Rate and Rate-Determining Step of Hydrogen-Atom-Induced Strand Breakage in Poly(U) in Aqueous Solution under Anoxic Conditions

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Dedicated to Professor Dr. D. Schulte-Frohlinde *on the Occasion of His 60th Birthday*

Pulse Radiolysis, Poly(U), Hydrogen-Atoms, Strand Breakage, DNA Models

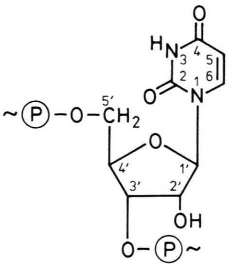
The rate constant for strand breakage in poly(U) after reaction with hydrogen atoms in deoxygenated aqueous solution has been determined to be $k = 1.5\ \mathrm{s}^{-1}$ at pH = 4–5 and 24 °C. Dithiothreitol has been found to prevent strand break formation by reacting with H-adduct radicals of poly(U) with a rate constant of $5 \times 10^6\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$. It is concluded that the rate-determining step in H atom-induced strand breakage in poly(U) at pH ≦ 6 is the decay of uracil moiety H-adduct radicals via H-abstraction from the ribose moiety.

Introduction

Radiation-induced damage of DNA is considered to be mainly responsible for cell inactivation by high-energy radiation. If the DNA damage is caused by the reaction of radiation-generated radicals with DNA, the lesions are said to be produced by the indirect effect. An important type of lesion which can be formed indirectly are strand breaks, *i.e.* cleavages of the main chain of DNA.

In earlier investigations of strand-breaking processes poly(U) (**1**) has been used as a DNA model [1–3] because it is simpler in many respects: it is single stranded at room temperature, it has no base stacking [4] and it carries only one kind of base (uracil). It has turned out that strand breaks in poly(U) are formed predominantly via base radicals as intermediates, i.e. a reaction has to occur in the course of which the radical site is transferred from the base to the main chain.

H atoms for example induce strand breaks in poly(U) with 19% efficiency [2]. As was deduced from the low yield of H_2 [5], H-abstraction by H atoms from the sugar moiety takes place only to a very small extent (≦ 5%). Accordingly, the first step in the reaction of H atoms with poly(U) is addition to the double bond of the uracil moiety of poly(U). Therefore uracil H-adduct radicals (**2**) have to be considered as the first intermediates in the strand breaking process.



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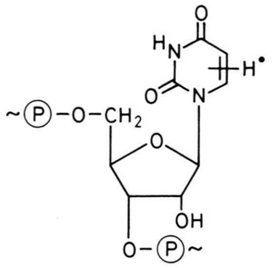

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With OH radicals the situation has been found to be similar [2], i.e. radicals formed by OH reaction with the uracil moiety have been shown to produce strand breaks. In this case evidence has been presented that the rate of strand break formation is determined by the (acid-catalysed) hydrolysis of a sugar-derived radical [1]. The results implied: (i) that the transfer of the radical site to the sugar phosphate backbone consists of an H-abstraction by base radicals from the ribose moiety, most likely from C(4′) or C(2′), thereby forming the corresponding radicals **3** and **4** (U denotes the uracil moiety of poly(U)) and (ii) that this H-abstraction of the OH adduct radical is fast, *i.e.* it is not rate-determining. It has further been shown that the ribose-derived radicals which produce strand breaks

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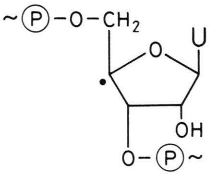

C(4′) radical

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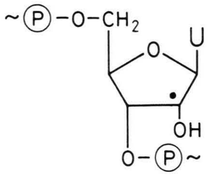

C(2′) radical

4

react with dithiothreitol (DTT) with an average rate constant of 1.7×10^6 M^{-1} s^{-1} [1].

Evidence for the transfer of the radical site from a uracil moiety to a ribose moiety has also been found with poly(U) in the presence of oxygen [3, 6] under conditions of OH radical initiation. Here the rate-determining step is H-abstraction at the ribose moiety by a base derived peroxyl radical [6].

In this work it will now be shown that strand break formation in poly(U) after H atom initiation is similar in mechanism but different in rate and rate-determining step as compared to OH radical initiation.

