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Sensing Amyloid-β Aggregation Using Luminescent Dipyridophenazine Ruthenium(II) Complexes

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Supporting Information

ABSTRACT: The aggregation of amyloid-β (Aβ) peptides has been associated with the onset of Alzheimer's disease. Here, we report the use of a luminescent dipyridophenazine ruthenium(II) complex to monitor Aβ fibrillization. This complex is not photoluminescent in aqueous solution nor in the presence of monomeric Aβ, but it presents a strong photoluminescence in the presence of Aβ fibril aggregates. One of the advantages of this metal complex is its large Stokes shift (180 nm). Furthermore, the long-lived photoluminescence lifetime of this ruthenium complex allows its use for the detection of fibrillar proteins in the presence of short-lived fluorescent backgrounds, using time-gating technology. We will present evidence of the advantages of dipyridophenazine ruthenium(II) complexes for monitoring protein fibrillization in highly fluorescent media.

Amyloid-β (Aβ) is an extracellular peptide fragment, thought to play an important role in the pathology of Alzheimer's disease (AD). AD is rapidly becoming a leading cause of death in the United States; an estimated 11−16 million people are expected to be affected by 2050.[1] The amyloid cascade hypothesis suggests that the transition of monomeric Aβ to fibrillized-Aβ plays an important role in the pathology of AD, but the exact nature of Aβ toxicity is controversial.[2] Much work has been done in characterizing the transition of monomer to fibril, and perhaps the mostly widely used monitoring tool is thioflavin T (ThT).[3] ThT is a benzothiazole dye (Figure 1) that has minimal fluorescence (excitation 440 nm/emission 480 nm) in aqueous solution or in the presence of monomeric Aβ; however, its fluorescence increases by several orders of magnitude in the presence of fibrils. ThT being an amyloid binding dye, its derivatives have found important applications as potential *in vivo* diagnostic agents.[4]

Dipyridophenazine ruthenium(II) complexes have been used in a wide variety of applications including DNA detection,[5,6] cell imaging,[7] DNA cleavage,[8] and photoinduced electron-transfer reactions,[9] among others. Recently, we reported that some of these complexes have the ability to disperse single-walled carbon nanotubes in aqueous solutions.[10] In this work, we report the light-switching properties of $[Ru(bpy)_2(dppz)]^{2+}$ (bpy = 2,2′-bipyridine; dppz = dipyrido[3,2-*a*:2′,3′-*c*]phenazine) when in contact with fibrillar Aβ. $[Ru(bpy)_2(dppz)]^{2+}$ displays large Stokes shifts (180 nm) and long photoluminescence (PL) lifetimes, making it an ideal probe in highly fluorescent environments, both *in vitro* and *ex vivo*. The PL properties of dipyridophenazine

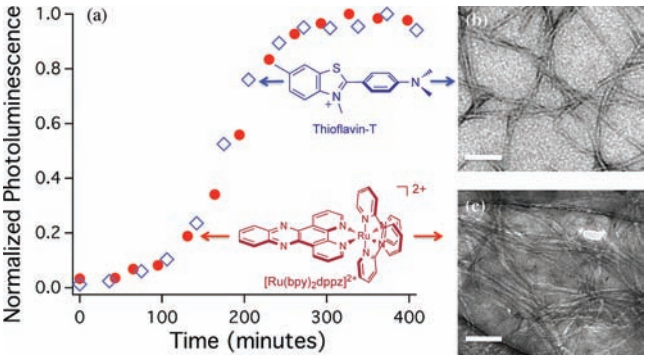

Figure 1. (a) Aβ fibrillization assay with ThT (blue ◇) and $[Ru(bpy)_2(dppz)]^{2+}$ (red ●). Right: TEM images of Aβ fibrils after 7 h incubation in the presence of (b) ThT and (c) $[Ru(bpy)_2(dppz)]^{2+}$. Scale bar = 100 nm.

Ru(II) and Ru(III) complexes have been characterized with some proteins, such as bovine serum albumin,[11,12] α-chymotrypsin,[13] and certain transcription factors,[14] but to the best of our knowledge, $[Ru(bpy)_2(dppz)]^{2+}$ light-switching properties have never been used to study peptide aggregation in real time.

The PL response of $[Ru(bpy)_2(dppz)]^{2+}$ upon protein aggregation is shown in Figure 1a. A typical sigmoidal behavior is observed for the PL of $[Ru(bpy)_2(dppz)]^{2+}$, in agreement with the fluorescence behavior of ThT. Generally, a lag phase is seen where Aβ monomers and soluble oligomers are present (throughout this paper we use the term "Aβ monomers" when referring to a freshly prepared non-aggregated Aβ solution which would contain a high concentration of monomeric Aβ but also some Aβ oligomers), and PL is not observed. After a few hours, protein aggregation starts, with the formation of small fibril aggregates which act as seeds for the further assembly of Aβ monomers into aggregated fibrillar structures. This aggregation and fibril elongation phase is relatively fast and followed by a leveling off of the PL once most of the monomers have been assembled into fibrils. The PL intensity of $[Ru(bpy)_2(dppz)]^{2+}$ increases up to 50-fold in the presence of Aβ fibrils. The formation of fibrils is confirmed by TEM images obtained from aliquots taken from the assays after 7 h (Figure 1b,c).

