POSSIBLE NATURE OF THE RADIATION-INDUCED SIGNAL IN NAILS: HIGH-FIELD EPR, CONFIRMING CHEMICAL SYNTHESIS, AND QUANTUM CHEMICAL CALCULATIONS

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Exposure of finger- and toe-nails to ionizing radiation generates an Electron Paramagnetic Resonance (EPR) signal whose intensity is dose dependent and stable at room temperature for several days. The dependency of the radiation-induced signal (RIS) on the received dose may be used as the basis for retrospective dosimetry of an individual's fortuitous exposure to ionizing radiation. Two radiation-induced signals, a quasi-stable (RIS2) and stable signal (RIS5), have been identified in nails irradiated up to a dose of 50 Gy. Using X-band EPR, both RIS signals exhibit a singlet line shape with a line width around 1.0 mT and an apparent *g*-value of 2.0044. In this work, we seek information on the exact chemical nature of the radiation-induced free radicals underlying the signal. This knowledge may provide insights into the reason for the discrepancy in the stabilities of the two RIS signals and help develop strategies for stabilizing the radicals in nails or devising methods for restoring the radicals after decay. In this work an analysis of high field (94 GHz and 240 GHz) EPR spectra of the RIS using quantum chemical calculations, the oxidation–reduction properties and the pH dependence of the signal intensities are used to show that spectroscopic and chemical properties of the RIS are consistent with a semiquinone-type radical underlying the RIS. It has been suggested that semiquinone radicals formed on trace amounts of melanin in nails are the basis for the RIS signals. However, based on the quantum chemical calculations and chemical properties of the RIS, it is likely that the radicals underlying this signal are generated from the radiolysis of L-3,4-dihydroxyphenylalanine (DOPA) amino acids in the keratin proteins. These DOPA amino acids are likely formed from the exogenous oxidation of tyrosine in keratin by the oxygen from the air prior to irradiation. We show that these DOPA amino acids can work as radical traps, capturing the highly reactive and unstable sulfur-based radicals and/or alkyl radicals generated during the radiation event and are converted to the more stable o-semiquinone anion-radicals. From this understanding of the oxidation–reduction properties of the RIS, it may be possible to regenerate the unstable RIS2 following its decay through treatment of nail clippings. However, the treatment used to recover the RIS2 also has the ability to recover an interfering, mechanically-induced signal (MIS) formed when the nail is clipped. Therefore, to use the recovered (regenerated) RIS2 to increase the detection limits and precision of the RIS measurements and, therefore, the dose estimates calculated from the RIS signal amplitudes, will require the application of methods to differentiate the RIS2 from the recovered MIS signal.

INTRODUCTION

Ionizing radiation generates numerous reactive species in the living organisms. Among these reactive species are short-lived radicals that are capable of damaging DNA or eliciting chronic immune responses. The severity of these radiation-induced toxicities is generally dose dependent. Therefore, with appropriate dosimetric methods, some predictable outcomes (i.e. survival) can be assessed from the radiation exposure in individuals. We seek the ability to accurately provide dose estimations in the event of nuclear weapon catastrophes or accidental radiation exposures from nuclear power plants. In both cases, mass exposure of individuals to radiation is expected to occur and the consequences of the exposure need to be rapidly and accurately assessed in order to provide the appropriate medical care⁽¹⁾. One approach to estimate dose is

through the use of ‘personal dosimeters’ that use a dose dependent, radiation-induced biological or chemical change as the basis for measuring and estimating an individual's dose⁽²⁾. Of these, the dosimetry methods that are based on physical or chemical changes in biological materials, so called physical dosimetry, are capable of reflecting irradiated dose dependent changes directly and not confounded by the radiation-induced inherent biological process (i.e. repair) or contributions from non-radiological sources⁽¹⁾.