Results and Discussion

a) Kinetics of the conductivity increase

Aqueous, Ar-saturated solutions containing *t*-butanol (2×10^{-2} M) and polyuridylic acid (poly(U); 2×10^{-4} M in nucleotide subunits) have been pulse-irradiated with 0.4 μs electron pulses and the conductivity change of the solution has been recorded as a function of time after the pulse, see *e.g.* Fig. 1. It can be seen, that the conductivity increases to a constant value within a couple of seconds.

From standard competition kinetics it is estimated that in such solutions ≈ 98% of the OH radicals react with *t*-butanol that at pH = 4 about 94% of the solvated electrons (e^-_{aq}) react with protons to give H atoms* and that more than 90% of the H atoms react with poly(U). In the estimations the same rate constants from the literature were used as mentioned in [2], except for k(H+poly(U)), where the average value of 5.5×10^8 $\text{M}^{-1}\text{s}^{-1}$ taken from recent investigations was used [5]. From this it follows, that the conductivity increase shown in Fig. 1 is mainly initiated by H atoms. As has been demonstrated earlier [1, 7], the conductivity increase is the result of strand break formation: with decreasing chain length the activity of the counterions of poly(U) and accordingly also the conductivity is increased.

The overall first half-life of the conductivity build-up amounts to $t_{1/2} = 0.45$ s. It was found to be independent of dose/pulse in the range 3.5−28.0 Gy. This shows, that the conductivity build-up is determined by first-order reactions. However, similarly as has been found earlier with N_2O-saturated [1] and O_2-containing [6] solutions, the kinetics of the conductivity increases cannot be described by one first-order rate constant only. The rate of the conductivity build-up decreases with increasing time. The second and third half-lives for example have been found to be 0.6 s and 0.8 s respectively.

Right after the pulse there has also been observed a fast initial increase of conductivity with $t_{1/2} \leqq 5$ ms. It contributed approximately 3−6% to the total conductivity increase except at the lowest pH value measured (pH = 2.6) where it made up ≈ 20%. This fast component will not be discussed in this work and the half-lives presented always refer to the slow and predominant part of the build-up.

b) pH dependence of the rate

Figure 2 presents the pH dependence of the first-order rate constant $k = t_{1/2}^{-1} \cdot \ln 2$ of the conductivity increase observed in pulse radiolysis of Ar-saturated solutions containing poly(U) (2×10^{-4} M) and

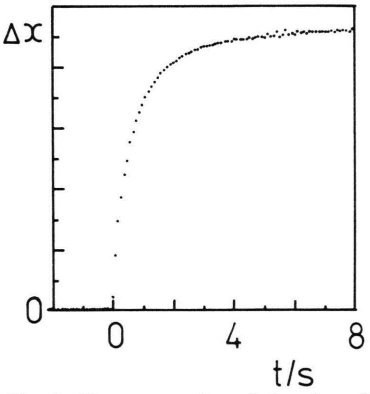

Fig. 1. Computer plot of the time dependence of conductivity (in rel. units) as observed in pulse radiolysis of an aqueous, Ar-saturated solution of poly(U) (2×10^{-4} M in nucleotides) and *t*-butanol (2×10^{-2} M) at 24 °C and pH = 4.0 after a 0.4 μs pulse of 6.8 Gy.

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* Upon irradiation of diluted aqueous solutions OH radicals, H atoms and e^-_{aq} are formed with yields of around 2.8×10^{-7} M · Gy^{-1}, 0.57×10^{-7} M · Gy^{-1} and 2.7×10^{-7} M · Gy^{-1} respectively.

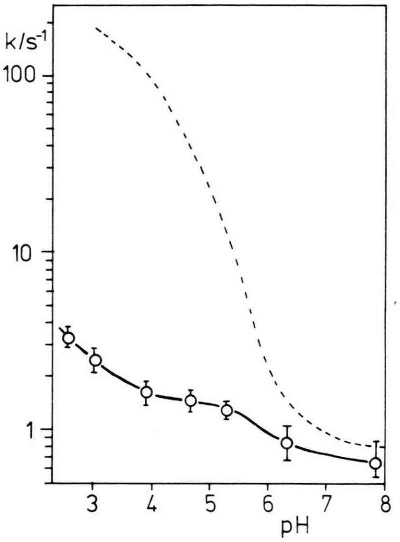


Fig. 2. pH Dependence of the rate constant $k = t_{1/2}^{-1} \cdot \ln 2$ of the slow component of the conductivity increase observed in pulse radiolysis of Ar-saturated aqueous solutions containing poly(U) (2×10^{-4} M) and *t*-butanol (2×10^{-2} M) at 24 °C and a dose/pulse of 9 Gy, solid line. The dashed line indicates the corresponding dependence found with N_2O saturated solutions without added *t*-butanol [1].

