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Chemical Molecule-Induced Light-Activated System for Anticancer and Antifungal Activities

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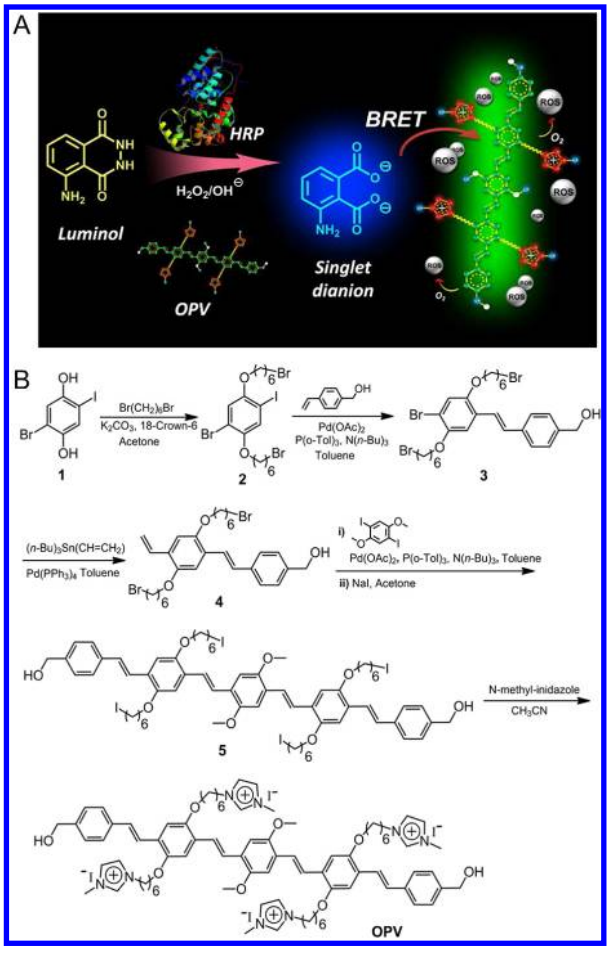
Supporting Information

ABSTRACT: Except for chemotherapy, surgery, and radiotherapy, photodynamic therapy (PDT) as new therapy modality is already in wide clinic use for the treatment of various diseases. The major bottleneck of this technique is the requirement of outer light source, which always limits effective application of PDT to the lesions in deeper tissue. Here, we first report a new modality for treating cancer and microbial infections, which is activated by chemical molecules instead of outer light irradiation. In this system, in situ bioluminescence of luminol can be absorbed by a cationic oligo(*p*-phenylene vinylene) (**OPV**) that acts as the photosensitizer through bioluminescence resonance energy transfer (BRET) process. The excited **OPV** sensitizes oxygen molecule in the surroundings to produce reactive oxygen species (ROS) that kill the adjacent cancer cells in vitro and in vivo, and pathogenic microbes. By avoiding the use of light irradiation, this work opens a new therapy modality to tumor and pathogen infections.

Since first used in clinical trials in the 1980s,[1] photodynamic therapy (PDT) has led to new therapy modalities apart from chemotherapy, surgery, and radiotherapy. PDT is already widely used in clinic for the treatment of a variety of tumors and ophthalmology and dermatology-related diseases.[2,3] The photosensitizer, light, and molecular oxygen are the primary three components in PDT to produce reactive oxygen species (ROS) that are toxic and kill cells. The principal advantage of PDT is its spatial selectivity for diseases treatment as the photosensitizer is activated only under light excitation with selected wavelength.[4,5] However, the requirement of outer light source always limits effective application of PDT to the lesions in deeper tissue since the light does not penetrate far through due to the absorption and scattering by biological tissues.[6] Although infrared light can reduce this shortcoming, unfortunately the development of photosensitizers with highly efficient absorption of infrared light (such as two-photon photosensitizers) has proved a challenge.[7] Thus, the development of novel PDT system that is independent of light irradiation can provide a new therapy modality for various diseases.

