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# NMR signal enhancement > 50000 times in Fast Dissolution Dynamic Nuclear Polarization.

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**Herein we report the synthesis and the study as polarizing agent for fast dissolution DNP of a novel mixed biradical with a BDPA and TEMPO radical units covalently bound by an ester group (BDPAesterTEMPO). Such a biradical exhibits an extremely high DNP NMR enhancement > 50000 which constitutes one of the largest signal enhancement observed so far, to the best of our knowledge.**

Dynamic nuclear polarization (DNP)[1] is used for enhancement of nuclear magnetic resonance signals in both solid and liquid samples. The introduction of dissolution DNP (dDNP) by Golman and co-workers[2] and low temperature magic angle spinning DNP (MAS-DNP) by Griffin and co-workers,[3] have demonstrated the applicability and potential of DNP to modern MRI and solid-state NMR. DNP enhanced NMR has enabled studies of previously unreachable systems, such as material surfaces[4], low concentration biological samples[5] or has been used for metabolic imaging applications.[6,7,8]

The nature of the polarizing agent has a crucial role in the efficiency of DNP. In the case of dDNP, the effort has been focused in monoradicals, mainly trityl radicals like the well-known Ox63,[2] PTM[9] or BDPA[10] derivatives. The typical mechanism of choice to transfer electron magnetization is the "solid-effect" (SE),[11] and the ideal radical for such a mechanism will be a monoradical with a narrow EPR line like Ox63, however it has been shown to form oligomers that may limit its efficiency.[12] On the other hand, biradicals are of considerable interest as polarizing agents for MAS-DNP experiments.[13,14] The development of high-field MAS-DNP has mainly focused on the cross-effect (CE) mechanism[15,16,17] which is one of the mechanisms for which a large signal

enhancement is expected for biradicals, since typical SE enhancements are considerably lower.[13] Up to now, the most successful biradicals so far, used for MAS-DNP in solid-state NMR applications, particularly to study biological solids, new materials and studies of material surfaces[3,4,18] are based on two radicals of the same type (nitroxides) combined in the proper orientation and distance.[14,19,20] It is known that the ideal CE polarizing agent would be a biradical with an EPR spectrum consisting of two sharp lines separated by the Larmor frequency of the nuclei to be polarized, $\omega_{0S1}-\omega_{0S2} \approx \omega_{0I}$. However, only a few known radicals exhibit spectral narrow lines, e. g. trityl radicals and BDPA radical derivatives, which have similar isotropic $g$-values.[21] As TEMPO derivatives have a broad line with significant spectral density at a frequency separation matching $\omega_{0I}$, using a narrow line radical such as trityl or BDPA together with TEMPO could be a reasonable approximation. On the other hand, it is known that for an efficient cross effect mechanism, the dipolar coupling, in conjunction with the $J$-coupling, leads to the state mixing that is critical for it.[17] Some mixed nitroxide-trityl biradicals had been described as EPR sensors of the redox state of cells[22] or others[23] but had not been tested for DNP. The Griffin group has demonstrated the goodness of using some physical mixtures of two different radical species[13,24,25] and, recently, the use of a mixed biradical for MAS-DNP.[26]

Under dDNP, the efficiency of TEMPO-based biradicals is not optimal due to the difficulties to efficiently irradiate the full broad EPR lineshape at low temperature under static conditions but mixed biradicals have to be explored. Taking into account all these data, we recently proposed the first mixed biradical as polarizing agent for dDNP based on trityl and TEMPO radical units covalently bound (PTM-TEMPO biradical),[27] which was the first mixed biradical used for DNP NMR applications, with very high signal enhancement. Such a biradical presented very weak coupling interaction $J$ between both radical units, as well as weak dipolar interaction, and the mechanism operating in the polarization process was most probably thermal mixing (TM), as the optimal concentration of the sample was very high (90 mM). Such results provided out

the interest in preparing and studying new biradicals made with trityl or BDPA and with nitroxide moieties showing both stronger *J*-coupling interaction and dipolar coupling, critical for efficient cross effect mechanism.

