Unusual EPR Profile Obtained by Laser Photoreaction of Cardiovascular Tissue

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Using spin trap methods, laser generated free radicals from cardiovascular tissue in high viscosity blood solution have been studied by EPR. The EPR spectra obtained are compared with spectra of free radicals produced by both cw and pulsed excimer laser irradiation. Nitroxide spin adducts produced by excimer laser irradiation show an unusual EPR hyperfine structure. In addition, the EPR spectra obtained by continuous wave (cw) laser irradiation are different from those obtained by excimer laser irradiation. The observed β-hydrogen hyperfine coupling for pulsed laser is approximately 1 Gauss smaller than those for cw laser irradiation. The present EPR results provide firm evidence that different free radical reaction intermediates are produced for each laser mode.

The detection and characterization of transient free radical intermediates generated by laser photoablation of biological tissue are essential to the understanding of the mechanism of laser–tissue interaction. Recently, there has been a great interest in studying the formation and determination of laser ablated molecular intermediates in the area of laser medicine.[1-5] Electron paramagnetic resonance (EPR) techniques using spin trap methods have been employed to determine the nature of free radical fragments which are formed by laser photoablation processes.[3-4] By examining the ablated fragments, it may also be possible to trace back the molecular details of the initial photoproduced events of laser ablation on the tissue surface.

Several experimental attempts have been made to elucidate the mechanism of laser interaction with tissue samples. For example, photoacoustic spectra of normal artery wall and of atherosclerotic plaque are reported by Singleton et al.[1] Threshold fluences (in units of [J cm^{-2}]) for excimer laser ablated gaseous products were calculated from the photoacoustic spectrum. Acoustic measurements of vascular tissue by pulsed laser has been performed by Cross et al.[6] They succeeded in measuring pulsed laser generated thermoelastic stress-waves on a 10 nanosecond time scale using acoustic transducers. Clarke et al.[4] suggested that by comparing two different energy sources, pulsed laser and ultrasound, it could be determined if free radicals are produced by the photoacoustic shock wave following the laser pulse. Gaseous products accompanying cw and pulsed laser ablation of cardiovascular tissue[2] and arterial wall[1] have been determined by gas chromatography. The pulsed laser ablation at high fluence of tissue results in plasma emission. Some of the emitted species have been identified, such as atomic calcium, C_2, and CN.[5] The results imply that radical fragments are common products of secondary reactions occurring in the plasma and should not be used to support mechanistic models for laser ablation. Hence, the formation and characterization of the fragments has not been well established.

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Nitroxide spin traps have great potential for application to biological systems. A number of biological systems have recently been investigated to probe the structure of biological free radicals.[7-10] As an extension, the free radicals produced by laser irradiation of tissue samples in high viscosity blood media are investigated. We hope to extend our findings to achieve better understandings of how free radicals are produced and what types of free radicals are produced by laser irradiation of cardiovascular tissue. The results are discussed in terms of the various laser wavelengths and a correlation between the laser generated free radicals produced by the action of continuous wave (cw) vs. pulsed laser irradiation.

Materials and Methods

Tissue sample preparation has been described in elsewhere.[1] A spin trap reagent: *N*-(4-pyridylmethylene)-*t*-butylamine *N,N'*-dioxide (4-POBN) was purchased from Aldrich Chemical Co. and used as is. The water soluble spin trap reagent 4-POBN was chosen because of its stability, solubility in water, and insensitivity to light. 4-POBN was dissolved in canine blood to a concentration of 50 mM (M=mol dm^{-3}). The canine blood containing heparin was used without deoxygenation and centrifuging or filtration in this in vitro experiment. The size of myocardium sample was approximately the diameter of an EPR tube (5 mm) and the tissue sample was mounted in the bottom of the EPR tube. The sample was in contact with a fused silica optical fiber connected to the laser and was directly irradiated through the optical fiber by the output of a focused laser beam.[3] The blood solution without tissue sample was also irradiated through the optical fiber coupled with the laser beam. The laser output power from the optical fiber was kept at ca. 0.3 W. Right after laser irradiation of the tissue sample, the spin trap-blood solution was immediately transferred to an EPR aqueous quartz cell and mounted in the EPR cavity.

