A simple double quantum coherence ESR sequence that minimizes nuclear modulations in Cu^{2+} -ion based distance measurements

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Abstract: Double quantum coherence (DQC) ESR is a sensitive method to measure magnetic dipolar interactions between spin labels. However, the DQC experiment on Cu^{2+} centers presents a challenge at X-band. The Cu^{2+} centers are usually coordinated to histidine residues in proteins. The electron–nuclear interaction between the Cu^{2+} ion and the remote nitrogen in the imidazole ring can interfere with the electron–electron dipolar interaction. Herein, we report on a modified DQC experiment that has the advantage of reduced contributions from electron–nuclear interactions, which enhances the resolution of the DQC signal to the electron–electron dipolar modulations. The modified pulse-sequence is verified on Cu^{2+} –NO system in a polyalanine-based peptide and on a coupled Cu^{2+} system in a polyproline-based peptide. The modified DQC data were compared with the DEER data and good agreement was found.

Keywords: ESR spectroscopy, Distance measurement, DQC, Electron–nuclear interaction, Electron–electron dipolar interaction

1. Introduction

In the last decade, pulsed electron spin resonance (ESR) has emerged as an excellent tool for mapping distances in a variety of soluble proteins [1, 2], DNAs [3, 4], RNAs [5, 6] and even membrane proteins [7-9]. Two techniques have been developed for the 12–100 Å distance range: double electron–electron resonance (DEER) [10, 11] and double quantum coherence (DQC) [12, 13]. In the DEER experiments, the local dipolar field due to the coupled spin-partner is inverted using a second *mw* channel, and the duration of the pulses is usually longer than 8 ns [14-17]. The echo is then modulated by the dipolar frequency providing sensitivity to distances. In DQC all pulses are applied at the same frequency, and it is important that all pulses should be intense in order to excite as many spins as possible [14, 18, 19].

Most of the ESR distance measurements have relied on the use of the methanethiosulfonate nitroxide spin-label, which is chemically attached to cysteine residues in the protein by site-directed spin labeling (SDSL) [20, 21]. However, with SDSL, one has to insure that only the cysteine residues of interest in the protein will be labeled, and thus the other cysteine residues in the protein have to be mutated. This constraint complicates the sample preparation. In addition, it is more difficult to label buried cysteine residues, so mostly solvent exposed cysteine residues are labeled. Hence, recently the DEER and DQC methods have been extended to the application of paramagnetic metal ions [22-28]. [ENREF 20](#) More than 30% of proteins in the cell exploit one or more metal ions to perform their specific functions, and over 40% of enzymes contain metal ions. Thus, proteins, especially enzymes can have natural binding sites for paramagnetic metal ions, decreasing the need for labeling. However, DEER and DQC experiments on metal ions are challenging given the lower sensitivity. Nevertheless, our group [26, 27, 29, 30] and others [17, 23, 31-37] have made tangible progress.

DEER has significant advantages for Cu^{2+} based experiments given that nuclear interactions are easier to reduce and due to the ease of implementation on commercial instruments. Nevertheless, the dipolar modulation depth of the DQC is larger [30, 38] as compared to the DEER. One major issue in Cu^{2+} -DQC is the substantial contribution of the electron–nuclear (EN) interaction between the electron spin and neighboring nuclear spins, such as ^{14}N and ^1H , that are present in the coordinating amino acid residues. At X-band (ca. 9.6 GHz), these EN interactions, especially the electron– ^{14}N nuclear interaction below 4 MHz, swamp the modulation due to the electron–electron dipolar (EED) interaction and make it difficult to measure the interspin distance [39].

At higher magnetic fields, the peaks from unfavorable EN interactions are shifted to higher frequencies [40, 41]. However, most experiments are performed at X-band, and therefore new approaches are required to reduce the signal due to the EN interactions.

Herein, we report on a modified DQC experiment that has the advantage of reduced contributions from EN interactions. Hence, the EED interaction between coupled copper centers is better resolved.

