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FULL PAPER

Light-triggered Nitric Oxide Release Photosensitizer to Combat Bacterial Biofilm Infections

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Abstract: Bacterial biofilms are a serious global health concern, often responsible for persistent infections. New strategies to prevent and treat bacterial infections via eradication of the biofilms are urgently needed. A novel ruthenium-based compound was reported in this study that functions as both a boronic acid-decorated photosensitizer (PS) and a light-triggered nitric oxide (NO) releasing agent. The compound could selectively attach to the bacterial membrane and biofilms. And it is highly potent to eradicate *P. aeruginosa* biofilms via the simultaneous release of NO as well as reactive oxygen species (ROS), which is more effective than clinical antibiotics tobramycin. The compound also has excellent bacterial specificity and shows no significant cytotoxicity to human cells. The results reveal potential applications of this innovative dual-functional photoactivated ruthenium compound to combat bacterial biofilm infections.

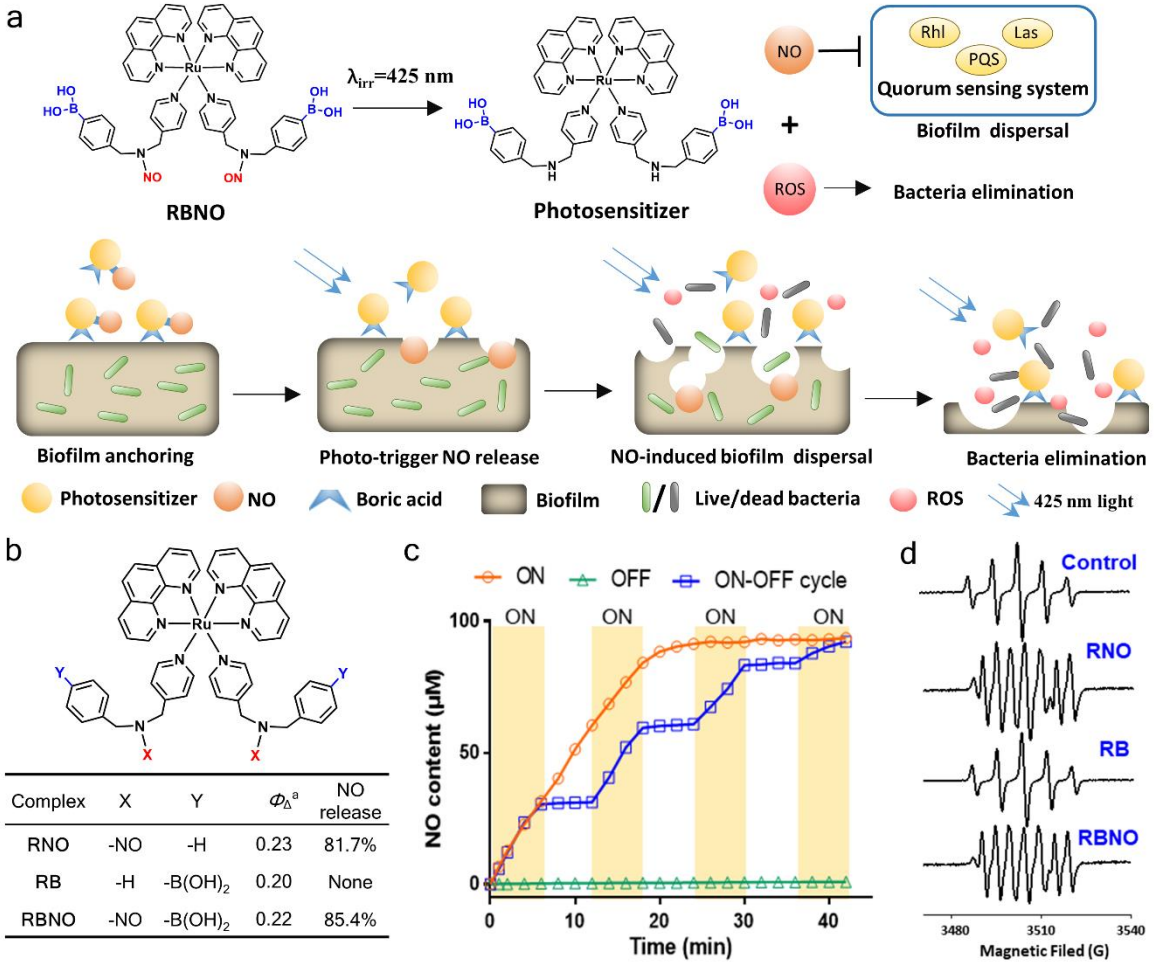
Introduction

Antimicrobial resistance (AMR) has become on the greatest threats to gloabl health.[1] One important cause of AMR and bacterial persistent infection is the capability of bacteria to form biofilm. It is reported that up to 80% of human bacterial infections are biofilm associated.[2] Biofilms are composed of bacteria that are encased in self-generated extracellular polymeric substances (EPS), including polysaccharides, proteins, extracellular DNA, lipids as well as bacterial decomposition substances.[3-4] The thick EPS matrix provides protection to the biofilm bacteria against high dose of antibiotics. Therefore, bacteria in biofilms can be up to 1000 times more resistant to antibiotics than their planktonic counterparts.[5] Opportunistic human pathogen *Pseudomonas aeruginosa* is particularly notorious for its ability to form biofilms. The bacteria could form biofilm on endotracheal tube and associated with the ventilator-associated pneumonia (VAP), which causes a significant higher mortality rate for patients in intensive care unit (ICU).[6] Due to the novel coronavirus (2019-nCoV) outbreak, it is estimated that there will be an increasing rate of VAP in ICU patients. Therefore, the development of new anti-biofilm agents for the treatment of biofilm infections remains a priority, especially during the coronavirus disease pandemic.

