Simulation of EPR spectra as a tool for interpreting the degradation pathway of hyaluronan

Simulation of EPR spectra as a tool for interpreting the degradation pathway of hyaluronan

Elena Busi,[a,*,1] Valter Travagli[b,1], Iacopo Zanardi[b], Alessandro Gabbrielli[b], Riccardo Basosi[a]

[a]Dipartimento di Chimica and [b]Dipartimento Farmaco Chimico Tecnologico, Università degli Studi di Siena, Viale Aldo Moro, 2 53100 Siena, Italy

** Corresponding author. Elena Busi, Viale Aldo Moro, 2 Tel + 39 0577 234261, fax + 39 0577 234239, e-mail: busie@unisi.it*

[1] *Both the authors equally contributed to this work*

Abstract

EPR spin trapping is the one of choice techniques to identify free radicals, and often used in the study of biological systems. However its sensitivity can result in a typical complicated EPR spectrum. The accurate simulation of these systems is essential for correct identification of the radical species, whenever more than one species contributes to the spectrum. Programs implementing the. linear combination of single simulations allow interpretation of EPR spectra, without modifying experimental conditions.

In this study this approach was used to investigate the influence of the ferrous ion and the role of oxygen as well on the formation of transient radical species in the whole mechanism of hyaluronan degradation. Degradation was carried out under different environmental conditions (air, O_2, Ar, N_2, N_2+CO_2) and EPR spin trapping studies were performed. The advantages of the simulation of multiple species EPR spectra procedure were applied to the obtained results and some aspects of hyaluronan degradation mechanism were elucidated. The depolymerization reaction pathway has been defined according to two possible subsequent steps: the first is consistent with formation of an amidyl radical that induces a series of strand scissions which stabilize at two different levels of molecular weight. The second step occurs when the molecular weight is lower than before and two different adducts are generated.

1. Introduction

EPR spin trapping is the first choice technique to identify free radicals. However in biological systems a very difficult kind of spectra are often obtained, due to the large number of signals generated by entrapment of transient radical species. In the present study the simulation of multiple species EPR isotropic spectra, via linear combination of single simulations, was used to correctly interpret all the spectra components. Two spin traps were used: the phosphorylated nitrone DEPMPO, a quite stable and versatile spin trap for a variety of radicals [1-3] and 2-methyl-2-nitroso propane (MNP). This last was chosen in order to extend the opportunities of entrapping all the possible present radicals, as MNP is particularly suitable for carbon and proton centered radicals. The combined study of DEPMPO and MNP was already successful in letting us understand the involvement of free radicals in other different degradation mechanisms.[4, 5]

The system to be investigated is the hyaluronan (HA) degradation mechanism: hyaluronan is a linear polysaccharide, made up of repeating disaccharide units of glucuronate and N-acetyl-d-glucosamine linked by alternating β(1→3) and β(1→4) glycosidic bonds.[6-8]. HA conformations in aqueous solution have been extensively investigated and hydrogen bonds have been found essential for obtaining secondary and tertiary structures. In particular, secondary structures are formed as tape-like two-fold helixes. Hydrogen-bonding interactions together with hydrophobic interactions and electrostatic repulsions lead to formation of tertiary structures in terms of HA matrices. All these aspects in physiological fluids are responsible for the mechanical functions of HA, like that of synovial fluid for shock-absorbing, structure-stabilising and protective roles as well as the more recent concerns in terms of morphogenesis, regeneration, wound healing and tumour invasion.[9] As expected, any HA inherent instability modifies these structures leading to an alteration of the HA peculiar functions. Glycosaminoglycan modification is of considerable biological significance, as the extracellular matrix plays a

key role in modulating the behavior of cells.[10] Low-molecular-mass HA oligosaccharides are known to modify macrophage expression of chemokines, cytokines, and growth factors, as well as cell-surface markers[11-15] and can attenuate the proliferation of endothelial, fibroblast, and smooth muscle cells.[16] HA molecules of lower molecular mass accumulate at sites of inflammation and tissue injury [14,17-19] as modified/fragmented versions of other matrix do. Metal ions also bind to this material[20] strongly interacting with hydrogen bonded structure. Significant levels of metal ions have been detected at sites of inflammation and macromolecule degradation,[21,22] modifying the bio-rheological properties. For these reasons the HA stability is an issue of primary interest. In rheumatoid synovial fluid, the HA weight average molecular weight is only about 4.8 MDa compared to the one of about 7 MDa in normal fluids.[23, 24] As far as it might concern irreversible denaturation, it also might be a consequence of enzymatic[25] and chemical degradation.[26] In the latter case, in fact the influence of iron is well known..[27-33] It was previously shown that one-electron reduction of glycosaminoglycan chloramides by low-valent transition metal ions (e.g. Cu^{+} and Fe^{2+}) initiates fragmentation of these polysaccharides *via* amidyl radicals formation .[34,35] Recently, HA hydrogels at concentration below the physiological conditions were incubated under different atmospheric conditions to investigate iron speciation influence on the HA degradation together with the role of the molecular oxygen. The obtained results as electrophoretic mobility and viscometric variations were reported.[36] In the present paper, chemical degradation of HA under different atmosphere conditions (air, N_2, N_2+CO_2, Ar) is evaluated. The aim of the present study on HA hydrogels is devoted to obtain useful information about:

i) influence of ferrous ions as well as of oxidative species leading to formation of hydroxyl radicals on HA degradation;

ii) nature of species formed during degradation in absence or presence of O_2;

iii) mechanism of HA degradation, as detectable by EPR spin trapping..

