

**Recent applications of triarylmethyl (TAM) derivatives as Electron Paramagnetic
Resonance (EPR) spin labels in biomacromolecular structural studies**

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Abstract

Triarylmethyl (TAM) radicals, also known as trityl radicals, have found wide applications in Electron Paramagnetic Resonance (EPR), EPR imaging (EPRI), and Dynamic Nuclear Polarization (DNP). Of particular interest is the use of TAM as a spin labels/tag to study biomacromolecular structure and dynamics using various pulsed EPR techniques, a field initiated by the Hubbell and Schiemann groups. The long relaxation times of TAM spin labels as compared to the traditional nitroxide and paramagnetic metal ion spin labels/probes allow for inter-spin distance measurements under conditions above the cryogenic temperatures, leading to protein structure and conformational dynamics information under the physiological conditions. In addition, the high spectral intensity of TAM spin labels can enhance the percentage of spins being affected by microwave pulses commonly used in pulsed EPR spectroscopy, allowing for distance measurement in samples at low concentrations. The distinct spectral feature and minimal overlap with other commonly used spin centers also opens an avenue for orthogonal spin labeling and selective measurement of distances in protein samples. More recently, the enhanced protection of the radical center enhances the stability of the spin center under the reducing conditions in cells, leading to in-cell distance measurements, one further step toward distance measurement in proteins under the native conditions. The current review in this Special Issue aims to concisely highlight one of Prof. Wayne Hubbell's contributions to spin labeling and EPR methodological development, followed by the applications of TAM spin labels in recent biomacromolecular studies. The goal is to generate further excitement in the use of TAM spin labels to probe biomacromolecular structure and dynamics for various purposes.

Introduction

Triarylmethyl (TAM) radicals, also known as trityl radicals, have found wide biomedical and clinical applications based on Electron Paramagnetic Resonance (EPR) imaging (EPRI), due to its extremely narrow linewidth and thus, high contrast with respect to the backgrounds of biological tissues.[1-5] In the early 2010s, the Schiemann and Hubbell groups almost simultaneously reported utilizing TAM radical as spin labels for pulsed EPR-based inter-spin distance measurements.[6, 7] In comparison to the classic nitroxide spin labels invented by Hubbell and coworkers,[8, 9] TAM-based spin label in principle should possess extended spin lattice and spin-spin relaxation times as reflected by the narrow linewidth ($\sim 1\text{-}5$ G for TAM vs $\sim 70\text{-}75$ G for nitroxide spin labels). This privilege allows for inter-spin distance measurements to be carried out at temperatures way above the traditional, cryogenic temperatures, ideally the physiological temperatures of proteins.[10, 11] Although the most rapid EPR sample freezing rate has reached to μs scale as compared to the ms range using the classic, nitrogen-based freezing,[12, 13] removal of the need of rapid freezing would remove concerns on the potential conformational changes of proteins due to freezing.[14-17] Thus, in spite of the potential technical barriers of attaching a TAM spin label to a protein, TAM-based spin labeling and EPR studies were still explored.

Then, the high spectral intensity under the same spin concentration as compared to nitroxide or paramagnetic metal ions- based spin centers was recognized and employed to enhance the sensitivity of pulsed EPR distance measurement. Such high spectral intensity directly led to significantly enhanced modulation depth and/or absolute spectral signal, which allows for distance measurement for samples at much lower concentrations than other spin centers. The distinct spectral feature and minimal overlap with nitroxide and paramagnetic metal ion-based spin centers

also allows for orthogonal spin labeling and selective measurement of distances among different spin centers (attached to different areas of the same target protein), offering more structural information on one labeled protein sample.[18-21] More recently, the potentially enhanced protection of the radical center by the triaryl groups was recognized as an effective approach to enhance the stability of the spin center under the reducing conditions in cells, leading to in-cell distance measurements in proteins.[16] In-cell protein studies are necessary as compared to the studies in dilute solutions because this is an environment where proteins actually function in nature. Here in this Special Issue we aim to briefly highlight one of Prof. Wayne Hubbell's contributions to spin labeling and EPR methodological development, the ways to attach TAM site-specifically to a site of interest with minimal disturbance to the host biomacromolecule and demonstration of its application in protein science, followed by the applications of TAM spin labels in other recent protein/biomacromolecular studies.