t-butanol (2×10^{-2} M) at 24 °C (solid line). As a comparison, the dashed line shows the corresponding pH dependence which is measured when the conductivity increase (and hence strand break formation) is initiated by OH radicals, *i.e.* in N_2O-saturated solution in the absence of *t*-butanol [1].

It is seen that the rate constant of strand break formation for H atom initiation is lower than for OH radical initiation. This applies especially for pH = 3.5−5.5 where the rate constant for OH initiation decreases drastically with increasing pH whereas with H atom initiation a decrease of only a factor of 1.7 has been found (Fig. 2). There is considerable uncertainty associated with the values at pH ≧ 6 because of the low yield of H atoms (see below) and the contribution of OH radicals which are left to react with poly(U). However for pH > 6 the two curves tend to be parallel or even to approach one another.

c) Magnitude $\Delta\varkappa$ of the conductivity increase

In going from pH = 4 to pH = 7 the magnitude $\Delta\varkappa$ of the conductivity increase has been found to decrease steadily. This is expected because the conversion of solvated electrons into H atoms becomes less effective at lower H^+ concentrations.

In Ar-saturated solutions without added *t*-butanol at 24 °C and pH = 4 a fast conductivity increase with $k \approx 100\ \mathrm{s}^{-1}$ has been observed followed by a slower increase with $k \approx 1.5\ \mathrm{s}^{-1}$. At pH = 3 the corresponding rate constants were $k \approx 200\ \mathrm{s}^{-1}$ and $k \approx 2.5\ \mathrm{s}^{-1}$, respectively. At both pH values and at the lowest dose/pulse values used (2.5 Gy) the fast component contributed ≈ 70% to the total conductivity increase, the slow component ≈ 30%. At higher doses/pulse the contribution of the slow component has been found to decrease.

In Ar-saturated solutions under the above conditions 46% of the primary water radicals which react with poly(U) are OH radicals and 54% H atoms. OH radicals give rise to conductivity increases formed with rate constants around $k = 100\ \mathrm{s}^{-1}$ and $k = 200\ \mathrm{s}^{-1}$ at pH = 4 and pH = 3 respectively [1]. These rate constants have been found for the faster component. H atoms produce conductivity increases which agree with those found for the slow component. The two components are then assigned to conductivity increase and strand break formation generated by OH radicals (fast component) and H atoms (slow component).

41% of the OH radicals and 19% of the H atoms form strand breaks (and conductivity increase) when reacting with poly(U) in aqueous solution under ^{60}Co-γ irradiation conditions [2]. If these efficiencies apply also to pulse-radiolytic conditions it is expected that the OH radicals contribute to $0.46 \times 0.41/(0.46 \times 0.41 + 0.54 \times 0.19) \times 100 = 65\%$ to the conductivity increase, the H atoms to 35%.

The comparison with the experimental values shows, that the measured contribution of the slow component is somewhat lower. This may be partly a result of the relatively small rate constant for the reaction of H atoms with poly(U) (see above). Under pulse-radiolytic conditions and at a poly(U) concentration of 2×10^{-4} M a mutual recombination of H atoms to give H_2 cannot be totally avoided even at the lowest dose of 2.5 Gy which has been used. At this dose the calculated half-lives for the reaction of H atoms with poly(U) are 6.4 µs and for recombination 90 µs ($k(\mathrm{H} + \mathrm{H}) = 1.3 \times 10^{10}\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$ [8] has been used). From this one roughly estimates that ≈ 7% of the H atoms do not react with poly(U). The expected contributions for the fast and the slow component thus corrected are then 67% and 33% respectively. The similarity of these values to the measured contributions is viewed as evidence that also under pulse radiolytic conditions around 19% of the H atoms produce strand breaks.

