min of the 1 hr incubation it was set for long wavelength UV light (peak intensity at 366 nm) and for the following 30 min was set to short wavelength (254 nm). The lamp was placed 10 cm from the samples which were prepared as

above, except that they were prepared in UV-permeable micro cuvettes. For comparison 4 samples were prepared with 1 mM FeS_{eq} + pDNA and wrapped in aluminium foil, 4 pDNA controls were placed under the UV lamp and 4 pDNA controls were also wrapped in aluminium foil.

The EPR data were collected on an X-band JEOL spectrometer (JES-2RE2X) operating at 100 kHz field modulation with a cylindrical cavity. All spectra were recorded at 298K and manipulated using the JEOL data analyser program (Version 1.3).

2.7 Electron microscopy.

Samples of pDNA from blanks and FeS experiments were prepared as for the electrophoretic gels and observed on a Philips EM 208 transmission electron microscope. The samples were pipetted onto a droplet of 1 mg mL-1 cytochrome C (Sigma-Aldrich ™) to spread the pDNA and isolating individual molecules. The droplet was sampled with a carbon-coated Cu grid and rotary shadowed with Pt with a vacuum evaporator (Edwards Auto 306).

2.8 Control reactions.

A large number of control reactions were performed over and above those described in this paper and these are reported in Hatton (2007). In particular, we found no reaction between pDNA and aqueous sulfide under anoxic conditions. Attene-Ramos et al. (2007) reported damage to wild DNA at H_2S concentrations ≤ 1 μmol. L^{-1} in oxidizing systems and related this to sulfoxide radicals. We found that anoxic aqueous sodium sulfide solutions only affected pDNA at pH >11

and showed, using NaoH blanks, that this was due to the effects of high OH^- concentrations on DNA concentrations in the presence of Na+ ions (Dore 1972).

We examined the effects of Mohr's salt on pDNA to see whether there was a reaction with aqueous Fe(II). In the presence of the reducing agent Ti(III) citrate or a free radical trap (DMSO) no relaxation of pDNA was observed. We conclude that there is no reaction with aqueous Fe(II). We also looked at the effects of aqueous Fe(III) on pDNA. Again we found no reaction, which is consistent with the relaxation of pDNA in Fe soutlions being initiated by trace molecular oxygen.

3. Results

3.1 Preliminary radical trapping results.

Results for the photolysis of diphenyl disulfide with DMPO (500µL 1mg mL^{-1} DMPO and 100µl of 6mM diphenyl disulfide, both in hexane) are illustrated in Fig 2.

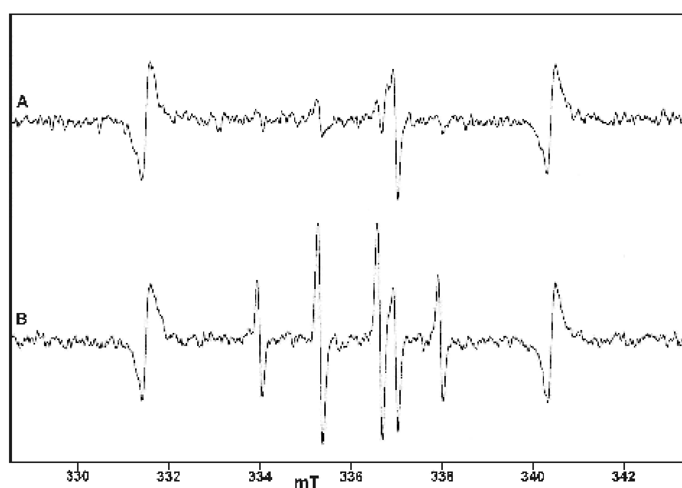

Fig 2. ESR spectra for trapping of RS• resulting from photolysis of diphenyl disulfide in hexane. A) Background spectrum for DMPO, hexane and the silica sample tube. B) Resonances spectra of spin trapped RS• superimposed on the background spectrum.

Photolysis of diphenyl disulfide with an Hg-Xe lamp results in the formation of a weak spin adduct with DMPO. The spin adduct is short lived and decays rapidly once irradiation of the sample was stopped. The results are consistent with the observations of Ito and Matsuda (1984) and confirm trapping of R-S• species by DMPO. Results for the reaction of Mohr's salt with H_2O_2 with DMPO are illustrated in Fig 3.

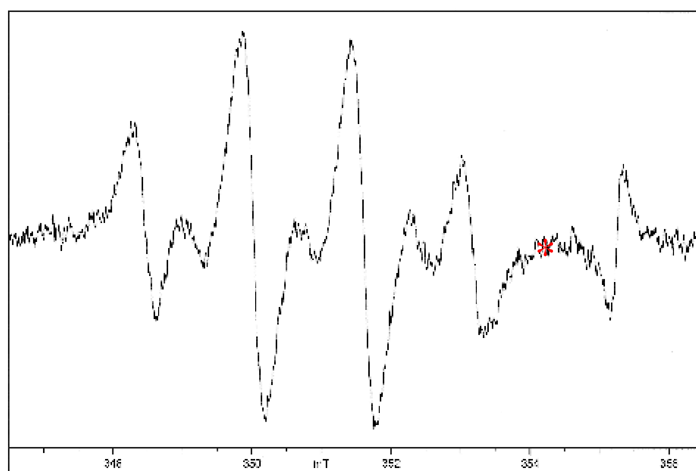

Fig 3. ESR spectrum for trapping of OH• produced by a Fenton's reaction of Mohr's salt in water in silica tubes. * = cavity reference marker. 10 G = 1mT.

Experiments using FeS_m were problematic because of the reactivity of FeS_m coupled with the difficulty of tuning the ESR cavity in the presence of both FeS_m and water. Fenton's reaction of H_2O_2 with Fe(II) readily forms OH• and a coupling constant of 16G was determined by simulation of the spectrum for the spin adduct of OH• with DMPO.