Attaching a TAM to a biomacromolecule for EPR studies: for macromolecules such as rigid polymers and nucleic acids which can be modified during their synthesis, TAM spin centers can be covalently attached at desired positions. Attaching TAM to a protein is a challenge due to the high hydrophobicity of the aromatic groups. An approach developed by Hubbell and coworkers is to attach a thiopyridine group to TAM, followed by a click reaction between thiopyridine and the protein cysteine. An advantage of this approach is to allow for a rapid quantification of the labeling efficiency by assessing the amount of the colored leaving group 2-thiopyridone. Still, at the high label concentrations needed for the reaction to complete a significant amount of protein precipitated. A suitable solution is to immobilize the target protein onto a solid support such as sepharose beads, which prevents protein precipitation during the reaction with the hydrophobic TAM radical. An additional bonus is that immobilization eliminates the rotational tumbling of the

protein, which also reduces the motional averaging of the dipolar interactions between the spin centers and thus enhances the relaxation time, both benefiting the inter-spin distance measurement. This approach was exemplified on a globular model protein, T4 phage lysozyme (T4L), revealing distances at near room temperature consistent with the crystal structure of the protein.[6]

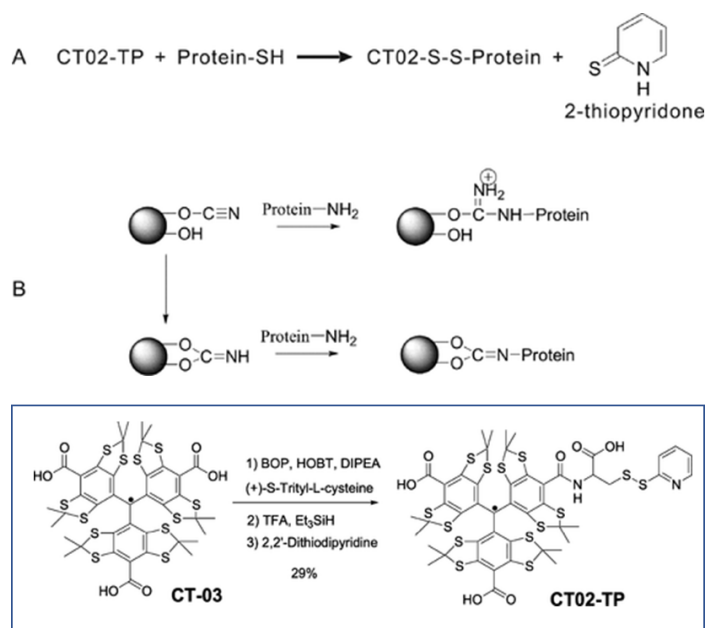


Figure 1. A modified TAM spin label (inset) can react with a protein thiol (A) and generate a 2-thiopyridone which can be quantified optically. (B) Protein immobilization reaction to a solid support. Reprinted (adapted) with permission from *J. Am. Chem. Soc.* 2012, 134, 24, 9950–9952.

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Other leaving groups have also been reported in the literature. For example, the classic, methanethiolsulfonate group can be attached to TAM (Figure 2) and react with a protein cysteine, also forming a disulfide bond.[22] In addition, the thioether-forming maleimide group can react with a cysteine residue in a protein. The resultant spin label sidechain is stable under reducing conditions, an advantage over the disulfide bond especially suitable for in-cell distance

measurement.[23, 24] Another alternative to label a cysteine via a thioether bond was detailed by Schiemann and coworkers, wherein a 3-butenyl group (Figure 2) was involved in the reaction with a cysteine -SH; the formed sidechain was stable under reducing conditions.[25] If a cysteine is not available to be labeled (ca. in a cysteine-rich protein), then the copper (I)-catalyzed alkyne-azide cycloaddition (CuAAC) reaction can be used to attach a modified TAM label (with a propargyl group; Figure 2) to an unnatural amino acid, such as azido-phenylalanine, which can be placed at a residue of interest in the target protein.[25]

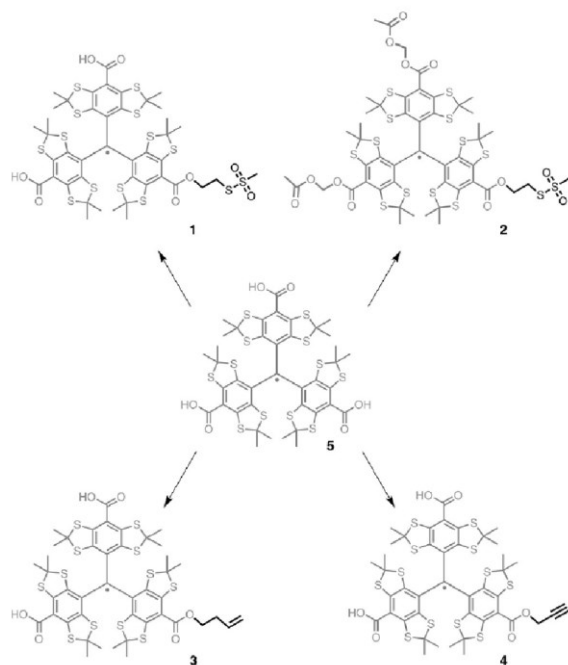


Figure 2. The synthesis of 4 TAM spin labels from the TAM precursor. Reprinted (adapted) with permission.[25]