EPR measurements were made with a Varian E 104 X-band spectrometer. EPR spectra were recorded at room temperature. The recording conditions were the following: microwave power, 20 mW; microwave frequency, 9.42 GHz; modulation amplitude, 1.0 G; modulation frequency, 100 kHz; time constant, 1.0 s; scan rate, 20 Gauss per minute (1 Gauss=10^{-4} T). The resonance fields were measured relative to the standard free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH).

Results

The background signals of the spin trap reagent (4-POBN) and the 4-POBN dissolved in blood solution are shown in Fig. 1(A) and (B), respectively. As evidenced by Fig. 1(A) and (B), there is no EPR detectable background signal from the spin trap reagent as well as spin trap-blood solution. The EPR signal of spin trapped free radicals generated by cw 514 nm irradiation of cardiovascular tissue is a doublet of triplet as shown in Fig. 1(C). The laser irradiation of spin trap-blood solution, without tissue sample, irradiated by cw 514 nm argon ion laser is shown in Fig. 1(D). Figure 1(D) shows a nitrogen dominated triplet overlapping with a weak doublet of triplet. This spectrum is indicative of the decomposition of 4-POBN such as di-*t*-butylnitroxide ($a^N \approx 17$ Gauss). These experimental procedures were repeated for cw 351 nm and pulsed 308 nm irradiation. The obtained results are shown in Figs. 2 and 3, respectively. The EPR spectra observed for both cw 514 nm and 351 nm irradiation of tissue sample are very similar as shown in Figs. 1(C) and 2(C), respectively. Both EPR spectra are dominated by a nitrogen triplet hyperfine pattern and further split by β-hydrogen.

Figure 3 shows that the trapped free radicals produced by pulsed UV laser ablation of the tissue sample are not the same as those by the irradiation of spin trap-blood solution. The EPR spectrum obtained on pulsed excimer laser irradiation of tissue under high viscosity blood solution is not a simple nitrogen triplet signal, as shown in Fig. 3(C). This EPR signal is a weak boublet. In addition, the previously obtained EPR spectra for pulsed 308 nm and 351 nm irradiation of tissue sample under low viscosity cyclohexane show a simple three line pattern.[3] In contrast, the EPR spec-

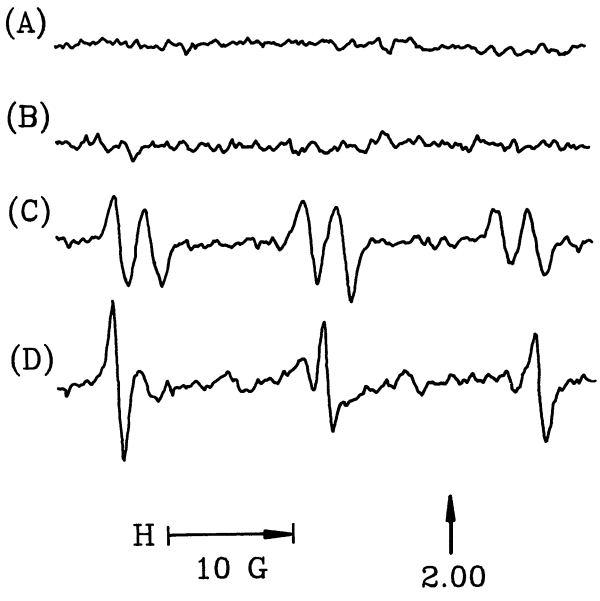


Fig. 1. Observed EPR spectra for cw 514 nm: (A) 4-POBN reagent before laser irradiation; (B) 4-POBN dissolved in blood before irradiation; (C) laser irradiation of myocardium in 4-POBN blood solution; and (D) irradiation of 4-POBN blood solution without myocardium. The calibrated g-value, 2.00$_0$, is marked. (Not DPPH, g=2.0036) EPR conditions are in the experimental section.