Several ‘personal’ physical dosimetry methods have been developed that take advantage of radiation-induced free radicals generated in calciferous or hardened human tissues, such as teeth or finger and toe nails^(2, 3). These methods rely on signals that are stable enough to be used for the assessment of the dose received⁽¹⁾. While the nature of the EPR signal

generated by the ionizing radiation in teeth and bones was established decades ago⁽⁴⁾, for many years the nature of the most stable radical, generated by γ -radiation in nails, was a subject of numerous debates^(5, 6). When the nails are γ -irradiated to doses of 5–35 Gy, whether at the temperature of liquid nitrogen or at room temperature, a broad spectral component is observed. The spectrum was initially attributed to different types of sulfur-centered radicals^(5, 6). This assignment is supported by numerous g -factor measurements compared to quantum chemical calculations and by experimental methods (including chemical synthesis of known sulfur-centered radicals which gave similar EPR-spectra)⁽⁶⁾. However, the nature of the long-lived singlet observed at X-band (9 GHz) is much more difficult to identify. It is suggested that the observed singlet originates from a sulfonyl radical, being transformed from a sulfur peroxy radical⁽⁵⁾, or as originating from a melanin semiquinone radical⁽⁶⁾. In this article we summarize the outcomes of numerous experiments, including high-field (94 and 240 GHz) EPR measurements combined with quantum chemical calculations of possible radicals, wet chemistry experiments, and studies of the oxidation–reduction and base–acid properties of the stable radical in nails, to support the hypothesis that the RIS originates from a semiquinone radical formed on DOPA amino acids which are present in keratin proteins in nails.

The radical(s) that give rise to the RIS signal in irradiated nails are expected to be in the form of an inherently stable secondary radical structure, perhaps a resonance stabilized radical, which is further stabilized in radical ‘trapping sites’ within the tightly bundled keratin fibrils. There are at least five different RIS signals identified⁽⁵⁾. However, only two RIS signals, the RIS2 and RIS5, are observed at doses that are in the clinically relevant range of 10 Gy and under. At X-band these are singlet signals with a 1.0 mT linewidth centered at an apparent g -value of 2.0044. The RIS2 is a quasi-stable signal that fades with time following radiation exposure. The fade rate is dependent on the water content and temperature of the nail, and has a limited dependence on oxygen^(7, 8). However, the RIS2 signal is stable when nail clippings are stored at -20°C ⁽⁹⁾. The RIS5 is a radical which is stable even under high water content of the nail and is also stable to temperatures as high as 150°C ⁽⁵⁾. The difference in the stabilities of the radicals underlying the RIS2 and RIS5 signals is likely based on the depth of the radical within the keratin fibrils, which may play a role in limiting accessibility of RIS5 radicals to potential reactants. Even so, the fading of the RIS2 following radiation exposure presents a challenge in the use of this signal in gaining an accurate estimation of dose through EPR nail dosimetry. It may be possible, though, to address this challenge through a more thorough

understanding of the chemical nature of the RIS signal in irradiated nails.

The problem of characterizing the RIS

The nature of the radical(s) trapped in the keratin matrix from which the RIS originates are difficult to characterize from the viewpoint of X-band EPR. The RIS is a 1.0 mT singlet without detectable hyperfine couplings, and is produced at concentrations that are not sufficient for use in ENDOR qualitative techniques to determine the presence of small hyperfine splittings. At Q-band (35 GHz) a difference in the apparent g -value for the RIS2 ($g = 2.005$) and RIS5 ($g = 2.004$) are detected⁽⁵⁾, suggesting a difference in the nature of the radicals underlying the two RIS species. Further elucidation of the possible chemical nature of the radicals underlying the RIS signals is described here based on quantum chemical calculations (ORCA program)⁽¹⁰⁾, comparison to g -factors of radicals with known g -tensor and hyperfine structure, and similarities in chemical properties.

MATERIALS AND METHODS

Preparation of nail samples

Nail and hair clippings are collected from 15 healthy volunteers. Prior to use in irradiation or nail treatment studies, nails clippings are sized to the dimensions of the sample holder, X-band ($3 \times 3 \text{ mm}^2$), W-band ($0.4 \times 1 \text{ mm}^2$), and multi-frequency EPR ($0.1 \times 0.3 \text{ mm}^2$) and then pre-conditioned by soaking in deionized water to remove the mechanically-induced EPR signals (MIS) in the nail samples generated by the harvesting cut, followed by drying, generally under air for 30 min or nitrogen gas for 1 h. For qualitative analysis of the singlet component of the MIS, nail samples are sized for W-band measurement but instead of soaking the samples in water, the nail samples are alternatively left in the dark at ambient conditions for 24 h before EPR measurement to allow for the decay of two other MIS spectral components in the samples^(5, 7, 8). Hair samples are sized at 5 mm lengths and used untreated.