The fluorescent behavior of ThT is modulated by rotation between its benzothiazole moiety and dimethylaniline group.[15] In aqueous solution the rapid rotation between these two groups provides a non-radiative deactivation pathway rendering a

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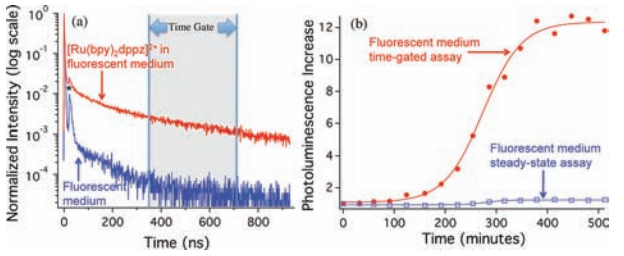

Figure 2. (a) Time-decay transients for rhodamine B fluorescent medium (blue line) and $[Ru(bpy)_2(dppz)]^{2+}$ in the presence of fibrillar Aβ in rhodamine B fluorescent medium (red line). Peak marked with asterisk at 21 ns is a detector artifact. (b) Aβ fibrillization assay with $[Ru(bpy)_2(dppz)]^{2+}$ in rhodamine B fluorescent medium using a steady-state fluorometer (blue line) and a time-resolved fluorometer using time-gating from 350 to 700 ns after light excitation (red line).

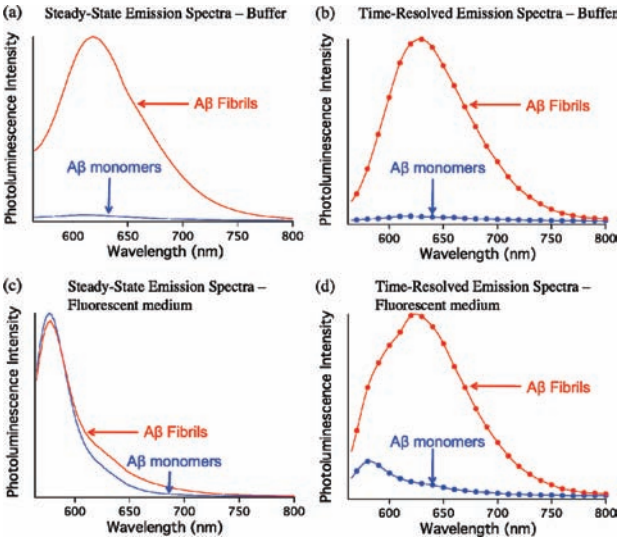

Figure 3. (a) Steady-state photoluminescence and (b) TRES of $[Ru(bpy)_2(dppz)]^{2+}$ in the presence of Aβ monomers before incubation (blue lines) and Aβ fibrils after incubation (red lines) in buffer solution. (c) Steady-state photoluminescence and (d) TRES of $[Ru(bpy)_2(dppz)]^{2+}$ in the presence of Aβ monomers before incubation (blue lines) and Aβ fibrils after incubation (red lines) in rhodamine B fluorescent medium. The spectra "before incubation" are at 0 min and "after incubation" are after 500 min of incubation.

non-fluorescent state; when bound to fibrillar Aβ (or any other confined media), the rigid environment precludes the rotation, resulting in an increase in fluorescence emission.[16] The light-switching properties of $[Ru(bpy)_2(dppz)]^{2+}$ can be attributed to a completely different pathway, which is likely related to its mechanism in DNA.[5] In aqueous solution, $[Ru(bpy)_2(dppz)]^{2+}$ does not display any PL due to the population of a low-lying dark state.[17] In the presence of double-stranded DNA, $[Ru(bpy)_2(dppz)]^{2+}$ interacts with the major groove, which produces a change in microenvironment that favors the population of a luminescent state. It is likely that the light-switching behavior observed in the presence of Aβ fibrils is related to that in the presence of DNA. Therefore, we propose that $[Ru(bpy)_2(dppz)]^{2+}$ interacts strongly with the fibril framework, which changes the polarity of the microenvironment felt by the dppz ligand, favoring the luminescent state. The absence of PL in the presence of monomeric Aβ is likely due to a poor interaction between $[Ru(bpy)_2(dppz)]^{2+}$ and the Aβ monomer.