2. Materials and Methods

2.1 Sample preparations

Polyalanine-based peptide sample: The sequence of the alanine-based peptide is PPHGGGWPAATAAKAAAKCAATAKA (P, proline; H, histidine; G, glycine; W, tryptophan; A, alanine; K, lysine; C, cysteine). This peptide was synthesized at the peptide synthesis facility, University of Pittsburgh. The lysine residues in this peptide increase the solubility of the peptide in aqueous solutions. The PHGGGW on one end of the peptide serves as a copper binding sequence. The purpose of the cysteine residue close to the other end of the peptide is to

covalently attach the (1-oxy-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate spin label (MTSSL), using site-directed spin labeling (SDSL). The spin-labeled alanine-based peptide was mixed with 25 mM *N*-ethylmorpholine (NEM) buffer and 25% glycerol at a pH value of 7.4–7.6. The concentration of the peptide sample was 1.2 mM. Isotopically enriched $^{63}\text{CuCl}_2$ was dissolved in the same buffer as for the peptide solution with the same pH value to make the Cu^{2+} stock solution. Cu^{2+} was then mixed with the peptide solution in a 1:1 Cu^{2+} -to-peptide ratio. Based on the binding affinity of Cu^{2+} to the PHGGGW segment, more than 99% of the Cu^{2+} was bound to the peptide.

Polyproline-based peptide sample: The sequence of the polyproline-based peptide is PPHGGGWPPPWGGGHPP. This peptide was synthesized at the peptide synthesis facility, University of Pittsburgh. The polyproline-based peptide was mixed with 25 mM NEM buffer and 25% glycerol at a pH value of 7.4–7.6. The concentration of the peptide sample was 1.0 mM. Isotopically enriched $^{63}\text{CuCl}_2$ was dissolved in the same buffer with the same pH value to make the Cu^{2+} stock solution. Cu^{2+} was then mixed with the peptide solution with a 5:1 Cu^{2+} -to-peptide ratio to ensure that Cu^{2+} occupied both PHGGGW sites.

2.2 ESR spectroscopy

All DQC and DEER experiments were done at 20 K on a Bruker ELEXSYS E580 spectrometer (~9.68 GHz) using a Bruker ER4118x-MD-5 probehead with a split ring resonator (5 mm sample access).

The constant time four-pulse DEER experiment $\pi/2(\nu_{\text{obs}})-\tau_1-\pi(\nu_{\text{obs}})-t'-\pi(\nu_{\text{pump}})-(\tau_1+\tau_2-t')-\pi(\nu_{\text{obs}})-\tau_2(\nu_{\text{obs}})-\tau_2$ -echo was used. A two-step phase cycle was employed on the first pulse [42]. The echo was measured as a function of t' , while τ_2 was kept constant to eliminate relaxation effects. For the polyalanine-based peptide, the pump frequency, ν_{pump} , was set to the maximum of

the nitroxide spectrum, and the observer frequency, ν_{obs} , was 260 MHz higher. The observer $\pi/2$ and π pulses had a length of 10 and 20 ns, respectively, and the π pump pulse had a length of 16 ns, the dwell time was 12 ns. For the polyproline-based peptide, the pump pulse was set to the maximum of the copper spectrum, and the observer pulse was 100 MHz higher. The observer $\pi/2$ and π pulses had a length of 16 and 32 ns, respectively, the π pump pulse had a length of 16 ns, and the dwell time was 20 ns.

The six-pulse DQC-ESR sequence was implemented in the following manner. A 256-step phase cycle (64×4) was used to select the correct pathway. The DQC-echo was integrated after the sixth pulse. The pulse lengths were 8 ns for $\pi/2$ pulses, 16 ns for π pulses, and 32 ns for 2π pulse. The initial delays t_p and t_1 were both 80 ns and the delay t_2 was 200 ns. The time t_p was stepped by 8 ns for a total of 360 points. The repetition time was 3 ms for the polyproline-based sample, and 20 ms for the polyalanine-based sample. A video bandwidth of 20 MHz was used.

Data Processing: The DEER data were analyzed with the DeerAnalysis2013 program, using the Tikhonov regularization.

Fourier transformation (FT): The real FT was performed on the time-domain DEER and DQC data. The resolution of the DQC spectrum is 0.3 MHz, while the resolution of the DEER spectrum is 0.6 MHz. The frequency of the dominant peak is consistent given the resolution.

3. Results and discussion

The six-pulse DQC sequence is presented in Fig. 1A. This is the asymmetric pulse DQC sequence [19, 29], which yields better signal to noise ratio for samples with limited relaxation time compared to the symmetric DQC sequence. However, a disadvantage is that it is not dead-time free compared to symmetric DQC sequence.

