

Original Article

# Calibration ruler for CW-EPR distance measurement using diradical molecule of rigid structure

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## Abstract

Many experimental factors and uncontrollable factors may introduce errors in the distance measurement by continuous wave electron paramagnetic resonance. To deal with this problem, several C<sub>60</sub> nitroxide diradical adducts with rigid structure and definite molecular dimension were used as distance calibration rulers. Based on the improvement of distance calculation program via adding simulation programs of experimental spectra and dipolar broadening function, respectively, the distance calibration method was developed under different conditions such as different solvent, solution concentration, measuring temperature, and microwave power. As a result, stable distance calibration rulers were established within the range of 8–13 Å. The distance calibration effect was evaluated resulting in a corresponding distance measurement precision of 0.84 Å. The results suggested that the influence of non-dipolar spectral broadening factors could be overcome, and the established experimental and calculation methods were suitable to a wide range of situations. The developed method will ensure more accurate and objective distance measurement in biomacromolecular analysis.

**Key words:** biomacromolecules, distance measurement, diradical, distance ruler, EPR

## Introduction

The study of structures and functions of biomacromolecules (e.g. proteins) is an important topic in modern biology research [1]. The rapid development of structural methods, such as nuclear magnetic resonance and X-ray crystallography, results in a nearly exponential increase of solved protein structures [2–6]. Recently, site-directed spin labeling in combination with electron paramagnetic resonance (SDSL-EPR) spectroscopy has emerged as an efficient tool to complement the above methods by detecting motions and structures of biomacromolecules in solution that is similar to the physiological environments [7–11].

One of the most important features of SDSL-EPR is the capability of wider range distance measuring between spins selectively labeled on biomacromolecules. Normally, the explanation of molecular mechanism highly depends on the accuracy and objectivity of distance measurement between significant sites. Unfortunately, there are still a few unsolved problems with SDSL-EPR distance measurement due to the diversity of biology samples [3]. Continuous wave EPR (CW-EPR) distance measurement depends on the spectral broadening caused by dipolar interaction [12]. However, in practical studies, there are many other experimental factors that can cause spectral broadening, therefore influencing the accuracy of distance measurement. It is difficult to

take all these factors into consideration in routine experiments. Therefore, it is necessary to establish distance calibration rulers serving as CW-EPR distance correction tools when measuring biomolecules distance. Furthermore, the uncertainty due to the flexible link between the spin label and the protein increases the degrees of freedom of nitroxide side chain R1 [3]. In general, interpretation of EPR data from R1 in terms of protein structure and dynamics requires knowledge of the rotamers and internal dynamics of the side chain itself. Accordingly, a limited rotamer library has been defined to addressing the inherent distributions and dynamics of the nitroxide side chain [13,14]. With appropriate knowledge of the rotameric states of R1, analysis of the distances and distance distributions can be obtained with higher accuracy by using more accurate method. Thus, it also makes sense to establish distance calibration rulers to improve the accuracy and objectivity of EPR distance measurement.

Nitroxide diradical molecules with rigid structure, defined molecular dimension, and stable structure are good candidates. However, there is very limited work related to this purpose. In the pioneering study of Rabenstein and Shin, an EPR ‘spectroscopic ruler’ was developed using a series of  $\alpha$ -helical polypeptides to test the Fourier deconvolution distance measurement (FDDM) method [15]. DeRose and coworkers advanced the spectroscopic ruler in a model RNA duplex system to predict the conformational changes [16]. Although they both demonstrated that SDSL-EPR is applicable to bimolecular structure prediction, there was a defect that helical structure was taken as distance calibration ruler. The flexibility and dimensional instability of the helical structure may produce uncertainties with increasing length or in different solvent, which would result in uncertainty in distance calibration. Therefore, it is necessary to set up more stable and practical distance calibration rulers for structure analysis of biomolecules.

Recently, a series of  $C_{60}$  nitroxide diradical bisadducts, where the relative position of the unpaired electron couple is exactly determined, have already been synthesized and studied by EPR [17–20]. In this work, several rigid  $C_{60}$  nitroxide diradical bisadducts were synthesized as distance calibration rulers. Experimental method was established to measure the spectral broadening caused by dipolar interactions between spins in solution samples within the range of CW-EPR distance measurement, and the distance calculation program was improved. The results of distance calibration effect demonstrated that it is necessary and feasible to set up distance calibration rulers with nitroxide diradical molecules of rigid structure and fixed distance. By using the objective distance calibration rulers, the developed method will ensure more accurate and precise distance measurement in biomolecular analysis by overcoming the influence of non-dipolar spectrum broadening factors.

