



## Sensitivity Enhancement in Solution NMR *via* Photochemically Induced Dynamic Nuclear Polarization

Jonghyuk Im and Jung Ho Lee\*

Department of Chemistry, Seoul National University, Seoul 08826, South Korea

Received Jan 15, 2017; Revised Feb 10, 2017; Accepted Mar 02, 2017

**Abstract** Enhancements in NMR sensitivity have been the main driving force to extend the boundaries of NMR applications. Recently, techniques to shift the thermally populated nuclear spin states are employed to gain high NMR signals. Here, we introduce a technique called photochemically induced dynamic nuclear polarization (photo-CIDNP) and discuss its progresses in enhancing the solution-state NMR sensitivity.

**Keywords** Photo-CIDNP, Solution NMR Sensitivity, Radical Pair Mechanism, Solid-state Photo-CIDNP

### NMR Sensitivity in Solution

In the initial days of NMR, its sensitivity was just enough to detect 55 M bulk water.<sup>1</sup> Since then, NMR has experienced many technical breakthroughs that now enable analysis of sub-millimolar biomolecules (e.g., proteins, RNA, DNA, and their complexes) at atomic resolution. The advancements in NMR techniques strongly correlate with the enhancements in NMR sensitivity. For example, superconducting magnets, cryoprobes, and Fourier transform NMR,<sup>2</sup> each led to dramatic enhancements in NMR sensitivity and revolutionized our way of doing NMR experiments. In addition, development of isotope labeling schemes, pulse sequences (e.g. indirect detection methods, transverse relaxation optimized

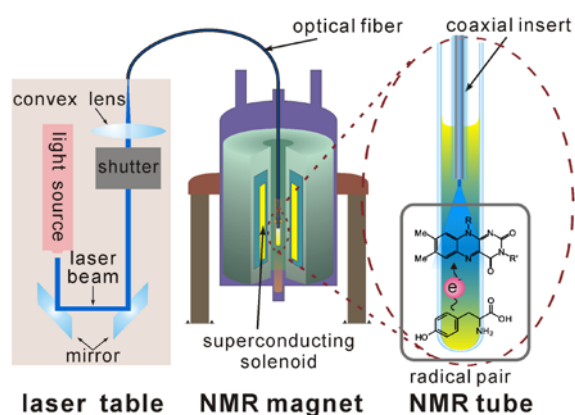
spectroscopy),<sup>3-5</sup> and signal processing schemes, all culminated in the current state-of-the-art NMR sensitivity.<sup>6</sup> All these methods focus on either increasing NMR signal or reducing noise, both of which lead to enhanced NMR sensitivity.

For spin=1/2 nuclei (e.g. <sup>1</sup>H, <sup>13</sup>C, <sup>15</sup>N),  $\alpha$  and  $\beta$  nuclear spin states are the eigenstates of the Zeeman Hamiltonian. Even in high-field magnets, the  $\alpha$  and  $\beta$  states are nearly equally populated due to their similar energy levels, and the difference in population gives rise to NMR signals. Perturbing the Boltzmann distribution of nuclear spin states has the potential to greatly enhance NMR signals, and the hyperpolarization techniques<sup>7-11</sup> are based on this principle. Despite the success of these techniques, they are usually not effective in solution state,<sup>6</sup> where the majority of NMR experiments are performed.

### Photochemically Induced Dynamic Nuclear Polarization (Photo-CIDNP)

Chemically induced dynamic nuclear polarization (CIDNP) was first observed in chemical reactions.<sup>12, 13</sup> Nuclear spins can influence the course of chemical reactions that involve radical pairs as intermediates. As a result, nuclei with different spin states can be sorted into different reaction products. Later, it was shown that light irradiation can cause CIDNP by promoting the formation of transient radical pairs.<sup>14</sup>

\* Correspondence to: **Jung Ho Lee**, Department of Chemistry, Seoul National University, Gwanak-ro 1, Gwanak-gu, Seoul, 08826, South Korea, Tel: 82-2-880-4363; E-mail: jung.ho.lee@snu.ac.kr



**Figure 1.** A photo-CIDNP setup. Light is guided into an NMR sample through an optical fiber. The sample-end of the fiber is protected by a coaxial insert. When a dye gets excited by light, it extracts an electron from an oxidizable molecule upon collision, and a radical pair is transiently generated. Nuclear-spin-state-dependent recombination of radical pairs gives rise to the photo-CIDNP effect.