ESR analysis of a blue-green solution sodium tetrasulfide in dimethyl formamide returned a strong ESR spectrum centred on 355mT which is described by a single Lorentzian

shape with no fine structure (see also Levillain et al. 1997). Calibration of the Na_2S_4 spectrum against 2,2-diphenyl-1-picrylhydrazyl hydrate returned a g value of 2.0319, similar to the value of 2.029 for Li_2S_3 determined by Levillain et al (1997). For a free electron in space the value is 2.0023, so a shift to positive g of 2.0319 is entirely what would be expected due to the slight increased in orbital angular momentum. An identical experiment performed using a solution of sodium tetrasulfide in water returned no measurable ESR signal. Photolysis of the same solution also produced no detectable resonance.

Due to the difficulty of tuning the ESR instrument on a suspension of FeS_m in water to test reaction (4), we elected to use photolysis of aqueous sodium tetrasulfide solution with DMPO in order to attempt to trap the radical products of its breakdown. For this we used initially 200µL of 50mM Na_2S_4 with 200µl of 20mM DMPO. For this, both with and without UV photolysis, we observed no measurable signal.

Fig 4. Replicate ESR spectra for trapping of a radical species produced by photolysis of sodium tetrasulfide in water using DMPO. The horizontal reference bar is in Gauss units where 10 G = 1mT.

Addition of a further 30µL of undiluted DMPO showed no signal without photolysis, but with photolysis a very weak spectrum centred on 339.5mT was observed (Fig 4). The coupling constant was determined to be 15G, which is different to that determined for OH• radicals

trapped by DMPO during a Fenton's reaction in an aqueous medium. Repeated photolysis of the same solution resulted in even weaker or undetectable ESR signals.

3.1 Effect of varying FeS_{eq} concentrations.

An increase in the proportion of relaxed pDNA occurred on incubation with FeS_{eq} concentrations as low as 0.1 mM (Figs. 5 and 6). In these systems, 0.1mM approaches the minimum S(-II) concentration which can be used robustly. Below this value, contamination by higher valence sulfur species, partly though natural equilibration processes, risks reaching significant proportions of the total dissolved sulfur concentration.

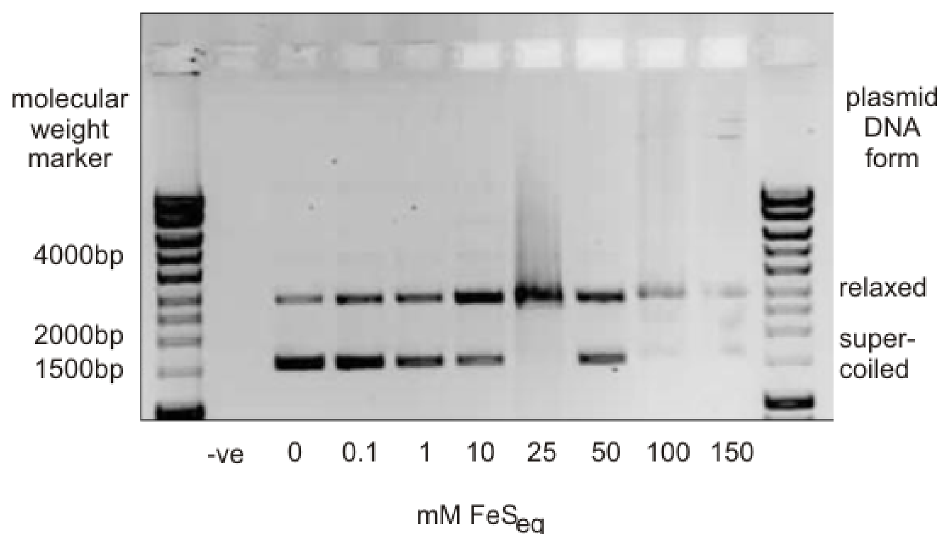

Fig 5. Image of a typical electrophoretic gel of FeS + pDNA. FeS_{eq} concentrations are given below the image of the gel, the positions of the relaxed and super-coiled plasmid and the size of the molecular weight markers are indicated. The concentration of pDNA detected in the positive control (0mM FeS_{eq}) is 17.2 ug mL^{-1}

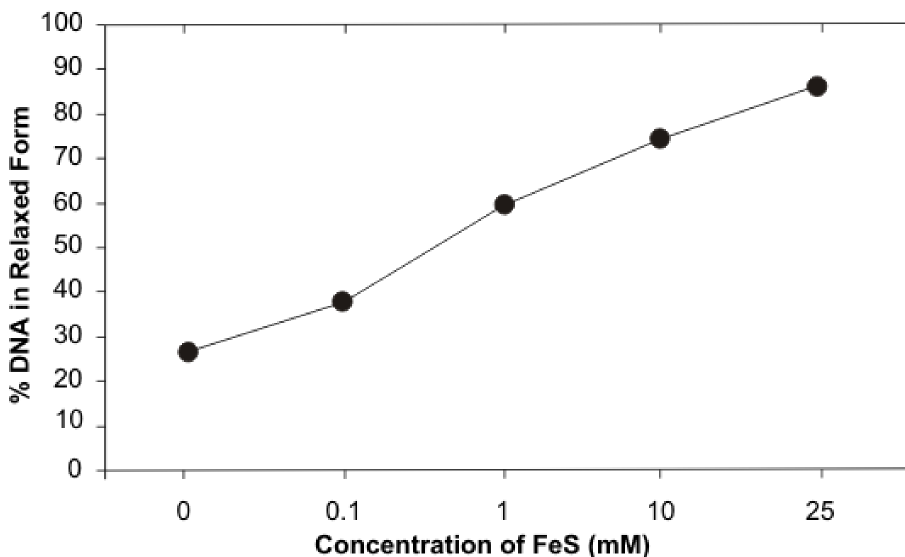

Fig 6. Graph showing FeS-induced relaxation of plasmid DNA as a function of FeS_{eq} concentration. 12 samples, with differing concentrations of pDNA(1.3-78.5 µg mL^{-1}), at each FeS concentration (0.1, 1 10, 25 mM) were run on 6 different gels. Percentage relaxation was calculated, for each sample, from the amount of pDNA in the relaxed state as a percentage of the total of both the relaxed and super-coiled bands. The percentage of pDNA relaxed increased with FeS_{eq} concentration. Quantification was performed using BioLine hyper ladder 1™.