## Materials and Methods

### Synthesis and identification of $C_{60}$ monoradical and diradical adducts

Synthesis and purification of  $C_{60}$  monoradical and diradical adducts have been described in literatures [17–19]. In this work,  $C_{60}$  monoradical and diradical adducts were synthesized and purified by a modified procedure of Maggini *et al.* [17] and Schick *et al.* [18].  $C_{60}$  (360 mg, 0.5 mM) in chlorobenzene (PhCl) solution (80 ml) was treated with acetone (6 ml) and 2-aminoisobutyric acid (DMG, 209 mg, 2 mM) at 180°C for 2 h in a 150 ml pressure glass tube. The mixture of NH-fulleropyrrolidines was concentrated and purified by flash chromatography on Silica flash column from unreacted  $C_{60}$  [eluent:

toluene (PhMe),  $R_f = 0.93$ , 90 mg], from the monoaddition product 2,2,5,5-tetramethyl-fulleropyrrolidine [eluent: PhMe/ethyl acetate (EA) 95/5,  $R_f = 0.60$ , 97 mg] and, partially from traces of higher molecular weight adducts. Three fractions of a brown-orange solid were obtained. The first one, eluted after the monoaddition product with a mixture of PhMe/EA 9/1, consisted mainly of trans bisadducts 4, 5, and impurities, whereas the second eluted mixture contained impurities and triple adducts, and the last one contained equatorial bisadduct 6 [eluent: PhMe/EA 7/3,  $R_f = 0.08$ , 32 mg]. The mixture of trans bisadducts 4 [eluent: PhMe/EA 7/3,  $R_f = 0.41$ , 13 mg], 5 [eluent: PhMe/EA 7/3,  $R_f = 0.35$ , 40 mg], and impurities was purified again by Silica flash column chromatography. Oxidation with MCPBA in PhMe at room temperature produced the binitroxides 1, 2, and 3 that were purified by semipreparative HPLC on a Silica column [5  $\mu$ m (Phenomenex Primesphere), 250  $\times$  10 mm<sup>2</sup>, PhMe/EA mixtures]. The monoradical and nitroxide diradical adducts were identified by comparison of their UV–Vis absorption spectra, EPR spectra, and mass spectra.

### CW-EPR spectroscopy

#### CW-EPR sample preparation

The  $C_{60}$  radical adduct samples were dissolved and diluted in PhMe or PhCl, respectively (concentration:  $6 \times 10^{-4}$  M,  $1.2 \times 10^{-4}$  M,  $6 \times 10^{-5}$  M). And then, the solutions were carefully deoxygenated by repeated freeze–pump–thaw cycles and sealed under vacuum in 2 mm ER221TPX quartz capillary tubes.

#### CW-EPR spectroscopy measurements

CW-EPR measurements were performed on an X-band CW-EPR spectrometer (A300; Bruker, Cologne, Germany), equipped with an ER4111VT temperature variation apparatus and an ER4123D dielectric cavity. The spectra were recorded at different temperatures (297, 200, 150, and 130 K). The experimental parameters were 100 kHz modulation frequency, 1 Gs modulation amplitude, 200 Gs magnetic scanning, and 180 s scanning time. The microwave power was set at 1, 5, and 10 mW, respectively.

### Fourier deconvolution distance measurement

Fourier deconvolution distance measurement (FDDM) method [15], developed by Rabenstein and Shin, assumes that the diradical spectrum  $D(B)$  can be described as a convolution of corresponding monoradical spectrum  $S(B)$  with a dipolar broadening function  $M(B)$  by Equation (1) [15].

$$D(B) = \int_{-\infty}^{\infty} S(B')M(B' - B) dB' \quad (1)$$

Using the convolution theorem,  $M(B)$  is then described by Equation (2) [15].

$$M(B) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\infty} \exp\left(2\pi i \omega B \frac{D^*(\omega)}{S^*(\omega)}\right) d\omega \quad (2)$$

where the superscript \* represents the Fourier transformed function, and  $\omega$  stands for the inverse variable of  $B$ . Thus, the average splitting  $\langle 2B \rangle$  over the distribution is obtained from Equation (3) [15].

$$\langle 2B \rangle = \frac{\int_{-\infty}^{\infty} |2B| \cdot M(B) dB}{\int_{-\infty}^{\infty} M(B) dB} \quad (3)$$