Here, we report a novel PDT system in which the photosensitizer is activated by chemical molecules instead of light source (Scheme 1A). To demonstrate the concept, luminol, hydrogen peroxide, and horseradish peroxidase (HRP) were used as bioluminescent molecules and a cationic oligo (*p*-phenylene vinylene) (**OPV**) was used as the photosensitizer (see its chemical structure and synthesis in Scheme 1B). Luminol emits a striking blue bioluminescence at the maximum of 425 nm in the presence of oxidizing agent (such as hydrogen peroxide) and HRP.[8,9] **OPV** exhibits a broad absorption (350–550 nm) and a maximal emission at 550 nm in water. They

Scheme 1. (A) Schematic Illustration of the BRET System for PDT; (B) Synthesis of Cationic OPV as Photosensitizer



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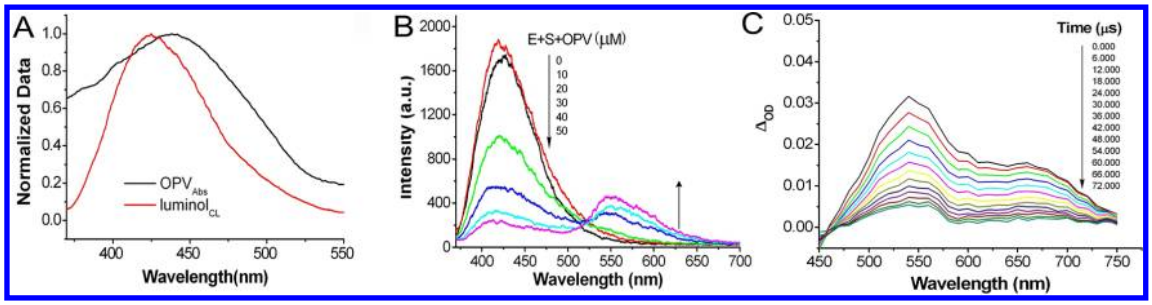

Figure 1. (A) Normalized absorption of **OPV** and luminescence spectra of luminol. (B) Luminescence spectra of luminol in the presence of various concentrations of **OPV**. [OPV] = 0−50 μM, [HRP] = 0.003 mg/mL, [luminol] = 0.2 mM, [4-iodophenol] = 0.5 mM, [H_2O_2] = 0.5 mM. (C) Transient absorption spectra of **OPV** in argon-saturated ethanol. Delay time: 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72 μs.

meet the spectral overlap requirement for bioluminescence resonance energy transfer (BRET) as the donor−acceptor pair (Figure 1A).[10,11] Thus, the bioluminescence from luminol is absorbed by **OPV** to make BRET take place due to the electrostatic interactions between the negatively charged luminol oxidation product (dianion) and cationic **OPV**, and then the excited **OPV** sensitizes oxygen molecules in the surroundings to produce ROS. It is noted that the HRP did not bind to cells (Figure S1). The cationic **OPV** could bind to the negatively charged cells and fungi and subsequently kill the cancer cells and pathogenic microbes. To the best of our knowledge, this is the first reported PDT system based on BRET without requirement for outer light source, although the BRET systems have been explored for in vivo imaging and biological detections.[12−15]

To prove the BRET process, the bioluminescence of luminol was measured in the presence of **OPV** with varying concentrations. As shown in Figure 1B, the addition of **OPV** leads to a dramatic decrease of the luminescence intensity at 425 nm (corresponding to luminol); at the same time, the luminescence intensity at 550 nm (corresponding to **OPV**) gradually increases with increasing **OPV** concentration (0−50 μM). This result confirms the occurrence of the BRET. Because of the important role of the long-lived triplet state in the generation of toxic ROS, we investigated the transient absorption spectra of **OPV** to demonstrate the presence of triplet excited state. As shown in Figure 1C, direct excitation of **OPV** in argon-saturated ethanol can generate a long-lived transient absorption characterized by two bands with absorption maxima at around 540 and 660 nm, which correspond to the triplet−triplet absorption and the radical cation of **OPV**, respectively. The triplet lifetime from the decayed transient absorption was determined to be 44 μs. 2,7-dichlorofluorescein diacetate (DCFH-DA) was utilized to further verify the production of ROS.[16] Conversion of DCFH-DA into 2,7-dichlorofluorescin (DCFH) was followed by transformation into highly fluorescent 2,7-dichlorofluorescein (DCF, quantum yield: 90%) in the presence of ROS. As shown in Figure S2, upon irradiating DCFH in the presence of **OPV** under white light (400−800 nm), an apparent emission at 525 nm (characteristic of DCF) was detected (excitation: 488 nm) and the fluorescence intensity was much higher than that of the control group without **OPV**. It is noted that **OPV** itself can not be excited at 488 nm. These results demonstrate the significant generation of ROS originated from **OPV**.