Photodynamic therapy (PDT) is a spatiotemporally precise, controllable and non-invasive method that is clinically approved for the treatment of cancer.[7-8] In PDT, light and photosensitizer (PS) is used in conjugation with oxygen molecule to produce short-lived and highly toxic reactive oxygen species (ROS), which subsequently elicit cell death.[9-10] The strategy has also been applied for bacterial infection treatment. However, a much higher concentration of PS are required to eradicate mature biofilms than planktonic bacteria, which usually leads to side effect due to the cytotoxicity of PS with high concentration.[11-12] Recent studies demonstrate that exogenous addition of nitric oxide (NO) stimulates *P. aeruginosa* motility and biofilms dispersal by lowing c-di-GMP levels.[13-15] Similar biofilms dispersal by NO are also observed for *Staphylococcus aureus* and various single and multispecies biofilms.[16-17] It is indicated that NO is a potential biofilm dispersal agent. However, relatively short half-lives of current NO donor compounds and difficulties in targeted delivery of NO to biofilm have hindered their practical clinical use.[18-19]

We envision that the combination of PDT and NO anti-biofilm properties will be a potential novel strategy to combat bacterial biofilm infection. NO could stimulate bacterial motility and liberate planktonic bacteria from biofilms. The free-floating bacteria are subsequently eradicated by elevated ROS generated by PS (Figure 1a). With this strategy, the PS concetration could be dramatically reduced to avoid side effects. Therefore, we synthesized a novel boronic acid-decorated Ru(II)-based photosensitizer with light-responsive NO release property. The boronic acid enables the compound to selectively anchor to *P. aeruginosa* biofilms, which contain a high proportion of extracellular polysaccharides.[20]

Photoirradiation triggers the stimultaneous release of NO and ROS from the compound to effectively eradicate the matured biofilm. Importantly, this novel compound could eliminate 90% of *P. aeruginosa* biofilms with a low concentration of 20 μM and exhibits no toxicity to mammalian cells. The proof-of-concept studies here demonstrate the potential of NO-releasing Ru(II) photosensitizer as a new type of antibiofilm agent for therapeutic use.



Complex	X	Y	Φ_Δ[a]	NO release
RNO	-NO	-H	0.23	81.7%
RB	-H	-B(OH)$_2$	0.20	None
RBNO	-NO	-B(OH)$_2$	0.22	85.4%

Figure 1. Light-triggered NO release from Ru(II) compounds. (a) The diagram shows **RBNO** with NO releasing property functions as a photosensitizer to eradicate *P. aeruginosa* PAO1 biofilms. (b) Chemical structure and photochemical properties of synthesized Ru(II) compounds. [a] $[Ru(bpy)_3]Cl_2$ (Φ_Δ = 0.18) in aqueous solution is used as a standard reference. (c) NO releasing profile of **RBNO** (50 μM) under continuous (orange) and pulsed (blue) photoirradiation measured with Griess reagent. (d) EPR spectra of PTIO (40 μM) and different Ru(II) compounds (20 μM) in PBS with 425 nm light (20 mW cm^{-2}) irradiation for 20 min.