The averaged spin–spin distance  $\langle r \rangle$  can be obtained from the algorithm that  $\langle r \rangle$  is positively proportional to  $\langle 2B \rangle^{1/3}$  as shown in

Equation (4).

$$\langle r \rangle \propto (2B)^{1/3} \quad (4)$$

### Spectrum simulation-FDDM

The spectrum simulation-FDDM (SS-FDDM) method was developed in this work in order to reduce the calculation errors. Based on the principle of FDDM method, SS-FDDM contains two added modules, i.e. spectra simulation module and broadening function fitting module, by which the errors caused from spectrum center shift of mono- and diradical and other contrived resources could be significantly avoided [21], therefore, the accuracy and precision of distance measurement could be improved. The spectra simulation module was developed by means of EasySpin (<http://www.easyspin.ethz.ch>) software [22,23], including baseline correction, background subtraction, high-frequency noise reduction, and spectral simulation. The spectral simulation approaches contain EasySpin approach for parameters and

lineshape fitting and Gaussian approach for lineshape fitting. The broadening function fitting module was developed according to Gaussian fitting algorithm and by summarizing the variation of  $M(B)$  obtained from different diradical distance, which could get a more objective  $M(B)$  function. First,  $M(B)$  was calculated by deconvolution of simulated diradical spectrum and corresponding monoradical spectrum followed with upgraded Gaussian fitting. The fitting process can be described by Equation (5).

$$M(B) = \sum_1^n a_n e^{-((B-1023)/b_n)^2} + c \quad (5)$$

where  $a_n$ ,  $b_n$ , and  $c$  represent the fitting parameters. The number of  $n$  will be determined according to the fitting effect of  $M(B)$ . On the other side,  $M(B)$  was simulated by superimposed Gaussian functions followed with convolution with simulated monoradical spectrum to fit diradical spectrum. The diradical distances were calculated from  $M(B)$  generated in both ways and calibrated by theoretic values. Briefly, the process of SS-FDDM method is shown in Fig. 1.

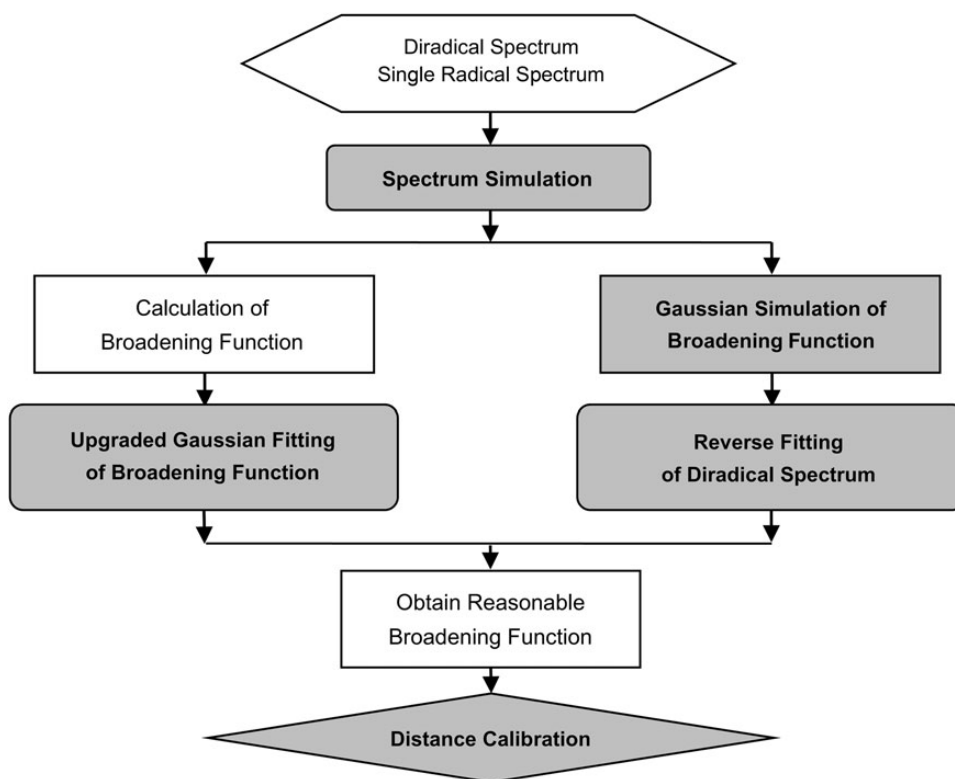
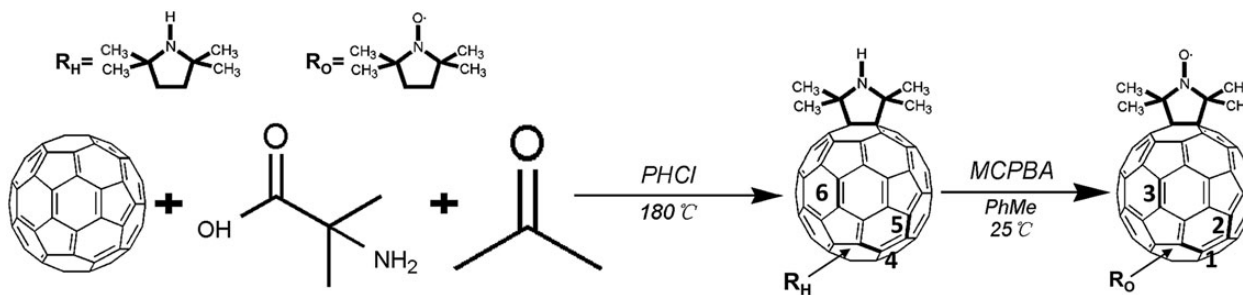