It is also possible to label liganucleotides using TAM-based radicals for distance measurement. A typical labeling reaction[26] is to convert the original -OH group into its ester, i.e. 1-piperazinecarboxylate (Figure 3A), which can then react with TAM-Cl to introduce the TAM side chain. The linker can be shortened by reacting the -NH₂ group in a base with the TAM-Cl (Figure 3B).

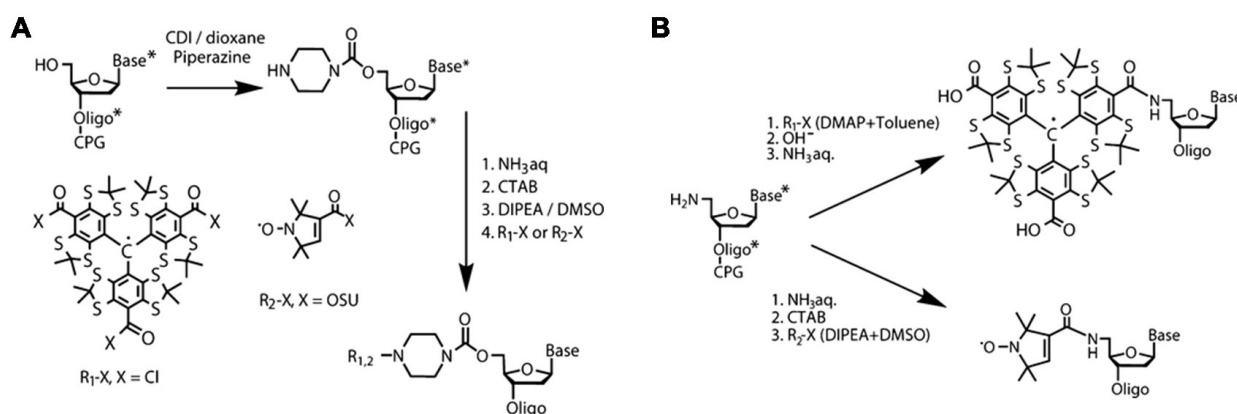


Figure 3. Two ways to attach TAM spin labels to oligonucleotides with different linker lengths.

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For a more detailed summary of labeling strategies we refer to the literature.[10] Depending on the target macromolecule, purpose of the experiment, and measurement conditions, a proper labeling strategy can be selected. The reactions discussed above are all reasonable to carry out except for the chemo-selectivity issue (i.e., only one -OH or -COOH of TAM is modified) which can be addressed via concentration control (i.e., a 1:1 modifying reagent:TAM stoichiometry will be applied by controlling the concentration of reagent that modifies the TAM).

Room temperature distance measurements based on the long T₂ (T_m) of TAM:

Perhaps the most intriguing use of the TAM spin labels is to carry out long-range (a few nm) distance measurement in proteins and other biomacromolecules at room temperature using various pulsed EPR techniques.[10] This approach avoids the need of freezing conventionally used to extend the phase memory times of other spin labels/probes (nitroxides, Cu^{2+} , Gd^{3+} , etc). Removing this requirement also removes the potential concerns on the conformational changes of a biomacromolecule during freezing, facilitating distance measurements under physiological conditions of the biomacromolecules of interest.