Gamma ray and UVA exposures

Hair samples or nail samples, which are pre-conditioned by soaking in water 10–20 min and then air dried for 30 min prior to irradiation, are either gamma irradiated (^{137}Cs , 0.9 Gy/min) to obtain the combined RIS2+RIS5 signal or exposed to UV light under ambient conditions to obtain the UVA-induced signal (340–400 nm, fluence rate of 3.5 mW/cm^2 for 2 h) or UVB-induced signal (280–320 nm, fluence rate of 12.2 mW/cm^2 for 1 h) using an 8 watt 3UV lamp (UVP, LLC). EPR signals are measured at 9.4 or

94 GHz for each sample before and after each exposure dose to gamma irradiation or UV light.

Nail treatments

For the acid–base test model, matched nail clippings sets from the same donors are freshly cut using a blade. Blades are used instead of scissors to minimize the formation of mechanically-induced EPR signals (MIS) in the nail samples. The clippings are divided into two sample sets, soaked in either aqueous KOH (pH 12) or HCl (pH 2) for 20 min to remove the MIS and alter the pH of the local environment of the keratin proteins. The clippings are then dried for 1 h under nitrogen and irradiated to a dose of 6 Gy. EPR signals are measured at X-band (9.4 GHz) for each sample before and after irradiation.

Testing of the oxidation–reduction properties of the RIS2 signal is conducted on irradiated nail samples after removal of the RIS2 by soaking/drying prior to treatment with either potassium iodide (KI) (Sigma-Aldrich) or potassium ferricyanide [$K_3Fe(CN)_6$] (Sigma-Aldrich). Nail samples are prepared by cutting into small pieces and then soaking in water for 10 min to eliminate MIS. After drying for 1 h under nitrogen and confirming a low background signal, they are irradiated to different doses (2, 6, or 20 Gy). After the RIS singlet is recorded as the post-irradiated spectrum, the RIS2 in the nails is removed by soaking in water for 10 min and drying for 1 h under nitrogen. Treatment of the post-irradiation soaked nails is conducted by soaking for 10 min in either the reducing agent 0.1 M KI in 0.1 N KOH medium or the oxidizing agent 0.1 M $K_3Fe(CN)_6$ in 0.1 N KOH medium, followed by drying under nitrogen gas for 1 h prior to acquiring X-band (9.4 GHz) EPR measurements as the post-treatment spectrum. Recovery of the RIS singlet is calculated from the ratio of the mass normalized signal amplitudes in the post-treatment and post-irradiated spectra.

Testing of the oxidation properties of the MIS singlet signal is conducted on unirradiated nail samples from four donors. Nail samples are prepared by cutting into small pieces ($3 \times 3 \text{ mm}^2$), holding overnight in the dark to allow for fading of two MIS signals leaving only the MIS singlet which was measured at X-band (9.4 GHz) as the post-cut spectrum. The samples are soaked in water for 10 min to eliminate MIS followed by drying for 1 h under nitrogen and confirming a low background signal. The nails are then soaked for 10 min in the oxidizing agent 0.1 M $K_3Fe(CN)_6$ in 0.1 N KOH medium, rinsed three times in deionized water, dried under nitrogen gas for 1 h, followed by EPR measurement of the post-treatment MIS singlet signal. Recovery of the MIS singlet is calculated from the ratio of the

mass normalized signal amplitudes in the post-treatment and post-cut spectra.

Covalent binding of a semiquinone to keratin proteins in nails is achieved by oxidatively coupling catechol to nail proteins, forming a protein bound semiquinone radical on proteins⁽¹¹⁾. Briefly, pre-conditioned nail clippings are placed into a solution of freshly made o-semiquinone in base media (pH = 12). That solution is made by mixing a solution of 0.3 M catechol (Sigma-Aldrich) with 0.3 M solution of $K_3Fe(CN)_6$ in base media. After holding in this solution for 10 minutes, the nails are removed and washed three times in distilled water (to eliminate all the unbounded o-semiquinone anion-radical) before drying in air for several hours prior to EPR measurement at 240 GHz.