Since the dose rate in pulse radiolysis ($\approx$ 10 Gy per 1 μs pulse) is many orders of magnitude higher than during ^{60}Co-γ irradiation (8×10^{-3} Gy s^{-1} has been used in [2]) it is concluded that the 19% efficiency of H atoms for strand break formation is nearly independent of dose rate under the experimental conditions described (*i.e.*, for example, low ionic strength). This provides evidence that the strand breaks are formed in first-order process and that second-order reactions which could possibly compete with strand break formation are of minor importance.

d) Addition of dithiothreitol (DTT)

When DTT is added in small amounts to Ar-saturated solutions of poly(U) (2×10^{-4} M) and *t*-butanol (2×10^{-2} M) at pH = 4 it has been observed that the size $\Delta\varkappa$ of the conductivity increase decreases (see Fig. 3, right ordinate, cross symbols). The dashed line indicates the calculated decrease of $\Delta\varkappa$ expected for scavenging of H atoms by DTT. The line was calculated using rate constants given in the legend of Fig. 3. As can be seen from a comparison of the dashed line and the solid line connecting the crosses the observed decrease is much larger than can be explained by mere scavenging of H atoms by DTT. This indicates that poly(U) radicals react with DTT and are prevented from giving rise to conductivity.

It is notheworthy that in our experiments (similar to earlier ones for N$_2$O-saturated solutions [1]) the half-value concentration of DTT (the concentration at which $\Delta\varkappa$ has decreased to one half of the value without DTT, 3×10^{-7} M, see Fig. 3) is less than one half of the concentration of H atom derived poly(U) radicals after a dose of 8 Gy ($\simeq 10^{-6}$ M). This is possibly a result of the fact that only one kind of the poly(U) radicals react with DTT, *e.g.* those radicals which give rise to strand breaks. Their concentration amounts to $0.19 \times 1.3 \times 10^{-6}$ M $= 2.5 \times 10^{-7}$ M. Another explanation may be found in the assumption that one molecule of DTT can prevent more than one poly(U) radical from producing a strand break.

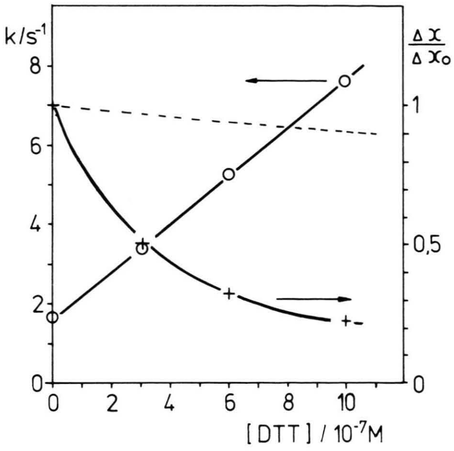


Fig. 3. Rate constant $k = t_{1/2}^{-1} \cdot \ln 2$ (left ordinate) and relative size $\Delta\varkappa/\Delta\varkappa_0$ (right ordinate) of the conductivity increase as a function of the concentration of added dithiothreitol (DTT). Ar-saturated aqueous solutions containing poly(U) (2×10^{-4} M) and *t*-butanol (2×10^{-2} M) at 22 °C, pH = 4.0 and dose/pulse = 8 Gy. $\Delta\varkappa_0$ denotes the size of the conductivity increase in the absence of DTT. The dashed line shows the expected decrease of $\Delta\varkappa$ if only those H atoms contribute to the conductivity increase which react with poly(U) and not with DTT (calculated on the basis of $k(\mathrm{H} + \mathrm{poly(U)}) = 5.5 \times 10^8\ \mathrm{M}^{-1}\,\mathrm{s}^{-1}$ and $k(\mathrm{H} + \mathrm{DTT}) = 1 \times 10^{10}\ \mathrm{M}^{-1}\,\mathrm{s}^{-1}$; the latter value is an upper limit which was estimated from the rate constants of H atoms with different sulfur containing compounds [18]).

On adding DTT it is observed further that in the presence of DTT the rate constant for the conductivity build-up is increased. The observed rate constant $k = t_{1/2}^{-1} \cdot \ln 2$ is a linear function of the concentration of DTT, see solid line in Fig. 3 connecting the circles. This behaviour can be described by

$$k = k_1 + k_2\,[\mathrm{DTT}] \tag{1}$$

where k_1 denotes the rate constants for strand break formation in the absence of DTT and k_2 represents the average rate constant for the reaction of those poly(U) radicals with DTT which would produce strand breaks in the absence of DTT (it is assumed that DTT can react only with a radical intermediate with a rate constant of the order given below). A behaviour according to equation (1) is only expected if the decay of the poly(U) radicals to form strand breaks is the rate determining step of the conductivity increase and of strand break formation (see [1] for details of the kinetic treatment).