Upon attachment to a protein, the phase memory time (T_m) of the TAM spin labels is usually slightly reduced as compared to free TAM radicals in solution but still long enough to carry out double electron-electron resonance (DEER)/double quantum coherence (DQC) experiments covering the distance range up to a few nm distance (pulse sequences see Figure 4). For example, Hubbell, Freed, Zweier, and coworkers attached two TAM spin labels to two double cysteine mutants (with different spin-spin distances) in a model protein immobilized on a solid support and carried out DQC experiment at 4 °C and 17.2 GHz using facilities at the National Biomedical Resource for Advanced Electron Spin Resonance Spectroscopy (ACERT), Cornell University. DQC was selected over DEER due to the narrow linewidth of the TAM on the protein and solid support (which is slightly broader than free TAM in solution but not broad enough for a sufficient separation of the pump and observe pulse frequencies in DEER).[6, 27] In this case (TAM-labeled protein immobilized on a solid support), the T_m is on the order of $\sim 1 \mu\text{s}$ at 4-25 °C, sufficient for measuring distances up to 2-2.5 nm. Using a model protein with known structure, TAM-TAM distances of 1.8 and 2.1 nm were obtained, which are close to the Ca-Ca distances of the labeled site (the bulky TAM sidechains are in parallel with each other; thus, the measured TAM-TAM distance is close to the Ca-Ca distance), suggesting the success of TAM in near-room

temperature distance measurement using DQC. The small symmetric spin density delocalization of TAM could cause a minor deviation from the point-dipole approximation as compared to nitroxide spin labels.

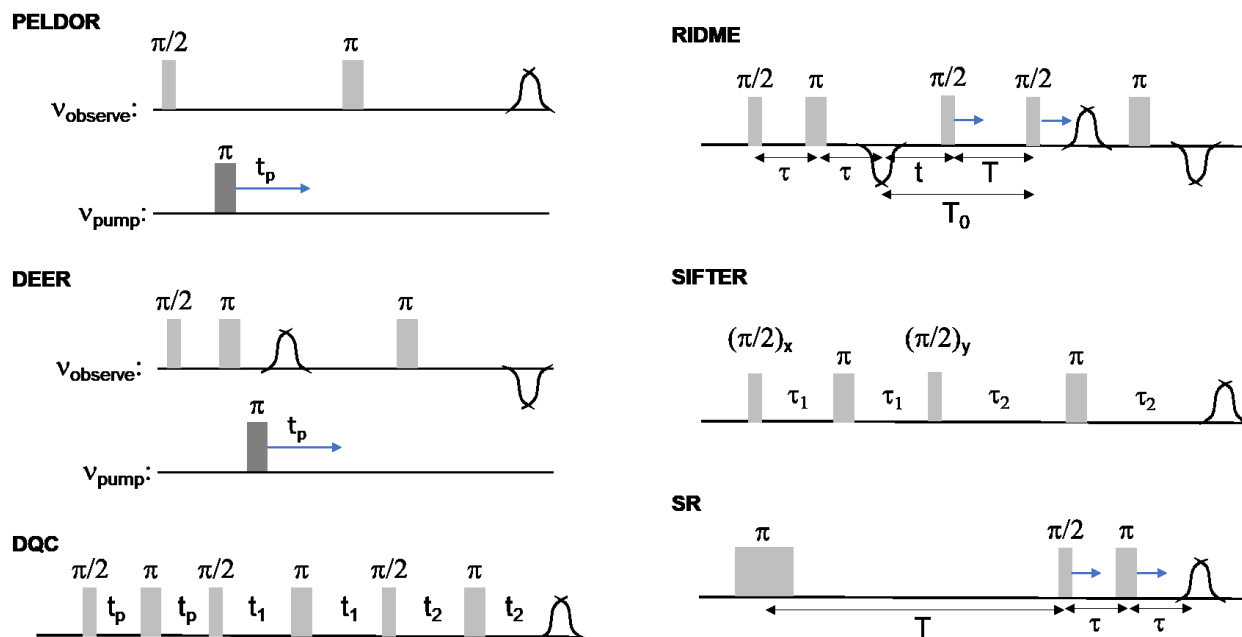


Figure 4. Pulse sequences involved in this review.

TAM-labeled DNA duplexes immobilized in trehalose and nucleosil have also shown long ($\sim 2 \mu\text{s}$) T_m at even higher temperatures (310 K or 37 °C; D_2O was employed to help enhance the T_m), which allowed for DQC measurements to reveal a TAM-TAM distance of $\sim 4.6 \text{ nm}$, which is consistent with model distances based on the rigid, duplex structure, as well as DEER and DQC measurements at 80 K upon rapid freezing of free DNA samples without immobilization.[10, 28] This work also proved that weak electrostatic interactions between TAM-labeled DNA duplex and sorbent do not influence the structure and orientation of the TAM labeled DNA helix.[28] Other single-frequency-based EPR techniques for long-range distance measurement, such as relaxation

induced dipolar modulation enhancement (RIDME) or single-frequency technique for refocusing dipolar coupling (SIFTER; Figure 4), have also been used as alternatives of DQC to measure inter-TAM distances at 298 K and 310 K upon immobilization in trehalose and nucleosil.[10, 19] The T_m at 298 K was $\sim 2.5 \mu s$ which led to time domain evolution of $\sim 3 \mu s$ to be obtained, which allows for measuring average distances at ~ 4.0 - 4.2 nm. In principle, the maximum distance via room temperature five-pulse RIDME could be ~ 5.7 nm under the condition of the $3 \mu s$ time domain evolution.

