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Fractional Spin-Labeling of Polymers for Enhancing NMR Sensitivity by Solvent-Free Dynamic Nuclear Polarization

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Dynamic nuclear polarization (DNP)[1] combined with magic angle spinning (MAS)[2] can—under favorable conditions—enhance the nuclear spin polarization, that is, the difference between the populations of the Zeeman levels $|\alpha\rangle$ and $|\beta\rangle$ of a spin $I=^1/_2$ by up to two orders of magnitude ($\varepsilon_{DNP} \leq 10^2$) with respect to the Boltzmann distribution at thermal equilibrium at ca. 100 K, while accelerating relaxation and hence reducing recovery delays by more than an order of magnitude ($\kappa = R_{DNP}/R_1 = T_1/\tau_{DNP} > 10$), thus providing a means of shortening measurement times by up to five orders of magnitude. Where suitable solvents can be found, typical enhancements for ^{13}C or ^{29}Si are between $10 < \varepsilon_{DNP} < 100$ after cross-polarization from protons. However, line-broadening may offset those gains to some extent and part of the NMR signals may be bleached out by the presence of free radicals. At $T = 100$ K and $B_0 = 9.4$ T (400 MHz for protons), the DNP enhancement ε_{DNP} arises predominantly from the cross effect (CE),[3] which is induced by the microwave saturation of the electron spin resonance (ESR) transitions of stable biradicals, such as 1-(TEMPO-4-oxy)-3-(TEMPO-4-amino)propan-2-ol (TOTAPOL).[4] Until now, most studies have relied on solutions of free radicals in glass-forming solvents that are suitable to dissolve molecules,[5] to suspend fibers and nanocrystals or to wet porous powders,[6] in view of obtaining a spatially homogeneous distribution of free radicals in a frozen glassy state. It turns out to be far from trivial to find a solvent that forms a homogeneous glass at about 100 K, and that allows one to achieve high concentrations of the sample of interest. Many samples of biological origin (e.g. membrane proteins, peptide fibrils or protein microcrystals) cannot be dissolved or suspended to reasonable concentrations in any solvent. If the solubility is limited to *s* %, the DNP enhancement should be $\varepsilon_{DNP} = 100/s$ to break even, and more to justify the effort. By wetting of porous powders with solutions of free radicals, one can enhance the NMR signals of molecules grafted onto their surfaces.[7] The absence of solvent avoids exerting any forces that would cause the grafted molecules to stand up or to lie down on the surface, or, where applicable, interfere with catalytic activity. For these reasons, we set out to develop a method for DNP of dry samples that do not require any solvents.

In solution-state NMR, the covalent attachment of paramagnetic spin labels with unpaired electrons to biomolecules can accelerate their nuclear spin-lattice relaxation rates, thus making it possible to shorten the recovery delays between scans and accelerate the acquisition of NMR spectra.[8] If spin labels can accelerate the return of nuclear spins to their thermal equilibrium, they should also be able to enhance the nuclear spin polarization by DNP in the solid state, in analogy to liquid-state Overhauser effects on spin-labeled molecules[9] and to radicals embedded in glassy frozen solutions. Spin labels allow one to obtain long-range distance constraints,[10] particularly in large proteins.[11]

Herein, we demonstrate DNP-MAS in a solvent-free amorphous powder of the decapeptide Fmoc-Gly-Ala-Arg(Pbf)-Gly-Asp(O*t*Bu)-D-Phe-Lys(*Z*)-Arg(Pbf)-Gly-OH) (**DP**), of which a fraction $f \leq 16\,\%$ was labeled by covalent attachment of the biradical TOTAPOL (**DP***). Such peptides may be relevant for therapeutic approaches. For instance, a decapeptide fragment of Alzheimer's Aβ peptide shows antitumoral activity towards breast cancer cells.[13] It has also been shown that the decapeptide H_2N-RRYIRRYMRR-Ac inhibits HIV-1 entry into human helper T cells.[14] The therapeutic potential of such biopolymers makes the development of suitable analytical methods desirable. The nonapeptide GARGDFKRG (**NP**, Scheme 1) was produced by solid-phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin as solid support. The sequential introduction of the amino acids GARGDFKRG was performed by successive coupling steps promoted by *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) and 1-hydroxybenzotriazole (HOBt) as coupling reagents followed by the removal of the fluorenylmethoxycarbonyl (Fmoc) protecting group in the presence of piperidine. Finally the nonapeptide **NP** was cleaved from the resin under acidic conditions. The free biradical TOTAPOL was synthesized according to the procedure described by Griffin.[4b] It reacts with the terminal