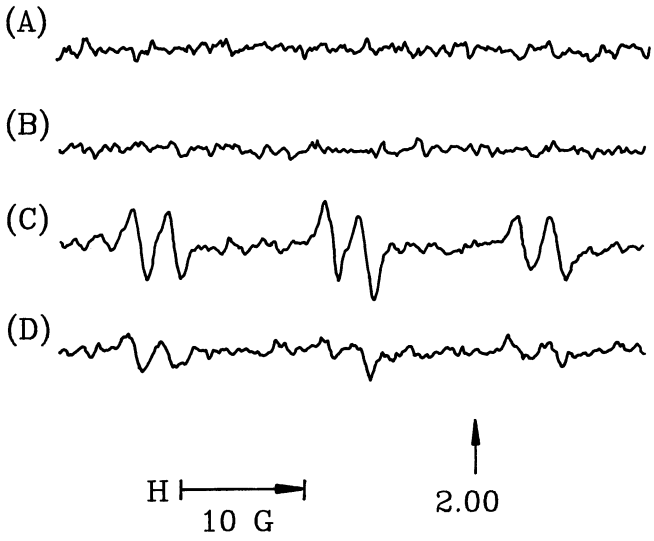


Fig. 2. Observed EPR spectra for cw 351 nm irradiation: (A) 4-POBN reagent before laser irradiation; (B) 4-POBN dissolved in blood before irradiation; (C) laser irradiation of myocardium in 4-POBN blood solution; and (D) irradiation of 4-POBN blood solution without myocardium.

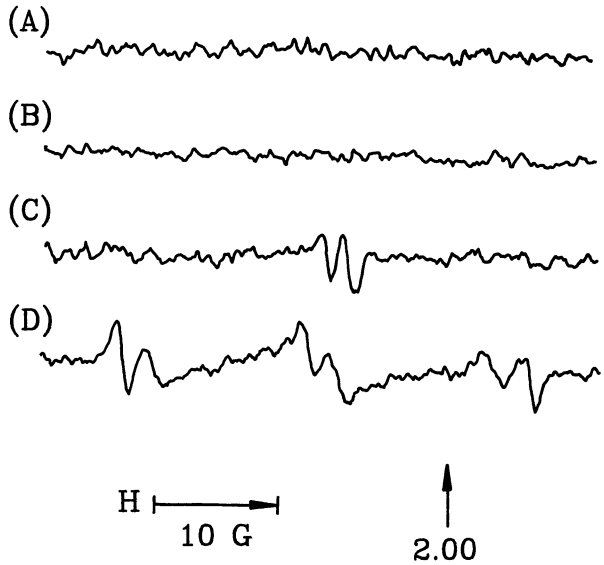


Fig. 3. Observed EPR spectra for pulsed 308 nm: (A) 4-POBN reagent before laser irradiation; (B) 4-POBN dissolved in blood before irradiation; (C) laser irradiation of myocardium in 4-POBN blood solution; and (D) irradiation of 4-POBN blood solution without myocardium.

trum obtained from excimer laser irradiation of blood solution shows a broad nitrogen dominated hyperfine structure (Fig. 3(D)). This EPR pattern presumably consists of di-*t*-butylnitroxide which is formed by photodecomposition 4-POBN and blood cell.

Comparison of the EPR spectra for trapped free radicals generated by laser irradiation of tissue sample is shown in Fig. 4. We immediately recognize three major EPR spectral differences between cw and pulsed laser irradiation. First of all, the hyperfine structure obtained for pulsed laser irradiation is obviously distinct from those obtained for cw laser irradiation. Second, the center line for pulsed laser irradiation is shifted towards high magnetic field. Thus, the *g*-value associated with the photogenerated free radicals is smaller than those for cw laser irradiation. Third, the hyperfine splitting for pulsed excimer laser photolysis is smaller (ca. 1 Gauss) than those for cw laser irradiation, as listed in Table 1. In addition, the pulsed 308 nm ablation of tissue sample did not produce thermal damage to the target site or the surrounding blood solution. However, cw 514 and 351 nm irradiation of the tissue sample resulted in thermal damage to the tissue target site and formed a thermally damaged cloud in the surrounding blood solution.

