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Molecular “Light switch” [Ru(phen)\textsubscript{2}dppzidzo]\textsuperscript{2+} Monitoring the Aggregation of Tau

Xing Gao, Tian-Ming Yao,* Hai-Liang Huang, Lin-Lin Wang, Jun-Liang Yao, and Shuo Shi*

Monitoring aggregation of tau protein is a key protocol for illuminating pathogenic mechanism of Alzheimer’s disease. In the present article, [Ru(phen)\textsubscript{2}dppzidzo]\textsuperscript{2+}, a “light switch” ruthenium (II) complex, was presented as a new monitoring probe for aggregation of tau R3 peptide, the third repeat of tau microtubule-binding domain. Having little impact on the aggregation process, large fixed Stokes shift, small background luminescence, made the complex a better probe for monitoring the aggregation process and quantitatively detecting tau filaments, while compared to Thioflavin S, a commonly used fluorescent dye for staining neurofibrillary tangles and monitoring tau aggregation. Furthermore, a long luminescence lifetime of this complex could also expand its potential usage in detection of tau filaments in the presence of short-lived fluorescent backgrounds.

Introduction

Intracellular neurofibrillary tangle (NFT) is a major defining neuropathological feature of Alzheimer’s disease (AD). Tau protein, which stabilizes microtubules in axon of neurons, is the main component of neurofibrillary tangle core.\textsuperscript{1, 2} The amount of tau aggregation products including paired helical filaments (PHFs) and straight filaments (SFs) is closely correlated with cognitive impairment of AD patients.\textsuperscript{3-6} Thus, sensing the tau assembled products and monitoring the aggregation process in real time are very important for the diagnosis, and systematic in vitro researches for illuminating pathogenic mechanism of AD.\textsuperscript{7, 8}

Thioflavin S (ThS), a methylated, sulfonated polymerized primulin product, is the most commonly used fluorescent dye to stain NFTs structures and other fibril structures like Amyloid-β (Aβ) aggregates in postmortem examination of AD brain tissues. Another important role of ThS was monitoring the aggregation of tau protein or the filament formation of tau microtubule-binding domain (MBD) in vitro.\textsuperscript{7-11} The emission intensity and emission wavelength maximum of ThS is dependent on the solvent polarity or dielectric constant, and a decrease in polarity leads to an increase in fluorescence intensity for ThS.\textsuperscript{12} Although the mechanism of tau-ThS complex formation is still unknown, a molecular modelling result shows that ThS could intercalate into β-sheets in tau PHF core protofibrils through electrostatic, hydrophobic and Van der Waals interactions.\textsuperscript{13} However, studies have shown that ThS-positive amyloid deposits are neurotoxic to mice with AD, and as an anionic dye, ThS is able to induce the tau aggregation in vitro.\textsuperscript{13, 14} Furthermore, the fluorescence background of ThS was very high, and it was inconvenient to observe the fibril from the bright background. Thus, ThS is imperative as a monitoring probe for tau aggregation, and a new luminescent dye is urgently needed.

“Light switch” ruthenium (II) complexes like [Ru(L)\textsubscript{2}dppz]\textsuperscript{2+} were used to sense the mismatch of double-stranded DNA,\textsuperscript{15} distinguish the G-quadruplexes architectures form other kind of DNAs.\textsuperscript{16-17} For instance, [Ru(L)\textsubscript{2}dppz]\textsuperscript{2+} binding to biomolecules in many different ways, generally, the DNA or aggregated proteins provide a hydrophobic cavity that can shield the dppz ligand from water, which restores the luminescence of the complexes and generates the “light switch” effect.\textsuperscript{22, 23}