EPR instrumentation and settings

X-band EPR spectra are acquired using a Bruker EMX Micro spectrometer equipped with a standard rectangular cavity resonator. High frequency (HF) EPR spectra are acquired using custom-built W-band (94–95 GHz) spectrometer⁽¹²⁾ with hyperbolic-cosine waveguide and 5-loop-4-gap resonator^(13, 14) or multi-frequency EPR instrument⁽¹⁵⁾. X-band (9.4 GHz) settings: sweep width 15.0 mT, modulation frequency 100 kHz, modulation amplitude 0.5 mT, microwave power 0.0225–5.0 mW, time constant 40.96 ms, sweep time 40.96 s, and 10 scan averages. Instrument settings for the W-band (94 GHz) settings: sweep width 300 G, modulation frequency 18.3 kHz, modulation amplitude 0.5 mT, microwave power 0.05 mW, sensitivity 500 μV , time constant 200 μs , sweep time 90 s, and 16 scan averages. Settings for the multi-frequency EPR instrument set at 240 GHz were: sweep width 70.0 mT, modulation frequency 96 kHz, modulation amplitude 0.3 mT, microwave power 10 mW, time constant 300 ms, sweep time 140 s, and 100 scan averages.

Data analysis

Field position standardization at W-band is achieved using 3 of the 6 background Mn^{2+} lines in the nail spectrum. g -Values are obtained from measurement of magnetic field and microwave frequency; measurements of the g -values were to ± 0.0001 . Microwave power saturation analysis is based on the changes in the signal intensity of the spectral line located at $g = 2.005$ as a function of the square-root of the microwave output power.

Quantum mechanical calculations are conducted using ORCA⁽¹⁰⁾ to calculate g -values for a number of radicals types as shown in Table 1. The simulations are conducted using the B3LYP method and TZVP basis set; the `eprnmr` g -tensor command is used to calculate g -tensors following structural optimization.

Table 1. *g*-Tensors for select sulfur-oxygen, phenoxy, benzyl, and semiquinone radicals and radiation-induced signal (RIS) in nails.

Radical	<i>g</i> -Tensor components			Method
	g_x	g_y	g_z	
Methyl sulfonyl ($\text{CH}_3\text{-SO}_2\bullet$)	2.0108	2.0075	2.0022	Calculations ^a
Methyl sulfonyl ($\text{CH}_3\text{-SO}_2\bullet$)	2.0094	2.0056	2.0027	Single-crystal experiment ^(16, 17)
Methyl sulfonyl (H-bonded)	2.0107	2.0073	2.0022	Calculations ^a
Cysteinyl sulfonyl ($\text{Cys-SO}_2\bullet$)	2.0105	2.0076	2.0023	Calculations ^a
Phenyl sulfonyl ($\text{C}_6\text{H}_5\text{SO}_2\bullet$)	2.0101	2.0062	2.0023	Calculations ^a
Phenylmethyl sulfonyl ($\text{C}_6\text{H}_5\text{CH}_2\text{SO}_2\bullet$)	2.0104	2.0079	2.0021	Calculations ^a
Methyl thiylperoxy ($\text{CH}_3\text{SOO}\bullet$)	2.0328	2.0084	2.0017	Calculations ^a
Cysteine sulfinyl ($\text{Cys-SO}\bullet$)	2.0228	2.0122	2.0022	Calculations ^a
Sulfuric ($\text{HSO}_3\bullet$)	2.0122	2.0112	2.0056	Calculations ^a
p-Methylbenzyl ($\text{CH}_3\text{C}_6\text{H}_4\bullet$)	2.0031	2.0022	2.0017	Calculations ^a
p-Methylphenoxy ($\text{CH}_3\text{C}_6\text{H}_4\text{O}\bullet$)	2.0109	2.0049	2.0022	Calculations ^a
Tyrosyl ($\text{TyrC}_6\text{H}_4\bullet$) (no H-bonding)	2.0091–2.0089	2.0042–2.0050	2.0021–2.0025	Experiment ⁽¹⁸⁾
Tyrosyl ($\text{TyrC}_6\text{H}_4\bullet$) (H-bonded)	2.0076	2.0043	2.0022	Experiment ⁽¹⁸⁾
Tyrosyl ($\text{TyrC}_6\text{H}_4\bullet$) (H-bonded, HCl)	2.0067	2.0042	2.0023	Experiment ⁽¹⁹⁾
Tyrosyl ($\text{TyrC}_6\text{H}_4\bullet$) (H-bonded)	2.0074–2.0081	2.0047–2.0049	2.0025	Calculations ⁽¹⁸⁾
o-Semiquinone ($\text{RC}_6\text{H}_3\text{O}_2\bullet$)	2.0063	2.0053	2.0022	Calculations, unbound
o-Semiquinone ($\text{RC}_6\text{H}_3\text{O}_2\bullet$)	2.0051	n.d. ^b	2.0022	Melanin in hair (exp) ^c
o-Semiquinone ($\text{RC}_6\text{H}_3\text{O}_2\bullet$)	2.0060	2.0044	2.0023	Chemical synthesis, keratin bound (exp) ^c
RIS2, RIS5	2.0059	2.0046	2.0023	γ -Irradiated nail (exp) ^c

^aThis study.^bn.d. – not determined.^cexp – experimental results, this study.