In the untreated control (Fig 4, 0 mM) pDNA existed mostly in the super-coiled form (lower band). At FeS concentrations between 0.1 mM and 25mM, an increase in the proportion of the relaxed pDNA (upper band) was observed which was proportional to the concentration of FeS_{eq}. When incubated with 25 mM FeS_{eq} there was no detectable super-coiled pDNA on the gel. However, super-coiled DNA is present at higher concentrations. At 25, 50, 100 and 150 mM both relaxed and super-coiled pDNA are visible, although the bands were smeared, broken and at a lower intensity than the positive control. Lateral streaks can be seen above the bands at $\geq$ 25 mM.

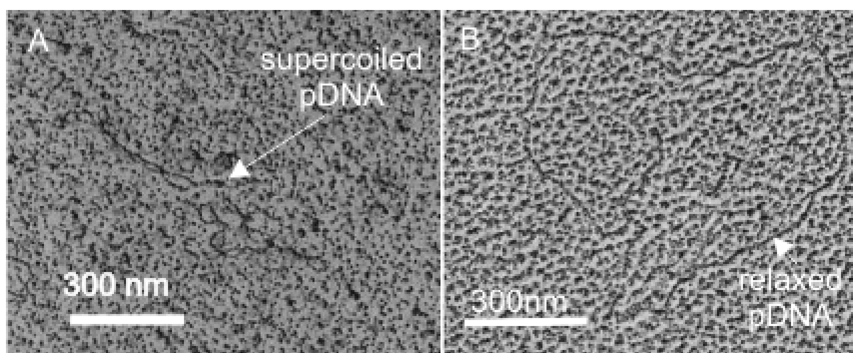

Fig 7. Electron micrographs of pDNA. A. The supercoiled form in the blank. B. The relaxed, circular form in 10mM FeS_{eq} solutions.

In order to confirm this result, samples of pDNA from the blanks and from FeS solutions were examined by electron microscopy. As shown in Fig.7, the electron images revealed that the supercoiled pDNA in the blank had relaxed to the circular form in the FeS solutions, in accordance with the electrophoresis results.

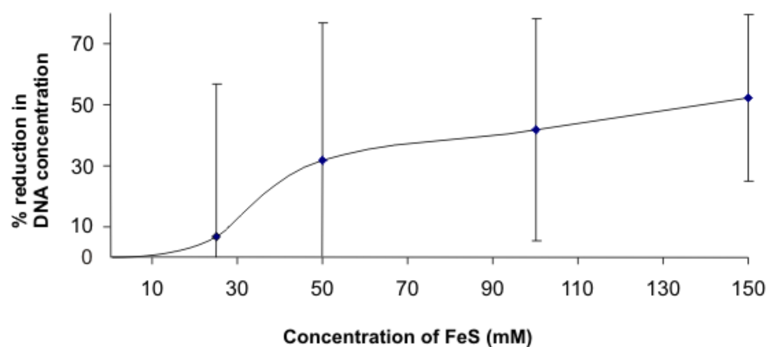

Fig 8. Graph showing that the total pDNA concentration decreases with increasing FeS_{eq} concentration. Reduction in DNA concentration was calculated for each sample from the total of the pDNA in the relaxed and super-coiled band for each FeS_{eq} + pDNA preparation subtracted from the total pDNA in the corresponding FeS_{eq}-free control. Each point is the mean of 12 at pDNA at concentrations of 1.3-78.5 μg mL^{-1} against FeS_m concentration (25, 50, 100 and 150 mM). Error bars are ± 1 standard deviation.

Over the concentration range 0.1 to 10 mM the total DNA concentration remained approximately constant. However, FeS_{eq} concentrations ≥25 mM caused a significant drop in total DNA concentrations: the intensity of both the super-coiled and relaxed pDNA decreased (Figs. 5 and 8). When incubated with 100 or 150 mM FeS_{eq} only 60% of the pDNA could still be detected (Fig. 8).

3.2 Effect of varying pDNA concentrations.

In other gels, prepared under the same conditions but with a lower initial pDNA concentration, the pattern was different. Complete relaxation of the pDNA was observed at 25 mM FeS_{eq} in most cases. However, above this, depending on the pDNA concentration, a complete loss of both supercoiled and relaxed pDNA occurred. The total amount of pDNA relaxed was dependent on the initial pDNA concentration: the more pDNA in the reaction vessel the more was relaxed across the FeS_{eq} concentration range 100-150 mM (Fig. 9).

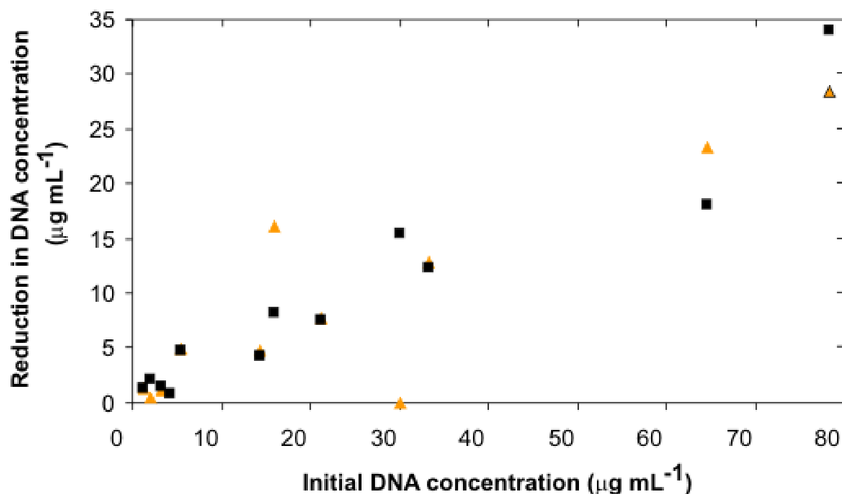

Fig 9. Graph showing total reduction in pDNA concentration against initial DNA concentration at 100 mM (▲) and 150 mM (■) FeS_{eq}. Each point represents the reduction in DNA concentration, calculated for each sample from the total of the pDNA in the relaxed and super-coiled band for each FeS + pDNA preparation subtracted from the total pDNA in the corresponding FeS-free control.