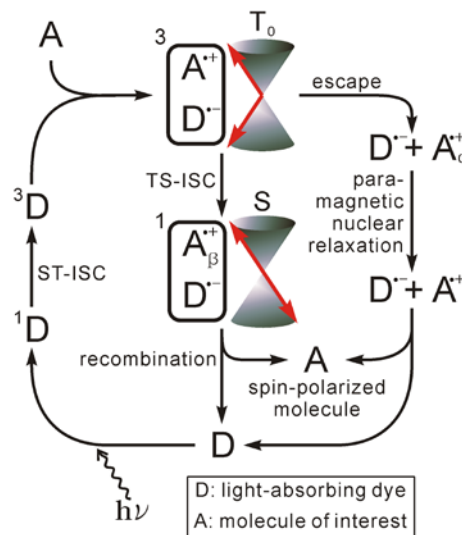
This effect is called photochemically induced dynamic nuclear polarization (photo-CIDNP).<sup>15-17</sup> The timing of the radical pair formation and the ensuing nuclear spin polarization can be controlled, e.g., just before a 90° pulse is applied in an NMR magnet. For proteins, a radical pair is generated between a light-absorbing dye and an aromatic amino acid, which induces nuclear spin polarization in both counterparts. Light is guided into an NMR sample through an optical fiber. Laser triggering and shutter opening can control the irradiation timing of a pulsed laser and a continuous-wave (CW) laser, respectively (Fig. 1).

In addition to enhancing NMR sensitivity, photo-CIDNP monitors the solvent-accessibility of aromatic amino acids in solution, since the excited dye molecules are required to be in spatial proximity to the oxidizable molecules, in order to generate radical pairs. Therefore, the photo-CIDNP signals indicate the extent of solvent exposure of the aromatic amino acids in well-ordered proteins, which provide information on biomolecular structures and interactions.<sup>16,17</sup> In addition, photo-CIDNP is suitable for the mechanistic studies of protein folding,<sup>18,19</sup> where the degree of solvent exposure of hydrophobic residues continuously change over the folding

process. Furthermore, it has been demonstrated that protein dynamics can be probed by photo-CIDNP either by measuring the nuclear spin relaxation rates in transiently generated radical pairs<sup>20</sup> or by observing the photo-CIDNP phase inversions in fluorine-substituted proteins.<sup>21</sup>

### Radical Pair Mechanism (RPM)

How does the transient radical pair formation lead to the polarization of nuclear spin states? Most of the photo-CIDNP effects observed in solution can be understood in terms of radical pair mechanism (RPM) suggested by Kaptein<sup>22</sup> and Closs<sup>23</sup> (Fig. 2).

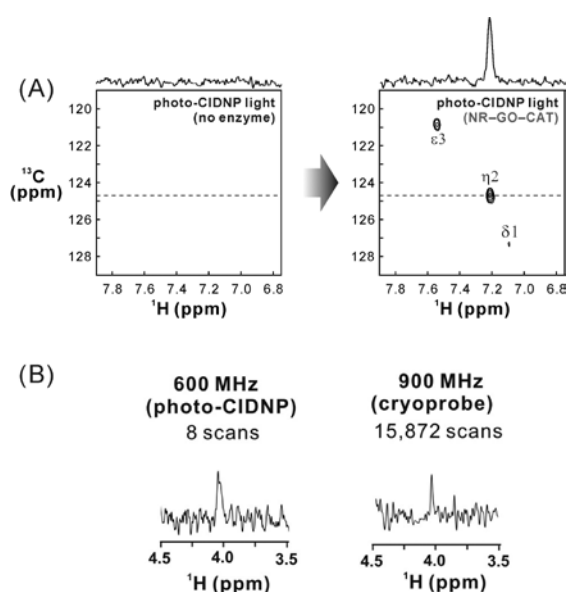


**Figure 2.** Radical Pair Mechanism. Upon light absorption ( $h\nu$ ), a dye (D) gets excited to a  $^1D$  singlet excited state, which experiences ST-ISC (singlet-to-triplet intersystem crossing) to become a long-lived  $^3D$  triplet excited state.  $^3D$  can oxidize A (e.g., polypeptides with aromatic amino acids) upon physical contact and form a triplet radical pair ( $T_0$ ). This pair can either experience TS-ISC (triplet-to-singlet intersystem crossing) to become a singlet radical pair (S) or can escape and lose coherence. If a molecule with a nucleus in the  $\beta$  spin state, i.e.,  $A_\beta$ , undergoes accelerated TS-ISC and  $A_\alpha$  experiences decelerated TS-ISC, the  $\beta$  spin is likely to be preserved after recombination, while the  $\alpha$  spin is likely to experience paramagnetic relaxation. This results in a  $\beta > \alpha$  spin polarization. The red arrows indicate unpaired electrons in radical pairs.