2. **Materials and Methods**

2.1 Biopolymer

HA used for the study (TRB Chemedica SA, Vouvry, Switzerland) was extracted from culture medium of Streptococcus Equi and was of pharmaceutical and medical device grades. The weight-average of the molecular weight of the HA samples were 1.2 MDa. The desired amount of HA in order to obtain a final concentration of 1%w/w (hereinafter referred to as **HA1%**) was dissolved overnight in the dark in saline phosphate buffer pH 7.4, in two steps. First, about half of the solvent was added in the morning. The remaining solvent was gently poured after 8 h and the resulting hydrogel was kept overnight at the dark at + 4 °C. For EPR studies, a 10-fold dilution (w/w) of **HA1%** with buffer was obtained, doubling the two steps reported above.

2.2 Chemicals

N-acetylglucosamine (NAG), desferrioxamine (DFO), 2-Methyl-2-nitroso propane (MNP), hydrogen peroxide, $FeSO_4$, and ascorbic acid and were purchased from Sigma-Aldrich. Glucuronic acid (GA) was purchased from Fluka. All substances were used without any further purification.

5-Diethoxyphosphoryl-5-methyl-1-pyrroline N-Oxide (DEPMPO) spin trap was synthesized in our laboratory following the procedure reported in literature.[37]

2.3 Degradation protocol

The ferrous sulphate solutions 40 mM were prepared in phosphate buffer 50 mM pH 7.4. All the system was in de-aerated condition. Briefly, **HA** was purged with the

various gases through a needle inserted at the bottom of the container to remove as much dissolved O_2 as possible. This purge was performed for one hour prior to each experiment at a positive pressure of 1.5 bar. At this point, ferrous sulphate degrading solution (50 μL), previously purged in the same manner, was added through the needle. Purge conditions were continued during the experiment by a gas-filled balloon inserted at the top of the sealed system. Parafilm® was used to seal the open areas, making the purge more effective. All the experiments were conducted with simultaneous stirring at a temperature of 22.0 ± 0.2 °C. As positive controls, air-stream and O_2 medical grade, were adopted while as negative control Ar (99.99%) was used. As far as N_2 atmosphere is concerned, technical N_2 (declared purity of 99.5%) and a binary system 95:5 of research nitrogen (purity: 99.9995%) and carbon dioxide (N_2+CO_2) were used.

2.4 EPR Spin Trapping Experiments

Spin trap solutions were prepared immediately before each experiment and stored in the dark, saturated by the atmosphere scheduled for the experiment. The MNP solution was obtained by carefully dissolving at 45°C, in the dark, for 30 minutes. Buffer used for dissolving MNP, was previously conditioned in a convenient atmosphere for the experiment. Degradation of HA in the presence of spin traps was carried out with the following procedure: 0.1 mL of 0.1% or 1% of HA in conditioned phosphate buffer were placed in a Eppendorf vial. 0.1 mL of 20 mM spin trap in conditioned buffer (DEPMPO or MNP) 18 mM were added, caring that no contamination of O_2 occurred during the addition. After two hours of incubation the reaction mixture was introduced in Suprasil quarts tube and followed by EPR measurements. EPR spectra obtained for all experiments were stable for hours and recorded with 15-20 scans. All blanks were performed in the same conditions (buffer and DEPMPO or NMP, buffer and HA).

Chemical oxidation of monomers (GA and NAG) was performed producing hydroxyl radical via a Fenton reaction and the conditions were as follows: 0.1 mL of 1 mM of monomer in phosphate buffer were placed in a Eppendorf vial. 0,1 mL of 18 mM DEPMPO in distilled water, 0.05 mL 0.5 mM ferrous sulphate in distilled water and 0.05 mL of H_2O_2 in phosphate buffer were added.

2.5 EPR measurements and simulations

Instrument conditions were as follows: X-Band EPR e500 Bruker Elexys, modulation amplitude 1 G, time constant 1.28 ms, receiver gain 85 dB, microwave power 1.2 mW to prevent saturation effects. Simulations of EPR spectra were obtained using a computer program made in our laboratory.[4]

2.6 pH measurements

Sample *p*H in various atmospheres were performed at the end of incubation condition. *p*H's were measured using a Ino Lab WTW *p*Hmeter, equipped with a VWR electrode.

2.7 Viscosity measurements

After purging operations previously indicated, the **HA** viscosity values are evaluated by Viscomate VM-10AL (CBC Materials CO., Ltd Japan), and the mean values ± SD are calculated. Thus, the viscosity at this point is considered 100% and the degradation profile was assessed by measuring the viscosity changes as a function of time.

According to the aim of this investigation, the molecular weight of the degraded HA samples was not evaluated.

3. Results and Discussion

The presence of a transient radical species was checked and studied, to verify a possible common mechanism for the different atmosphere environments, i.e. Ar, air, N_2 and N_2+CO_2.

First, it was verified that in absence of Fe(II), even in traces, the degradation reaction does not take place. In fact incubating HA1% in air atmosphere and monitoring the viscosity (as reported in experimental section) the initial viscosity changes from 100% to 10% in about 10 hours. This evidence shows that a depolymerization of HA occurs. The viscosity, in the same conditions, remains constant if, before the incubation, an excess of dexferoxamine (a strong Fe(II) chelator) is added.