Figure 1. Process frame of SS-FDDM The dark marked frames indicate the added modules.



Scheme 1. Structure and synthesis of  $\text{C}_{60}$  nitroxide diradical isomers 1, 2, and 3

## Distance calibration

In order to comprehensively study experimental factors influencing CW-EPR distance measurement and to exactly measure the spectral broadening caused by interactions between spins in solution samples, the diradical distances of  $C_{60}$  nitroxide diradical bisadducts under different measuring conditions that may be mainly concerned during biomolecule experiment were measured. The selected experimental factors were solution concentration, measuring temperature, solvent type, and microwave power. The final experimental distance results were obtained by averaging three independent experiments under the same conditions. The obtained distances were analyzed to determine the best measuring condition and then the corresponding distance calibration method under the given condition was established.

## Results

### $C_{60}$ monoradical and diradical adducts

One  $C_{60}$  monoradical adduct and three diradical isomers with different distances between the two radicals were constructed as distance rulers (Scheme 1) [19].

**Table 1. The spin–spin distances obtained by standard MM calculation [20]**

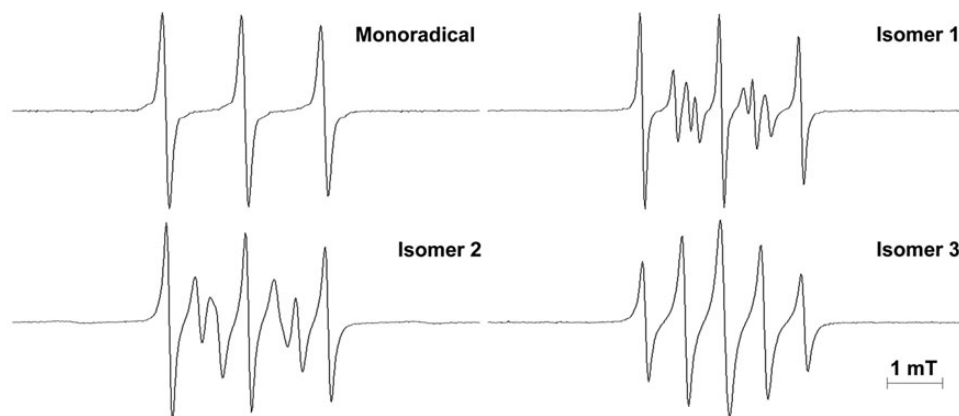
Isomer	1	2	3
Spin–spin distance (Å)	12.84	11.13	9.73

As is reported, the structures of  $C_{60}$  nitroxide diradical isomers have been clearly defined, which mean that nitroxide diradicals are placed at fixed distances and orientations and the relative position of the unpaired electron couple is exactly determined [20]. The theoretical values of spin–spin distance were obtained by a standard molecular mechanics (MM) program, shown in Table 1, all of which were in the range of CW-EPR distance measurement. To confirm the product molecules, we compared the EPR spectra recorded at 298 K and UV–Vis spectra of monoadduct and bisadducts (shown in Figs. 2 and 3) with those reported in literatures [17–19]. They were consistent with each other correspondingly, confirming that we got the correct products with definite molecular dimension, stable property, and rigid structure, which meet the requirement for a distance calibration ruler.