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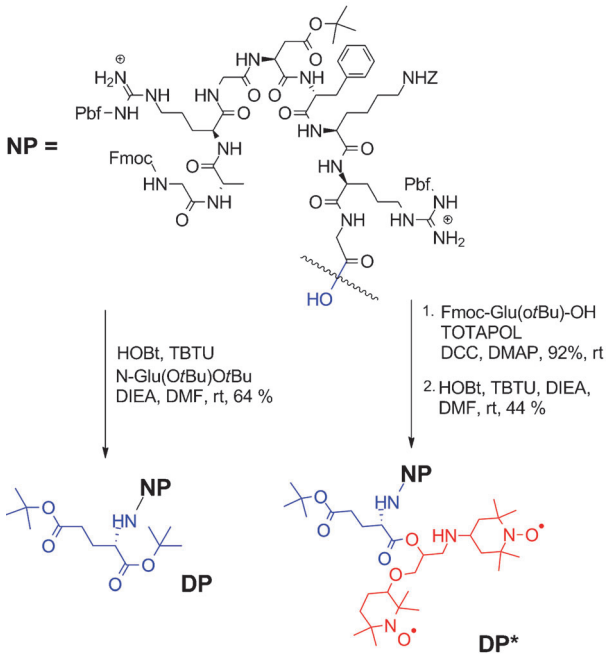

Scheme 1. Polycrystalline solvent-free powder of a peptide (in this case a decapeptide (**DP**) obtained from a nonapeptide (**NP**) precursor) is mixed with a fraction $0 \leq f \leq 16\,\%$ of the same peptide that has been labeled by covalent attachment at its C-terminal end of a polarizing agent such as the biradical TOTAPOL (**DP***). This allows one to enhance the signals by DNP, without requiring any solvent that forms a glassy solid near 100 K. (DIEA: *N*,*N*-diisopropylethylamine, Pbf: 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl, Z: benzyloxycarbonyl, Fmoc: fluorenylmethyloxycarbonyl, HOBt: 1-hydroxybenzotriazole, TBTU: *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate.)

Fmoc-Glu(O*t*Bu)-OH amino acid using *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP) to provide the corresponding ester in 92% yield. After deprotection of the amine under basic conditions the biradical was coupled with the nonapeptide **NP** in the presence of TBTU/HOBt to give the spin-labeled decapeptide **DP*** in 44% yield. The non-labeled decapeptide **DP** was obtained by coupling the nonapeptide **NP** with the amino acid N-Glu(O*t*Bu)-O*t*Bu.

Figure 1 presents the ESR spectrum of decapeptide **DP** mixed with a fraction $f = 8\,\%$ of labeled peptide **DP***, measured at $T = 296$ K with an X-band Bruker ESR spectrometer ($f_{\mu w} = 9.78$ GHz). The ESR spectrum is typical for a binitroxide and it

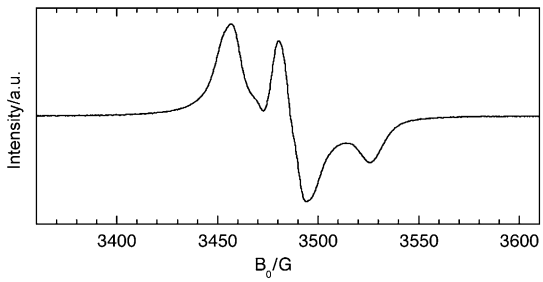

Figure 1. X-band ($f_{\mu w} = 9.78$ GHz) ESR spectrum at $T = 296$ K of a polycrystalline sample **S5** consisting of the decapeptide (**DP**) mixed with a fraction of $f = 8\,\%$ of the same decapeptide labeled by covalent attachment of TOTAPOL (**DP***).

is comparable to frozen glassy solutions of water/glycerol mixtures doped with TOTAPOL.[4b] The electron spin ***g*** factors and hyperfine couplings to ^{14}N nuclear spins of TOTAPOL are not significantly affected by the linkage to the nonapeptide **NP**. From these ESR features, we expect the DNP process to occur predominantly via CE.