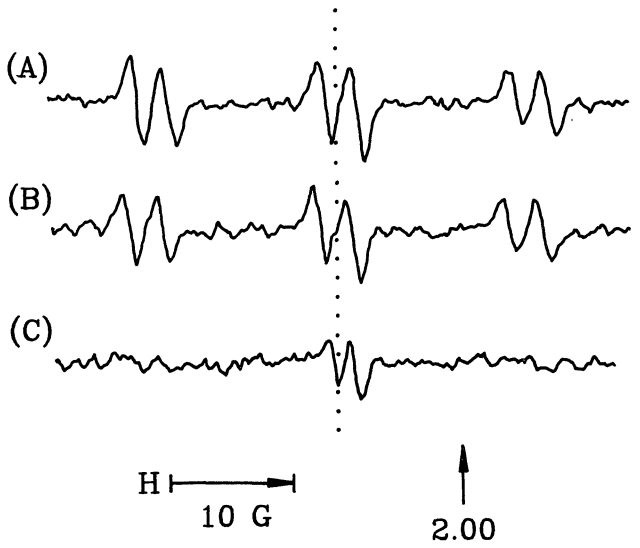


Fig. 4. Comparison of the EPR spectra for (A) cw 514 nm, (B) cw 351 nm, and (C) pulsed 308 nm laser irradiation of myocardium in the presence of 4-POBN in blood. The vertical dotted line represents a reference line. The calibrated *g*-value, 2.00$_0$, is marked. (Not DPPH, g=2.0036).

Table 1. EPR Parameters of Trapped Free Radicals by 4-POBN

R·	a^{N}_{NO} [b)]	a^{H}_{β} [b)]	Comments
Unidentified	15.77±0.07	2.66±0.06	cw 514 nm irrad.
Unidentified	15.63±0.09	2.86±0.06	cw 351 nm irrad.
Unidentified	—	1.78±0.08	pulsed 308 nm irrad.
H	16.2	10.2	Ref. 16
LOO	15.8	2.56	Ref. 17[a)]

a) Lipid peroxy radical. b) Units in Gauss.

Addition comparison of the EPR spectra for cw 514 nm, 351 nm, and pulsed 308 nm irradiation of blood solution in the presence of 4-POBN is shown in Fig. 5. Figure 5(A) shows that the decomposition of spin trap results in a sharp three line pattern building on a weak doublet of triplet signal. Changing the laser wavelength to 351 nm a weak doublet of triplet signal is observed. The main spectral differences between (A) and (B) in Fig. 5 are due to the light absorption property of canine blood. The absorption spectrum of canine blood is shown in Fig. 6. A peak at 412 nm is

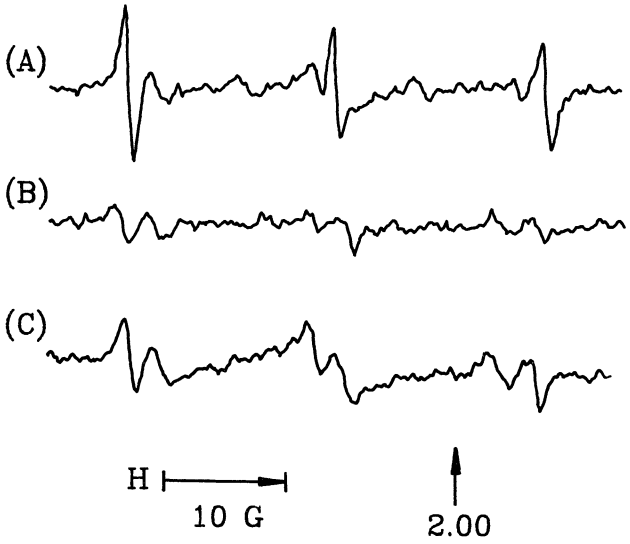


Fig. 5. Comparison of EPR spectra for (A) cw 514 nm, (B) cw 351 nm, and (C) pulsed 308 nm laser irradiation of blood solution in the presence of 4-POBN.

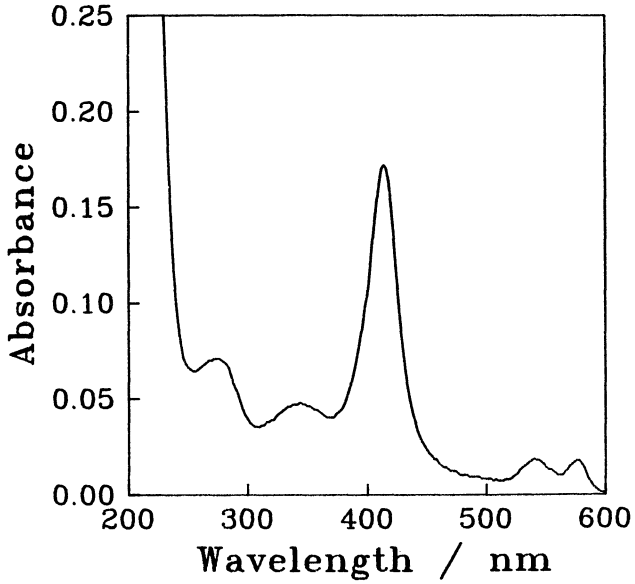


Fig. 6. The canine blood absorption spectrum.

