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A quenched binuclear ruthenium(II) dimer activated by another photosensitizer†

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A binuclear ruthenium(II) dimer (BiRD) bridged by an alkene linker was synthesized that was labile to reactive oxygen species generated by another photosensitizer. Compared to the monomeric Ru(II) complex, the BiRD had attenuated fluorescence and singlet oxygen production which could be restored by a longer-wavelength photosensitizer. This two-step amplification strategy demonstrates proof-of-principal for photosensitization chain-reactions.

Photosensitizers produce hyperstoichiometric quantities of reactive singlet oxygen upon irradiation with light of the appropriate wavelength. This suggests that they could be useful for sensitive detection applications. Indeed, the ultrasensitive luminescent oxygen channeling immunoassay is based on the use of nanoparticle-entrapped photosensitizers to generate singlet oxygen that activates analyte-tethered luminescent particles.[1] More recently, the past decade has seen the discovery and implementation of numerous activatable photosensitizers that increase their production of singlet oxygen in response to diverse chemical stimuli or biomolecule recognition.[2] While most of these have been intended for use as therapeutic photodynamic therapy agents, these could also have potential for use as *in vitro* detection agents. Unfortunately, the detection of singlet oxygen is challenging and photosensitizers are typically used at concentrations that are orders of magnitude higher than fluorophores.[3] Here, we develop a two-component system based on an activatable photosensitizer that itself is activated by reactive oxygen species (ROS) generated by another photosensitizer and demonstrate proof-of-principle for a chemical amplification system.

A Ru(II) complex was selected as the basis for the photosensitizer-activated photodynamic molecular beacon. Ru(II) complexes are stable, have high singlet oxygen quantum yields and importantly for this work have relatively blue-shifted absorption around 450 nm. This spectral absorbance range enables a second, red-shifted photosensitizer to be irradiated without directly exciting the shorter wavelength ruthenium complex. We synthesized bis(2,2′-bipyridine)-(5-aminophenanthroline) Ru(II) as the Ru(II) monomer (Fig. 1A) from *cis*-dichlorobis-(2,2′-bipyridine) Ru(II) and 1,10-phenanthrolin-5-amine (5phen) according to the literature.[4] The product was confirmed with mass spectrometry and NMR.

To generate a quenched photosensitizer that could be activated by reactive oxygen species, we created a self-quenched

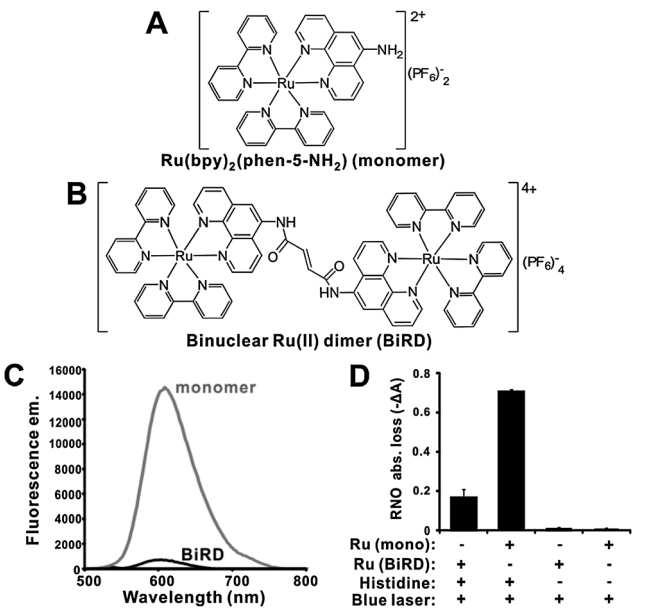

Fig. 1 Generation of a self-quenched binuclear Ru(II) dimer (BiRD). (A) Ru(II) monomer. (B) BiRD. (C) Emission spectra of absorbance-matched Ru(II) monomer and BiRD at 450 nm excitation (50 μM in D_2O). (D) $^1O_2$ generation in monomer and BiRD based on RNO beaching in D_2O. Concentrations were 100 μM RNO, 50 μM monomer, 50 μM BiRD and 25 mM histidine. RNO bleaching was assessed at 440 nm following 10 minutes of Ru(II) irradiation with a 450 nm, 300 mW laser in a 96 well plate. Mean ± std dev. for n = 3.