The electron-electron dipolar modulation part of this signal is given as follows:

$$V_{EED} = \sin(\omega_{dd}t_2)\sin(\omega_{dd}(t_p + dt)).$$

t_2 is constant and should be larger than the ring-down time of the resonator and shorter than the relaxation time. Herein, it was set to 200 ns. The signal is modulated by the dipolar frequency as a function of t_p+dt . In order to understand the effect of the EN interaction on the DQC signal, we carried out a simple analysis using the product operator formalism for two coupled electron spins of $S=1/2$ and a nuclear spin of $I=1/2$. The DQC signal (V_{DQC}) contains information regarding the electron–electron dipolar interaction (V_{EED}) and the electron–nuclear interaction (V_{EN}), where $V_{DQC} = V_{EED}V_{EN}$. The analysis, detailed in the Supplementary data shows that $V_{EN} = A(\omega_\alpha, \omega_\beta, t_p, t_2) + B(\omega_\alpha, \omega_\beta, t_p, t_1, t_2)$, where A and B are two functions of the characteristic nuclear frequencies ω_α and ω_β . The full expression for each of these functions is given in the Supplementary data. However, *a change of the flip-angle of the fourth pulse* (cf. p_4 in Fig. 1A) from π to 2π leads to the following equation:

$$V_{MDQC} = \{V_{EED}[1-2\sin^2(2\Omega_s t_1)]\} \cdot [A(\omega_\alpha, \omega_\beta, t_p, t_2) + B(\omega_\alpha, \omega_\beta, t_p, t_1, t_2)\cos(2\Omega_s t_1)]$$

where the subscript MDQC denotes the modified DQC, and Ω_s is the (angular) frequency offset.

Now the B -term is multiplied by an off-resonance term.

The large spectral width of the Cu^{2+} spectrum and the presence of non-ideal pulses in DQC leads to large off-resonance effects, where $\Omega_s \approx \pi/(4t_\pi)$, and t_π is the length of the π pulse in the modified DQC experiment (see the Supplementary data, page 13 for the full derivation) [18]. Thus, for the ratio of $t_1/t_\pi = (2n+1)$, where $n = 0, 1, 2, 3, \dots$, $\sin(2\Omega_s t_1) = \pm 1$ and $\cos(2\Omega_s t_1) = 0$, so the final modified DQC signal is reduced to $V_{MDQC} = V_{EED}A(\omega_\alpha, \omega_\beta, t_p, t_2)$. Hence, the nuclear modulation effects of the off-resonance spins are reduced. The term $A(\omega_\alpha, \omega_\beta, t_p, t_2)$ yields peaks at the combination frequencies (i.e. $\omega_\alpha \pm \omega_\beta$), (see SI). For $I=1/2$ nuclei, such as protons, $\omega_\alpha \approx \omega_\beta$,

this term should result in mainly a peak around 28 MHz at X-band. For $I=1$ nuclei, such as ^{14}N , the existence of only the A -term in the modified DQC signal should lead to a reduction in the peak intensities in the 0–6 MHz region. This phenomenon is similar to the blind spot effect observed in three-pulse electron spin-echo envelope modulation (ESEEM) [39]. The blind-spot effect was also suggested previously by Borbat et al. for the six-pulse DQC experiment for matrix nuclei, where there the researchers succeeded to remove ESEEM modulations by just varying the t_1 value [18].

In order to check if the signal from the EN interaction is reduced also for an $I=1$ system involved non-matrix nuclei, simulations using the density matrix formalism were carried out, since the product operator formalism is harder to apply for an $I>1/2$ system. Here, the nuclear quadrupole interaction was added to the system, as described in the Supplementary data.

Fig. 1. (A) Six-pulse DQC pulse sequence. (B) Simulated DQC spectra with two different lengths of the fourth pulse (p_4) for the case of two copper ion centers separated by 1.5 nm. Each center was assumed to be coordinated to a histidine residue. The EN interaction with ^{14}N of histidine results in dominant components in the 0–5 MHz region for the case of $p_4=\pi$. These effects are reduced when the fourth pulse is a 2π pulse, for $t_1/t_\pi = 5$ ($t_1=80$ ns, $t_\pi=16$ ns).

Fig. 1B shows the simulation results for the case in which p_4 is a π pulse, and a 2π pulse, respectively. The simulations were performed for the case of two Cu^{2+} centers separated by 1.5

nm, which corresponds to an EED interaction of ~15 MHz. Each copper center was considered to be coordinated to one imidazole ring. Typical values of the quadrupolar and asymmetry parameters for equatorially coordinated imidazole were used in these simulations (see the Supplementary data) [43]. The simulations show a pronounced reduction in the electron- ^{14}N nuclear contributions to the DQC signal upon a change in p_4 from π to 2π . In the modified DQC experiment the EED interaction remains, as is shown in the conserved 15 MHz peak in Fig. 1B.