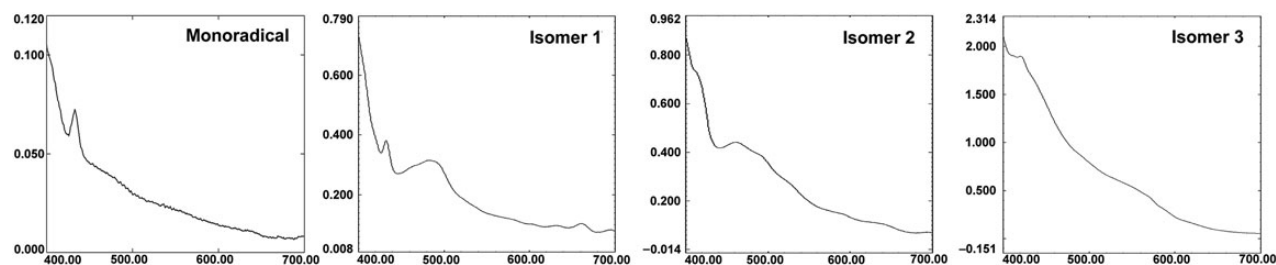
### Distance calibration

#### Acquisition of powder EPR spectra by low-temperature experiment

CW-EPR distance measurement needs to get powder spectra by means of low-temperature experiment to eliminate the isotropic average effect from molecule tumble movement. To investigate the temperature influence on spectral broadening and to determine a suitable temperature range to get complete powder spectrum, experiments at different temperatures (130, 150, and 200 K) were carried out. At 130 and 150 K, the spectra showed an obvious anisotropic spectrum characteristic and intensity-dependent spectral broadening (Fig. 4A,B), suggesting that the powder spectra could be obtained at such temperature. At 200 K, the EPR spectra showed a partial isotropic shape caused by



**Figure 2. EPR spectra of  $C_{60}$  monoradical and diradical isomers 1, 2, and 3** All spectra were recorded at 298 K in PhCl solution with  $6 \times 10^{-5}$  M concentration. The experimental parameters were 1 Gs modulation amplitude and 1 mW power. The spectral characteristic showed the structure difference of  $C_{60}$  monoradical and diradical isomers.

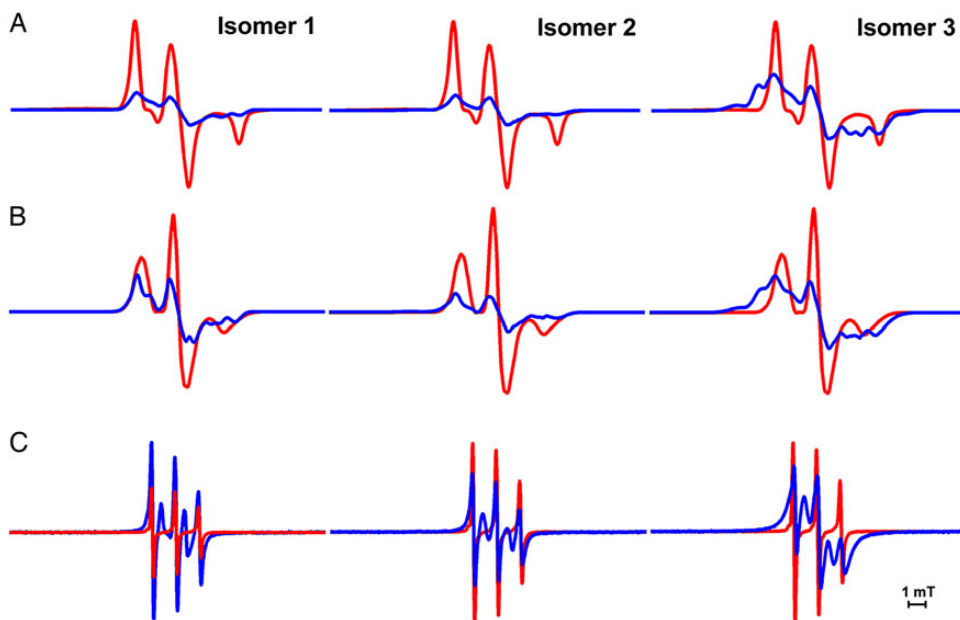


**Figure 3. UV–Vis (400–700 nm interval) of  $C_{60}$  nitroxide monoradical and diradical isomers 1, 2, and 3** All spectra were recorded at room temperature in PhMe solution. The spectral characteristic showed the structure difference of  $C_{60}$  monoradical and diradical isomers.