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dimer connected by an alkene bond, forming the binuclear ruthenium(II) dimer (BiRD) as shown in Fig. 1B. Alkene bonds are known to be labile to oxidative agents.[5] We generated the alkene-linked BiRD by first dimerizing 5phen using a fumaryl linker. The dimeric 5phen was then converted to the BiRD with *cis*-dichlorobis(2,2′-bipyridine) Ru(II) in a similar method as the monomer. The identity of the BiRD was confirmed with mass spectrometry and NMR. The absorption spectra of the monomer and BiRD are shown in Fig. S1 (ESI†).

Photosensitizer dimers are able to self-quench with respect to fluorescence and singlet oxygen generation when linked in close proximity.[6] The fluorescence of the absorbance-matched BiRD was attenuated approximately 20 fold compared to the monomeric Ru(II) complex, as shown in Fig. 1C. Generation of singlet oxygen was next assessed using the method of chemical bleaching of the *p*-nitrosodimethylaniline (RNO) chromophore.[7] In this assay, histidine is used as an intermediate which reacts with the singlet oxygen to generate a *trans*-annular peroxide which can react with other species including the RNO indicator. As shown in Fig. 1D, using absorbance-matched Ru(II) complexes, upon irradiation with a blue 450 nm excitation laser, singlet oxygen generation was significantly attenuated in the BiRD compared to the monomer. Histidine was required to achieve bleaching of the RNO. Thus, the BiRD displayed the expected properties of attenuated fluorescence and singlet oxygen generation.

The BiRD was next assessed for activation following exposure to photosensitization by a second photosensitizer. To avoid directly exciting the BiRD, a longer wavelength photosensitizer was selected, since photosensitizers can generally only be excited at wavelengths shorter than their emission. As shown in Fig. 2A, the commonly used photosensitizer methylene blue was used since it has a red-shifted absorption peak around 670 nm. In D_2O, methylene blue has a singlet oxygen quantum yield of 0.52,[8] whereas the Ru(II) monomer singlet oxygen quantum yield was determined to be 0.16 (see ESI†). However, at the methylene blue excitation wavelength, the BiRD exhibited negligible excitation. The spectral location of the blue laser used to excite the BiRD and the red laser used to excite methylene blue were separated by over 200 nm. As shown in Fig. 2B, when methylene blue and the BiRD were incubated together and exposed to the red laser, an 8 fold restoration in the BiRD fluorescence was achieved. Without the presence of methylene blue, direct irradiation of the BiRD with the red laser had no effect on BiRD activation. As shown in Fig. 2C, both histidine and methylene blue were required to achieve BiRD activation. Histidine likely plays the same role as in RNO bleaching in trapping the singlet oxygen in a reactive *trans*-annular peroxide. A photocleavage product from activation of BiRD could be directly observed using HPLC (Fig. S2, ESI†). The smaller mass of the emerging peak was consistent with the assumption that the BiRD is cleaved at the alkene bond. However, further work is required to accurately elucidate the cleavage mechanism. But clearly the BiRD could selectively be activated by a longer wavelength photosensitizer. The BiRD demonstrated activation by sub-nanomolar levels of methylene blue (Fig. S3, ESI†).