However, the percentage pDNA relaxed was constant across initial pDNA concentrations of 0.1, 1 and 10 mM FeS_{eq} (Fig. 10). On average 26.5 % of pDNA was relaxed in the FeS_{eq}-free control which rose to 37.7, 59.4 and 74.3 % for 0.1, 1 and 10 mM FeS_{eq} respectively.

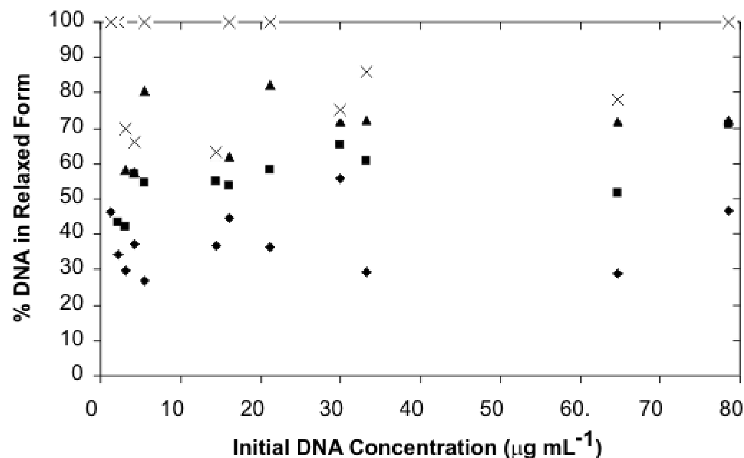

Fig 10. Graph showing FeS-induced relaxation of plasmid DNA as a function of pDNA concentration. 12 samples, with differing concentrations of DNA (1.3-78.5 μg mL^{-1}), at FeS_{eq} concentrations (0.1, 1 10, 25 mM, ♦,■, ▲, x respectively) were run on 6 different gels. Each point shows the percentage relaxation for each FeS + pDNA preparation, calculated from the amount of pDNA in the relaxed state as a percentage of the total of both the relaxed and corresponding super-coiled bands. Initial DNA concentration was calculated from the total of the relaxed and super-coiled bands in the untreated positive control which contained relaxed DNA due to the extraction technique. The percentage of pDNA relaxed was independent of initial pDNA concentration. Quantification was performed using BioLine hyper ladder 1™.

3.3. Radical reactions

The effect of 2.8 M DMSO on the reaction at 100 and 150 mM FeS_{eq} (Fig. 11) was to partially inhibit both the relaxation and loss of pDNA. More DNA is visible in the DMSO gels and more of it is in the super-coiled form A DMSO control showed no effect on FeS-free pDNA. At a lower concentration of FeS_{eq} (1 mM), 2.8 M DMSO completely inhibited the relaxation of pDNA .

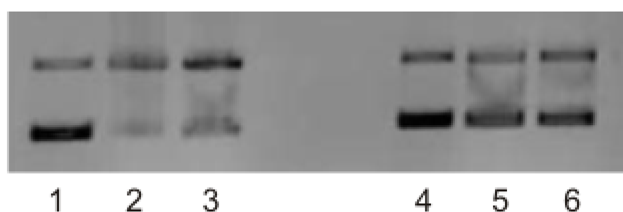

Fig 11. Effect of a free radical scavenger (DMSO) on the pDNA-FeS reaction: 1) positive control, 2) pDNA plus 100mM FeS_{eq}, 3) pDNA plus 150mM FeS_{eq}, 4) DMSO control: DNA plus 20% DMSO, 5) pDNA plus 100mM FeS_{eq} and 20% DMSO, 6) pDNA plus 150mM FeS_{eq} and 20% DMSO

The relaxation of pDNA induced by 0.1 mM FeS_{eq} was not inhibited by 1000 units mL^{-1} catalase (Table 1).

Table 1. Total pDNA and the percentage relaxed for FeS + pDNA reactions in the presence and absence of 1000 µg mL^{-1} catalase. Results represent the mean of four preparations.

	0.1 mM FeS_{eq}	0.1 mM FeS_{eq} + catalase	F value
% pDNA relaxed	46.44	39.70	5.09

The effect of catalase was analysed at 0.1 mM FeS_{eq} as enzymes suffer inhibition at high salt concentrations. In the 0.1 mM FeS_{eq} control the mean percent of pDNA relaxed was 46.4 %; in the FeS_{eq} + catalase sample a mean of 39.7 % of pDNA was relaxed. A one way ANOVA shows an F value for $p \leq 0.05$ of 6.0. Therefore, the small reduction in percentage pDNA relaxed in the catalase samples is insignificant.

Neither conducting the electrophoretic gels under anoxia (Fig. 12) nor the addition of Ti (III) citrate (Fig. 13) prevented the FeS-induced relaxation of plasmid DNA or the reduction in DNA intensity at high FeS_{eq} concentrations. A near complete loss of DNA is seen on incubation with 100 and 150 mM FeS_m and a progressive increase in the proportion of relaxed pDNA was observed from the positive control to the 50 mM sample in both experiments.