In order to check if the hyperfine and nuclear quadrupole interactions affect the EN signal of the modified DQC as compared to the normal DQC, we carried out simulations at different values of the hyperfine tensor and quadrupole parameters for ^{14}N nuclei coordinated to Cu^{2+} . In all of the simulations we observed a reduction in the EN signal upon changing from the normal DQC to the modified DQC sequence as presented in Fig. 2.

Fig. 2. Modified DQC spectra simulations on ^{14}N nuclei coordinated to Cu^{2+} . The initial delay t_p is 80 ns, t_1 is 80 ns, and the delay t_2 is 200 ns. The time t_p is stepped by 8 ns for a total of 1024 points. $\pi/2/\pi/2\pi$ pulse lengths are 8/16/32 ns ($t_1/t_\pi = 5$, $t_1=80$ ns, $t_\pi=16$ ns), and A_N , K , and η are varied. The Euler angles transform the hyperfine interaction into the principal axis system ($0^\circ, 10^\circ, 0^\circ$), and the Euler angles transform the nq_i into the principal axis system ($0^\circ, 10^\circ, 0^\circ$).

In order to confirm that the modified DQC pulse sequence reduces the signal from the $\text{Cu}^{2+}-^{14}\text{N}$ interaction, the experiment was first conducted on a polyaniline-based peptide, with one Cu^{2+} center and one nitroxide label.

Fig. 3A presents the structure of the polyaniline-based peptide. Fig. 3B shows the normal and the modified DQC and DEER time-domain signals of the polyaniline-based peptide at 3450 G, with the corresponding spectra presented in Fig. 3C. Due to the dead-time of the asymmetric

DQC pulse sequence, the data is plotted from 300 ns. The normal DQC spectrum is not well resolved and is composed of several frequencies, including components between 0–2 MHz and components between 3 and 5 MHz (Fig. 3C). These signals in the spectrum are due to the superposition of the EN (^{14}N) interaction and EED interaction between the two Cu^{2+} centers. In addition, proton modulations around 14 and 28 MHz are clearly observed (Fig. 3D). Due to the EN interaction, it is hard to resolve the EED interaction between the electron spins and to extract the distance.

Fig. 3. (A) Polyalanine-based peptide structure. (B) Normal DQC, modified DQC, and DEER signals of the polyalanine-based peptide (solid line). Both DQC and DEER measurements were performed on the same sample with similar concentration. The fast modulations in the DQC signal are from protons and are not noise. The dashed line corresponds to the DQC and DEER simulations. Modulations due to the EED interaction are clearly observed in the modified DQC experiment. The DQC signal was acquired at the maximum of the nitroxide spectrum (8 scans $\times$ 256 step phase cycle). The initial t_p was 80 ns, and was stepped by 8 ns. $t_1=80$ ns, $t_2=200$ ns, $\pi/2$ / π / 2π pulse lengths were 8/16/32 ns. The pump pulse of the DEER experiment was set to the maximum of the nitroxide spectrum, and the observer pulse was 260 MHz higher. The observer π pulse was set to 20 ns, and the pump π pulse was 16 ns. The dwell time was 12 ns. (132 scans $\times$ 4 step phase cycle) (C) The corresponding spectra (real FT). The inset shows the distance distribution obtained from the modified DQC simulation. (D) real FT spectra.

The dipolar coupling modulation is clearly resolved in the modified DQC signal and consistent with the DEER signal. Moreover, a reduction in the contribution of the matrix proton ESEEM signal is also observed in the modified DQC (Fig. 3D). Simulations carried out on the modified DQC signal, taking into account only the EED interaction, gave a distance distribution with a

mean of 2.6 nm and a standard deviation of 0.4 nm (dashed line in Fig. 3B). The distance distribution is presented in the inset of Fig. 3C. The DEER signal was analyzed here with the DeerAnalysis program, ignoring orientational effects. A similar distance distribution was also obtained earlier for this polyalanine-based peptide, using a series of DEER experiments at different frequency offsets [27]. There it was reported that, due to the flexibility of the peptide, almost no orientation dependence was observed.