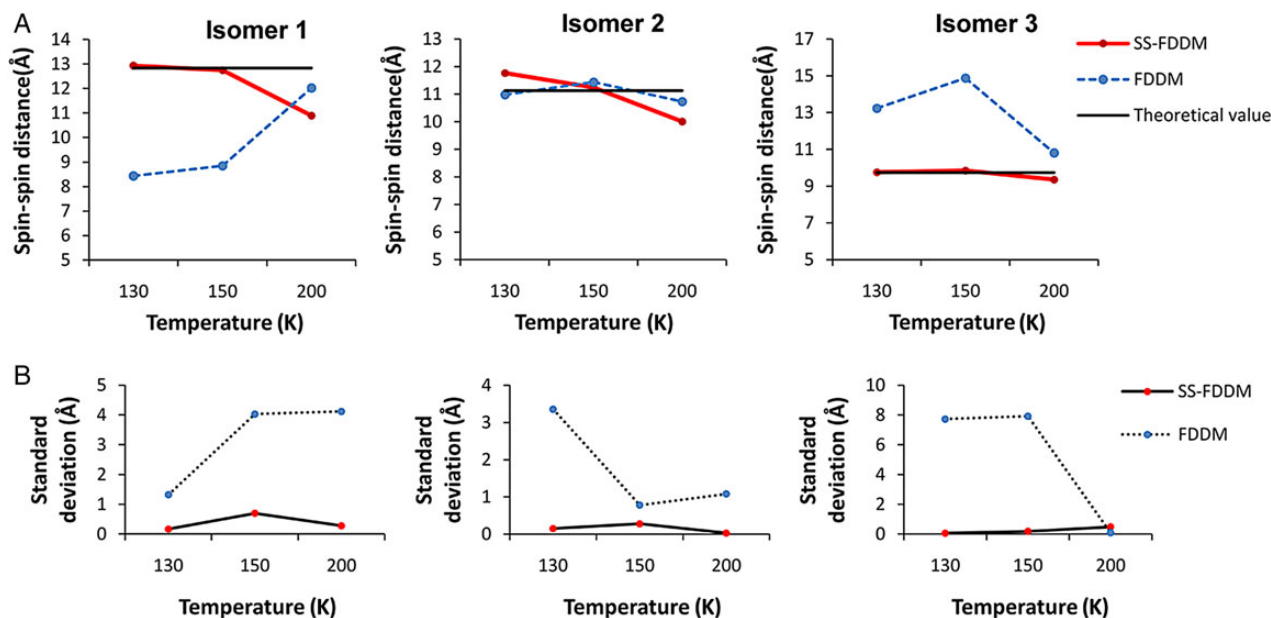
molecular motion because the toluene was in the semi-frozen state at such temperature (Fig. 4C). Thus, distance inaccuracy may occur and special distance calculation method needs to be developed over such temperature to extract the electron dipolar interaction more objectively.

#### Analysis of distance calculation methods

All EPR spectra, measured under different conditions, were analyzed by FDDM and SS-FDDM method separately. The measured spin-spin distance of  $C_{60}$  nitroxide diradical adducts under different temperature and corresponding standard deviation was analyzed to determine



**Figure 4.** Low-temperature EPR spectra of  $C_{60}$  monoradical (red) and diradical (blue) adducts shown in the first-derivative mode with the best-fit simulations (A), (B), and (C) show the spectra recorded at different temperature of 130, 150, and 200 K, respectively. All spectra were recorded in PhMe solution with  $6 \times 10^{-4}$  M concentration. The experimental parameters were 1 Gs modulation amplitude and 5 mW power. The simulation parameters of single radical spectra at 130 K were  $lwpp$ : 0.68 and 0.12 mT;  $g$ : 2.0084, 2.0084, and 2.0012;  $A$ : 0.76, 0.44, and 3.37 T.  $lwpp$  contains Gaussian peak-to-peak linewidths and Lorentzian peak-to-peak linewidths. For  $C_{60}$  monoradical and diradical isomers, the spectra showed different spectral broadening at different temperature. All spectra shown were normalized to the same number of spins.