In all cases discussed above, protein immobilization was critical to avoid the rapid protein rotational tumbling which shortens the dipolar coupling frequency. The accuracy, sensitivity, and resolution of TAM-based room temperature distance measurement in proteins/DNAs were comparable to those of nitroxide spin labels for DQC, RIDME, and SIFTER (with low sensitivity in DEER). DQC, RIDME, SIFTER, and DEER all report the distribution of distances, allowing for structural ensemble and/or conformational flexibility detection.

Room temperature distance measurements based on the long T_1 of TAM:

Another approach to measure intra-protein distances relies on the distance dependence of the paramagnetic relaxation enhancement (PRE) of a slowly-relaxing species due to the presence of a fast-relaxing species. PRE has been applied at low temperatures using nitroxide as the slowly relaxing center and Cu^{2+}/Fe^{3+} as the fast-relaxing one with a complete theory developed to compute for the average nitroxide-metal distances (the rigid motional limit).[20, 29-34] In principle, the same strategy is applicable to room temperature distance measurements, as long as the fast motional limit is applied. This concept of PRE at room temperature has been proved on Cu^{2+} -nitroxide spin pairs as early as 2006.[30] However, the relaxation times of nitroxides are often very short at room temperature, which reduces the upper limit of measurable distances. In fact, the

relatively short nitroxide T_1 limited the measurable range to less than 4 nm even if the intrinsic motion of the nitroxide spin labels is reduced (via modification of the spin label structure).[29] The long spin-lattice relaxation times of most TAM-based EPR spin labels at room temperature[34] offer an opportunity to measure long-range intra-protein distances according to the PRE of the TAM spin-lattice relaxation rate due to the presence of a nearby, fast-relaxing spin center (ca. transition paramagnetic metal ions). The long TAM radicals, even after attachment to a protein immobilized on a solid support, can offer a 12 μs T_1 as determined by saturation recovery (SR) EPR (Figure 4) and thus, extending the upper limit of measurable average intra-protein distances to ~ 5 nm if Cu^{2+} is employed as the fast-relaxing species at 298 K, comparable to the distance range based on room temperature distance measurement using TAM and DQC/RIDME, as demonstrated by Hubbell and coworkers.[35] Using a short loop inserted to the model protein (lysozyme) as the Cu^{2+} binding site, average Cu^{2+} -TAM distances from ~ 35 Å to ~ 40 Å were determined depending on labeled sites and position of loop insertion.[35] The sensitivity of the TAM-based PRE measurement is relatively high due to the long T_m of TAM at room temperature and thus, the relatively large spin echo in SR measurement.

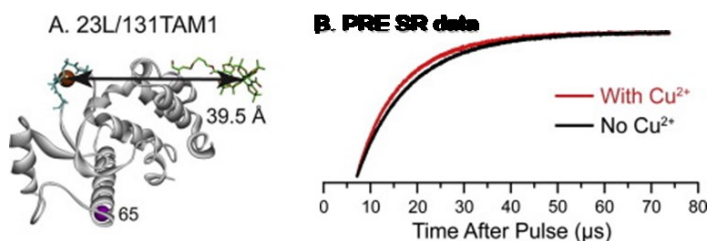


Figure 5. (A) Illustration of the distance between the Cu^{2+} loop and TAM spin label in the model protein. (B) The PRE effect on SR data of TAM upon Cu^{2+} binding. The T_1 is ~ 12 μs without Cu^{2+} , allowing a ~ 40 Å distance to be measured at room temperature. Reprinted (adapted) with permission.[35]

Of course, such PRE-based distance measurement does not provide the distance distribution between TAM and Cu^{2+} spin centers. A practice, however, is to measure DEER at low temperature and use the obtained distance distribution to back calculate the average TAM/nitroxide- Cu^{2+} distance, which in fact offers an opportunity to prove the proper use of the fast and rigid motional limit of the PRE theory.[29, 35] In this work, the model protein labeled by both nitroxide and Cu^{2+} was in solution and immobilized on a solid support, followed by freezing to 77 K before DEER measurement. The obtained DEER distance distribution in each case was used to calculate the average nitroxide- Cu^{2+} distances. Although almost identical distance distribution was obtained from DEER, the calculated distance using the fast-motional approximation matches the average distance based on the PRE of nitroxide when the protein was in solution. The calculated distance using the rigid-motional approximation matches the average distance based on PRE of nitroxide when the protein was immobilized,[29] confirming the success of this strategy.