Therefore this makes evident that the presence of Fe(II) is fundamental for the occurring of the degradation reaction.

To reveal and identify short-life radical species and to follow the degradation reaction of HA , EPR spin trapping in presence of $FeSO_4$ was applied. Furthermore controls with **HA1%** in Ar and in air atmosphere were performed, to check the possible variation of transient species composition, with a change in viscosity.

Since all blank EPR experiments (i.e. HA and spin trap, in absence of iron species; HA and iron, in absence of spin traps; spin trap and iron in absence of HA) have shown to be EPR silent, formation of a radical species can be only ascribed to radical intermediates formation due the degradation reaction.

Changing spin trap, concentration of HA, atmosphere conditions, the entrapped species are different and all results are summarized in the table 1.

Spin Trap	Atmospher/ conditions	Reagents/ concentration	Trapped Species
MNP	N_2+CO_2	0.1% HA	H· + DTBN
MNP	Ar	0.1% HA	H· + DTBN
MNP	Ar	1% HA	H· + DTBN
MNP	N_2	0.1% HA	DTBN

MNP	Air	0.1% HA	none
MNP	Air	1% HA	H· + DTBN
DEPMPO	N_2+CO_2	0.1% HA	none
DEPMPO	Ar	0.1% HA	none
DEPMPO	Ar	1% HA	none
DEPMPO	N_2	0.1% HA	A + B
DEPMPO	Air	0.1% HA	A + B
DEPMPO	Air	1% HA	A + B
DEPMPO	Air/Fenton	1mM NAG	A=NAGO$^{\cdot}$+$^{\cdot}$OH
DEPMPO	Air/Fenton	1mM GA	GAO$^{\cdot}$+$^{\cdot}$OH

Table 1 – Summary of spin trapping agents, system conditions and entrapped species.

In Figure 1, the EPR spectrum in Ar atmosphere in presence of MNP paired with its simulation is reported.

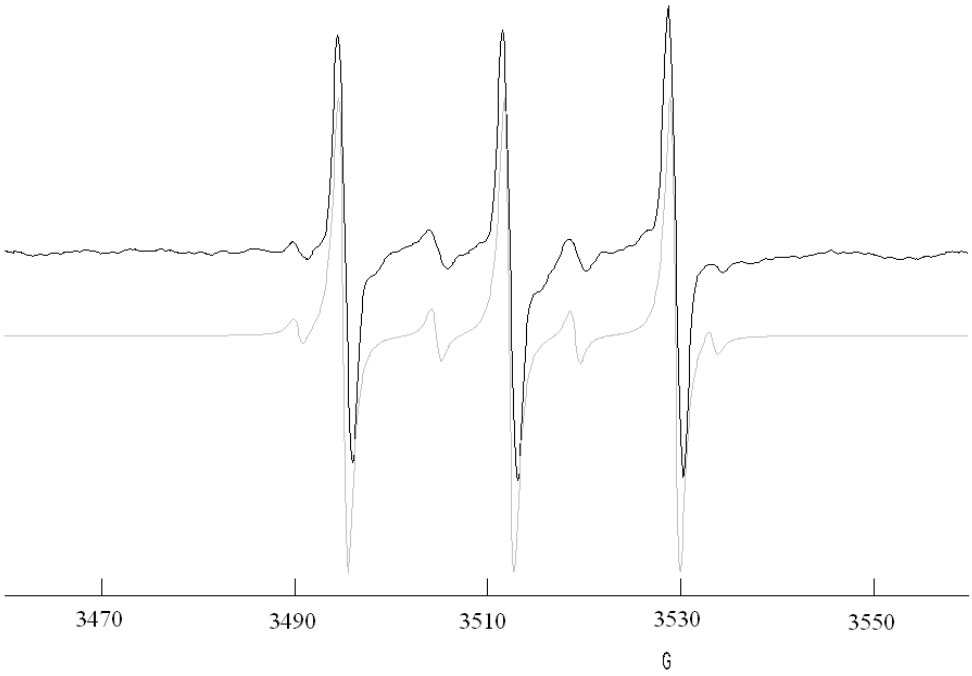


Fig. 1. Experimental EPR X-band spectrum of MNP-adducts of incubated HA0.1% in Ar atmosphere, in presence of MNP (black line), paired with the simulation (grey line) which gave the best fit. For experimental details and spectrometer settings see Experimental Section. Representative spectra from multiple experiments are shown. The simulated spectrum is obtained by linear combination of two spectra: simulation spectrum of a species I (10%) and of a species II (90%), whose corresponding magnetic

parameters are:: species I (A_N = 14.4±0.2 G; A_H = 14.0±0.2 G); species II (A_N = 14.2±0.2 G).

Species I showed the same magnetic parameter of the adduct of MNP with the H· radical.[36, 38] This result is consistent with a mechanism in which, as a first step, the abstraction of an H· from the polymer takes place.

Species II appeared to have the same magnetic parameters of DTBN, as reported in literature,[36, 39] which is a degradation species formed during the incubation in presence of other radicals.

The same experiments in presence of DEPMPO did not give a detectable EPR spectrum. This result means that in complete absence of O_2, no O-centered radical intermediates was found in the degradation reaction.