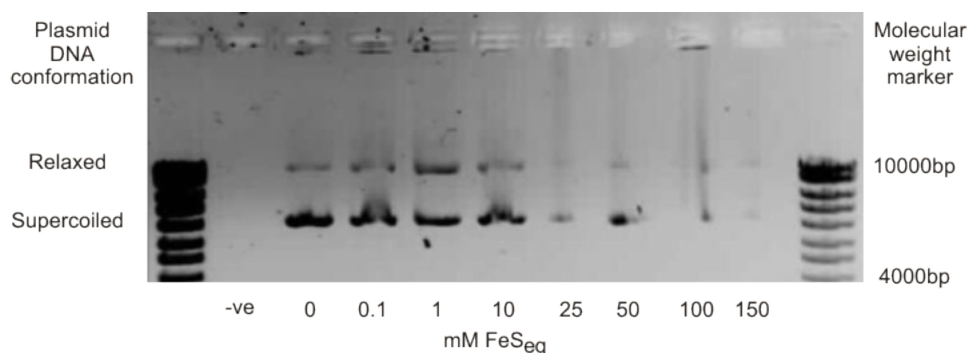

Fig 12. Image of an electrophoretic gel of FeS + pDNA conducted entirely in an anoxic cabinet at ≤ 1 ppmv O_2. FeS_{eq} concentrations are given below the image of the gel, the positions of the relaxed and super-coiled plasmid and the size of the molecular weight markers are indicated.

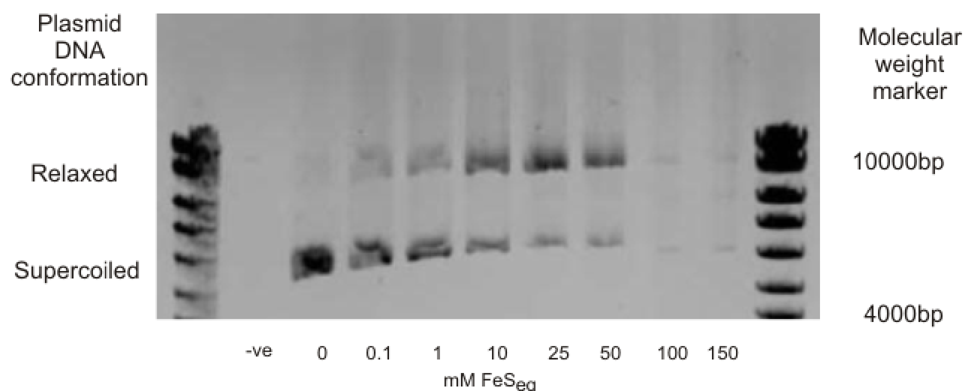

Fig 13. Image of an electrophoretic gel of FeS + pDNA + Ti(III) citrate . FeS_{eq} concentrations are given below the image of the gel, the positions of the relaxed and super-coiled plasmid and the size of the molecular weight markers are indicated.

Ultraviolet light caused no significant change in the total amount of pDNA or the percentage of the relaxed form in either the FeS samples or the FeS-free controls.

Formaldehyde (2 mM) caused no inhibition of the FeS-induced relaxation of pDNA. 47.4 % of pDNA was relaxed on incubation with 1 mM FeS_{eq} and 47.4 % was relaxed when 2 mM formaldehyde was added to the solution.

4. Discussion

On incubation with FeS solutions, plasmid DNA experience strand breaks causing it to unwind and migrate through an electrophoretic gel at a reduced rate. FeS_{eq} concentrations < 1 mM, are sufficient to induce strand breaks. The degree of relaxation increases with FeS_{eq} concentration over the range 0.1-25 mM. At $FeS_{eq} \geq 25$ mM an additional reaction occurs: the intensity of both the relaxed and super-coiled pDNA bands was reduced and the bands appeared smeared and fragmented.

In the solutions with 0.1 mM and 1 mM FeS_{eq} the S(-II) and Fe(II) concentrations are below the measured solubility of mackinawite so no precipitate was expected or observed to

form. The Fe(II) and S(-II) species were in solution mostly as aqueous clusters which can be represented as the monomer FeS^0. At this concentration and pH the speciation is determined by

$$FeS^0 + H^+ = Fe^{2+} + HS^- \qquad (3)$$

for which log K is 2.2 (Rickard et al. 2006). In the samples in which S(-II) and Fe(II) concentrations are ≥10 mM, FeS_m precipitates and the speciation is determined by Equation 2.

Aqueous FeS_{aq} clusters are not the reacting species since formaldehyde, which prevents their formation (Rickard et al. 2001), had no inhibitory effect. Despite much research on the toxicity of metals there is no known method for aqueous Fe^{2+} to directly cause DNA damage. As noted in 2.8, anoxic aqueous S(-II) does not cause relaxation of super-coiled DNA at these concentrations (Hatton 2007).

4.1. Radicals

We trapped both hydroxyl and sulfenyl radicals with DMPO. In DMF, sodium tetrasulfide is characterised by a simple Lorentzian curve, consistent with a radical anion with an electron delocalised over several sulphurs. The absence of fine structure in the ESR spectrum is expected because the S nucleus has no magnetic moment (^{32}S, I = 0; ^{33}S, I = 3/2 but only 0.75% abundant). No similar result was observed for tetrasulfide in water, in the absence of a spin trap. Photolysis of sodium tetrasulfide solution in water with elevated concentrations of DMPO results in a weak ESR spectrum which is progressively weaker as photolysis continues. These observations indicate that i) DMPO may be consumed during the production of the spin adduct, or that ii) only very small quantities of radical are produced or that iii) the radical produced is extremely transient in nature and consequently reacts only at the site of production. The latter

case is consistent with the requirement in our experiments for extremely high concentrations of DMPO in order to detect the presence of a photolytically produced sulfide radical as a spin adduct.