Orthogonal spin labeling and selective distance measurement:

The distinct absorption range and spectral lineshape of TAM spin labels as compared to other commonly used spin center/probes offer an opportunity to perform orthogonal spin labeling and selective distance measurement using multi-frequency pulsed EPR techniques such as DEER (also known as pulsed electron-electron double resonance, or, PELDOR; pulse sequence see Figure 4). Being able to carry out orthogonal spin labeling and selective distance measurement allows for the simultaneous determination of multiple distances on one biomacromolecule sample. This is an advantage in comparison to the needs of multiple samples each labeled with the same probe between different sites of interest in a protein. The relatively narrow linewidth of the TAM

spin center as compared to other spin probes often results in high spectral intensity and thus, a higher chance of being excited by a microwave pulse in DEER/PELDOR, leading to higher modulation depths of the DEER/PELDOR traces and thus, higher sensitivity. Note that because of the short relaxation times of the alternative spin label (other than TAM) at room temperature, the selective distance measurement using TAM and other spin centers/probes is usually carried out at cryogenic temperatures.

The selective distance measurement in TAM-nitroxide spin pairs has been shown in several works. To ensure the co-presence of both TAM and nitroxide spin labels, a common approach is to include these at the desired location of a model molecule or DNA during their synthesis.[7, 26, 36] Then, PELDOR/DEER measurements can be carried out to selectively “invert” (via the pump pulse) and “observe” the TAM and nitroxide centers, respectively. Note that TAM is often excited by the pump pulse so that the modulation depth of the obtained DEER/PELDOR signal is maximized due to the high chance of inversion of the TAM spin labels even using a regular 10-20 ns inversion pulse at tens of MHz away from the observe pulses. The experimentally observed modulation depths are on the order of 70-80%,[7, 26, 36] significantly higher than the 20-40% scale if nitroxides are being inverted. Note that for TAM doubly labeled compounds, both the pump and observe pulses were applied to TAM absorption spectrum (field-swept electron spin echo, FS ESE), resulting in shallower modulation depths (~15-40%).[36] Distances of up to 5 nm were determined in these DNA or synthetic rigid model molecules, which were further confirmed by modeling. It is also possible to probe the orientation effect in DEER/PELDOR using these (rigid) synthetic molecules by adjusting the frequencies of the observe pulses to probe nitroxide while keep pumping TAM.[36] The high modulation depth makes it easier to distinguish the subtle differences in time domain traces when a number of observe frequencies (differing from the pump

frequency by 30 to 90 MHz with a 10 MHz interval) were applied. The success of resolving both inter-spin, TAM-nitroxide distance distribution and the relative orientation of the spin centers was also confirmed by modeling.[36]

When attempting to perform orthogonal labeling in a protein, different labeling strategies are often needed to distinguish the attachment of a TAM and nitroxide spin center,[37, 38] or, if one site can be selectively labeled prior to derivatization. For example, a nitroxide and a TAM spin label were attached to the outer membrane cyanocobalamin (CNCbl) transporter BtuB (with TAM specifically attached to residue 188 of BtuB) while TEMPO-CNCbl as a labeled substrate analog non-specifically attached.[39] Then, nitroxide-nitroxide and TAM-nitroxide distances were selectively measured by adjusting the frequency of the pump and observe pulses in PELDOR/DEER experiments, so that both pump and observe pulses were adjusted to match frequencies to excite only nitroxides in the former case while these pulses were adjusted so that nitroxide was observed while TAM was pumped in the latter case. The resultant DEER traces showed enhanced modulation depth when TAM was pumped as compared to that when nitroxide was pumped (2-4 folds higher). More importantly, the distance between TAM and nitroxide in the same membrane protein was successfully measured, leading to key structural information depicting the protein function.[39] In a more recent work, in addition to the TAM or nitroxide labeled BtuB and nitroxide-labeled CNCbl, two other binding partners, TonB and BtuF were separately labeled with Gd^{3+} or a nitroxide. Thus, a triple labeling system was created to probe the interactions among the four parties by selectively pump and observe different spin centers to selectively measure the distances between specific interaction partners, an elegant application of orthogonal spin labeling and DEER EPR.[22]