In the present study, [Ru(phen)\textsubscript{2}dppzidzo]\textsuperscript{2+} (dppzidzo = dipyrido-[3,2-a:2’,3’-c]phenazine-imidazolone), an excellent ruthenium (II) “light switch” complex characterised in our lab,\textsuperscript{24} is reported as a new luminescent monitoring probe for aggregation of tau R3 peptide, the third semi-conserved repeat of tau microtubule-binding domain a representitive and convenient substitute for full length tau in vitro.\textsuperscript{25-28} Here, as a positively charged dye, [Ru(phen)\textsubscript{2}dppzidzo]\textsuperscript{2+} has little impact...
on tau R3 peptide aggregation process. It is also possible to quantitatively detect the amount of tau R3 filaments just using the luminescence intensity of the complex due to its “light switch” effect. Furthermore, the complex may have potential usage in detection of tau filaments in the presence of short-lived fluorescent backgrounds. Finally, a possible binding mode between $[\text{Ru(phen)}_2\text{dppzido}]^{2+}$ and tau R3 short filament is presented, which delivers an insight into the tau aggregation monitoring.

Results and Discussion

Monitoring Tau R3 aggregation by luminescence

Thioflavin S is a methylated, sulfonated polymerized primulin preparation whose structure has not been characterized yet.\textsuperscript{9, 12} Dissolved in water, ThS exhibited a strong emission at around 450 nm (Figure 1A, red curve). In the presence of tau R3 filaments, the emission spectrum of ThS displayed a red shift and a new stronger peak at around 500 nm emerged (Figure 1A, black curve), which made ThS a noteworthy clinical diagnosis of AD as well as for qualitative neuropathological investigations.\textsuperscript{7} For the emission intensity at 500 nm of ThS arose as the aggregation of tau R3 peptide was taking place, it seemed reasonable to use ThS as monitoring agentia in characterizing the transition of tau monomer to fibrils. Dissolved in water, $[\text{Ru(phen)}_2\text{dppzido}]^{2+}$ was weakly emissive (Figure 1B, red curve). In the presence of aggregated R3 peptide, the complex exhibited a large luminescence intensity growth (Figure 1B, black curve), and no obvious spectrum shift or other emissions was observed. However, it still needed to be answered whether the complex was able to monitoring the aggregation process of tau.

![Figure 1](image1.png)

Figure 1 (A) Luminescence spectra of ThS (10 μM, excitation at 400 nm) in the absence and presence of tau R3 filaments (15 μM); (B) Luminescence spectra of $[\text{Ru(phen)}_2\text{dppzido}]^{2+}$ (10 μM, excitation at 404 nm) in the absence and presence of R3 filaments.

Then, luminescence kinetics experiments were carried out. Mixed with R3 peptide and heparin, ThS displayed uninterrupted fluorescence growth in the first 40 min of R3 peptide assembling (Figure 2, black curve). The luminescence growth retardation indicated the terminal of tau R3 aggregation. Similarly, $[\text{Ru(phen)}_2\text{dppzido}]^{2+}$ displayed luminescence growth when incubated with R3 peptide and heparin (Figure 2, red curve). However, it took about 60 min for the aggregation to reach the terminal, which meant the increasing rate of Ru (II) complex was smaller than that of ThS. When R3 peptide was incubated without ThS or Ru (II) complex (Figure S1), the growth rates of both dyes were very close, and the equilibrium time was about 60 minutes. Tau R3 peptide aggregation reached the reaction equilibrium much earlier in the presence of ThS than interacting with Ru (II) complex, which meant ThS might accelerate the aggregation process. To settle down the contradiction, more detailed studies were carried out.