To directly visualize the binding of cationic **OPV** to live cells, confocal laser scanning microscopy (CLSM) was utilized. As shown in Figure 2A, the **OPV** only binds to the membrane of HeLa cell after incubating for 30 min. Zeta potentials (ζ) were

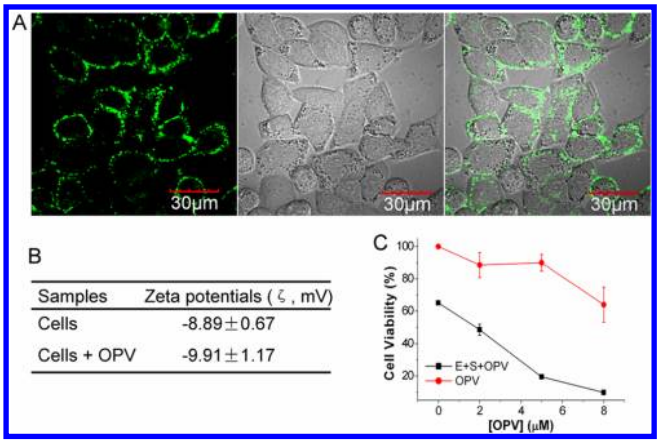

Figure 2. (A) CLSM images of HeLa cells incubated with **OPV**. The fluorescence of **OPV** is highlighted in green. Left, fluorescence image; middle, phase contrast bright-field image; right, overlap image. (B) ζ-potentials of HeLa cells before and after incubating with **OPV**. Measurements were performed in 10 mM PBS solution at 25 °C. (C) Cell viability of HeLa cells after incubation with **OPV** in the absence and presence of luminol luminescence system (E+S). Values are expressed as means ± SD (n = 3, P < 0.01). [**OPV**] = 0−8 μM, [HRP] = 0.01 mg/mL, [luminol] = 0.5 mM, [4-iodophenol] = 1.25 mM, [H_2O_2] = 0.4 mM.

attained to gain more insights into the interactions of **OPV** with live cells. No obvious change of ζ-potentials was observed for the HeLa cells before and after adding **OPV** (Figure 2B), which indicates that the **OPV** might insert into the lipid bilayer of the cells because of its high charge density and comparable linear length.[17] On the basis of the affinity of cationic **OPV** toward cells, viability analysis of **OPV** to HeLa cells was determined using a standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium hydrobromide) assay with and without luminol luminescence system (HRP, 4-iodophenol, luminol and hydrogen peroxide, namely, E+S). As shown in Figure 2C, less cytotoxicity was observed when the **OPV** concentration is less than 8 μM without luminol luminescence system. While in the presence of the luminol luminescence system, **OPV** displays more prominent cytotoxicity. We also examined the decrease of HeLa cell viability with increasing **OPV** concentration and less than 10% viability was obtained. These results show that the cytotoxity of **OPV** in the presence of luminol luminescence system is more effective toward HeLa cells than the case without luminol luminescence system thereby indicating the anticancer mechanism by BRET. It is noted that the luminol luminescence system (E+S) itself could kill approximately 30% cells due to the toxicity of H_2O_2. Following the cancer cell

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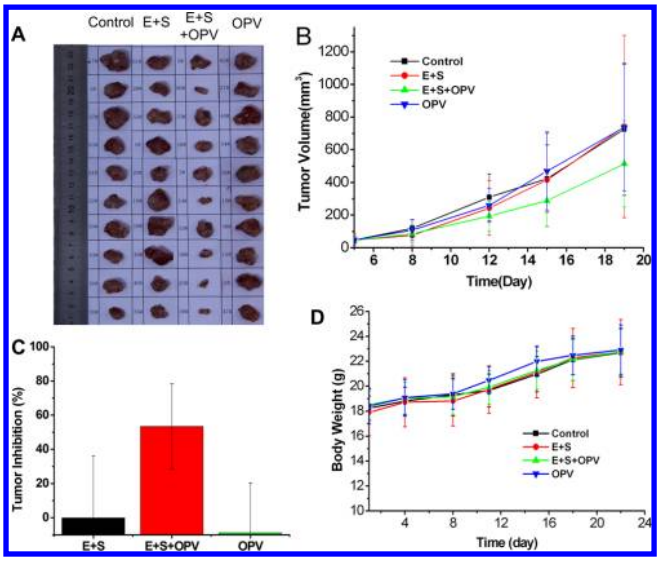