DMSO traps a number of free radicals including, but not only, hydroxyl radicals. DMSO inhibits the relaxation of pDNA in the presence of FeS (Fig.11). This suggests that the FeS - pDNA reaction involves free radicals. Other attributes of this reaction are consistent with the involvement of free radicals. These include the observed relaxation proportionality with pDNA concentrations at any given FeS_{eq} concentration (Fig. 14). This is consistent with a radical-mediated reaction since the radicals can react in a number of ways other than with pDNA and the probability of a reaction with pDNA is dependent on the concentration of pDNA.

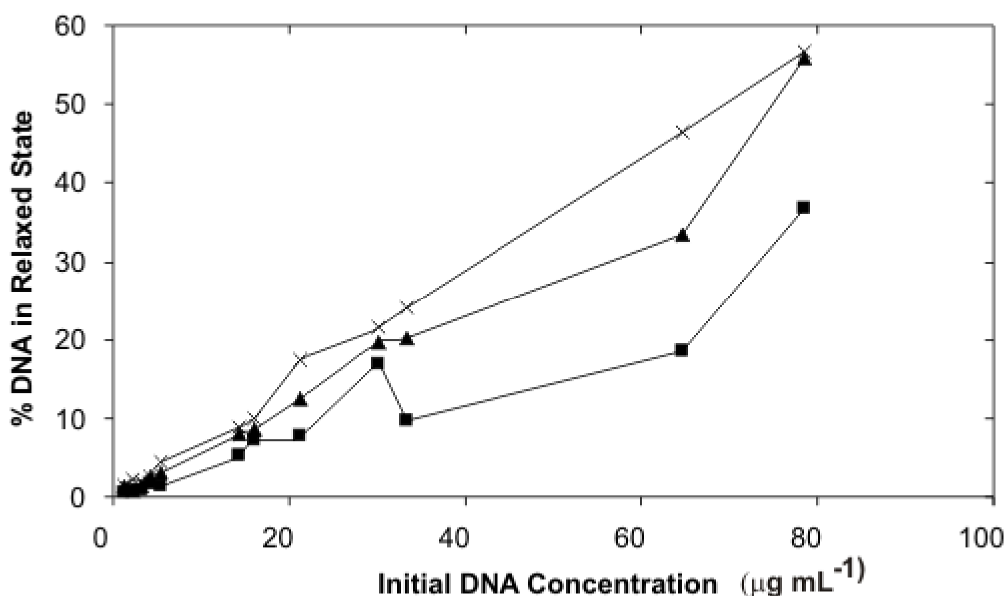

Fig 14. A chart showing the extent of relaxation of super-coiled plasmid DNA by FeS. pUC18 at varying concentrations (4.2-78.5µg mL^{-1}) was incubated with 0.1, 1 and 10mM FeS_{eq}. The graph shows the amount of pDNA in the relaxed state as a percentage of the total pDNA measured in each lane. The % relaxed pDNA in the untreated control has been subtracted from each measurement.

Our problem with the FeS-pDNA reaction is that free radicals are not detected, at least with conventional electron spin resonance spectroscopy (ESR) and the readily available spin traps. This is partly a technical problem: as we showed with the DMPO experiments, the ESR resonator was difficult to tune with both FeS_m and water in the cavity. We found no radical spin adduct produced by FeS_m in the absence or presence of hydrogen peroxide which is consistent with the results of Borda et al. (2001) – although Borda et al. suggested that this was because FeS_m does not induce hydrogen peroxide production in the first place. However, we found that when hydrogen peroxide was present, there was a Fe(III) signal suggesting that the Fenton reaction was occurring. This in turn implies that the radicals present were undetectable because of a combination of low free radical concentration and adduct formation or signal generation being interfered with by the presence of FeS_m precipitates or S(-II) which reacts with OH•.

Obviously, ESR spectroscopy can only be applied to systems in which the rates of radical decay and radical formation keeps the concentration of free radicals above the detection limit. If the free radicals are highly reactive, for example, and have short half-lives their concentrations may be too low for detection but still be adequate to nick the DNA molecules. In this sense, pDNA can act as a sensitive detector of free radicals.

Ultraviolet radiation enhances the effect of the Fenton reaction by reducing Fe(III) allowing it to react again with O_2 and/or by decomposing H_2O_2 into radicals in a non-iron dependent reaction (Ghaly et al. 2001). A similar effect was not found in this study, possibly because FeS_m strongly absorbs UV. The abundance of FeS_m over Fe(III) and H_2S_2 appears to have prevented UV light from reducing Fe(III) and decomposing H_2O_2. We were in an experimental Catch-22 situation (Heller 1961): in order to generate sufficient concentrations of

free radicals we needed a high FeS concentration. However, the high FeS concentration produced extensive nanoparticulate FeS_m which adsorbed UV.

The problem of identifying free radicals in the absence of direct ESR evidence is a familiar one. In this case, indirect evidence needs to be used. For example, Tapley et al.(1999) , did not specifically identify or trap HS• radicals. The Tapley et al. reaction for the generation of HS• radicals on the oxidation of sulfide requires a metal catalyst. We showed that the Tapley et al. reaction is not involved in the FeS – pDNA reaction since neither the reducing agent, Ti(III) citrate, nor conducting the experiment in anoxic conditions inhibited the reaction.

The Fenton reaction is the Fe^{2+}-catalysed decomposition of hydrogen peroxide into hydroxyl radicals. These hydroxyl radicals react with the back-bone of DNA causing toxic effects *in vivo* (Birnboim 1986; Imlay et al. 1988) and relaxation of pDNA *in vitro* (Henle et al. 1999; Lloyd and Phillips 1999). *In vitro* studies have shown that H_2O_2 is produced in a solution of Fe^{2+} in the presence of O_2 and will decompose into hydroxyl radicals (Toyokuni and Sagripanti 1992). Sulfide reacts with molecular oxygen, scavenging it from the system and thereby preventing reaction of Fe^{2+} with oxygen which is required for H_2O_2 generation.