Impacts of ThS on R3 aggregation

The difference of luminescence kinetics results between ThS and $[\text{Ru(phen)}_2\text{dppzido}]^{2+}$ reminded us that, ThS and other anionic dyes were able to induce the tau aggregation in vitro.\textsuperscript{13} To investigate the impacts of ThS on the heparin induced tau R3 peptide aggregations, fluorescence spectrum assay and TEM experiments were carried out. When ThS was added to R3 solution before incubation (Figure 3A, black curve), its fluorescence intensity was much larger than added after the incubation, which meant that ThS might have accelerated the aggregation of R3 peptide. The result was backed up by the visualized observations using TEM. In the absence of ThS (Figure 3B), R3 filaments were twisted together as reported.\textsuperscript{11} With the addition of 10 μM of ThS for incubation (Figure 3C), the R3 peptide aggregated into large plaques, which looked much thicker and was still growing at the edge. As an anionic dye, ThS was able to bind to the positively charged protein. The β-sheet structures of the tau PHF core were also a conceivable binding site for ThS.\textsuperscript{15} Both fluorescence and TEM results indicated that ThS was clearly an accelerator to tau R3 aggregation, which made the monitoring by ThS fluorescence impricable and unreliable.

$[\text{Ru(phen)}_2\text{dppzido}]^{2+}$ monitoring R3 aggregation

The same experiments were carried out with $[\text{Ru(phen)}_2\text{dppzido}]^{2+}$. The afterwards added ruthenium (II) complex exhibited about 95 percentage of luminescence intensity compared to the pre-added, which strongly indicated that the aggregation of tau R3 peptide was not accelerated or slowed down by the complex (Figure 4A). To testify the impacts of $[\text{Ru(phen)}_2\text{dppzido}]^{2+}$ on the aggregation morphology of R4 peptide, electron micrographs were also measured. The similar
characteristics in Figure 4B and 4C strongly indicated that [Ru(phen)\textsubscript{2}dppzidzo]\textsuperscript{2+} could hardly affect the aggregation of tau R3 peptide. According to the luminescence and TEM results, it was clear that [Ru(phen)\textsubscript{2}dppzidzo]\textsuperscript{2+} was a much more appropriate and accurate probe in monitoring the aggregation of tau protein. Light scattering experiment was also carried out, and the result was consistent with luminescence and TEM results (Figure S2).

Another advantage of [Ru(phen)\textsubscript{2}dppzidzo]\textsuperscript{2+} for monitoring R3 aggregation was its large Stokes shift (about 206 nm, Figure 5A), which made it easier to distinguish the emission light from the excitation light and eliminate background luminescence. This made it possible for [Ru(phen)\textsubscript{2}dppzidzo]\textsuperscript{2+} to have linear response to R3 aggregations from 5 to 100 μM (Figure 5B). For ThS, it did not possess a fixed characteristic Stokes shift in the excitation or emission spectra, which result in very high background fluorescence (Figure 1A), and make it unsuitable for quantitative analysis in solution.\textsuperscript{9} [Ru(phen)\textsubscript{2}dppzidzo]\textsuperscript{2+} had a longer luminescence lifetime (τ = 154 ns, Figure 5C) than ThS (τ = 1.4 ns), indicated that the complex might have potential usage for detection of tau filaments using time-gating technology.\textsuperscript{20} [Ru(phen)\textsubscript{2}dppzidzo]\textsuperscript{2+} was a racemic complex, thus, the different conformation of [Ru(phen)\textsubscript{2}dppzidzo]\textsuperscript{2+} was able to distinguish the chiral properties of the proteins. No obvious differences were observed between Δ and Λ-[Ru(phen)\textsubscript{2}dppzidzo]\textsuperscript{2+} with R3 peptides (Figure S4), which indicated that the complex was not binding with the chirality region of the protein.