RESULTS AND DISCUSSION

Quantum chemical approach from the top: what is the RIS radical?

In determining the nature of the radical(s) underlying the RIS2 and RIS5, spectroscopic and chemical properties of the radical(s) are considered. In Figure 1 is a comparison of the spectra acquired at W-band (94.0693 GHz) for the RIS2 (Figure 1A) and RIS 5 (Figure 1E). These spectra are found to be virtually identical with common *g*-tensors of $g_x = 2.0059$, $g_y = 2.0046$ and $g_z = 2.0023$, suggesting that the radical species underlying the two RIS signals are the same. This result differs from a previous study where the apparent *g*-values of the RIS2 and RIS5 differed slightly⁽⁵⁾. The reason for the difference in the spectroscopic results between the previous work and what is presented is unknown. However, the results presented here are verified in 30 nail sample sets from 15 different donors. Therefore, given the consistency of the *g*-value measurements between sample sets, and the higher spectral resolution provide by W-band EPR experiment, the results support a hypothesis that the two RIS signals arise from the same radical species. The difference in the chemical properties

(e.g. stabilities) may be the location of the radical centers within the nail keratin fibrils.

Regarding the type of radical underlying the RIS, these radicals are not likely to be carbon-centered radicals given the stability of the RIS radicals in the presence of oxygen and the large difference in *g*-tensors from known carbon radicals, including benzyl radicals. The lack of nitrogen hyperfine couplings indicates that the radical is not nitrogen centered, such as tryptophanyl radicals. However, other radical sites, which include sulfur and oxygen centered radicals such as sulfur-oxygen (sulfinyl, sulfonyl and sulfuryl radicals) and oxygen centered aromatic radicals (phenoxy, semiquinone, and semiquinone anions), are relatively stable and have similar *g*-values. In Table 1 are possible radical centers that could be formed in irradiated keratin proteins.

A number of sulfur-oxygen radicals are simulated using ORCA to calculate the *g*-tensors for these radical types and compared to the *g*-tensors measured for the RIS radicals. A comparison of the g_x -tensors in Table 1 shows a large difference in *g*-tensors for the sulfinyl, sulfonyl, sulfuryl and thiyl-peroxy radicals in comparison to the g_x value of 2.0059 for the RIS radical. Phenoxy-type radicals, such as the

Figure 1. Comparison of nail spectra acquired at W-band (94.0693 GHz) showing the equivalence of the RIS2+RIS5 from a 50 Gy dose (A), the singlet component of the MIS (B), the UVA (C) and UVB (D) induced signals, and the RIS5 (E). The g -tensors measured from these spectra are: $g_x = 2.0059$, $g_y = 2.0046$, $g_z = 2.0023$. The RIS5 spectrum is obtained after soaking a 50 Gy gamma-irradiated nail clipping for 15 min in water, followed by dry under vacuum overnight. Three of the six lines from the Mn^{2+} spectrum are seen at 3344.2, 3354.0 and 3363.8 mT.

tyrosyl radical, have a g_x component of the g -tensor that varies from 2.0066–2.0091. This variability is due to the sensitivity of the g -tensors on the local environment, specifically on H-bonding of the radical. Under conditions of no H-bonding the g_x values range from 2.0087 to 2.0089, but under H-bonding conditions the values decrease to 2.0066–2.0076^(18–20). The lowest value of 2.0066 is achieved under strong H-bonding conditions and even under this condition this g_x component of the tensor does not match the g -tensor component for the RIS. Therefore, of the sulfur-oxygen and phenoxy-type radical radicals that could be formed in irradiated keratin proteins, only a strongly H-bonded tyrosyl radical has g -tensors that approach those for the RIS but are still not a good candidate for the RIS.