To begin with, an NMR sample for a photo-CIDNP experiment is placed inside an NMR magnet and light is guided into the sample in a timely manner (Fig. 1). The sample contains light-absorbing dyes (D) and oxidizable molecules of interest (A), e.g., proteins with tryptophan, tyrosine, and histidine. Upon laser excitation, the dye gets excited to a singlet state ( $^1D$ ), which experiences singlet-to-triplet intersystem crossing (ST-ISC) to form a triplet state ( $^3D$ ). This triplet-excited dye ( $^3D$ ) has a long enough lifetime to diffuse and encounter the solvent-exposed aromatic amino acids in proteins. Upon encounter, an electron is transferred from the aromatic amino acid to the excited dye forming a triplet radical pair  $^3[D^{\bullet-}A^{\bullet+}]$ . Among the many amino acids, only the aromatic ones are capable of forming radical pairs because only these have oxidation potentials that are low enough to donate electrons to the excited dyes.<sup>24</sup> A triplet radical pair can experience triplet-to-singlet intersystem crossing (TS-ISC) to form a singlet radical pair  $^1[D^{\bullet-}A^{\bullet+}]$ , and it is only in this singlet state that the recombination takes place. Note that the intersystem crossing (ISC) occurs only when the two radicals are separated and experience negligible repulsive exchange interaction such that the energies of the singlet (S) and triplet ( $T_o$ ) electronic states are nearly degenerate. Here,  $S = (\alpha_D\beta_A - \beta_D\alpha_A) / \sqrt{2}$  and  $T_o = (\alpha_D\beta_A + \beta_D\alpha_A) / \sqrt{2}$ , where  $\alpha_D$  denotes an unpaired electron in the dye D in the  $\alpha$  spin state, and  $\beta_A$  denotes an unpaired electron in the molecule A in the  $\beta$  spin state. The rate of the intersystem crossing depends on the spin states of the nuclei in D and A, or more accurately, in  $D^{\bullet-}$  and  $A^{\bullet+}$ , by isotropic hyperfine coupling of the nuclear spins to the unpaired electron spins.

To illustrate the source of nuclear spin polarization, let us assume that the ISC is accelerated if a certain nucleus of a molecule A is originally in the  $\beta$  spin state ( $A_\beta$ ), and the ISC is decelerated if this nucleus is in the  $\alpha$  spin state ( $A_\alpha$ ), through hyperfine coupling. Because the ISC is relatively fast for the radical pair with  $A_\beta$ , this nuclear spin state is likely to be preserved during TS-ISC and recombination. In contrast, the relatively slow ISC for the radical pair

with  $A_\alpha$  can lead to dissociation of radicals  $D^{\bullet-}$  and  $A_\alpha^{\bullet+}$  followed by paramagnetic relaxation of  $A_\alpha^{\bullet+}$  to nearly equal populations of  $A_\beta^{\bullet+}$  and  $A_\alpha^{\bullet+}$ . The radicals of random phase later reencounter  $[D^{\bullet-}A^{\bullet+}]$  and return to their original states D and A (Fig. 2). Therefore, if the  $\beta$  spin state of the nucleus is preserved and the  $\alpha$  spin state of the nucleus relaxes to  $\alpha$  and  $\beta$ , the nuclear spin polarization of  $\beta > \alpha$  is established.



**Figure 3.** Method developments continuously push the limits of achievable NMR sensitivity by photo-CIDNP in solution. (A) 2D photo-CIDNP NMR spectra of a 20  $\mu$ M polypeptide sample without (left) and with (right) nitrate reductase (NR) to oxidize the reduced flavin dye, and glucose oxidase (GO) and catalase (CAT) to remove oxygen. (B) Photo-CIDNP experiment with a fluorescein dye enables rapid analysis of a 1  $\mu$ M tryptophan sample. Adapted with permission from (A) J.H. Lee and S. Cavagnero, *J. Phys. Chem. B* **117**, 6069 (2013) and (B) Y. Okuno and S. Cavagnero, *J. Phys. Chem. B* **120**, 715 (2016). Copyright 2013 & 2016 American Chemical Society.