Here we describe a novel mixed biradical (the second used for dDNP in the literature) based on BDPA and TEMPO radical units covalently bound by an ester group which shows both stronger *J*-coupling interaction and dipolar coupling than PTM-TEMPO biradical. Such BDPAesterTEMPO biradical exhibits an extremely high DNP NMR enhancement which constitutes one of the largest signal enhancements observed so far in dDNP.[28] Up to now the best polarizing agent or the golden standard for dDNP applications involving the polarization of $^{13}$C nuclei is the commercially available Ox63, in which the transfer takes place through the SE mechanism, showing enhancements of the NMR signal by > 10000 times.[2]

The reaction between 4-oxo-TEMPO and methyl 2-(methylsilyl)acetate, through a Wittig-type reaction, allowed us to incorporate at the 4-position an exocyclic double bond bearing an ester functional group (Scheme 1). The reaction was performed in anhydrous THF at -78ºC obtaining the desired product **1** with high yield (75%) as orange oil which eventually solidifies. Then, the ester derivative **1** was hydrolyzed to the corresponding acid product **2** with KOH in a mixture of MeOH/water in warm conditions (35-38 ºC) for 6 h.
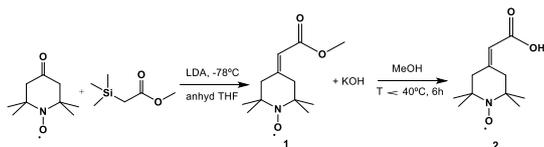


**Scheme 1** Synthetic scheme of 4-(2-methoxycarbonylmethylidene)-TEMPO radical (**1**) and of 4-carboxymethylidene-TEMPO (**2**).

The synthesis of biradical BDPAesterTEMPO (**5**) involved the esterification reaction between the benzyl alcohol derivative of BDPA, BA-BDPA (**3**)[10b] and 4-carboxymethylidene-2,2,6,6,-tetramethyl-1-piperidyloxyl (**2**, Scheme 2). The reaction was performed using DCC/DMAP in anhydrous DCM for 16 h and under Ar atmosphere. After chromatographic purification, monoradical **4** was isolated as an orange pure solid product (Y: 46%). The conversion of monoradical **4** to biradical **5** was done by treatment of the former with DBU base followed by an AgNO$_3$ one-electron oxidation. It should be mentioned that we always obtained biradical **5** with some amount of monoradical **4**. The sample used to perform the DNP studies contained 29 % of monoradical **4** (see below). A pure sample of monoradical **4** was also examined to discard its possible effect in the polarization process of **5**.
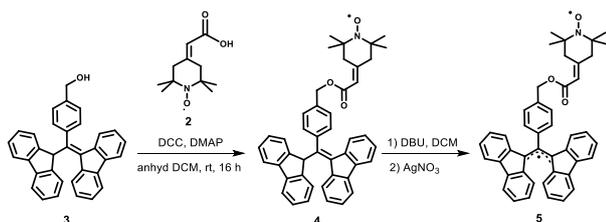