From the slope of the straight line in Fig. 3 a bimolecular rate constant of $k_2 = 5 \times 10^6\ \mathrm{M}^{-1}\,\mathrm{s}^{-1}$ is obtained.

e) Rate-determining step of the conductivity increase

There are three consecutive reactions which finally result in strand breakage:

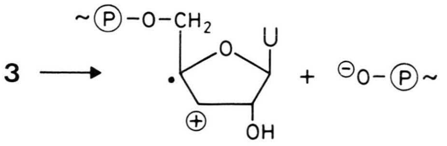
(i) H-addition to the uracil moiety;
(ii) H-abstraction of a uracil H-adduct radical from the sugar moiety;
(iii) decay of a sugar radical leading to strand breakage.

The question as to which of these reactions can be rate-determining will now be discussed:

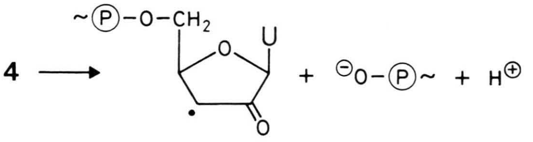
ad (i): H atoms react with poly(U) nearly exclusively *via* addition to the C(5)−C(6) double bond of the uracil moiety of poly(U) [5], whereby adduct radicals **2** with the free spin either at C(5) or C(6) can be formed. It has been shown that in poly(U) 60% of the addition occurs at C(5), *i.e.* mainly C(6)-centered radicals are formed [9]. Hence the first reaction step leading to H atom-induced strand break generation in poly(U) is the formation of one of these radicals. This H-addition cannot be rate-determining, because from the derived half-life of 6.4 µs (see results) a pseudo unimolecular rate constant of $1.1 \times 10^5\ s^{-1}$ is calculated for this reaction. This is several orders of magnitude higher than the rate constants observed for strand break formation.

ad (ii): As a second chemical reaction step the formation of strand breaks requires a reaction of the uracil H-adduct radicals with the sugar phosphate backbone of poly(U). An abstraction of one of the H atoms of the ribose moiety of poly(U) by the uracil radicals is the most likely type of reaction for this second step. This would lead to a carbon-centered radical at one of the carbons C(1′) to C(5′) of the ribose moiety. Mechanisms for the formation of strand breads in first-order reactions starting from radicals C(4′) (formula **3**) and C (2′) (formula **4**) have been advanced [10−12], whereas no such mechanisms are known for radicals at C(3′) and at C(5′) in deoxygenated solutions. Radical C(1′) could lead to strand breaks but only with lower rates. Hence C(2′) and C(4′) are the most likely radical intermediates in strand break formation.

ad (iii): The third and last chemical reaction step in strand break formation is the heterolytic cleavage of the radicals C(4′) (reaction (1)) or of C(2′) (reaction (2)) thus producing the strand break by mechanisms that are fairly well understood (for reviews see [13−15]).



(1)



(2)

From the results of the experiments involving addition of DTT it has been concluded that the lifetime of a poly(U) radical determines the rate of strand break formation. If the lifetime of the C(4′) radical would be rate-determining − the one possibility of reaction step (iii) − the same rate constant for strand break formation should be observed for OH radical and for H atom initiation, since in the case of OH radical initiation the decay of the C(4′) radical has been postulated to be rate-limiting. However, both rate constants differ and have a different dependence on pH (see Fig. 2). Furthermore small amounts of DTT (3×10^{-5} M) do not prevent OH-induced strand break formation at pH = 4 [16] (probably because the C-4′ radical decays too fast at pH = 4, see dashed line in Fig. 2). In contrast still smaller concentrations of DTT have been found to prevent strand breakage after H atom initiation, see further above. Hence the decay of the C(4′) radical as rate-determining step is excluded. Also the lifetime of the C(2′) radical is not likely to be rate-determining because it is not assumed that it decays more slowly than the C(4′) radical [17]. There remain only the lifetimes of the uracil moiety radicals C(5) and C(6) with respect to H-abstraction from the ribose moiety of poly(U) as rate-limiting step, *i.e.* reaction step (ii). It is presently not known from which of the two base adduct radicals the strand breaks are originating. The C(6) radical is formed to a greater extent [9] and hence may be regarded to be the more likely candidate. However contributions from the C(5) radical are not excluded. Experiments are in progress to distinguish between these two possibilities.