The DNP measurements were performed at $T = 100$ K and $B_0 = 9.4$ T (400 MHz for protons) under MAS conditions (spinning frequency 10 kHz) with a Bruker Avance solid-state DNP NMR spectrometer equipped with a 263 GHz gyrotron[15]. Several mixtures of the decapeptide **DP** with increasing fractions $0\,\% \leq f \leq 16\,\%$ of labeled peptide **DP*** were prepared (see Experimental Section) and loaded in 3.2 mm outer diameter (OD) sapphire rotors. The ^{1}H spin-lattice relaxation rates $R_1(^1\mathrm{H}) = 1/T_1(^1\mathrm{H})$ strongly depend on the fraction f, indicating that ^{1}H spin-lattice relaxation is driven by the paramagnetic centers and is not limited by spin-diffusion, which would be affected by the high ^{1}H spin density. This is in contrast to glass-forming solvents that are typically deuterated to 90%. Proton DNP was combined with cross polarization (CP) from protons to the naturally abundant ^{13}C spins of the decapeptide. The proton DNP build-up rates $R_{DNP}(^1\mathrm{H}) = 1/\tau_{DNP}(^1\mathrm{H})$ and enhancements $\varepsilon_{DNP}(^1\mathrm{H})$ depend on the fraction f. Whereas the proton build-up rates $R_{DNP}(^1\mathrm{H})$ and spin-lattice relaxation rates $R_1(^1\mathrm{H})$ were equal for all samples, which is characteristic for DNP via CE,[16] while one would expect $R_{DNP}(^1\mathrm{H}) > R_1(^1\mathrm{H})$ if thermal mixing or the solid effect mechanism were dominant. We observed rather modest enhancements $\varepsilon_{DNP}(^1\mathrm{H}) \leq 4$ detected indirectly after CP from ^{1}H to ^{13}C for $f = 8\,\%$ and an acceleration of the proton spin-lattice relaxation rates $R_1(^1\mathrm{H})$ by a factor of up to $\kappa = 11$ for $f = 16\,\%$ with respect to the undoped decapeptide with $f = 0$ (Figure 2).

The definition of enhancement factors is not always clear in the literature. We therefore propose to define an enhancement factor ε_{global} that comprises: 1) the enhancement ε_{DNP} with respect to the thermal Boltzmann polarization $P_0 = \tanh(\hbar\omega/2k_BT)$ at the same static field and temperature, measured with long recovery delays ($d_1 > 5T_1 \geq 5\tau_{DNP}$), 2) the enhancement of the DNP build-up rate with respect to the spin-lattice relaxation rate without polarizing agent ($\kappa = R_{DNP}/R_1^{undoped}$), 3) the fraction $\varepsilon_{dilution}$ (vol/vol) of molecules of interest, and 4) ε_{bleach}, which accounts for the bleaching of nuclear spins due to the proximity of the electron spins (usually in a radius of a few Å). In our solvent-free study, ε_{bleach} was estimated to be at worst $\varepsilon_{bleach} = (1-f)$. For a conventional DNP experiment performed on a powder of density d of a molecule with a molar mass M dissolved at a concentration c in a glassy frozen solution, the fraction of NMR-visible molecules is $\varepsilon_{dilution} = Mc/d$. As an example, a 1 M solution of sodium acetate ($M = 82\,\mathrm{g\,mol^{-1}}$, $c = 10^{-3}\,\mathrm{mol\,cm^{-3}}$, and $d = 1.52\,\mathrm{g\,cm^{-3}}$) is characterized by $\varepsilon_{dilution} = 0.054$, whereas our solvent-free approach gives $\varepsilon_{dilution} = 1$ and $\varepsilon_{bleach} \sim 0.92$ for sample **S1**, that is, a 15-fold gain.

The global DNP enhancement factor can be defined as shown in Equation (1):

$$\varepsilon_{global} = \varepsilon_{DNP}\varepsilon_{dilution}\varepsilon_{bleach}\sqrt{\kappa} \quad (1)$$