The DEER spectrum is broader than the DQC spectrum. In principle, this could be due to different orientational selectivity for DEER versus DQC. Both the DEER and DQC experiments shown in Fig. 3 were performed with similar pulse lengths. Additionally, such differences might be due to artifacts of discrete Fourier transformation in DEER since three full periods were acquired in DQC and only one in DEER. Thus, it is more likely that the acquisition of more periods in DQC led to a narrower spectrum. Indeed when we cut the modified DQC signal at 1500 ns and perform a Fourier transformation, a spectrum similar to the DEER spectrum is obtained.

The modified DQC signal was also acquired at various magnetic fields. In a region where the nitroxide and Cu^{2+} spectra do not overlap, the EED interaction should not be detected by the DQC experiment. The data are presented in Fig. 4. Indeed, Fig. 4 shows that at 3360 G and 2910 G, where the nitroxide and Cu^{2+} spectra do not overlap, only noise or a signal corresponding to 28 MHz proton modulations is observed.

Fig. 4. Modified DQC signals for the polyaniline based peptide at various magnetic fields. The initial t_p was 80 ns, and was stepped out by 8 ns. $t_1=80$ ns, $t_2=200$ ns, $\pi/2$ / π / 2π pulse lengths were 8/16/32 ns. The fast modulations in the DQC signal are from protons.

Fig. 5 shows the modified DQC signals with $t_1 = 80$ ns, and different pulse lengths for the polyaniline-based peptide. For $t_\pi = 16$ ns, the ratio $t_1/t_\pi = 5$, and thus the ^{14}N EN signal is reduced. For $t_\pi = 12$ and 24 ns, the ratio is 6.67 and 3.34, respectively, and thus ^{14}N nuclear modulation appears. In addition, the contribution of the ^1H EN signal is larger when this ratio is not kept.

Fig. 5. Modified DQC signal and the corresponding spectra (real FT). The initial delays t_p and t_1 were 80 ns and the delay t_2 was 200 ns. The time t_p was stepped by 8 ns for a total of 360 points. $\pi/2$ / π / 2π pulse lengths were: (a) 6/12/24 ns, (b) 8/16/32 ns, and (c) 12/24/48 ns.

The pulse sequence was also tested on a polyproline-based peptide with two Cu^{2+} centers. Fig. 6 shows the modified DQC (after 10 MHz low-pass filter) and DEER signals of the polyproline-based peptide. Herein, the DEER signal was acquired with a small resonance offset (100 MHz) and the pump pulse was set to the maximum of the Cu^{2+} spectrum as in the DQC experiment, in order to mimic the DQC conditions. Indeed, the modified DQC and the DEER signals show similar EED modulation frequencies of ~ 2 MHz. The DEER signal was simulated with the DeerAnalysis program, and the distance distribution that was obtained was used for the DQC simulations. The simulated DQC signal was in reasonable agreement with the experiment; however, as is presented in Fig.6 the simulations do not completely fit to the experimental data. Therefore, in order to obtain an accurate distance distribution and orientation of the interspin vector with the g -tensor of the copper center, the DQC and the DEER signals have to be acquired at various magnetic fields, and simulation taking into account orientational effects should be considered. Nevertheless, the data do illustrate the utility of the DQC experiments for such systems.

4. Conclusions

In this work, we demonstrate a strong motivation for the development of DQC. The modified DQC pulse sequence presented here succeeded to provide better resolution of the EED modulation than the conventional DQC sequence. Further improvements can be made by the use of narrower pulse lengths [40, 44, 45], as well as by understanding the role of orientational effects in DQC measurements [46].

Fig. 6. Modified DQC signal (red solid line) of the polyproline-based peptide after 10 MHz low-pass filter and the DEER signal (black solid line). The red dashed line corresponds to the DQC simulation. The DEER signal was simulated with the DeerAnalysis program, Tikhonov regularization (black dashed line). The insets show the structure of the polyproline-based peptide and the distance distribution obtained from the DQC simulations. The DQC was acquired at the maximum of the Cu^{2+} spectrum (16 scans $\times$ 256 step phase cycle). The initial t_p was 80 ns, and was stepped by 8 ns. $t_1=80$ ns, $t_2=200$ ns, and $\pi/2$ / π / 2π pulse lengths were 8/16/32 ns. The pump pulse of the DEER was set to the maximum of the Cu^{2+} spectrum (9900 scans $\times$ 4 step phase cycle), and the observer pulse was 100 MHz higher. The observer π pulse was 32 ns, and the pump π pulse length was 16 ns. The dwell time was 20 ns.

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Appendix A. Supplementary data. Details of the product operator formalism and the DQC simulations are described.

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