In another example, selective spin labeling was achieved by the simultaneous creation of a cysteine (E15C) to attach a TAM and two histidine (K28H and Q32H; dHis) mutations wherein the two His were placed to form a highly selective binding with a Cu^{2+} -NTA complex in a GB1 protein.[40] Note that this elegant strategy to include a Cu^{2+} spin center to the target protein has the advantage of minimal intrinsic length and flexibility of the Cu^{2+} spin probe in comparison to the Cu^{2+} loop and nitroxide spin labels.[40-43] DEER was then carried out at X-band frequencies by observing at the first local maximum of the TAM and pumping at the maximum of the Cu^{2+} -NTA complex.[18] The setup of the pump and observe frequencies could also be reversed but the smaller observe echo (low signal-to-noise ratio) and the long T_1 of TAM (requiring long shot repetition time, and thus, signal acquisition time) reduced the sensitivity of the DEER measurements. The obtained distance distribution was consistent with the structural model. Note that in this work TAM was ^{13}C -substituted ($^{13}\text{C}_1$ -mOX063-d₂₄) to broaden the field-swept (FS) spectrum so that TAM-TAM DEER measurement could be carried out at Q-band.[18] The ^{13}C satellite line is the key to broaden the absorption spectrum of the labeled protein so that the pump and observe pulse could be sufficiently separated (~ 0.5 mT away) at Q-band frequencies.[18] The obtained DEER evolution was ~ 2.5 μs with an average TAM-TAM distance of ~ 4 nm obtained. The obtained distance distribution was in good agreement with structural models.

It is also possible to not distinguish the spin centers but non-selectively label two cysteines in a protein if both the TAM and another spin center can react with a protein thiol group. For example, TAM and Gd^{3+} spin centers were non-selectively attached to two cysteines in two model proteins each with two cysteine residues separated by a few nm. Although the FS spectra of the two spin centers overlap (at W-band in this work), it was possible to selectively pump and observe each spin center for DEER measurements when a dual mode cavity was employed (due to the 520

MHz different between the maximum absorption of the TAM and Gd^{3+}). [44] The modulation depth was on the order of 0.2-0.3 at W-band and the signal-to-noise ratio was high, which indicates the high sensitivity of TAM- Gd^{3+} DEER measurement in proteins at W-band. As compared to Gd^{3+} - Gd^{3+} distances, the obtained TAM- Gd^{3+} distance distributions were relatively narrow using chirped pump pulses and were in good agreement with structural models. TAM alone or in combination with Gd^{3+} , thus, offers promising opportunities for distance measurements in proteins in vitro or in cells at W-band frequencies. [44]

In-cell distance measurement:

Due to the complexity in cellular components and their dynamic interaction under the highly crowded/confined environment, direct in-cell distance measurement of proteins or other biomacromolecules using EPR methods faces several challenges as compared to solution studies. The stability of the radical and the linkage of the spin label on the target protein are the primary concern, which can be overcome by using more stable spin centers such as Gd^{3+} or reduction-resistant nitroxides and linkages without disulfide bonds. The next concern is the sensitivity of the EPR measurements inside of a cell, which requires a sufficient spin concentration. Lastly, the phase memory time of a spin labeled protein inside of a cell is often shortened as compared to that in solution due to the presence of endogenous cellular paramagnetic metal ions (ca. Mn^{2+}) and possible local high spin concentrations due to cellular crowding.

TAM radicals and their derivatives as spin labels possess unique properties to potentially overcome these barriers. For example, the radical of TAM is surrounded/protected by three bulky aryl groups and is thus more stable in the reducing cellular environment. Thioether based labeling strategies have been developed for TAM derivatives to avoid the breakage of the disulfide-based spin labeling approach. Moreover, the narrow linewidth offers high sensitivity in EPR

measurements and the long T_m facilitates long-range distance measurement. The first example of in-cell TAM-based distance measurement was a RIDME study which revealed TAM-Fe³⁺ distances in CYP101 protein injected to *xenopus laevis* oocytes.[25] The TAM spin label was modified with a 3-butenyl group (also see Figure 2) so that no disulfide was involved in the linkage. Significantly reduced modulation depth (from ~30% to 4%) as compared to solution study was also observed due to “the difficulty in controlling the iron content of the CYP101 protein” as well as “the 40 μM Mn^{II} which may reduce the modulation depth because of relaxation enhancement of the Fe center compared to the in vitro measurement of the identically labeled protein”. The observed distance distributions were slightly broader in cells as compared to the in-solution study, indicating that the protein behaves slightly differently in crowded cells compared to dilute solutions.[25] Still, this TAM-based RIDME study presents significantly enhanced signal-to-noise ratio as compared to nitroxide spin labels and pointed out the potential use of TAM spin labels for in-cell EPR studies even at room temperature.