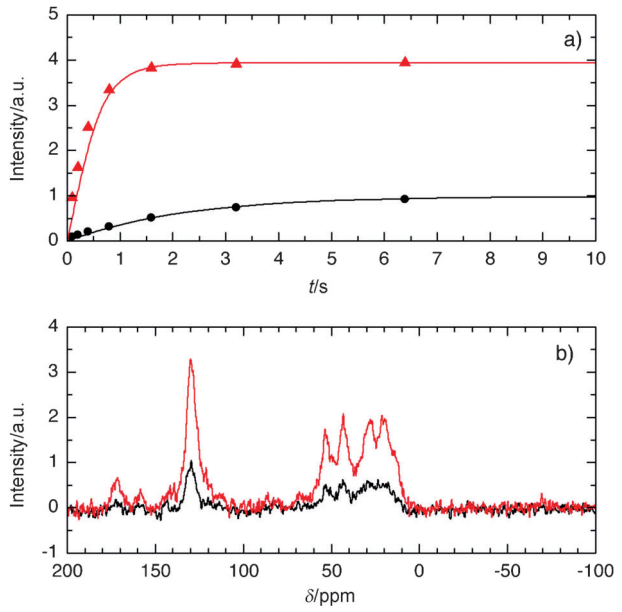

Figure 2. a) Build-up of the ^{1}H nuclear spin polarization (measured after saturation) in the decapeptide (**DP**) without (●) and with (▲) a fraction $f=8\%$ of labeled decapeptide (**DP***), irradiated with 5 W microwaves at 263 GHz, yielding build-up time constants of $\tau_{DNP}=2.17\pm0.12$ s and $\tau_{DNP}=0.39\pm0.01$ s for the diamagnetic and doped samples, respectively, that is, an acceleration by a factor $\kappa=5.6$. The proton relaxation rate $R_1(^1H)$ is also increased by a factor of $\kappa=5.6$. b) ^{13}C spectra obtained after cross polarization from ^{1}H to ^{13}C of sample **S5** ($f=8\%$) with microwaves ON (——) and OFF (——), giving a modest DNP enhancement of ε_{DNP} (^{1}H) ~ 4.

The time saving is proportional to ε^2_{global}. For sample **S6** (Table 1) $\varepsilon^2_{global}=100$, so that the experimental time is reduced from 12 h to about 7 min. Since **DP** is insoluble in water, and only slightly soluble (~1 mM) in DMF, dilution would give $\varepsilon_{dilution}\sim0.001$, which cannot be compensated by ε_{DNP}. For a 1 M sodium acetate solution ($\varepsilon_{dilution}=0.054$), one obtains $\varepsilon_{global}=4.7$ if $\varepsilon_{DNP}=50$ and $\kappa=3$.

Table 1. Composition of DNP samples investigated: polarization build-up times τ_{DNP}, enhancements ε_{DNP} and acceleration factors $\kappa=R_{DNP}(^1H)/R_1^{undoped}(^1H)$, and global enhancement factors ε_{global} [Eq. (1)].

Sample number	**S0**	**S1**	**S2**	**S3**	**S5**	**S6**
Labeled fraction f [%]	0	1	2	4	8	16
τ_{DNP} [s]	2.2[a]	1.6	1.1	0.47	0.4	0.2
ε_{DNP}	1	2.2	2.5	3.0	4.0	3.6
κ	1	1.4	2	4.7	5.5	11
ε_{global}	1	2.6	3.5	6.2	8.6	10.0

[a] For sample **S0**, $T_1(^1H)$ is given instead of τ_{DNP}.

Direct polarization of naturally abundant ^{13}C spins[16] did not show any significant enhancement $\varepsilon_{DNP}(^{13}C)$. Spin-lattice relaxation times $T_1(^{13}C)$ and DNP build-up times $\tau_{DNP}(^{13}C)$ are long [$T_1(^{13}C)>100$ s for all carbons even when $f=4\%$], so that direct ^{13}C polarization is not attractive. Moreover, the ESR features of TOTAPOL prove it to be a good DNP enhancement agent for protons, but not for ^{13}C. In fact, the two electrons of TOTAPOL have a difference of ESR frequencies that is close to the proton Larmor frequency, $|\omega_{e1}-\omega_{e2}|\approx\omega_H$.

In summary, we conceived a methodology to prepare dry solvent-free doped polymer samples suitable for DNP that circumvents the need of glass-forming solvents. This approach can be recommended for many non-soluble samples, including natural and synthetic polymers.

Experimental Section

DNP NMR: All measurements were performed with a 400 MHz Avance Bruker NMR spectrometer equipped with a low-temperature 100 K MAS probe coupled to a gyrotron delivering 5 W at 263 GHz. The samples were packed in 3.2 mm sapphire rotors without adding any solvent or polarizing agent. The spinning speed was 10 kHz in all cases and the nominal sample temperature was $T=98$ K. All ^{13}C spectra were recorded after cross polarization (CP) from protons with a contact time of 300 μs. The proton DNP enhancements $\varepsilon_{DNP}(^1H)$ were measured with respect to the thermal equilibrium signal at 98 K when the microwaves were switched off, with a long relaxation delay $T>5T_1(^1H)$ so that the proton polarization had fully recovered.

ESR measurements: Room-temperature X-band ESR experiments for the powdered sample were carried out on a Bruker ESP300E spectrometer equipped with a standard rectangular TE_{102} microwave cavity. An amount of ca. 5 mg of the powdered sample was transferred to a standard 4 mm outer diameter (OD) and 3 mm inner diameter (ID) clear-fused quartz ESR tube (Wilmad-Labglass, Vineland, NJ, USA, model 707-SQ-250M). The typical experimental parameters were: temperature 296 K, microwave frequency 9.78 GHz, microwave power 5.0 mW, modulation frequency 100 kHz, modulation amplitude 1.5 G = 0.15 mT, sweep width 250 G = 25 mT, receiver gain 2×10^3, time constant 21.0 ms, lock-in conversion time 81.92 ms, and time per scan 84 s. For each ESR trace, five field-swept ESR spectra were recorded and averaged.