When the degradation reaction was conducted in air, different results in the spin trap experiment were obtained in presence of both MNP and DEPMPO, even in the case of **HA1%**. In particular, experiments performed in presence of MNP showed the absence of adducts when they were performed in air atmosphere, while the same adducts of N_2+CO_2 or Ar of Figure 1 were found in case of **HA1%**. Therefore there is evidence that the presence of O_2 disturbed in some way adducts detection, but they become visible when protected by the viscosity of the **HA1%** solution.

The effect of oxygen on adducts detected with MNP is not surprising. This trap does not give readily detectable adducts with oxygen-centered radicals that are likely to form when carbon-centered radicals are generated in presence of O_2.

On the other hand, in presence of DEPMPO two radicals were trapped, as reported in Figure 2.

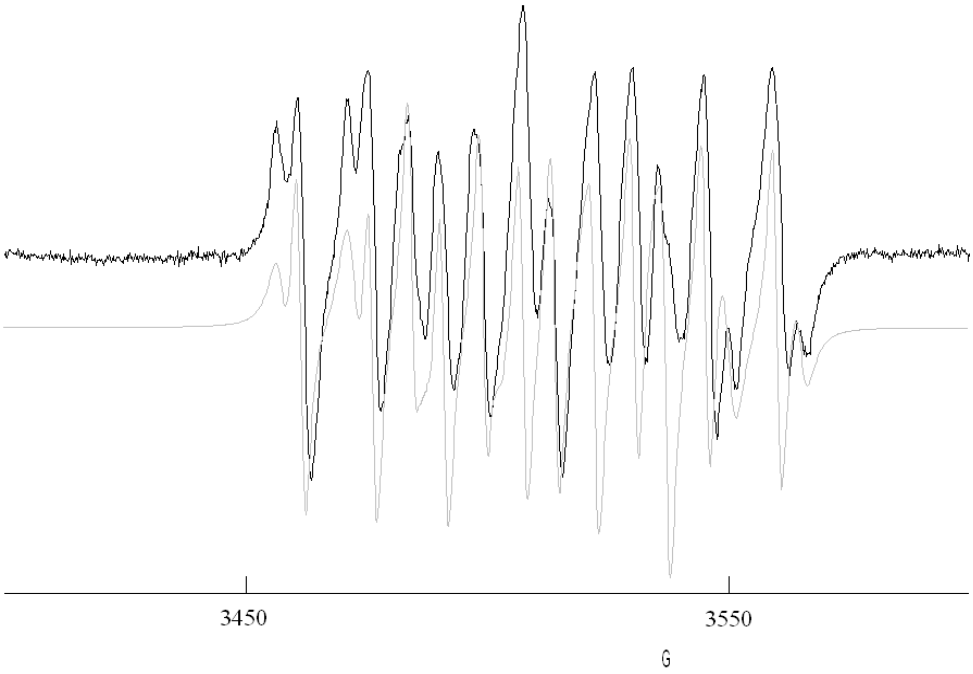


Fig. 2. Experimental EPR X-band spectrum of DEPMPO-adducts of incubated HA 0.1% in air atmosphere, in presence of DEPMPO (black line), paired with the simulation (grey line). For experimental details and spectrometer settings see Experimental Section. Representative spectra from multiple experiments are shown. The simulated spectrum is obtained by linear combination of the simulated spectrum of a species A (70%) and a species B (30%), whose corresponding magnetic parameters are: species A (A_N = 14.9±0.2 G; A_H = 23.0±0.2 G; A_P = 45.8±0.2G); species B (A_N = 14.4±0.2 G; A_H = 15.6±0.2 G; A_P = 63.0±0.2G).

The simulated spectrum is obtained by linear combination of two spectra of species A (70%) and species B (30%). In order to identify the two radical species, Fenton reactions were carried out on both GA and NAG monomers. Figure 3 shows the experimental spectrum paired with its best simulation obtained after the Fenton reaction in presence of GA.