Figure 3. (A) Representative photo of tumors collected from the tumor-bearing mice after 18 days treatments with new BRET system. (B) Tumor volume as a function of treatment time. Values are expressed as means ± SD The value of $P < 0.05$ of saline treatment vs treatment with the luminol luminescence system (E+S) and **OPV** was considered statistically significant. (C) Tumor inhibition of mice post 18 days treatment, 10 mice used per group. Values are expressed as means ± SD ($n = 10$, $P < 0.001$). (D) Body weight curves after various treatments. Values are expressed as means ± SD ($n = 10$, $P > 0.1$, no significant difference) [OPV] = 10 μM, [HRP] = 0.02 mg/mL, [luminol] = 2 mM, [PC_3SO_3Na] = 0.03 mg/mL, [H_2O_2] = 0.5 mM.

viability analysis, we investigated the toxicity of BRET system to normal human epithelial cells, including human kidney epithelial cells (HKC) and immortalized human epidermal cells (HaCaT). The MTT assay results (Figure S3) indicated that the BRET system was also cytotoxic to normal human epithelial cells, which means the BRET system has little specificity due to a lack of recognition groups to the cancer cells.

After verification by in vitro experiment, we then evaluated the antitumor therapeutic efficacy of the novel BRET system in HeLa cell tumor-bearing nude mice using intratumoral injection mode. Ten mice were used for each group, including the negative controls in the absence of **OPV** and luminol luminescence system. Tumor growth was monitored with digital caliper after treatment. As shown in Figure S3A, neither luminol luminescence system injection by itself (E+S: HRP, luminol, luminol signal enhancer and H_2O_2) nor **OPV** alone was able to inhibit the tumor growth, whiles the group injected with BRET system (E+S+**OPV**) shows a slower tumor growth in comparison to control group. As seen in Figure S3B, about 30% of tumor inhibition ratio was shown by the BRET treatment group (E+S+**OPV**) in comparison to luminol luminescence system (E+S) group, and the **OPV** group did not demonstrate any tumor inhibition. These results indicate that our new BRET system could inhibit the tumor growth in vivo. Furthermore, our BRET-based PDT treatment does not show toxic side effect as the body weight growth was not inhibited for mice in comparison to control group (Figure S4C), which indicates there is no apparent toxicity to the normal mice tissue though the BRET system has little specificity. It is reported that enhanced chemiluminescent reactions provide more intense, prolonged and stable light emission. Here the sodium 3-(10′-phenothiaziny1)propane-1-sulfonat (PC_3SO_3Na) is used as luminol signal enhancer instead of 4-iodophenol to improve the antitumor effect of our BRET system.[18,19] As shown in Figure S5, the addition of PC_3SO_3Na leads to a dramatic prolonged BRET signal up to 2 h. As shown in Figure 3A,B, the BRET system (E+S+**OPV**) shows a slower tumor growth in comparison to control group. As expected, the addition of luminol signal enhancer, PC_3SO_3Na, improves the antitumor activity of the BRET system and a 55% of tumor inhibition ratio was observed (Figure 3C). The addition of PC_3SO_3Na does not show toxic side effect as the body weight growth was not inhibited for mice in comparison to control group (Figure 3D). Athough the distribution in vivo of **OPV** was not investigated, nanoparticles prepared by other polymers have been observed mainly in the targeted region and the liver in vivo using intravenous injection mode.[20,21]