The Borda et al. (2001) result might be dependent on oxygen levels, FeS_{eq} concentration, pH or other factors. So we ran a series of experiments to examine these possibilities. Catalase did not inhibit the relaxation of pDNA at 0.1 mM; as expected H_2O_2 is not produced under these conditions because molecular oxygen is not available. This is supported by the observation that Ti(III) citrate did not have an inhibitory effect either (Fig. 13).

Titanium (III) citrate is a strong reducing agent whose presence makes significant oxidation of Fe^{2+} by O_2 less probable. The relaxation of pDNA also occurred when the experiment was carried out completely in an anoxic cabinet in which the concentration of O_2 did

not exceed 1 ppmv (Fig. 12), which further suggests that the Fenton reaction is not responsible for the observed DNA damage.

The experimental observations suggest that the reaction mechanism involves short-lived radicals generated from the FeS solution without the involvement of O_2 or H_2O_2.

Our preliminary study of the aqueous tetrasulfide solutions suggest that sulfide free radicals were produced by photolysis of Na_2S_4. This result is interesting because recalculations by Rickard and Luther (2007) of stabilities in the aqueous sulfide system, which include the Kamyshny dataset for polysulfides (Kamyshny et al. 2003) show that S_2(-I) species are common and that HS_2^- is the third most abundant sulfide species (after HS^- and H_2S) in most natural waters. Thus it is possible that sufficient S(-I) is in solution to produce small but significant quantities of sulfide free radicals.

We propose that Fe^{2+} decomposes hydrogen disulfide into sulfide radicals in a similar reaction to Fenton's:

$$Fe^{2+} + H_2S_2 \rightarrow Fe^{3+} + HS^- + HS\bullet \quad (4)$$

In this scheme disulfide is formed in an anoxic environment in a similar manner to the formation of peroxide from hydrogen peroxide in oxic systems. According to the calculations of Rickard and Luther (2007) the total polysulfide activity at pH 8 under the conditions of this experimentation is $< 10^{-12}$ with H_2S_2 making up less than 0.1% of the total polysulfide activity. This suggests that the activity of HS• in reaction (4) would be relatively low at equilibrium. HS• recombines to form H_2S_2 with a rate constant of 6.5×10^9 $M^{-1}s^{-1}$ (Mills et al. 1987). These kinetic data further suggest that HS• is a transient species at low concentration. However, a single free radical can nick a deoxyribose unit and cause the uncoiling of a DNA strand (Damiani et al. 1999).

In free radical terms, the analogy with the oxygen system is striking. Thus the sulfur analog of the peroxide free radical $\cdot\ddot{\underset{\cdot\cdot}{O}}::\ddot{\underset{\cdot\cdot}{O}}\cdot$ is the persulfide free radical $\cdot\ddot{\underset{\cdot\cdot}{S}}::\ddot{\underset{\cdot\cdot}{S}}\cdot$ and S_2^- is the supersulfide radical by analogy with the superoxide free radical O_2^-. The bisulfide free radical $\cdot\ddot{\underset{\cdot\cdot}{S}}:H$ is analogous to the hydroxyl radical OH•. And, of course, the fully protonated disulfide, H_2S_2, is the sulfur analog of H_2O_2, (Winnewisser et al. 2003). The formation of sulfide free radicals in aqueous solutions by a mechanism analogous to Fenton's reaction for oxygen free radicals, is further suggested by comparative frontier molecular orbital considerations. The highest occupied molecular orbital for H_2S_2 is -9.40 eV (Cheng and Hung 1996) which compares with -10.631 eV for H_2O_2 (Litorja and Ruscic 1998); the lowest unoccupied molecular orbital for H_2S_2 is -1.907 eV (Moran and Ellison 1988) which compares with -1.089 eV for H_2O_2 (Clifford et al. 1998).

The proposed decomposition of H_2S_2 by Fe^{2+} into HS• radicals is a new reaction, although not unexpected as H_2S_2 is analogous in many ways to H_2O_2 (Winnewisser et al. 2003). It is possible that other sulfide free radicals, such as $H_2S_2^-$ which is energetically favored (Mills et al. 1987) are possible reactants. However, DMPO should have trapped $H_2S_2^-$ if this was formed in significant quantities and we would have seen this since it would produce a distinct spectrum. Furthermore, the formation of $H_2S_2^-$ normally requires the pre-existence of HS● anyway which, as we have noted, is an extremely reactive species. Other known sulfur radicals, such as SO_3• and S_2O_3• (Norman and Storey 1971), seem improbable in the FeS – pDNA system.

The mechanism by which hydroxyl radicals create strand-breaks in DNA is by abstraction of one of the hydrogen atoms from the deoxyribose moiety. Balasubramian (1998) determined that OH• can react with any of the C-H bonds in the ribose or nucleobase constituent

of the molecule. HS• radicals can also abstract hydrogen atoms from organic chemicals (Berberova and Shinkar 2000) and would cause DNA scission in the same manner.

$$SH\bullet + R\text{-}CH\text{-}R \rightarrow H_2S + R\text{-}C\bullet + R \qquad (6)$$

The stoichiometry of this reaction means that the quantity of pDNA which was relaxed by FeS should be proportional to the initial quantity of pDNA in the reaction. This is exactly what we observe experimentally (Figure 14).

4.2. Surface interactions

The earlier study by Hatton and Rickard (2008) showed that FeS_m nanoparticles bind to DNA in a manner analogous to quantum dots. They showed that the moiety that allows for the interaction with nanoparticulate transition metal sulfides is the nucleobase and that coupling occurs through electrostatic interaction. In the present study with pDNA, a similar effect is observed. Thus, although it is possible that nicking by sulfide free radicals contributes to the reduction in pDNA concentration seen at ≥ 50 mM FeS_{eq} ,it is probable that the main process which causes DNA to be retained in the well is binding of the FeS_m nanoparticles with the pDNA. The lateral streaks above the relaxed pDNA band are the result of FeS_m nanoparticles binding to pDNA, increasing its molecular weight and/or decreasing its charge creating a species with a continuum of charge/mass values lower than that of natural pDNA. The faint, thin bands formed at 150 mM (Fig.5) are dimers or trimers of plasmids connected to each other by FeS_m particles.