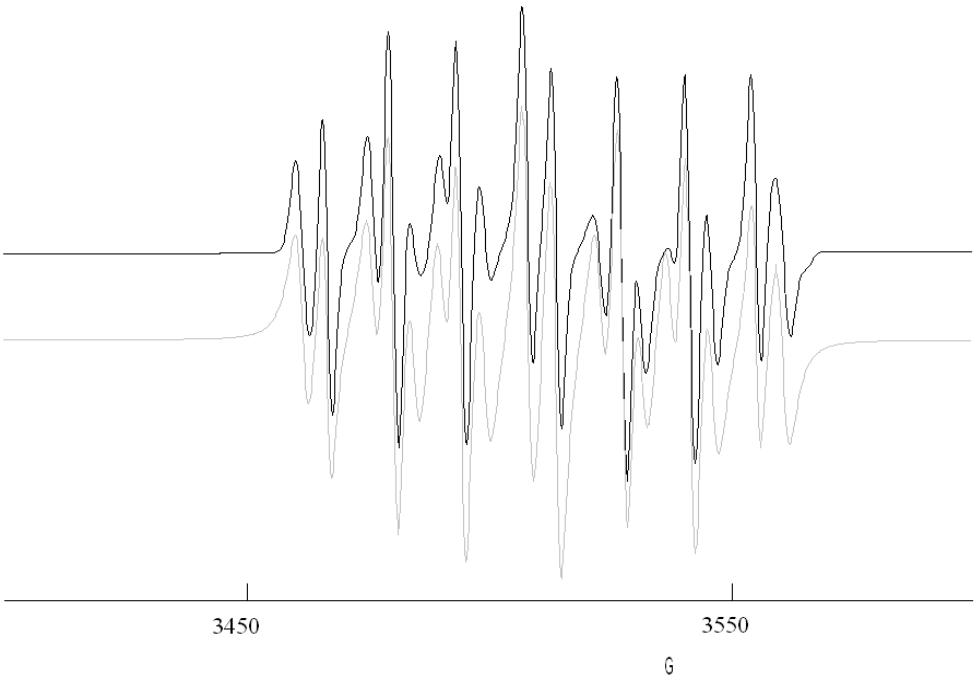


Fig. 3. Experimental EPR X-band spectrum of DEPMPO-adducts of incubated GA monomer in air atmosphere, in presence of DEPMPO (black line), paired with simulation (grey line). For experimental details and spectrometer settings see Experimental Section. Representative spectra from multiple experiments are shown. The simulated spectrum is obtained by linear combination of two spectra of the above mentioned species (30%), A_N *= 14.4±0.2 G;* A_H *= 23±0.2 G;* A_P *= 47.2±0.2G) and a DEPMPO-·OH (70%).*

The simulated spectrum was obtained by linear combination of the simulated spectrum of an unknown species (30%) and the DEPMPO-·OH (70%) species whose parameters are known from literature.[40] The DEPMPO-·OH species was formed during the Fenton reaction, because of the use in excess of H_2O_2.

Similar results are obtained in the case of Fenton reaction on NAG and reported in Fig.4.

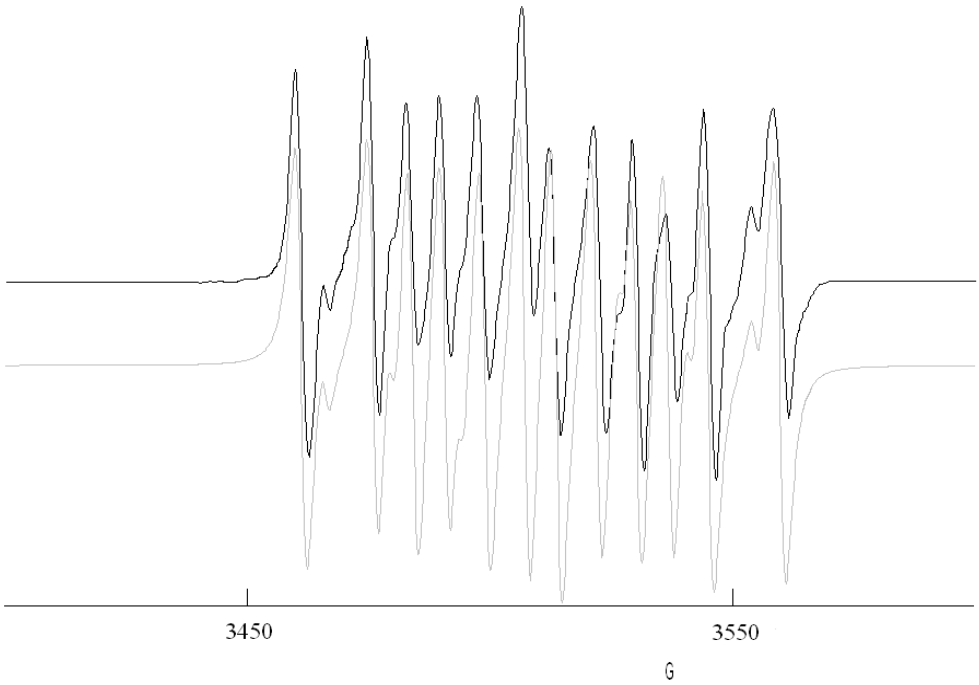


Fig. 4. Experimental EPR X-band spectrum of DEPMPO-adducts of incubated NAG monomer in air atmosphere, in presence of DEPMPO (black line), paired with its simulation (grey line). For experimental details and spectrometer settings see Experimental Section. Representative spectra from multiple experiments are shown. The simulated spectrum is obtained by linear combination of the simulation spectrum of DEPMPO-˙OH (20%) and an unknown species (80%) (A_N = 14.02±0.2 G; A_H = 13.2±0.2 G; A_P = 47.3±0.2G).

The simulated spectrum was obtained by linear combination of the simulated spectrum of an unknown species (80%) and the DEPMPO-˙OH (20%) species whose parameters are known from literature.[40] The unknown species shows the following parameters A_N = 14.02±0.2 G; A_H = 13.2±0.2 G; A_P = 47.3±0.2G, which are coincident with the species A previously entrapped in the HA degradation.

The case of technical N_2 atmosphere is somewhat intermediate between N_2+CO_2 and air atmosphere situation. Under this environment, O_2 was present in traces, but continuously supplied to the system by the connected flow.

Therefore in the presence of MNP, only DTBN was trapped because of O_2 reacted with H· before than the spin trap. In presence of DEPMPO both the species A and B were found, because the amount of O_2 was enough stoichiometrically.

As a summary of the EPR experiments regards degradation of HA and monomers NAG and GA in different atmosphere conditions were reported in Table 1.