### Efforts to Improve Solution-State NMR Sensitivity by Photo-CIDNP

Methods have been developed to further enhance the sensitivity of NMR, achievable by photo-CIDNP in

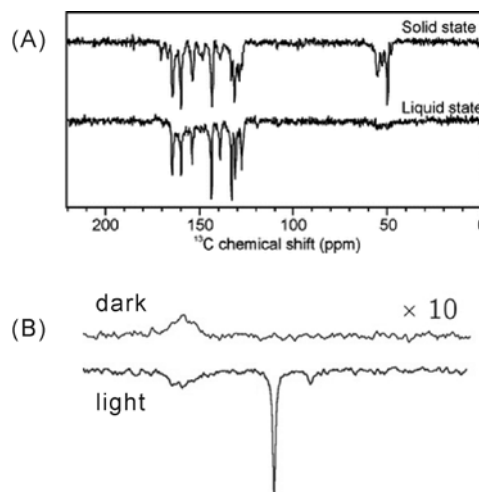
solution. First of all, pulse sequences were developed to combine the sensitive NMR detection of protons with the huge photo-CIDNP enhancements in heteronuclei.<sup>25,26</sup> In addition, methods to prolong the photo-CIDNP data acquisition time were developed to gain additional sensitivity by signal averaging and to perform multidimensional photo-CIDNP experiments. For instance, a tri-enzyme system has been developed to remove the singlet oxygen and to oxidize the reduced dye at the same time, thereby preserving the integrity of both the sample and the dye under repeated laser pulses.<sup>27</sup> This strategy led to a 48-fold enhancement in NMR sensitivity compared to that of a control SE-HSQC (sensitivity-enhanced heteronuclear single quantum coherence) experiment and enabled 2D photo-CIDNP experiments that use 20  $\mu\text{M}$  polypeptide samples (Fig. 3A). Furthermore, it is important to select light-absorbing dyes that are stable under high power laser pulses. Besides, when an excited dye generates a radical pair, the unpaired electrons in the dye and the sample radicals should have distinct  $g$ -values.<sup>16</sup> Recently, a fluorescein dye was found to be very effective in extending the photo-CIDNP detection limit down to 1  $\mu\text{M}$  sample concentration (Fig. 3B).<sup>28</sup> Additionally, stepwise-tapered optical fibers were found to be effective for optically dense samples with high dye concentrations.<sup>29</sup> Moreover, the time-resolved photo-CIDNP experiments using a pulsed laser can provide great enhancements by eliminating various cancellation and nuclear Overhauser effects, which take place in CW laser experiments.<sup>30,31</sup> The cancellation effect is observed when the nuclear spins of the escaped radicals do not experience fast enough paramagnetic relaxation.

### Solid-State Photo-CIDNP Mechanisms in Action

Photo-CIDNP was first detected in solid-state by McDermott and coworkers in a photosynthetic reaction center (RC).<sup>32</sup> Since then, numerous RCs in bacteria and plants have witnessed solid-state photo-CIDNP effects.<sup>33</sup> The RCs show over 10,000-fold enhancements in NMR sensitivity upon

light irradiation, and new mechanisms other than RPM were proposed to explain the solid-state photo-CIDNP observations.<sup>34-36</sup> The only other system that shows solid-state photo-CIDNP is the chromophore-binding domain of phototropins, namely, LOV1-C57S.<sup>37</sup>

Strikingly, an RC and a LOV2-C450A (another chromophore-binding domain) showed photo-CIDNP in solution, but their enhancement factors and phases could only be explained by the solid-state photo-CIDNP mechanisms. The enhancement factors are quite large for these systems where the RC showed an 10,000-fold NMR sensitivity enhancement<sup>38</sup> (Fig. 4A), whereas the LOV2-C450A



**Figure 4.** Solid-state photo-CIDNP mechanism in solution. (A)  $^{13}\text{C}$  photo-CIDNP NMR spectra of photosynthetic reaction center at 4.7 T under continuous illumination. (B)  $^{13}\text{C}$  photo-CIDNP NMR spectra of LOV2-C450A mutant of phototropin without (dark, top) and with (light, bottom) light irradiation at 7.1 T. Adapted with permission from (A) E. Daviso et al., *J. Am. Chem. Soc.* **133**, 16754 (2011) and (B) G. Kothe et al., *J. Phys. Chem. B* **118**, 11622 (2014). Copyright 2011 & 2014 American Chemical Society.