Although $[Ru(bpy)_2(dppz)]^{2+}$ has shown remarkable light-switching properties in the presence of Aβ fibrils, it would be desirable to explore whether its long PL lifetime is capable of increasing the signal-to-background ratio in the presence of strongly fluorescent environments. This is particularly important in the screening of potential drugs for the inhibition of Aβ fibrils formation. Potential drugs with fluorescence from 460 to 550 nm, such as curcumin and quercetin, would interfere with ThT assays, making assays unreliable.[18] The long PL lifetime of $[Ru(bpy)_2(dppz)]^{2+}$ makes it possible to use time-gating technology to selectively detect its PL even in the presence of a strong fluorescent background.[19]

In order to demonstrate if $[Ru(bpy)_2(dppz)]^{2+}$ is capable of detecting Aβ fibrillization in the presence of a strongly fluorescent background, an Aβ assay was prepared in the presence of rhodamine B, a bright fluorophore with a fluorescence maximum at 575 nm and a tail that extends up to 700 nm. Figure 2a shows the PL time-resolved transient of $[Ru(bpy)_2(dppz)]^{2+}$ in the presence of Aβ fibrils and the time-resolved transients of the rhodamine B fluorescent background. The lifetime of rhodamine B in buffer solution is 1.6 ns, while $[Ru(bpy)_2(dppz)]^{2+}$ with Aβ fibrils can be fitted to a biexponential function with lifetimes of 21 (19%) and 221 ns (81%), which indicates that the average PL lifetime of $[Ru(bpy)_2(dppz)]^{2+}$ (185 ns) is about 2 orders of magnitude larger than that of rhodamine B. Therefore, using a time-gating technique, it is possible to discard the first few nanoseconds after the excitation, where most of the rhodamine B fluorescence appears, and retain only the part where the PL of $[Ru(bpy)_2(dppz)]^{2+}$ is dominant. Figure 2b shows the Aβ growing assay detected by steady-state fluorescence spectroscopy and by using time-resolved gating. It is evident from Figure 2b that time-gating increases the signal-to-background ratio from 1.4 (steady-state) to 12 (time-resolved), allowing a reliable assay even in the presence of a strongly fluorescent background. Furthermore, it is observed that Aβ fibrillization in rhodamine B fluorescent medium starts about 60 min later than in buffer solution (Figure 1a). This delay is likely due to interaction of rhodamine B with fibrils, retarding their aggregation. It is important to notice that this observation is made possible by the improved resolution offered by the time-gated assay, which takes advantage of the long lifetime of the $[Ru(bpy)_2(dppz)]^{2+}$ probe.

Steady-state PL and time-resolved emission spectra (TRES) of $[Ru(bpy)_2(dppz)]^{2+}$ before and after Aβ fibrillization further portray the advantages of time-gating in these kinds of assays (Figure 3). Figure 3a,b shows the changes in the steady-state PL spectra and TRES before and after Aβ fibrillization in buffer, while Figure 3c,d shows the spectra for the sample in the presence of rhodamine B fluorescent medium. It is noticeable that the steady-state PL in Figure 3c is dominated by rhodamine B and not by the ruthenium probe PL. However, after time-gating from 350 to 700 ns, the TRES of $[Ru(bpy)_2(dppz)]^{2+}$ in the presence of rhodamine B (Figure 3d) is almost indistinguishable from the TRES (Figure 3b) and steady-state PL spectra (Figure 3a) of $[Ru(bpy)_2(dppz)]^{2+}$ in buffer. This further confirms that long-lived photoluminescent probes can be combined with time-gating techniques to effectively remove strongly fluorescent background and increase the sensitivity of Aβ fibril detection in highly fluorescent media.

In summary, we have demonstrated the potential of $[Ru(bpy)_2(dppz)]^{2+}$ complexes as a probe for Aβ fibrillization.

$[Ru(bpy)_2(dppz)]^{2+}$ possesses interesting photophysical properties such as large Stokes shifts, long lifetimes, and light-switching properties toward Aβ fibrils. This probe, in combination with TRES, can be used to distinguish the longer lifetime of the metal complexes from an intense yet short-lived luminescent background, allowing monitoring the transition of monomeric Aβ to Aβ fibrils. The long lifetimes of metal complexes such as $[Ru(bpy)_2(dppz)]^{2+}$ provide a unique opportunity to examine fibril formation in environments that are normally unsuitable for shorter-lived organic dyes such as ThT. This is particularly important for the evaluation of possible drug candidates with strong fluorescence signals that would interfere with ThT assays. These probes could also be potentially used in combination with fluorescence lifetime imaging microscopy (FLIM),[20] a technique that is gaining recognition due to its ability to discriminate microenvironments on the basis of lifetime rather than wavelength. The success of this technique depends in part, on the development of novel probes capable of making use of the time-discrimination power of the system. Long-lived probes such as $[Ru(bpy)_2(dppz)]^{2+}$ are desirable, since their long PL lifetimes would allow for time-gating discrimination of short-lived fluorescent backgrounds and suppression of scattered light[21] and could be even used to determine cell viability.[22] Furthermore, changes in microenvironment would have a bolder effect on the lifetime of long-lived probes, which would increase FLIM contrast.

■ ASSOCIATED CONTENT

Supporting Information. Materials and methods, spectra of ThT and $[Ru(bpy)_2(dppz)]^{2+}$, and complete ref 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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