Some conclusion can be draw from the experiments which led to two interesting results:

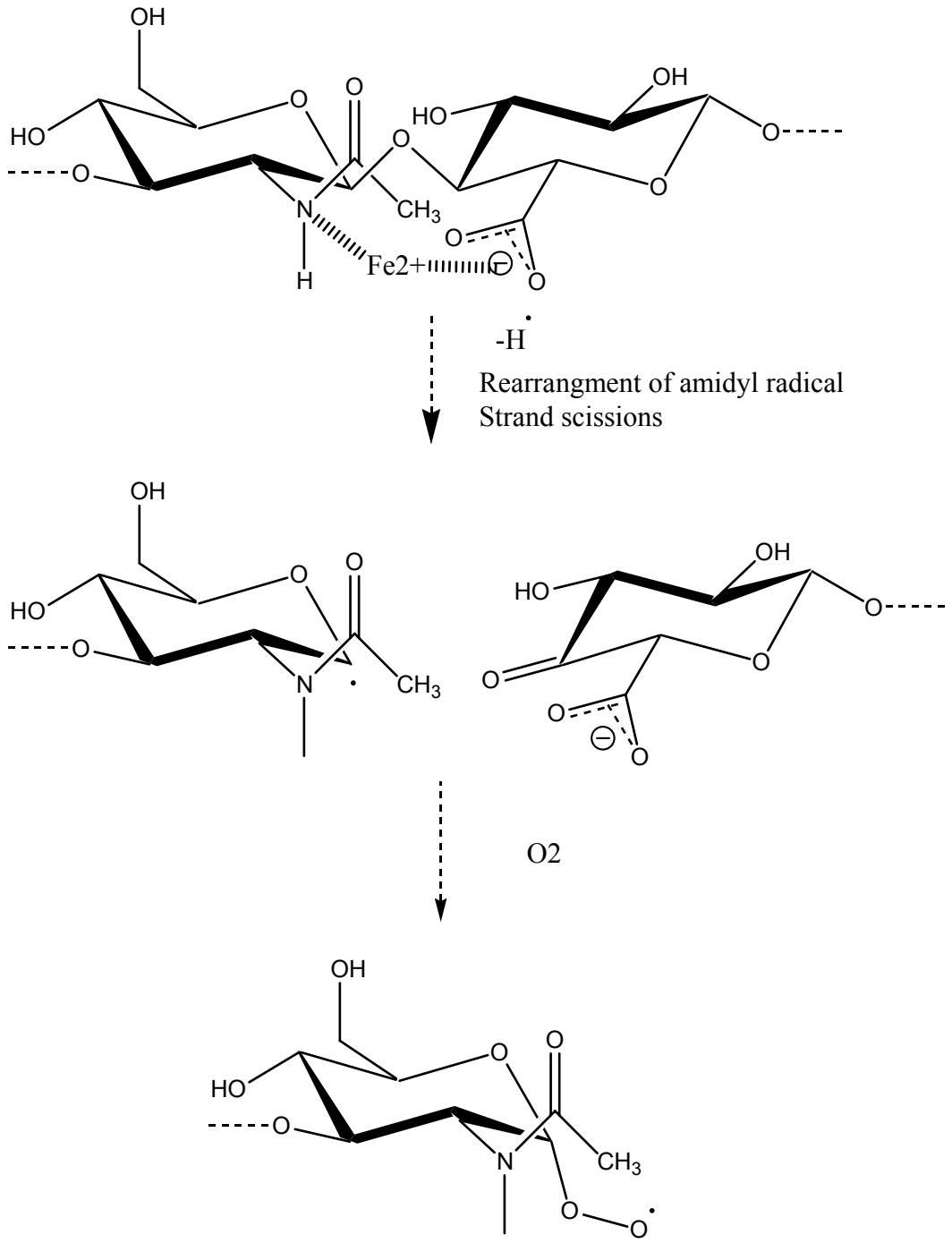
i) Both the trapped species, leading to fast motion EPR isotropic spectrum, are attributable to low molecular weight substances;

ii) The role of different atmosphere conditions on the molecular mechanism of degradation gives us the possibility of differentiating the degradation pathways.

This is in agreement with literature, as previously reported for HA in presence of NaOCl[36]. No radical was detected on the polymer with any kind of spin traps; the formation of an amidyl radical induces a series of strand scissions which might stabilize at two different levels of molecular weight: when the equilibrium is reached the depolymerization proceeds further, but with a change in the rate. It is apparent that, even if NaOCl is absent, the metal can act as a catalyst for the H· abstraction, probably because it locates between the amidyl–N and the glucuronic–COO^-, rupturing the H-bond with water.[41]

The O_2, when present, enters only at the end of the mechanism, when its reaction with the radical centers is kinetically favored, leading to the two low molecular radical species trapped with DEPMPO. One of these two species was found to be a radical intermediate of oxidized NAG (the same product found after Fenton reaction). This result means that the degradation of the polymer goes through a depolymerization of GA-NAG dimer blocks, as evidenced by the fact that one of the species entrapped during the degradation

of HA is found to be a radical intermediate of oxidized NAG. Such an interpretation is also in agreement with mechanisms previously reported in literature, in which it was proposed that the radical present in the system, after the strand scission, is centered on the NAG moiety.[35,36] The radical intermediate is probably derived by a different rearrangement of the amidyl radical, which can evolve through 1,5-hydrogen shift to a C-1 of NAG.[35] In fact, in aqueous solution a water molecule has been proposed to be involved in the secondary structure by forming an hydrogen-bonded bridge. This particular arrangement between the groups, allows the amide side chain to assume the *trans* conformation.[41] Iron can exchange with water, in a solvent exposed vulnerable position of the polymer, promoting the H· abstraction and strand scission. Scheme 1 reports the proposed mechanism for the strand scission reaction.