characteristic hemoglobin absorption. The relative intensity of absorption at 351 nm is six times stronger than that at 514 nm. Switching to pulsed 308 nm irradiation of canine blood, a broad doublet of triplet signal is observed without thermal damage to the site. The signal might be due to decomposition of blood cell as well as spin trap. This broad EPR signal might result from slow tumbling motion of the trapped free radicals. Slow molecular motion generally results in the broadness of EPR signals.[11] However, there is no similarity on the EPR signal between laser irradiation of the blood solution and laser ablation of the tissue sample.

Discussion

The mechanism of laser-tissue interaction has been the subject of great concern. The light delivery of cw or pulsed energy has quite different consequences in its effect on the tissue target site. A schematic illustration of laser-tissue interaction is presented in Fig. 7. In the case of light delivered in a cw mode, several groups have reported the extensive thermal damaged to the tissue target site.[2,3,12] In contrast, high energy excimer laser ablates the tissue with no evidence for thermal damage.[1-3,6,12] However, both laser modes cw and pulse involve the production of free radicals accompanying laser interaction with tissue. The species of free radicals are weakly dependent depending on laser wavelengths and strongly dependent on laser operational mode: cw and pulse.

A. CW Laser Irradiation. A photon 514 nm wavelength is equivalent to 2.4 eV. This energy is not enough to break the average C–C bond which is ca. 3.6 eV. The excited molecules at the target site undergo internal coversion to a vibrational excited states, and any subsequent decomposition can be considered as a thermal process in which the photons merely act as a source of thermal energy.[13] This heat accumulation of the tissue may play an important role of the free radical generation and propagation. As a matter of fact, sever

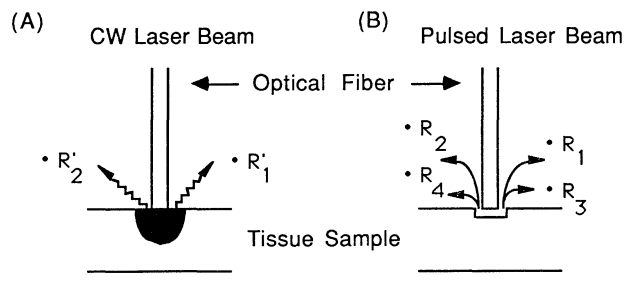


Fig. 7. A schematic illustration of short-lived free radicals produced by laser irradiation of the tissue sample. $\cdot R'_1$ and $\cdot R'_2$ represent cw laser generated free radicals, and $\cdot R'_1$, $\cdot R'_2$, $\cdot R'_3$, and $\cdot R'_4$ represent pulsed laser genertd free radicals where pulsed laser produces free radicals more efficient compared to cw laser (Ref. 3). (A) CW laser irradiation shows severe thermal damage to the target site. (B) Excimer laser irradiation shows clean tissue removal without thermal damage.

thermal damage to the tissue target site was observed for 514 nm laser irradiation of the cardiovascular tissue.

A photon energy of cw 351 nm wavelength is 3.9 eV. This laser also produces thermal damage to the tissue target site.[3] Thus, the fundamental radical generation scheme might be similar to that for cw 514 nm radiation. However, the tissue sample absorbs more cw 351 nm laser light than cw 514 nm.[14] Thus, the UV laser light excites the tissue sample more effectively and may break weak bonds of the protein sample. The slight differences (ca. 0.2 Gauss) in β-hydrogen splitting in the observed EPR spectra between cw 514 nm and 351 nm, as listed in Table 1, could explain the differences. Both EPR spectra obtained by two different cw laser wavelength irradiations of myocardium may not be distinguished clearly without additional characteristic EPR structure.

Therefore, the trapped radicals for cw visible and ultraviolet laser photolysis should have some similarities, and the free radicals are photothermally produced. Thermally excited water in the tissue and oxygen in blood is involved for their generation and propagation. Oxygen centered free radicals are the most likely produced. Hydrogen radicals may not be trapped because the typical hyperfine values for the trapped hydrogen radical ($a^N \approx 16.3$, $a_\beta^H \approx 10.2$ Gauss[15]) do not match the hyperfine values obtained by laser irradiation of tissue as listed in Table 1.