The comparatively small increase (*i.e.* by a factor of about two) of the rate constant at pH ≦ 4 (see Fig. 2) is not yet fully understood but could be the result of acid-induced changes of steric conditions in the polymeric chain that facilitate the H-abstraction by the uracil moiety radicals. A similar small increase of the rate constant with decreasing pH was also observed after OH initiation in oxygen containing solutions [6].

As to the question which of the sugar radicals, C(2′) or C(4′), are responsible for strand breakage: from Fig. 2 it is seen, that the rate constant for the decay of the C(4′) radicals (dashed line) and the rate constant for strand break formation after H atom initiation (solid line) approach each other at pH $\geqq$ 7. Here the rate-determining step might possibly change into the decay of the C(4′) radicals via reaction (1) because at pH $\geqq$ 6 reaction (1) becomes slow. This may be regarded as evidence that also at lower pH values the strand breakage proceeds via the C(4′) radical.

Summarizing: the decay of the H-adduct radicals proceeds via H-abstraction with the relatively small rate constant of $k = 1.5\ \mathrm{s}^{-1}$ at pH = 4−5. It is rate-determining in the strand breakage in this pH range. The rate constant of $k_2 = 5 \times 10^6\ \mathrm{M}^{-1}\,\mathrm{s}^{-1}$ obtained from the experiments with DTT is interpreted to be the rate constant for the reaction of the C(5) and/or the C(6) radical with DTT. It may further be noted that the assignment of the dashed line in Fig. 2 to the decay of the C(4′) or C(2′) radical implies that the uracil OH-adduct radicals react (by H-abstraction) faster by at least two orders of magnitude than the H-adduct radicals.

Conclusion

The H atom-initiated strand breakage proceeds by an addition of an H atom to the uracil moiety. The uracil H-adduct radicals **2** abstract an H atom from the sugar moiety. This reaction is rate determining in strand break formation at pH $\leqq$ 6. The resulting sugar radicals (**3** or **4**) decay *via* reactions (1) or (2) thereby forming the strand break. Although it is believed that the OH-radical-initiated strand breakage proceeds by an analogous mechanism, the rate-determining step is different. This is likely due to the fact that the uracil OH-adduct radicals undergo the H-abstraction reaction at least two orders of magnitudes faster than the H-adduct radicals which renders the decay of the sugar radicals rate-limiting in case of OH radical initiated strand breakage.

Experimental

The experiments were carried out using poly(U) (the potassium salt of polyuridylic acid) obtained from Boehringer, Mannheim. The substrate was dissolved in water purified by a millipore purification system type "milli-Q". The solutions were membranefiltered as described earlier [1]. The concentration of poly(U) in the final solution was estimated from the absorbance at 260 nm using a molar extinction coefficient of $9.7 \times 10^3\ \mathrm{M}^{-1}\,\mathrm{cm}^{-1}$ per nucleotide unit. The solutions were saturated with N_2O prior to use and the molecular weight was reduced by pre-irradiation at the ^{60}Co-γ source from its original value of typical $\overline{\mathrm{MW}}_\omega = 8 \times 10^5\ \mathrm{g\,mol}^{-1}$ to $\overline{\mathrm{MW}}_\omega \approx 1 \times 10^5\ \mathrm{g\,mol}^{-1}$ in order to avoid the contribution of conductivity decreases which are frequently observed at high molecular-weights and low doses. The N_2O was then removed by passing Ar through the solution. The pH values were adjusted using perchloric acid or potassium hydroxide solutions.

The pulse irradiations were carried out with electron pulses (2.8 MeV, duration 0.2−1.0 µs) generated by a Van de Graaff accelerator (High Voltage Engineering).

Conductivity measurements were made using an AC operated Wheatstone bridge at 10^5 Hz with a voltage of 40 V_{pp} across the electrodes.

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