Scheme 1. Proposed strand scission reaction an the basis of EPR results.

Subsequent strand scissions would lower the molecular weight of the polymer, until O_2 reacts with the radical centered on C1, giving one of the species entrapped with DEPMPO, that shows the same magnetic parameters of the entrapped species of the Fenton reaction on NAG. The hypothesis of a radical centered on the C1 position is supported by mass spectroscopy results,[31] in which Fenton oxidation main product for

NAG is 2-amino-2-deoxy gluconic acid (oxidation product of NAG aldehyde in C1 to corresponding acid), and by molecular modeling.[42]

The second entrapped radical might come from an alternative intramolecular rearrangement giving a C4 or C1 centered radical on the adjacent glucuronic acid residue,[35] similarly to the degradation of HA chloramides. However the C1 position seems somewhat less probable because the radical intermediate of the Fenton reaction does not match with this species: the C1 position is the most likely oxidized intermediate since the main oxidation product during the Fenton reaction on GA is glucaric acid as oxidation product of GA aldehyde in C1 to corresponding acid.[31]

Comparison of the intensities of the EPR spectra obtained via the Fenton reaction on GA and NAG proves that radicals entrapped during the degradation reaction of HA are not secondary (or breakdown species), as their concentration is about the same order of magnitude of the reactant species.

Moreover, if the degradation reaction is taken in presence of ascorbate, which is well known to be a radical and oxygen scavenger, the depolymerization of HA happens efficiently (in fact viscosimetric measurements show that initial viscosity decreases from 100% to 10% after 10 hours in air atmosphere). Although the degradation reaction occurs, in presence of ascorbate, no intermediate oxygen radical species can be trapped. This could mean that ascorbate reacts with radical intermediates before than spin traps and when the degradation reaction of HA is irreversible.

This fact is further confirmed by the entrapment of H· radical during the reaction in air and **HA1%** demonstrating that in aerobic environment the system involves an initial no-oxygen transient species, which is detectable, in this case, because of a reduced diffusion kinetics of O_2 in a more viscous environment, where the MNP is rather soluble.

Conversely, technical N_2 is a deceptive system, because the trace amount of O_2 (≈5 μM) in the total flux of hours, results enough for a stoichiometric reaction with H·

radical, but it is kinetically less efficient, therefore only the degradation compound of MNP adduct (DTBN) is detectable.

4. Conclusion

In conclusion, analyzing EPR spectra at different environmental conditions, the depolymerization mechanism of HA can followed in two steps:

i) In the first step, formation of an amidyl radical, catalyzed by the Fe^{2+} interacting with the glucurate COO^- moiety, induces a series of strand scissions: when the equilibrium is reached the depolymerization proceeds further, but with a change in the rate according to the different atmospheres.

ii) In the second step, when the molecular weight is lower, the reaction with O_2 is favored. Therefore, when O_2 is present, it reacts with the carbon centered radicals of the system, giving two different low molecular weight adducts. One of these adducts is the same of that obtained via a Fenton reaction on NAG, probably located on the C1 atom. The second adduct might then be ascribed to a different rearrangement of the amidyl radical on the NAG or GA moiety.

Abbreviations: HA, hyaluronic acid; NAG, N-acetylglucosamine; DEPMPO, 5-Diethoxyphosphoryl-5-methyl-1-pyrroline N-Oxide; MNP, 2-methyl-2-nitrosopropane; DFO, desferrioxamine; DTBN, di-tert-butylnitroxide radical; GA, Glucuronic acid; EPR, electron paramagnetic resonance;

Acknowledgments

This study was supported by the PRIN 2007, Ministero dell'Università e della Ricerca, Italia and PAR 2006 (Piano Ateneo della Ricerca, Università di Siena).

References

1 Roubaud V., Sankarapandi S., Kuppusamy P., Tordo P., Zweier J. L.: Anal. Biochem., **247,** 404-411 (1997)

2 Tuccio B., Lauricella R., Frejaville C., Bouteiller J. C.: J. Chem. Soc. Perkin Trans. 2, **15**, 295-298 (1995)

3 Karoui H. C., Hogg N., Frejaville C., Tordo P., Kalyanaraman B.: J. Biol. Chem., **271**, 6000-6009 (1996)

4 Busi E., Howes B. D., Pogni R., Basosi R., Tinoco R., Vasquez-Duhalt R.: J. Mol. Catal. B Enzym., **9**, 39-48 (2000)

5 Busi E., Sinicropi A., Terzuoli L., Marinello E., Basosi R.: Appl. Magn. Reson., **31** ,471-482 (2007)

6 Lapcíc L. J., Lapcíc L., De Smedt S., Demeester J., Chabrecek, P.:; Chem. Rev., **98**, 2663-2684 (1998)

7 Fischer E., Callaghan P. T., Heatley F. , Scott J. E.: J. Mol. Struct., **603**, 303-311 (2002)

8 Haxaire K., Maréchal Y., Milas M., Rinaudo M., Biopolymers (Biospectroscopy), **72,** 10-20 (2003)

9 Liao Y. H., Jones S. A., Forbes B., Martin G. P., Brown M. B.: Drug Deliv., **12**, 327-342 (2005)

10 Raines E. W.: Int. J. Exp. Pathol., **281,** 173-182 (2000)

11 Noble P. W. L., Lake F. R., Henson P. M., Riches D. W.: J. Clin. Invest., **91**, 2368-2377 (1993)

12 Horton M. R., McKee C. M., Bao C., Liao F., Farber J. M., Hodge DuFour J. P., Pure E., Oliver B. L., Wright T. M., Noble P. W.: J. Biol. Chem., **273**, 35088-35094 (1998)