The first in-cell DQC study on TAM spin labels was on a YopQ mutant in eukaryotic *xenopus laevis* oocytes at Q-band frequencies using the SLIM TAM spin label at cryogenic temperatures.[23] A similar linkage as in the case above was also employed to avoid degradation of the spin labeled sidechain under the reducing conditions, which was proved effective by the redox stability tests. Reduced T_m was also observed yet a short incubation (2 hours) made the spins more uniformly distributed, confirming that local crowding in cells could reduce T_m . [23] Enhanced SNR was also observed as compared to in-cell nitroxide-based EPR measurements. The distance distribution obtained from in-cell DQC measurement was different compared to in vitro studies,[23] suggesting a significant influence of the cellular environment of protein structure

(such as molecular crowding or binding of regulatory proteins) and highlighting the importance of carrying out in-cell studies when attempting to understand a protein.

A thorough recent in-cell distance measurement study demonstrated these points using two model proteins and human HeLa cells.[24] The T_m of TAM allowed for DEER traces to be measured for $\sim 5 \mu s$ in cells which resulted in near 5 nm local maximum in the distance distribution, consistent with the known structures of the proteins and distances obtained from DEER measurement on Gd^{3+} -based spin labeling. Orientation effects were avoided by using chirped pulses. A reduction of the modulation depth on in-cell DEER signal as compared to in solution studies was observed. Incubation of the labeled proteins with cells for 4 hours seemed to provide a good sensitivity to distance measurement, as further incubation resulted in a decrease in modulation depth (although a clear modulation and accurate distances could still be measured). The origin of the reduction in T_m in cells as compared to solution and Gd^{3+} labels was also identified/ suspected to be caused by the methyl groups. The main advantage of in-cell TAM is concluded as their high potential to carry out in-cell distance measurement at room temperature.[24]

Additional benchmarks on TAM-based EPR distance measurement.

Given the rising interest in using TAM as a spin label for various applications, there has been a critical need for further exploration of TAM spin labels in terms of methodological and theoretical studies. A comprehensive, systematic study on a comparison of PELDOR, RIDME, SIFTER, and DQC on TAM-based samples is a typical example. Different pulse sequences were applied to the model molecules contain 2 and 3 TAM centers to evaluate their performance on measuring inter-spin distances. Theoretical approaches were followed by thorough experimental studies to reveal not only the TAM-TAM distances but also the exchange coupling, pseudosecular

coupling and multi-spin effects.[45] The orientation effects in DEER for TAM-spin labeled biomacromolecules were also explored in several cases using high-frequency pulsed EPR.[46, 47] It is also possible to conduct “multiquantum counting” on oligomeric molecules labeled with TAM due to its narrow spectral width which allows for homogeneous excitation of all spins by the pulses.[48] The long transversal relaxation times of higher quantum coherences of TAM spin centers also helps the detection of multiquantum coherences, although certain challenges such as the influences of ESEEM and the difficult in precise phase settings to separate the higher quantum coherences need to be overcome.[48]

Conclusion

TAM as a spin label has been employed for the investigation of protein and DNA structure/dynamics for only slightly more than a decade, yet promising progress has been made with respect to labeling strategy, room temperature distance determination, orthogonal spin labeling and selective distance measurement, and in-cell distance measurement. Moreover, a whole set of theoretical studies were performed on various CW and pulsed EPR techniques using TAM-based radicals. Meanwhile, further improvement is needed to broaden the application of TAM-based spin labeling in EPR studies such as enhancing the reaction efficiency with proteins while reducing perturbation to proteins during labeling, the labeling selectivity when multiple different labels are needed in one biomacromolecule, and sensitivity enhancement in in-cell distance measurements. Nevertheless, TAM radicals as spin labels will be a powerful tool for spin labeling of proteins and other biomacromolecules for magnetic resonance studies including EPR and dynamic nuclear polarization (DNP).[23, 49]

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Declarations

Ethical Approval

Not applicable.

Competing interests

The authors claim no competing interests.

Authors' contributions

Z. A., A. M., P. Z., and Z. Y. wrote the main manuscript text and prepared figures. M.L. and Q.L. assisted in literature search and figure preparation. G. B. and W. B. and L. F. assisted in literature search and writing. All authors reviewed the manuscript.

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Availability of data and materials

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