showed up to a 6,000-fold enhancement<sup>39</sup> (Fig. 4B). The electron-electron-nuclear three-spin mixing (TSM) mechanism is mainly responsible for these observations. TSM is a coherent mechanism that transfers the high spin order of electrons to nuclei, mostly through anisotropic hyperfine interactions.<sup>36</sup> It is not surprising that the RC photo-CIDNP works

through this solid-state mechanism because its tumbling rate is too slow to efficiently average out the hyperfine anisotropy during the radical pair lifetime.<sup>38</sup> However, this is unlikely to be the case for a small ~17 kDa LOV2-C450A protein, where the angle between the symmetry axis of the hyperfine tensor and the external field is rapidly changing. Considering that the photo-CIDNP of many proteins with mass greater than 20 kDa can be readily explained by RPM,<sup>16</sup> intraprotein electron transfer, fixed orientation between D and A, or some other unknown factors may be crucial to trigger a solid-state photo-CIDNP mechanism in solution. Finally, although the solid-state mechanisms lead to high NMR sensitivity in solution, note that these experiments are performed in low magnetic fields to match the Zeeman energy to hyperfine coupling<sup>36</sup> (Fig. 4).

### Perspectives

Photo-CIDNP is one of the most promising tools to enhance NMR sensitivity in solution. It also provides information on the residue-specific solvent

accessibility of biomolecules. Considering that solid-state photo-CIDNP mechanisms, which have a potential to greatly enhance NMR sensitivity, can surprisingly be active even in small proteins in solution, and that there are yet unexplained photo-CIDNP observations,<sup>40</sup> an in-depth understanding of photo-CIDNP mechanisms may lead to even higher NMR sensitivity enhancements in solution.

Hyperpolarization methods have great potential to impart exceptional NMR sensitivity; however, their application is often restricted to limited samples. In practice, NMR users invest millions of dollars in purchasing superconducting magnets and cryoprobes to achieve only several folds of enhancements in 'general' NMR sensitivity. Thus, in addition to pursuing methods to shift the Boltzmann population of the nuclear spin states, it is important to devise ways to enhance NMR sensitivity that would benefit NMR users in general. In conjunction with the methods that can quickly give high-resolution NMR spectra provided that high NMR sensitivity is guaranteed,<sup>41,42</sup> NMR sensitivity enhancements in solution hold great promise for exciting applications in the future.

### Acknowledgements

This work was supported by Research Resettlement Fund for the new faculty of Seoul National University (305-20160036).

### References

1. F. Bloch, W. W. Hansen, and M. Packard, *Phys. Rev.* **70**, 460 (1946)
2. R. R. Ernst, G. Bodenhausen, and A. Wokaun, *Principles of nuclear magnetic resonance in one and two dimensions*, Vol. 14, Clarendon Press Oxford (1987)
3. G. A. Morris, and R. Freeman, *J. Am. Chem. Soc.* **101**, 760 (1979)
4. A. Bax, S. W. Sparks, and D. A. Torchia, *Methods Enzymol.* **176**, 134 (1989)
5. K. Pervushin, R. Riek, G. Wider, and K. Wüthrich, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 12366 (1997)
6. J. H. Lee, Y. Okuno, and S. Cavagnero, *J. Magn. Reson.* **241**, 18 (2014)
7. J. H. Ardenkjær-Larsen, B. Fridlund, A. Gram, G. Hansson, L. Hansson, M. H. Lerche, M. Thaning, and K. Golman, *Proc. Natl. Acad. Sci. U.S.A.* **100**, 10158 (2003)
8. B. M. Goodson, *J. Magn. Reson.* **155**, 157 (2002)
9. W. Happer, *Rev. Mod. Phys.* **44**, 169 (1972)