To show that the BRET system works with other kinds of pathogenic cells, we also investigated its antimicrobial activity toward *Candida albicans*, a kind of pathogenic fungi. Fungi have been recognized as major pathogen associated with chronic and ultimately fatal infections in critically ill patients, and they are more resistant to antifungal agents than bacteria.[22] Colony counting shows that in a low **OPV** concentration (1.0 μM), the killing efficiency reaches 98% in the presence of the BRET system (E+S+**OPV**). The control experiments exhibit that **OPV** alone only kills ~10% of the fungi, and the luminol luminescence system (E+S) did not inhibit the growth of *C. albicans* at all (Figure 4A,B). That is, our BRET system becomes much more potent at killing fungi, which results from the ROS generated by the BRET from luminol luminescence to **OPV**. To further evidence the high efficiency of the BRET

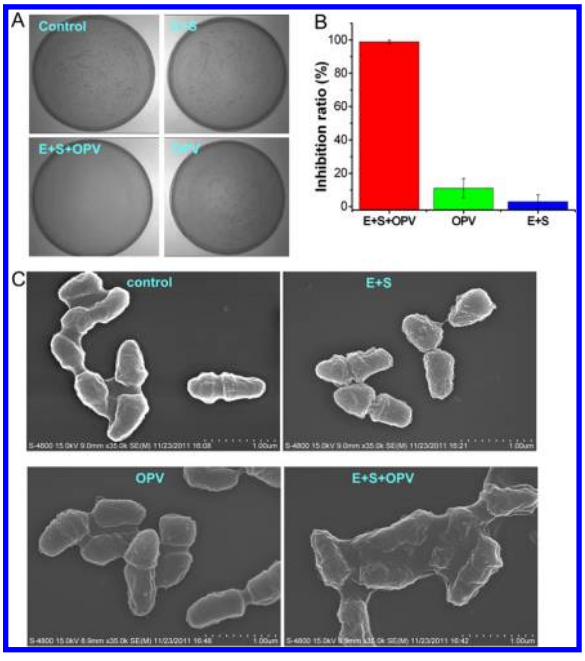

Figure 4. (A) Plate photographs for *C. albicans* on YTD agar plate treated with **OPV** in the absence and presence of the luminol luminescence system. (B) Biocidal activity of **OPV** toward *C. albicans* in the absence and presence of the luminol luminescence system (E+S). Values are expressed as means ± SD ($n = 3$, $P < 0.01$). [**OPV**] = 1.0 μM, [HRP] = 0.004 mg/mL, [luminol] = 0.2 mM, [4-iodophenol] = 0.5 mM, [H_2O_2] = 0.5 mM. (C) Morphology of *C. albicans* treated with **OPV** in the absence and presence of the luminol luminescence system.

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system for killing *C. albicans*, field-emission SEM was employed to observe the morphological changes of *C. albicans*. As shown in Figure 4C, clear edges and the surface integrity of *C. albicans* were observed. Control experiments exhibit that both luminol luminescence system (E+S) and **OPV** alone show less effect on the morphology of *C. albicans* membrane. Upon treatment with the BRET system, collapsed, split, and merged membranes were visualized. The direct visualization from SEM images is consistent with the results in antifungal experiments. The ζ-potentials of *C. albicans* were also measured to investigate the interaction between the fungi cell membrane and **OPV** (Table S1). However, no obvious change of ζ-potentials was observed for the *C. albicans* before and after adding luminol luminescence system (E+S), **OPV** and BRET system (E+S +OPV), which indicates that the **OPV** insert into the lipid bilayer of *C. albicans* membrane.[17]

In conclusion, a novel PDT system was developed in which the photosensitizer is activated by chemical molecules instead of outer light source. In this system, luminol, hydrogen peroxide, and HRP were used as bioluminescent molecules and a cationic oligo (*p*-phenylene vinylene) (**OPV**) was used as the photosensitizer. They meet the spectral overlap requirement for BRET as the donor−acceptor pair. The excited **OPV** by BRET from luminol sensitizes oxygen molecules in the surroundings to produce ROS that kill the adjacent cancer cells and pathogenic microbes. The BRET system can work in vivo even in the deeper tissue, which overcomes the drawback of the deep tissue penetration for PDT with light irradiation. To the best of our knowledge, this is the first reported PDT system for treating cancer and microbial infections that is independent of light irradiation. This work opens a new therapy modality to tumor and pathogen infections. We note that this strategy also has potential application in light irradiation-dependent regulation of signaling pathways and optogenetic control of biological events in vivo.[23] Future work will entail modification of the BRET system with recognition groups specifically binding to cancer cells to improve the specificity of this system.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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