Another class of radicals that can be formed in irradiated nails are of the semiquinone or semiquinone-anion type. Semiquinone radicals are proposed as candidates for the stable component of RIS, which attributed the semiquinones to melanin⁽⁶⁾. Through the use of high-field (94 GHz and in a separate experiment 240 GHz) EPR, the semiquinone radicals observed in irradiated nails can be distinguished from melanin-based semiquinone radicals based on the g -tensors of the two types of semiquinone radical spectra. In Figure 2 is shown the difference between the g_x component of the tensor of the RIS2 (2.0059) formed in irradiated nails (Figure 2B) and the melanin semiquinone in hair (2.0051) in Figure 2C. The melanin in hair is expected to be a good model for any trace amounts

of melanin that may exist in nails. Gamma-irradiation of the hair to a dose of 50 Gy produces a separate and distinguishable RIS2 signal as noted by the g_x component of the tensor under the melanin semiquinone spectrum shown in Figure 2A. Therefore, the high field EPR studies of the hair model suggest that the RIS2 is not due to a melanin semiquinone radical but rather to another type of semiquinone radical.

In Figure 1, the high field (94 GHz) EPR spectrum of the RIS in an irradiated nail clipping shows overlapping Mn^{2+} signals at 3344.2, 3354.0 and 3363.8 mT, which are inevitably present in hair and nails. The presence of the manganese ions prevents a full registration of the EPR spectrum at even higher frequencies. Attempts to record the spectrum of the stable mechanically induced signal at 150 and 300 GHz demonstrated only the presence of a minor MIS signal beneath the very strong signal of Mn^{2+} without the possibility to subtract the Mn^{2+} signal. Given the limited resolution of spectra acquired at 150–300 GHz the focus of the qualitative analysis of the RIS is conducted at 94 GHz.

Possible mechanism of the generation of o-semiquinone radical in keratin

One of the abundant amino acids in keratin is tyrosine. Tyrosine is essentially a phenol, which may be oxidized further to L-3,4-dihydroxyphenylalanine (DOPA). The oxidation pathway includes generation of semiquinone radicals in the intermediate steps as shown below:

DOPA is formed through either enzymatic or non-enzymatic reaction from tyrosine⁽²¹⁾. In fingernails it is likely that DOPA is formed from non-enzymatic pathways through oxidation of keratin protein in air (by reactive oxygen species) in the presence of redox metals like copper⁽²²⁾. Melanocytes in nails are quiescent and are predominantly DOPA-negative^(23, 24), therefore formation of DOPA in the nail through enzymatic pathways is not expected, except in cases of melanonychia where pigmentation of the nail is found⁽²⁵⁾. It is known that tyrosine can be oxidized to DOPA in wool through exposure to sunlight and UV light^(26, 27), and a similar photo-oxidation of tyrosine is expected in fingernails. In fact, we show that exposure of nails to

Figure 2. Comparison of melanin spectra acquired at W-band (93.0736 GHz) from a 50 Gy gamma-irradiated hair sample (A) to the RIS2 acquired in a nail sample gamma-irradiated to a dose of 50 Gy (B), both samples from the same donor. The spectra show a clear difference in the g_x components of g -tensors between the melanin (A and C) at 2.0051 and RIS2 (B) at 2.0059. The spectrum (A) was acquired from a hair sample after gamma-irradiation to a dose of 50 Gy. The arrow in the figure shows the appearance of the g_x component of the g -tensor from the RIS2 in the irradiated hair spectrum (A). Spectrum (C) corresponds to unirradiated hair.

UVA (Figure 1C) and UVB (Figure 1D) light gives rise to the same spectrum as found for the RIS and the stable MIS signals (see Figure 1). The process underlying the yellowing of wool keratin was investigated and it was found that the most important contribution was the oxidation of tyrosine to form DOPA intermediate products, as determined chromatographically⁽²⁶⁾. Additionally, the process of pyrocatecholes oxidation is well known^(11, 28) and includes as an intermediate step the production of semiquinones (which in basic medium forms a stable radical o-semiquinone anion radical, which may be trapped in the matrix of keratin as the RIS component). Cationic semiquinone radicals may be safely excluded since these are very unstable⁽²⁹⁾. Therefore, from the standpoint of the extremely low levels of melanin in nails and the oxidation of tyrosines in keratin to form DOPA supports the plausibility for o-semiquinone radical as the basis of the RIS signal through the direct radiolysis of DOPA or reaction of DOPA with radiation-induced radicals formed in keratin.