B. Excimer Laser Irradiation. A photon energy at 351 nm for pulsed laser is the same as that of the cw UV 351 nm. However, the pulsed laser beam is very is very intense, that is, a large number of photons are produced within a 20 nanosecond laser pulse. Such a direct impact of photons from the laser causes nonthermal ablation of the tissue samples and the expulsion of the fragments at supersonic velocities.[13] This pulsed laser excitation could have enough energy to fragment the tissue protein chains without thermal damage at the target site.

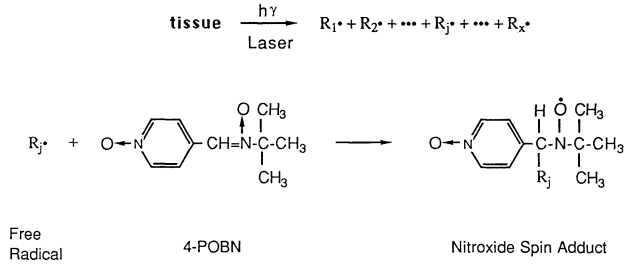
The present results show that there are clear differences in observed EPR spectra between cw and pulsed laser irradiation of the tissue sample under blood solution as well as laser irradiation of the spin trap-blood solution without the tissue sample, as shown in Figs. 4 and 5, respectively. The EPR spectra obtained by cw laser photolysis of the tissue sample show a doublet of triplets. On the other hand, the EPR spectrum obtained by excimer laser ablation has a unique profile (Fig. 4(C)). The unusual EPR profile for excimer laser excitation can be accounted for by the followings: First, recent laser photoablation studies have indicated that various molecules are produced by pulsed UV laser irradiation of biological material and organic polymer.[1-3,5,18,19] If the tissue sample is exposed to a pulsed UV laser beam, it will break down into various molecular fragments of the protein sample. These free radical fragments can subsequently react with 4-POBN and

become stable free radicals. Second, the various size of free radicals might be a key to the unusual EPR spectrum. The tumbling motion of a nitroxide spin adduct affects changes in EPR lines due to loss of spectral symmetry. If the size of the spin adduct is large, the rotational correlation time for the spin adducts increases considerably. Third, viscosity of the blood solution might contribute to the unusual hyperfine structure for excimer laser irradiation of tissue. The viscosity of blood is about 4.8 relative to water.[20] If a nitroxide spin adduct is immobilized due to the higher viscosity, the EPR spectrum loses the spectral symmetry of nitrogen hyperfine structure.[10, 21]

As conformation of the interpretation, Collagen as the model compound was irradiated by excimer laser in the same manner as the irradiation of the tissue samples. The EPR signal obtained by excimer laser ablation of collagen is identical to that of the tissue sample.[22] Therefore, carbon centered various free radicals from the tissue sample can be trapped by excimer laser.

Summary

Considering the above discussed, the possible free radicals for pulsed UV laser ablation are C-terminal with various chain molecules. Various free radicals are produced by pulsed and cw laser operational modes. The contingent reaction scheme is



where R_1·, R_2·, R_j·, and R_x·, represents unidentified free radical fragments formed on either cw or pulse laser irradiation of the tissue. Those free radicals react with 4-POBN and become stable nitroxide spin adducts. For cw laser irradiation, R· can be oxygen centered radicals. C-terminal R· are most likely produced by pulsed laser. The present EPR results suggest that the backbone of amino acids, lipids, or other biological building blocks are cleaved during the ablation. Moreover, the carbon centered long chain free radicals derived from peptide main chain scission were produced by Hg–Xe lamp photolyses[23] and C-terminal radicals are observed by high output power cw laser irradiation of the tissue samples.[24]

Finally, it is interesting to note that those different types of laser generated short-lived free radicals should be converted to stable free radicals by biochemical manner based on the species for practical applications. The present results do not provide full details regarding laser generated free radicals. In the presence of multiple free radicals such as in our system, the identification of unknown radicals becomes more difficult using conventional cw EPR techniques. It is important to see substantial hyperfine structures in the EPR spectrum of the spin adduct. In most cases the hyperfine structure is a key to identification of the trapped radicals. Therefore, it might be useful to employ other techniques to regain some missing details of the hyperfine other techniques to regain some missing details of the hyperfine structure of the spin adducts in order to provide full information regarding the laser generated free radicals.

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