Synthesis: Commercial reagents (Fluka, Aldrich, Bachem) were used without purification. Liquid/solid flash chromatography (FC) was performed with columns of silica gel (0.040–0.63 mm, using Merck No.9385 silica gel 60, 240–400 mesh). The eluent was a mixture of light petroleum ether (PE) and ethyl acetate (EtOAc) or a mixture of dichloromethane (DCM) and methanol (MeOH). TLC silica gel 60 F_{254} plates for reaction monitoring were purchased from Merck. Products were detected by UV light or made visible by staining with either Pancaldi reagent, $KMnO_4$, ninhydrine or phosphomolybdic acid. IR spectra were measured on a Perkin–Elmer-1420 spectrometer. ^{1}H NMR spectra were recorded on a Bruker-ARX-400 spectrometer (400 MHz) with δ(H) in ppm relative to the solvent's residual ^{1}H signal, using DMSO [δ(H) = 2.50] as internal reference. ^{13}C NMR spectra were recorded on the same instrument as above (100.6 MHz) with δ(C) in ppm relative to DMSO [δ(C) = 39.52] as internal reference. MALDI-TOF spectra were measured on a Axima-CFR+ spectrometer from Kratos, Manchester. The ESI-Q spectra were measured on a Finnigan SSQ 710C from Thermoquest, UK and the ESI-QT spectra on an Ultima spectrometer from Micromass, Manchester.

Synthesis of Fmoc-Gly-Ala-Arg(Pbf)-Gly-Asp(O*t*Bu)-D-Phe-Lys(*Z*)-Arg-(Pbf)-Gly-Glu-TOTAPOL (DP*): Fmoc-Gly-Ala-Arg(Pbf)-Gly-Asp(O*t*Bu)-D-Phe-Lys(*Z*)-Arg(Pbf)-Gly-OH (1 equiv, 225.5 mg, 0.12 mmol) was dissolved in DMF (0.6 mL) in the presence of HOBt (1.2 equiv, 19.5 mg, 0.144 mmol), TBTU (1.2 equiv, 46.2 mg, 0.144 mmol) and DIEA (4 equiv, 0.12 mL, 0.48 mmol). A solution of Glu(O*t*Bu)-TOTA-

POL (1 equiv, 70 mg, 0.12 mmol) in DMF (0.6 mL) was added. The reaction mixture was stirred until total conversion of the starting material at room temperature. DMF was removed under reduced pressure and ethyl acetate was added, causing the precipitation of the product. The precipitate was filtered on a Büchner funnel and washed with water and DCM. The product, an orange solid, was finally dried in vacuo (yield: 128 mg, 44%).

Synthesis of Fmoc-Gly-Ala-Arg(Pbf)-Gly-Asp(O*t*Bu)-D-Phe-Lys(*Z*)-Arg-(Pbf)-Gly-Glu(O*t*Bu)-O*t*Bu (DP): Fmoc-Gly-Ala-Arg(Pbf)-Gly-Asp-(O*t*Bu)-D-Phe-Lys(*Z*)-Arg(Pbf)-Gly-OH (1 equiv, 792 mg, 0.4 mmol) was dissolved in DMF (3 mL) in the presence of HOBt (1.2 equiv, 68 mg, 0.5 mmol), TBTU (1.2 equiv, 162 mg, 0.5 mmol) and DIEA (4 equiv, 0.3 mL, 1.7 mmol). A solution of Glu(O*t*Bu)-O*t*Bu (1 equiv, 124 mg, 0.4 mmol) in DMF (1.2 mL) was added. The reaction mixture was stirred for 2 h at room temperature. DMF was removed under reduced pressure and water was added. A precipitate was formed and was isolated by filtration on a Büchner funnel. The precipitate was washed with water, methanol and finally AcOEt to remove the excess of reagents. The red-brown product was finally dried in vacuo (Yield: 570 mg, 64%).

Sample Preparation: Five samples were prepared by mixing the unlabeled decapeptide (**DP**) and the labeled decapeptide (**DP***) with different molar fractions ($f = 1$, 2, 4, 8, 16%). The two decapeptides were mixed together and dissolved in DMF to ensure a good homogeneity of the samples. DMF was then removed by two successive co-evaporations with toluene. The resulting product was finally dried under vacuum for 24 h.

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