Results and Discussion

Molecular Design and Synthesis

The boronic acid-decorated Ru(II) compound with NO-releasable group **RBNO** (Figure 1b) and two appropriate control compounds **RNO** (without boronic acid group) and **RB** (without N-nitrosamine group) were synthesized as described in the supporting information. The functionalization of boronic acid group could faciliate biofilm-targeted binding of **RBNO**. Upon photoirradiation, NO would be readily released from **RBNO** in excited state through cleavage of N-nitrosamine bond (N-NO) as previously described.[21-23] Meanwhile, The resultant PS **RB** could generate $^1O_2$ via type II photodynamic process to eliminate bacteria (Figure 1a). The chemical structures of these compounds were characterized by the combination of ^{1}H NMR, ^{13}C NMR, electrospray ionization mass spectroscopy (ESI-MS) and elemental analysis. The electronic absorption spectra of the Ru(II) compounds were measured in PBS buffer. These complexes display typical metal-to-ligand charge-transfer (MLCT) absorption bands at 350~550 nm (Figure S1). The single oxygen quantum yields of Ru(II) compounds (Φ_Δ) upon excitation at 425 nm were determined using 9,10-anthracenediyl-bis(methylene)dimalonic acid in aqueous solution (Figure S2).[24] The relative Φ_Δ values of **RNO**, **RB** and **RBNO** were calculated to be 0.23, 0.20 and 0.22, respectively (Figure 1b).

Detection of controllable NO release

We then investigated whether photoirradiation could trigger NO release from the Ru(II) compounds. The released NO from Ru(II) compounds was measured using Griess reagent as described previously.[25] After continuous blue light (425 nm, 20 mW·cm^{-2}) irradiation for 20 min, NO could be efficiently released from **RBNO** (85.4%) and **RNO** (81.7%) (Figure 1b). We also investigated the kinetics of NO release from **RBNO** under continuous and pulsed (360 s/360 s on/off cycle) photoirradiation. As shown in Figure 1c, NO release from **RBNO** is fast and could finish within 20 min under continuous light irradiation (425 nm, 20

mW·cm^{-2}). Importantly, NO release is strictly coupled to photoirradiation. The released NO from Ru(II) compounds were also detected by electron paramagnetic resonance (EPR) using 2-phenyl-4,4,5,5- tetramethylimidazoline -1-oxyl-3-oxide (PTIO) as a radical scavenger. As shown in Figure 1d, the EPR spectrum of PTIO consists of typical five lines with intensities of 1:2:3:2:1. After photoirradiation, a distinctive change was observed in the EPR spectrum of PTIO mixed with **RBNO** and **RNO**, indicating the reaction of released NO with PTIO.[26] In contrast, no significant spectrum change was observed in the presence of **RB**. All these data suggested **RBNO** is dual-functional Ru(II) compound as a light-responsive NO donor as well as a photosensitizer.