13 Horton M. R., Boodoo S., Powell J. D.: J. Biol. Chem, **277,** 43757-43762 (2002)

14 McKee C. M., Penno M. B., Cowman M., Burdick M. D., Strieter R. M., Bao C., Noble P. W.: J. Clin. Invest., **98**, 2403-2413 (1996)

15 McKee C. M., Lowenstein C. J., Horton M. R., Wu J., Bao C., Chin B. Y., Choi A. M., Noble P. W.: J. Biol. Chem., **272**, 8013-8018 (1997)

16 West D. C., Kumar S.: Exp. Cell. Res., **183**, 179-196 (1989)

17 Balazs E. A., Watson D., Duff I. F., Roseman S.: Arthritis Rheum., **10**, 357-376 (1967)

18 Bjermer L., Lundgren R., Hallgren R. : Thorax, **44**, 126-131 (1989)

19 Horton M. R., Burdick M. D., Strieter R. M., Bao C., Noble P. W.: J. Immunol., **160**, 3023-3030 (1998)

20 Barbucci R., Magnani A., Lamponi S., Mitola S., Ziche M., Morbidelli L., Bussolino F.: J. Inorg. Biochem., **81,** 229-237 (2000)

21 Yuan X. M., Brunk U. T.: APMIS, **106**, 825-842 (1998)

22 Sayre L. M., Perry G., Harris P. L., Liu Y.,. Schubert K. A, Smith M. A.: J. Neurochem., **74**, 270-279 (2000)

23 Al-Assaf S., Navaratnam S., Parsons B. J., Phillips G. O.: Free Rad. Biol. Med., **40**, 2018-2027 (2006)

24 Prehm P., in: Biopolymers: biology, chemistry, biotechnology, applications, Polysaccharides I. Polysaccharides from prokaryotes, ed. E. J. Vandamme, S. De Baets, A. Steinbüchel, Wiley-VCH: Weinheim, vol. 5, pp. 379–404 (2000)

25 Maccari F., Tripodi F., Volpi N., Carbohydr. Polym., **56**, 55-63 (2004)

26 Šoltés L., Brezová V., Stankovská M., Kogan G., Gemeiner P.: Carbohydr. Res., **341**, 639-644 (2006)

27 Herp A., Harris M., Rickards T.: Carbohydr. Res., **16**, 395-407 (1971)

28 Kennedy J. F., Cho Tun H.: Carbohydr. Res., **22**, 43-51 (1972)

29 Wong S. F., Halliwell B., Richmond R., Skowroneck W. R.: J. Inorg. Biochem., , **14,** 127-134 (1981)

30 Uchiyama H., Dobashi Y., Ohkouchi K., Nagasawa K.: J. Biol. Chem., **265**, 7753-7759 (1990)

31 Jahn M., Baynes J. W., Spiteller G.: Carbohydr. Res., **321**, 228-234 (1999)

32 Yamazaki K., Fukuda K., Matsukawa M., Hara F., Yoshida K., Akagi M., Munakata H., Hamanishi C.: Pathophysiology, **9**, 215-220 (2003)

33 Kennett E. C., Davies M. J.: Free Rad. Biol. Med., **42**, 1278-1289 (2007)

34 Hawkins C. L., Davies M. J.: Free Rad. Biol. Med., 24**,** 1396-1410 (1998)

35 Rees M. D., Hawkins C. L., Davies M. J.: J. Am. Chem. Soc., **125**, 13719-13733 (2003)

36 Rees M. D., Hawkins C. L., Davies M. J.: Biochem. J., **381**, 175-184 (2003)

37 Frejaville C., Karoui H., Tuccio B., Le Moigne F., Culcasi M., Pietri S., Lauricella R., Tordo P.: J. Med. Chem., **38**, 258-265 (1995)

38 Thiery C. J., Agnel J. P. L., Frejaville C. M., Raffi J. J.: J. Phys. Chem., **87**, 4485-4488 (1983)

39 Arroyo C. M., Mak I. T., Weglicki W. B.: Free Radic. Res. Commun., **5**, 369-376 (1989)

40 Frejaville C., Karoui H., Tuccio B., Le Moigne F., Culcasi M., Pietri S., Lauricella R., Tordo P.: J. Chem. Soc. Chem. Commun., **15**, 1793-1794 (1994)

41 Blundell C. D., De Angelis P. L., Almond A.: Biochem. J., **396**, 487-498 (1996)

42 Adrian-Scotto M., Abdallah K. B., Mallet G., Vasilescu D.: J. Mol. Struct. (Theochem), **636**, 89-113 (2003)