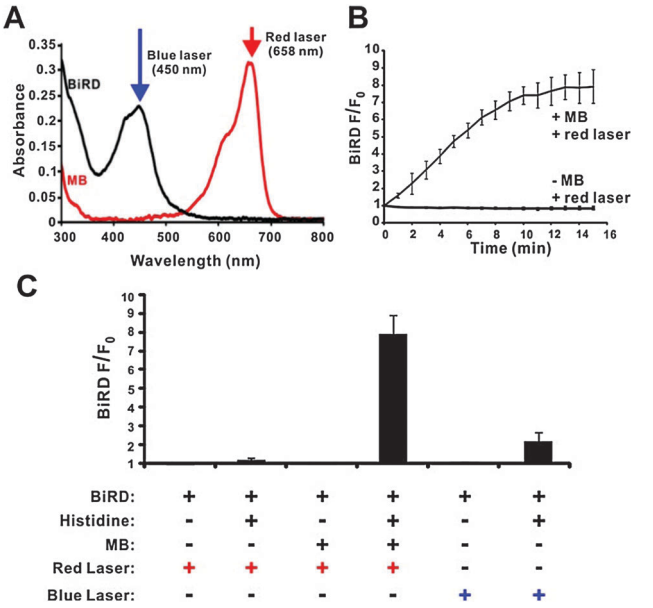

Fig. 2 BiRD activation *via* irradiation of another photosensitizer. (A) Absorption spectra of the BiRD and methylene blue. The laser excitation wavelengths corresponding to the excitation of the two dyes are indicated. (B) Fluorescence activation kinetics (assessed with 450 nm excitation and 600 nm emission) of the BiRD (50 μM) by methylene blue (20 μM) using irradiation with a red laser (300 mW) in D_2O. (C) Control experiments of BiRD activation by methylene blue in D_2O. Concentrations used were 50 μM BiRD, 1 mM histidine and 20 μM MB. Both laser fluences were 810 J cm^{-2} from lasers outputting 300 mW and irradiating the samples for 15 minutes. Mean ± std dev. for n = 3.

Next, the BiRD was used to develop a chemical amplification scheme. As shown in Fig. 3, conventional detection of photosensitizers using a chemical bleaching assay involves the single step of irradiating the photosensitizer in the presence of an indicator such as RNO (route I). By using a BiRD activation intermediate step as shown in route II, a chemical amplification system is created. The efficacy of the system is based on the fact that the initial photosensitization step in IIa need not bleach an indicator dye that itself need be detected, but rather only must activate the BiRD photosensitizer. The BiRD photosensitizer, is then irradiated to initiate a second step of chemical bleaching of an indicator dye (route IIb). 10 nM methylene blue was not a sufficient concentration to directly induce significant bleaching of the RNO indicator upon irradiation with a red laser (route I). This was not surprising, since photosensitizers are usually assessed at concentrations that are orders of magnitude higher (in the micromolar range). However, under the same irradiation conditions, 10 nM methylene blue could effectively activate the BiRD, as indicated by the increase in the BiRD fluorescence. The activated BiRD was then irradiated with a blue laser and resulted in the effective bleaching of the RNO indicator. Thus, using an intermediate chemical amplification effect with a ROS-sensitive photosensitizer offered improvement over conventional methodology.

In summary, we have developed a BiRD that is, to our knowledge, the first photosensitizer reported that itself is activated

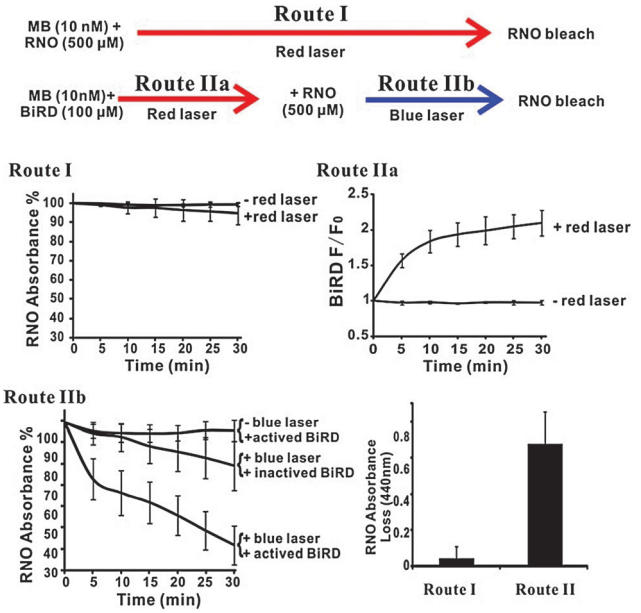

Fig. 3 A chemical amplification system based on BiRD activation. Nanomolar concentrations of methylene blue were used to either directly bleach RNO (route I) or to activate the intermediate BiRD photosensitizer, which then bleached the RNO (route II). 10 nM methylene blue and 0.5 M histidine in D_2O was present in all experiments. For route I and IIa, a 300 mW red laser was used and for route IIb, a 300 mW blue laser was used. Fluence was 1620 J cm^{-2}. Mean ± std dev. for n = 3.

by a second photosensitizer. By using a longer wavelength photosensitizer such as methylene blue, the BiRD could be activated by the second photosensitizer without any undesired activation by the longer wavelength laser. Fluorescence and singlet oxygen generation were restored following BiRD activation. Finally, proof-of-principle was demonstrated for a singlet oxygen chain reaction that made use of the BiRD as an intermediate photosensitizer. The use of chain-reaction photosensitization may be useful for analytical applications harnessing their hyperstoichiometric generation of singlet oxygen.

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