As one of the classical “light switch” Ru (II) polypyridyl complex, [Ru(phen)\textsubscript{2}dppz\textsubscript{2+} also showed luminescence growth while incubated with R3 peptide and heparin (Figure S5), however, its saturated intensity was much smaller than that of [Ru(phen)\textsubscript{2}dppzidzo]\textsuperscript{2+}. In other words, [Ru(phen)\textsubscript{2}dppzidzo]\textsuperscript{2+} had better sensitivity to tau R3 filaments than [Ru(phen)\textsubscript{2}dppz\textsuperscript{2+}. Tau R2 peptide and R2-C291A mutant peptide were also tested with [Ru(phen)\textsubscript{2}dppzidzo]\textsuperscript{2+}. In general, R2 peptide could also aggregate into filaments in the presence of heparin, but the aggregation speed in the first hour was slower than R3 peptide.\textsuperscript{25} R2-C291A mutant peptide was unable to dimerize through disulfide linkage, thus, the mutant aggregated much slower than R3 and R2 peptides; it could take days before the peptide aggregated into filaments.\textsuperscript{29,30} Due to different aggregation time of these three peptides, we were able to distinguish them just using the luminescence intensity of [Ru(phen)\textsubscript{2}dppzidzo]\textsuperscript{2+} (Figure S6).

**Molecular Modelling**

To gain insight into the interaction between R3 filament and [Ru(phen)\textsubscript{2}dppzidzo]\textsuperscript{2+}, molecular docking was carried out. 3D models of R3 PHF-core were built based on multiple-threading alignments by LOMETS (LOcal MEta-Threading-Server).\textsuperscript{31} Then, six R3 monomers were combined as a short tau R3 filament. Autodock vina program was applied as the docking strategy.\textsuperscript{32} During the docking process, the Ru (II) complex was regarded as rotatable and subjected to energy minimization. The complex molecule with the optimum orientation was docked into R3 domain and the results were imported into Discovery Molecular Modelling.
Figure 5 (A) Excitation and Emission spectra of [Ru(phen)₂dppzidzo]²⁺ (10 μM) interacting with aggregated R3 (15 μM); The Stokes shift of the complex was about 200 nm. (B) Luminescence of [Ru(phen)₂dppzidzo]²⁺ (10 μM) linear response to R3 aggregations; (C) Luminescence decays for ThS (τ = 1.4 ns, black curve) and [Ru(phen)₂dppzidzo]²⁺ (τ = 154 ns, red curve).

Studio V2.5 for further rendering. In Figure 6A, [Ru(phen)₂dppzidzo]²⁺ was calculated to insert into the gap between two R3 monomers, which might have frustrated the water molecules off the main ligand of the complex and resulted in the luminescence emission. The binding mode of the complex was quite different from that of ThS. According to Meraz-Ríos, ThS bound to the β-sheeted area of the protein, which was mainly consisted of positively charge residues. However, for the Ru (II) complex, it showed a larger trend to bind with the negatively charged residues (Figure 6B), which provided a hydrophobic cavity, shielded the dppzidzo ligand from water, and uprose the luminescence of [Ru(phen)₂dppzidzo]²⁺.

Figure 6 (A) [Ru(phen)₂dppzidzo]²⁺ binding with R3 formed PHF core; (B) Molecular surface of R3 formed PHF core colored by interpolated charge (blue represent positive charges, red signify negative charges).

Conclusions

In summary, we have demonstrated the potential usage of [Ru(phen)₂dppzidzo]²⁺ as a monitoring probe for tau R3 aggregation. The intriguing properties of [Ru(phen)₂dppzidzo]²⁺ such as large Stokes shifts, long lifetimes, small luminescence background, and for the most important little effect on the aggregation process itself, have made it valuable towards monitoring the tau aggregation process. These advantages also made it possible to quantitative analysis of tau R3 filaments just using the luminescence signal of [Ru(phen)₂dppzidzo]²⁺. The different electrostatic charges between [Ru(phen)₂dppzidzo]²⁺ and ThS, implied they might have different binding mode with R3 filament cores. With the assistance of molecular docking, we suggested that [Ru(phen)₂dppzidzo]²⁺ was aiming for the negative charged cavities on the protein surface, while ThS was targeting the β-sheeted structures. We believed that ThS could still be used as fluorescent dye staining NFTs in neurons, but tau aggregation monitoring should be replaced by [Ru(phen)₂dppzidzo]²⁺ or other luminescent dyes. We hope that our present work could be valuable for the detection of tau aggregation products.

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