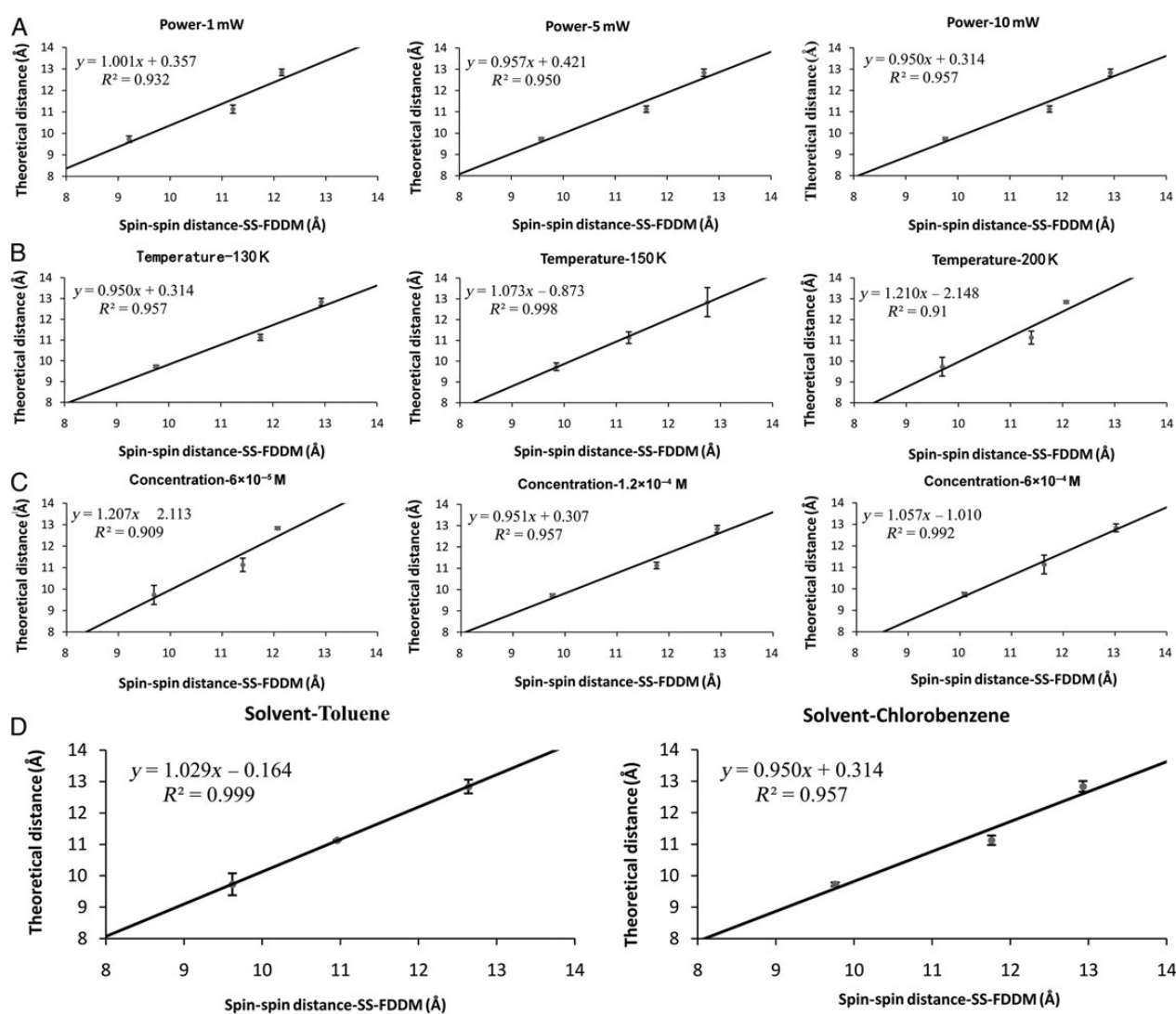
**Figure 5.** Distance calculating results of  $C_{60}$  diradical isomers by method of FDDM and SS-FDDM (A) Comparison of accuracy. (B) Comparison of standard deviation. The distance obtained from SS-FDDM method preferably agreed with the theoretical value. The standard deviation results of SS-FDDM method were smaller than that of FDDM method. The theoretical value was obtained by standard MM calculation. The corresponding experimental parameters were 1 Gs modulation amplitude, 10 mW power, and  $1.2 \times 10^{-4}$  M concentration.