4.3 Implications for natural systems

We used pDNA as a reactant since this is a useful test molecule for the study of nucleic acid reactions. The primary aim of the study was to investigate if FeS reacts with polynucleic acids and not, in the first hand, aimed at probing pDNA reactions in particular. We had already demonstrated (Hatton and Rickard 2008) that nanoparticulate FeS_m binds to chromosomal DNA, oligomeric DNA, RNA, deoxadenosine monophosphate, deoxyadenosine and adenine. The present study extends this to pDNA. The observation in the present study that FeS solutions cause relaxation of pDNA molecules at relatively low concentrations, suggests that a similar effect will occur with a range of biochemicals. The pDNA we used is the result of over 3Ga of biochemical evolution and, in a Darwinian system, it is to be expected that earlier versions of this molecule would have been less stable. The consequences of the reaction for the Early Earth environment are therefore interesting. On the one hand the interaction of FeS solutions and polynucleic acids would lead to their relaxation. On the other, this could speed up the development of more stable configurations in non-sulfidic environments.

Supercoiling of DNA affects nearly all DNA-protein transactions because it influences the energetics and physical structure of the DNA molecule. So the observation of relaxation of supercoiled forms through reaction with FeS solutions has direct implications to biochemistry. However, more significantly, the result demonstrates that FeS solutions have effects similar to those of oxygen-bearing free radicals and appear to be caused by an analogous sulfide free radical process. Conventionally, it is assumed that H_2S toxicity – through H_2S reacting with the metals of electron transfer proteins – is the cause of the limited survival of many organisms in

sulfidic environments. The results of this experimentation suggest that genotoxicity in FeS-rich systems is a further contributory factor. To survive in these environments organisms need to develop systems which protect and/or isolate their polynucleotides from FeS.

The FeS concentrations within modern sediments often exceed that of the experimental systems described in this study. We therefore expect that some part of the nucleic acid polymer extracted from such biomes to be more or less affected by reactions with FeS. This may contribute to a lack of precision in the identification of the microbial ecosystem based on molecular methods.

Populations of microorgansims may exchange genetic information very rapidly through horizontal gene transfer (Lorenz and Wackernagel 1994) , the insertion of viral and other mobile elements like plasmids (Banfield and Young 2009) as well as by hereditary processes. These processes are interesting in both understanding evolutionary processes as well as being exploited in genetic engineering. They result in the transfer of genetic material between entirely unrelated species. Kandhavelu and Vennison (2008) reported the results of an experimental study of pUC18 DNA persistence in soils. They showed that pDNA persisted up to 35 days and retained its functionality. Unfortunately, the definition of soil types was sketchy and mainly color-based. However, we note that their “black soil” – which is interesting in the context of FeS (cf. Rickard and Morse 2005)– was one of the more reactive. The persistence of DNA in soil suggests a route for horizontal gene transfer in soils and, hence, sediments and other natural systems. The reaction of FeS with nucleic acid polymers would suggest that FeS-rich natural systems would be less conducive to this process.

The suggestion of this study that sulfide free radicals are formed in FeS solutions may have widespread implications for the geochemistry of FeS environments. Sulfide free radicals

are extremely reactive and are likely to be involved in a number of processes. For example, they would contribute to the mackinawite – griegite transformation and could be involved in pyrite formation as well as transformations between sulfur-bearing species. However, more work needs to be done to isolate these radicals through the the use of alternative spin traps such as DMPO , DEMPO or TMPO which have shown some general advantages for trapping sulfur based radicals (Davies et al. 1987; Karoui et al. 1996).

Finally, there also appears to be potential biomedical implications. Although it is unlikely that few individuals, apart from sulfide geochemists, are regularly exposed to FeS, it would seem possibly unhealthy to ingest too much of this material over a prolonged period of time.

5. Conclusions

Iron(II) sulfide reacts with polynucleic acids in two ways: (1) at concentrations below the solubility limit for FeS_m, nicking of the DNA molecules resulting in relaxation to more stable configurations and a consequent modification of function; (2) at concentrations above the solubility limit, FeS_m nanoparticles bind to DNA which limits the ability of DNA to interact with other nucleic acids and amino acids (Hatton and Rickard 2008).

The agent involved in the reaction between solutions of Fe(II) and S(-II) and DNA appears to be sulfide radicals. These radicals are formed in a manner analogous to the Fenton reaction for oxygen radicals. We have only managed to collect indirect evidence so far for the involvement of these sulfide radicals.

On incubation with solutions of iron (II) sulfide, pDNA experiences strand-breaks which retard its migration through an electrophoretic gel. This reaction occurs when iron and sulfur concentrations are too low for nanoparticulate FeS_m to form. It is proposed that this is not caused by the Fenton reaction or iron-catalysed oxidation of sulfide but that it is sulfide radical-mediated. The sulfide radicals are produced through the Fe(II)-catalysed breakage of disulfide bonds akin to the iron-catalysed decomposition of H_2O_2 in the Fenton reaction. This reaction would occur in any solution that contains sulfide and iron and it does not require molecular oxygen to be present.

At low FeS concentrations this may actually have assisted in genome evolution by allowing nucleic acid strands to be broken and recycled allowing novel combinations to emerge. At higher FeS concentrations nucleic acids bind to FeS_m nanoparticles.

Acknowledgements

As with much else in modern aquatic geochemistry, this study was inspired by John Morse. John was struck by the way that many sulfide reactions in nature seemed to display similar attributes to classical radical reactions. During many conversations, we discussed ways in which this idea could be probed. The work was funded by a Charles Wright Scholarship to BH from Cardiff University and NERC grant NRE/L/S/2000/00611 to DR. We thank Professor A. Weightman for assistance in the DNA experimentation. Professor George Luther III made us aware of the MO data on sulfur and hydroxy radicals.

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