**Scheme 2.** Synthetic scheme for BDPAesterTEMPO biradical (**5**).

The EPR spectrum of the BDPA monoradical derived from **3** (BA-BDPA)[10b] shows the typical spectrum for BDPA monoradicals with a *g* factor of 2.0024 (Fig. S1). The hyperfine coupling constant with the 16 H atoms of the fluorenyl rings are $a_H$ (4H) = 1.99 G, $a_H$ (4H) = 1.83 G, $a_H$ (4H) = 0.54 G, $a_H$ (4H) = 0.37 G, and the total spectrum line width = 5.2 G. On the other hand, the EPR spectrum of the precursor monoradical TEMPO **4** shows the typical EPR spectrum of three sharp lines from the coupling of the electron with the nitrogen nucleus (I=1), with $a_N$ = 15.4 G and a *g* factor of 2.0064 (Fig. S1). The EPR spectrum of biradical **5** does not correspond to the sum of the EPR spectra of each radical unit separately but to three broad lines with a hyperfine coupling constant half the value of the corresponding to the monoradical TEMPO, i.e. $a_N$ = 7.82 G (Fig. S2). On the other hand, the linewidth of the three lines of the spectrum is lower ($\Delta$Hpp = 3.2 G) than that for the BDPA monoradical (5.2 G) and the typical hyperfine structure of BDPA radical is not resolved. These two features are explained because the hyperfine coupling constants with the 16 hydrogen atoms of the BDPA are also half the value than those for the monoradical one. This was determined by simulation (Fig. S2), showing 1/2 $a_H$ for BDPA as well as 1/2 $a_N$ for TEMPO, and, in addition, at an intermediated *g* value between both radical units (*g* = 2.0041, $a_H$ (4H) = 1.0 G, $a_H$ (4H) = 0.92 G, $a_H$ (4H) = 0.36 G, $a_H$ (4H) = 0.23 G, $a_N$ = 7.82 G). This means there is a strong *J*-coupling interaction between both radical units, where $|J| >> |a|$.[16] This was not the case of PTM-TEMPO biradical which showed two independent EPR lines of the two radical units separately, due to a very weak *J*-coupling. Along with these three broad lines there were three narrower lines characteristic of a TEMPO radical attached to a diamagnetic fragment (Fig. S2), which we attributed to unreacted monoradical **4**, and that was confirmed by HPLC analysis (see ESI†, Figs. S3-S5). Integration of the EPR signal indicates that there was 29 % of monoradical **4** present taking into account a per molecule analysis (see ESI†, Fig. S6). The frozen EPR spectrum of biradical **5** showed an $|\Delta m_s|$ = 2 transition at half-field (Fig. S7) confirming a dipolar interaction between both radical units,[29] that was not the case of PTM-TEMPO biradical.

The sample of BDPAesterTEMPO biradical **5** was tested as polarizing agent using as polarization sample a glassy mixture of [2-$^{13}$C]-acetone/sulfolane 1:1 (v/v). To determine the optimal frequency of polarization, a DNP microwave-sweep spectrum was registered with 100 µl of the acetone/sulfolane doped with 18 mM of biradical **5** (Fig. S8). It can be seen a narrow positive polarization peak at 94.090 GHz and a broad positive peak of 220 MHz width. This broad peak may be tentatively assigned to inefficient intermolecular CE between TEMPO moieties, while the narrow polarization feature at 94.090GHz may be attributed to intramolecular CE between the BDPA and TEMPO moieties. Similar observations have been reported by Griffin et al. for a trityl-TEMPO biradical[26] in MAS-DNP. The frequency of work was determined as the positive peak polarization P(+) = 94.090 GHz. In order to determine the optimal concentration of biradical **5** for the polarization of 2-$^{13}$C-acetone, polarizing building curves were determined at various radical concentrations: 5, 10, 20, 30, 40,

60 and 90 mM, prepared from a 120 mM stock solution of the biradical, at 3.35 T and 1.4 K (Fig. 1) and taking into account the 71% of biradical in the sample.
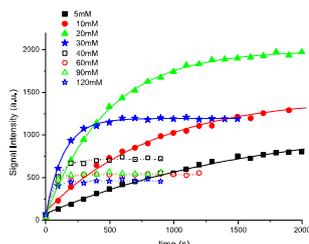


**Fig. 1** $^{13}$C polarization build-up curves of 100 µl samples doped with different concentrations of BDPAesterTEMPO biradical **5** in sulfolane:[2-$^{13}$C]acetone (1:1).

From Fig. 1 we can observe that the optimal concentration found for the BDPAesterTEMPO biradical **5** was 20 mM. It exhibits a shorter $\tau_{DNP}$ than Ox63. This suggests CE as the dominating mechanism operating for the polarization transference process. This is also shown in the plot of the time constants *versus* concentration (Fig. S9). The operative mechanism for the lower concentrations samples (5, 10, 20, 30, and 40 mM) probably corresponds to a CE mechanism, while that operating at larger concentrations (60 to 120 mM) could probably correspond to TM due to the high concentration.