the accuracy and stability of calculation method (Fig. 5). As is expected, the distance obtained from SS-FDDM method preferably agreed with the theoretical value obtained by Tinker MM program (Fig. 5A) and the standard deviation of SS-FDDM method was smaller than that of FDDM method (Fig. 5B). Although FDDM showed better agreement with theory at 200 K than SS-FDDM, SS-FDDM only showed disadvantages in this temperature point comparing to FDDM and gave smaller error at calculating shorter diradical distance for which the results from SS-FDDM at high temperatures could be corrected (Fig. 5A). The disperse effect of calculated values and theoretical values at 200 K point in Fig. 5A verifies that complete powder spectrum condition is necessary for distance calculation and that the following calibration process should comply with such prerequisite. The results demonstrate that the upgraded calculating method of SS-FDDM could give distances that better agree with the theoretical values, which could substantially improve the accuracy and stability of CW-EPR

distance calculation. Therefore, SS-FDDM is more suitable for the purpose of establishing distance calibration method in this work.

#### The effect of distance calibration ruler

According to the results of distance measurement under different conditions, distance calibration method and correction standard were established under conditions of different solvent, concentration, temperature, and microwave power in order to adapt to wider experimental conditions that might occur in biological experiments. As shown in Fig. 6, under each condition, the distance calibration had good linear correlation between measured and theoretical values, indicating that the established method is suitable for distance calibration under the experimental conditions and distance range. The current range of distance calibration is  $\sim 8\text{--}14$  Å and the precision of the distance measurement, if defined as the biggest standard deviation under all experimental conditions, is  $\sim 0.84$  Å (shown in Table 2).



**Figure 6. Distance calibration and correction curves under different conditions** Black line indicates the ideal correlation between measured and theoretical value. Experimental condition of each curve were (A) 1 Gs modulation amplitude, 130 K temperature,  $1.2 \times 10^{-4}$  M concentration, and PhCl solution at the same power; (B) 1 Gs modulation amplitude, 10 mW power,  $1.2 \times 10^{-4}$  M concentration, and PhCl solution at the same temperature; (C) 1 Gs modulation amplitude, 10 mW power, 130 K temperature, and PhCl solution at the same concentration; and (D) 1 Gs modulation amplitude, 10 mW power,  $1.2 \times 10^{-4}$  M concentration, and 200 K temperature in the same solution. The corresponding linear formulations and correlation coefficients ( $R^2$ ) are also displayed in the figure. All distance calibration and correction curves had good linear correlations.

**Table 2. The standard deviation results under different conditions**

Isomer	Standard deviation of spin–spin distance (Å)										
	Concentration (10 <sup>-4</sup> M)			Temperature (K)			MW power (mW)			Solvent	
	0.6	1.2	6.0	130	150	200	1	5	10	PhMe	PhCl
C <sub>60</sub> -1	0.84	0.17	0.18	0.17	0.70	0.28	0.15	0.40	0.17	0.07	0.38
C <sub>60</sub> -2	0.41	0.15	0.43	0.15	0.28	0.03	0.19	0.09	0.15	0.64	0.20
C <sub>60</sub> -3	0.48	0.06	0.09	0.06	0.18	0.48	0.15	0.09	0.06	0.71	0.42

## Discussion

CW-EPR distance measurement has been widely employed in research for determining the structure and conformational changes of biomacromolecules. However, the accuracy and stability of this technology was limited by some inherent defects. In this study, we proposed the idea of using rigid diradical molecules as distance ruler to calibrate EPR distance measurement. Based on the improvement of distance calculation program, several stable distance calibration rulers of diradicals with rigid molecule structure were established and the corresponding distance calibration effect was evaluated under different conditions that might be met in biomolecule measurement. With the help of these rulers, the accuracy and precision of measured distance by CW-EPR will be substantially improved in a certain range around 8–13 Å with a precision of 0.84 Å.

The molecule rigidity is a major prerequisite to establish a distance ruler. Consequently, the C<sub>60</sub> nitroxide diradical adducts were selected to be the calibration molecules in this work. The rigid structure, definite molecular dimension, and stable structure of these adducts make them excellent distance calibration rulers in the research of biomolecular structure.

The purpose of distance calibration is to more accurately determine the structure and conformational changes of biomacromolecules. Therefore, distance calibration and correction curves were established under conditions generally met in the study of biomacromolecules. The results showed that all distance calibration curves had good linear correlations, suggesting that the influence of non-dipolar spectral broadening factors could be overcome via distance calibration method and that the established experiment and calculation methods were suitable for a wide range of conditions.

At present, the distance calibration range is only ~8–13 Å because of the lack of longer nitroxide diradical molecules. Synthesis of double spin molecules with longer spin–spin distance will be needed to further extend the scale of distance ruler in future work.

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