Chemical properties of the stable component of RIS

As further support of the o-semiquinone anion-radical (the most stable of the semiquinone-type radicals) as the basis of the RIS, testing of the acid–base and oxidation–reduction properties of the RIS in irradiated nail samples test models is conducted. From the acid–base test model studies it is found that

under basic conditions, which favor the formation of the o-semiquinone anion, there is 1.6 times greater signal intensity for the RIS in 6 Gy irradiated nails (attributed to the recovery of the RIS2) compared to acid treated nails irradiated to the same dose. This result is likely due to the greater stability of the o-semiquinone anion radical compared to a semiquinone cation or neutral semiquinone radical. Because of the tight bundling of the keratin protein fibrils in the nail and the presence of small amounts of lipids⁽⁶⁾ it is likely that the acid and base treatments were not able to fully penetrate into the nail during the 20 min treatment time and, therefore, the differential in the RIS signal amplitude may actually be greater than that reported here. Even so, these results are consistent with the expected acid–base behavior of semiquinone radicals.

From the results of the oxidation–reduction RIS test model it is found that the treatment of the dried nails with the reducing agent, potassium iodide (KI), in basic medium resulted in no return of the RIS2 signal. Conversely, treatment of the dried nails with the oxidizing agent, $K_3Fe(CN)_6$, in basic medium restores the RIS2 signal in nail samples irradiated to doses of 2, 6 or 20 Gy. The restoration of the RIS2 signal under oxidizing conditions is consistent with the known chemistry of pyrocatechols, where the pyrocatechol is oxidized to the semiquinone. These results suggest that the nature of decay of RIS2 radicals in nails is reduction of the RIS2 radical to a dihydroquinone, rather than oxidation to the quinone. The expected dose response of the restored signal is inconclusive due to a high degree of variability in the recovered RIS2 signal amplitudes. However, with improvements in the nail treatment protocol there is the possibility of restoring the RIS2 signal after it has faded from the sample by treating the nails with an oxidizing agent. This approach offers the potential advantage to address the uncertainty in estimating dose from RIS2 measurements that arises from signal fading following exposure to radiation^(7, 30).

One confounding factor that would negate this advantage is the ability of the basic ferricyanide treatment to recover and directly measure the faded RIS2 is the simultaneous recovery of the MIS singlet signal. As is discussed above, the g -tensors of the MIS singlet match well those of the RIS2 and RIS5. Therefore, it is reasonable to assume that the chemical species underlying the MIS singlet is the same semiquinone radical as the RIS species. Therefore, the loss of the MIS singlet signal, either due to the natural fading in the nail or from the purposeful elimination of the signal through soaking of the nail clipping in water, is expected to be recovered during the basic ferricyanide treatment. This assumes that the fading of the MIS singlet occurs through the conversion of the semiquinone to a dihydroquinone. To test this, the spectra acquired from post-cut and post-treatment nail

samples (unirradiated) from four donors are compared. The results confirm the semiquinone nature of the radicals underlying the MIS singlet signal, where a full recovery ($111 \pm 12\%$) of the singlet is achieved through the treatment of the nail samples with basic ferricyanide. Given the full recovery of the MIS singlet following treatment of the nail samples it will not be possible to directly measure RIS signals recovered in irradiated clipped nails that have undergone treatment with basic ferricyanide.

Confirming the nature of the RIS through chemical synthesis

As additional support of an o-semiquinone radical based RIS signal, an o-semiquinone radical is covalently bond to the keratin matrix and the resulting spectrum and g -tensors are measured. To do so the process of oxidative coupling of the dihydroquinone to keratin proteins, described by Kalyanaraman⁽¹¹⁾, was chosen. The radical is generated according to the reaction:

The semiquinone function can be coupled to peptides or proteins in biological tissues through the formation of a covalent bond at the meta or para positions relative to the C-O• radical function⁽¹¹⁾:

It is expected that for nail keratin proteins the reaction will occur on the surface of the keratin fibrils. Here R represents the covalent bond formed between the semiquinone and the keratin protein. The most probable site of covalent bonding is to N-atom (from NH_2 group) or S-atom (from SH group)⁽¹¹⁾. Once formed, the radical is stable for years and can be kept in air or in a closed vial without oxygen removal, assuming storage is maintained under dry conditions.