The DNP efficiency of biradical **5** was compared with biradical PTM-TEMPO[27] as well as with the golden standard OX63 radical, at their optimal concentration. Moreover, it was very important to also study the precursor monoradical TEMPO derivative **4** to discard its possible effect in the polarization process, and it was studied at the optimal concentration determined for biradical **5**. Thus, we prepared different mixtures with the optimal concentration of each radical: 100 µl solution of sulfolane:[2-$^{13}$C]acetone (1:1) doped with 20 mM of biradical **5**, 90 mM of PTM-TEMPO biradical, 20 mM of monoradical **4**, and a 100 µl solution of d6-DMSO:[2-$^{13}$C]acetone (1:1) doped with 15 mM OX63. As can be observed in the polarization build-up curves (Fig. 2), the solid state polarization obtained for biradical BDPAesterTEMPO **5** is much higher than that obtained with PTM-TEMPO biradical as well as than with the golden standard OX63 radical, with large difference. On the other hand, the polarization level achieved with monoradical **4** was very low, as expected for a TEMPO monoradical, discarding its possible effect in the polarization process.
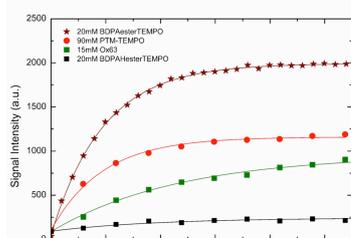


**Fig. 2** $^{13}$C polarization build-up curves at 3.35 T and 1.4 K of 100 µl solution of sulfolane:[2-$^{13}$C]acetone (1:1) doped with 20 mM of biradical **5** (red stars), 90 mM of PTM-TEMPO biradical (red circles), 20 mM of monoradical **4** (black squares), and a 100 µl solution of d6-DMSO:[2-$^{13}$C]acetone (1:1) doped with 15 mM OX63 (green squares).

After polarization, all the samples were fast dissolved into hot methanol and automatically transferred to a 600 MHz NMR spectrometer to perform the corresponding $^{13}$C NMR measurements. The overall transfer time was 6.5 s (5 s of transfer between DNP and NMR instruments, 1 s of sample stabilization and 500 ms of measurement time). The liquid-state signal enhancement (ε) was calculated by the acquisition of a conventional 1D $^{13}$C NMR experiment with 32 scans and using an excitation flip angle θ = 90º, for the thermal equilibrium samples and 1 scan and excitation flip angle θ = 10º the hyperpolarized samples (Table 1, Fig. 3). To measure the thermal sample, the hyperpolarized sample was maintained 5 minutes outside the magnet to ensure the complete relaxation. It can be observed in Table 1 and Fig. 3 that the corresponding liquid-state NMR enhancement ratio (ε) for biradical **5** was 50000, which means almost two times more enhancement than the only other mixed biradical described for dDNP (PTM-TEMPO) and much more than Ox63.
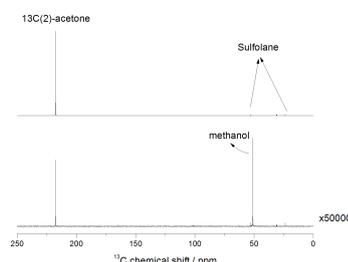


**Fig. 3** $^{13}$C NMR experiment with a single scan and using an excitation flip angle θ = 90º, for the hyperpolarized sulfolane:[2-$^{13}$C]acetone (1:1) doped with 20 mM of biradical **5** sample (up) and the thermal equilibrium sample enlarged 50000 times (down).

**Table 1.** Liquid-state NMR enhancement ratio (ε) of samples with biradical **5**, PTM-TEMPO, OX63 and monoradical TEMPO derived **4**.

| Radical | Pol. time [s] | Liquid-state enhancement (ε) |
|---|---|---|
| BDPAesterTEMPO **5**, 20 mM | 476 | 50000 |
| PTM-TEMPO 90 mM | 469 | 28750 |
| OX63 15 mM | 1169 | 18000 |
| Monoradical TEMPO **4**, 20 mM | 915 | 5000 |

In conclusion, we have shown an unprecedented increase in NMR signal-to-noise ratio achieved by a novel BDPAesterTEMPO mixed biradical in fast dissolution DNP experiments. It shows strong *J*-coupling interaction and dipolar interaction between both radical units and it is proposed to act as a cross effect polarizing agent as the optimal concentration of the biradical is low and exhibit shorter buildup time than those observed for monoradicals.

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