10. T. Maly, G. T. Debelouchina, V. S. Bajaj, K. N. Hu, C. G. Joo, M. L. Mak-Jurkauskas, J. R. Sirigiri, P. C. van der Wel, J. Herzfeld, R. J. Temkin, and R. G. Griffin, *J. Chem. Phys.* **128**, 052211 (2008)
11. J. Natterer, and J. Bargon, *Prog. Nucl. Magn. Reson. Spectrosc.* **31**, 293 (1997)
12. H. R. Ward, and R. G. Lawler, *J. Am. Chem. Soc.* **89**, 5518 (1967)
13. J. Bargon, H. Fischer, and U. Johnsen, *Z. Naturforsch. A* **22**, 1551 (1967)
14. M. Cocivera, *J. Am. Chem. Soc.* **90**, 3261 (1968)
15. L. T. Kuhn, *Hyperpolarization Methods in NMR Spectroscopy*, Vol. 338, Springer-Verlag, Berlin (2013)
16. J. Hore, and R. Broadhurst, *Prog. Nucl. Magn. Reson. Spectrosc.* **25**, 345 (1993)
17. R. Kaptein, K. Dijkstra, and K. Nicolay, *Nature* **274**, 293 (1978)
18. K. H. Mok, L. T. Kuhn, Martin Goez, I. J. Day, J. C. Lin, N. H. Andersen, and P. J. Hore, *Nature* **447**, 106 (2007)
19. K. H. Mok, and P. J. Hore, *Methods* **34**, 75 (2004)
20. O. B. Morozova, and A. V. Yurkovskaya, *J. Phys. Chem. B* **119**, 12644 (2015)
21. I. Kuprov, T. D. Craggs, S. E. Jackson, and P. Hore, *J. Am. Chem. Soc.* **129**, 9004 (2007)
22. R. Kaptein, and J. Oosterhoff, *Chem. Phys. Lett.* **4**, 195 (1969)
23. G. L. Closs, *J. Am. Chem. Soc.* **91**, 4552 (1969)
24. H. Seki, A. Takematsu, and S. Arai, *J. Phys. Chem.* **91**, 176 (1987)
25. J. H. Lee, A. Sekhar, and S. Cavagnero, *J. Am. Chem. Soc.* **133**, 8062 (2011)
26. A. Sekhar, and S. Cavagnero, *J. Magn. Reson.* **200**, 207 (2009)
27. J. H. Lee, and S. Cavagnero, *J. Phys. Chem. B* **117**, 6069 (2013)
28. Y. Okuno, and S. Cavagnero, *J. Phys. Chem. B* **120**, 715 (2016)
29. I. Kuprov, and P. Hore, *J. Magn. Reson.* **171**, 171 (2004)
30. O. B. Morozova, P. Hore, V. E. Bychkova, R. Z. Sagdeev, and A. V. Yurkovskaya, *J. Phys. Chem. B* **109**, 5912 (2005)
31. G. L. Closs, R. J. Miller, and O. D. Redwine, *Acc. Chem. Res.* **18**, 196 (1985)
32. M. G. Zysmilich, and A. McDermott, *J. Am. Chem. Soc.* **116**, 8362 (1994)
33. J. Matysik, A. Diller, E. Roy, and A. Alia, *Photosynth. Res.* **102**, 427 (2009)
34. T. Polenova, and A. E. McDermott, *J. Phys. Chem. B* **103**, 535 (1999)
35. A. McDermott, M. N. G. Zysmilich, and T. Polenova, *Solid State Nucl. Magn. Reson.* **11**, 21 (1998)
36. G. Jeschke, *J. Am. Chem. Soc.* **120**, 4425 (1998)
37. S. S. Thamarath, J. Heberle, P. J. Hore, T. Kottke, and J. Matysik, *J. Am. Chem. Soc.* **132**, 15542 (2010)
38. E. Daviso, G. J. Janssen, A. Alia, G. Jeschke, J. Matysik, and M. Tessari, *J. Am. Chem. Soc.* **133**, 16754 (2011)
39. G. Kothe, M. Lukaschek, G. Link, S. Kacprzak, B. Illarionov, M. Fischer, W. Eisenreich, A. Bacher, and S. Weber, *J. Phys. Chem. B* **118**, 11622 (2014)
40. V. I. Valyayev, Yu. N. Molin, R. Z. Sagdeev, P. J. Hore, K. A. McLaughlan, and N. J. K. Simpson, *Mol. Phys.* **63**, 891 (1988)
41. L. Frydman, T. Scherf, and A. Lupulescu, *Proc. Natl. Acad. Sci. U.S.A.* **99**, 15858 (2002)
42. Ě. Kupče, R. Freeman, and B. K. John, *J. Am. Chem. Soc.* **128**, 9606 (2006)