Although the exact bonding sites of the oxidatively-coupled semiquinone anion radical on the keratin cannot be established, there are some key observations from the spectrum that can be used to support the hypothesis of the semiquinone nature of the RIS. In the high-field (240 GHz) EPR spectrum of Figure 3, the lines are narrow and the spectrum is well resolved, indicative of a single radical contributing to the spectrum. This result is unlike that observed for melanin where there is an aggregation of differing radicals contributing to a broadened and relatively unresolved overall spectrum⁽³¹⁾. The g -tensor components, measured from the spectrum are: $g_x = 2.0060$, $g_y = 2.0044$, $g_z = 2.0023$. The slight difference in the g_x from that measured for the RIS may

Figure 3. High-field (240 GHz) EPR spectrum of o-semiquinone anion radical chemically bound to keratin protein in human nails⁽¹¹⁾ ($g_x = 2.0060$, $g_y = 2.0044$, $g_z = 2.0023$). The difference between the g -tensors of the RIS (see Table 1) and this radical may be attributed to the difference in location of the two radicals in the nails. While RIS is likely situated at a partially oxidized tyrosine residue, the position of chemical bond between this radical and keratin chain is difficult to predict, however, the influence of the side chain is small⁽³²⁾.

be explained by a dependence of the g -tensor on the attachment site of the oxidatively-coupled semiquinone radical, which will likely differ from the site of the RIS radical. The differences in these g -tensors fall well within the small variations in g_x component of the tensor that is observed for semiquinone radicals with differing side-groups, as determined from both quantum chemistry and experimental results⁽³²⁾. The spectra from the oxidatively-coupled semiquinone and RIS also show differing linewidths (0.8 mT for synthetic o-semiquinone and 1.0 mT for the RIS2). This may be partially explained, again, by the different attachment sites and the possibility of hydrogen bonding of one of the RIS radicals resulting in unresolved hyperfine structure to the spectrum. Finally, the microwave power saturation properties are compared between the synthesized radical and the RIS2. There is good agreement between the changes in signal amplitudes for both radicals as a function of the square root of the microwave power (Figure 4). Thus, the experimental parameters of a known semiquinone anion-radical covalently bond to keratin match well to those found for the RIS, supporting the assignment of the RIS as a semiquinone anion radical.

CONCLUSION

Through an analysis of the spectroscopic and chemical properties of the RIS signal and underlying radicals given here, support is provided for the assignment of the RIS as a DOPA o-semiquinone radical. The potential significance of this finding is

Figure 4. Microwave power saturation plots of the $\log_{10}$ (signal amplitude [arbitrary units]) as a function of the $\log_{10}$ (microwave power [mW]) for the o-semiquinone anion-radical attached to nail proteins and for 20 Gy RIS (predominantly the RIS2 with >20% RIS5). g -Factor at X-band is the same (2.0047). Line width is 0.8 mT for o-semiquinone and 1.0 mT for RIS. Modulation amplitude is 0.3 mT.

the redox property of the 1,2-dihydroquinone (catechol)-semiquinone-quinone couple and how this could be applied to addressing the problem of the RIS2 fading that can lead to the inaccuracy of the dose estimates calculated from the RIS2 signal amplitude^(7, 30). From the evidence presented, it appears that the fading of the RIS2 is the result of the one-electron reduction of the DOPA semiquinone to a catechol (1,2-dihydroquinone), which is EPR silent. This fading can lead to limitations in achieving both accuracy and precision of dose estimates calculated from the RIS2 (and RIS5 which underlies the RIS2) in nail dosimetry methods that rely mainly on the more intense RIS2 signal amplitude^(30, 33). The DOPA dihydroquinone in nails can be converted back to the semiquinone upon treatment with a one-electron oxidant, for example basic potassium ferricyanide, recovering the RIS2 signal. For this treatment method to be useful in the EPR clipped nail dosimetry assay it is important to establish, (1) stabilization of the recovered RIS2 signal, and (2) the full conversion of the DOPA dihydroquinone formed from the radiation-induced semiquinone back to the semiquinone without interference from any other dihydroquinone-to-semiquinone reactions that are formed through non-radiation processes (for example the MIS singlet). Although the oxidative treatment of the irradiated nail samples is able to recover and stabilize the RIS2 signal, it also results in the recovery of the MIS singlet. Because the chemical species underlying the MIS singlet is formed from the clipping of the nail when harvested from a donor, the signal will always be present in the

treated nail samples. Given the full recovery of the MIS singlet following treatment of the nail samples it will not be possible to directly measure the recovered RIS but will instead require an approach to separate the MIS singlet signal from the RIS signals in order to use the fully recovered RIS as a means to improve the estimation of radiation dose.

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