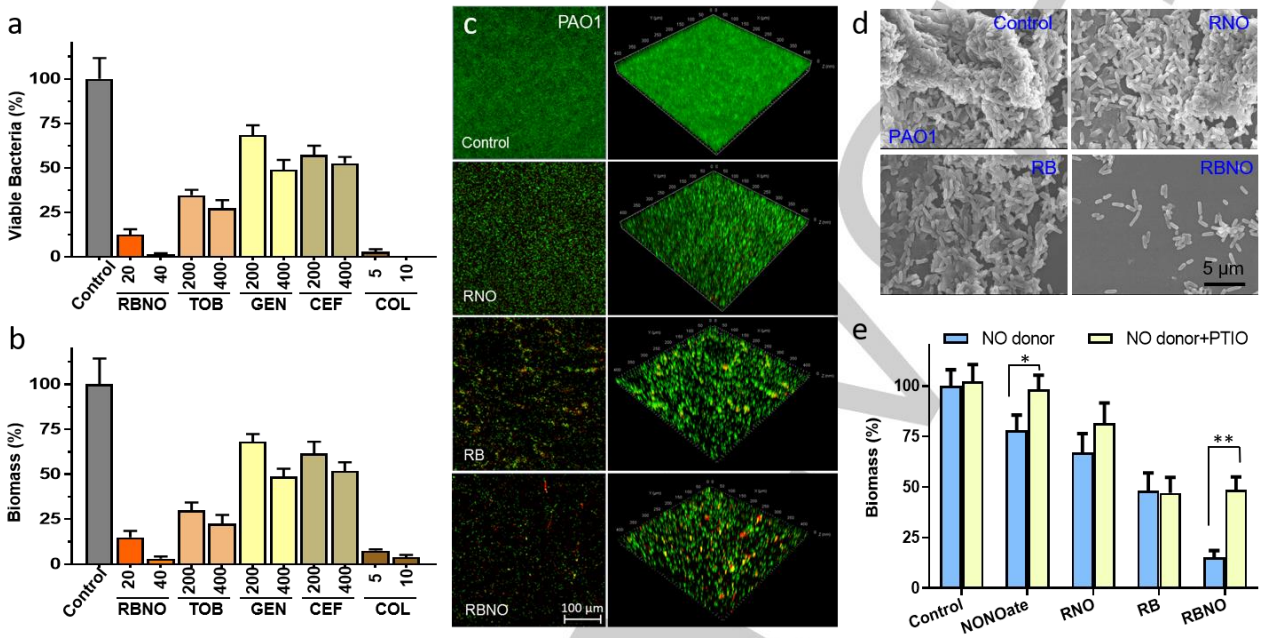


Figure 2. *P. aeruginosa* biofilms eradication activities of Ru(II) compounds. Bacterial viability (a) and biomass (b) of *P. aeruginosa* biofilms treated with various concentrations of tobramycin (TOB), gentamicin (GEN) ceftazidime (CEF), colistin (COL) and **RBNO** compounds. The biofilms and antibiotics were incubated in dark for 2 h. Biofilms incubated with **RBNO** were treated with photoirradiation for 20 min (425 nm, 20 mW·cm^{-2}). (c) Confocal laser scanning microscopy (CLSM) images of *P. aeruginosa* PAO1 biofilms after Ru(II) compounds treatment with photoirradiation. SYTO-9 is used to stain both live and dead bacterial cells (green) in biofilms. Propidium iodide stains dead cells (red). The merged images of green and red channels (left) and corresponding reconstructed 3D image (right) of bacterial biofilms were shown. (d) Representative scanning electron microscope (SEM) images of *P. aeruginosa* PAO1 biofilms after Ru(II) compounds treatment with photoirradiation. (e) The eradication of *P. aeruginosa* PAO1 biofilms treated with NONOate and Ru(II) compounds with or without 0.5 mM NO scavenger PTIO. The **RNO**, **RB** and **RBNO** treated groups were irradiated with photoirradiation (425 nm, 20 mW·cm^{-2}) for 20 min. All experiments are performed in triplicated and data are presented as mean ± sd. * means p< 0.05, ** means p< 0.01

The products of Ru compounds after photoirradiation were also analyzed (Figure. S3). In PBS solution, a slight decreasing of absorption band was observed for **RBNO** in the dark, which might due to partial dissociation of the monodentate ligand. When **RBNO** (30 μM) was exposed to the blue light (425 nm, 20 mW·cm^{-2}) for 20 min, UV-vis spectra indicated an significant decreasing of absorption band at ~300 nm and MLCT absorption bands between 400~500 nm (Figure S3f). Similar phenomena were also found for other two Ru compounds, **RB** and **RNO**. Liquid chromatography coupled to mass spectrometry (LC-MS) was employed to characterize the photoirradiation products (Figure S4). Before photoirradiation, there is only one peak (peak A) in the liquid chromatography with a retention time of 6.7 min, corresponding to the starting material **RBNO**. After 20 min photoirradiation, a dramatic decrease of the **RBNO** peak intensity in liquid chromatography was observed, indicating photodegradation of **RBNO**. Correspondingly, there are four new peaks appeared at 7.7 min (peak B), 10.4 min (peak C), 5.2 min (peak D) and 4.3 min (peak E). The peak B was assigned to **RB** $[Ru(phen)_2(L2)_2]^{2+}$, which is the product of **RBNO** after NO released. In the meantime, the dissociated ligand L2 (peaks C) was detected indicating the decomposion of Ru complex. The other two peak D and E have been assigned to mono-aqua product $[Ru(phen)_2(L2)(CH_3CN)]^{2+}$ (peak D) and di-aqua product $[Ru(phen)_2(CH_3CN)_2]^{2+}$, respectively. These results demonstrated that photoirradiation could lead to N-NO bond cleavage, which enabled NO release from **RBNO**.

Selective binding capacity toward bacteria and biofilm

Previous studies demonstrated that positively charged metal compounds could preferably absorb to the negatively charged outer membrane of Gram-negative bacteria via electrostatic interaction.[27] Meanwhile, decorated boronic acid group could facilitate Ru(II) compounds adhesion to bacterial peptidoglycan or lipopolysaccharide via boron-polyol based boronolectin chemistry.[28] Therefore, we postulated that the Ru(II) compounds decorated with boronic acid would preferably bind to

bacteria rather than mammalian cell. To test the hypothesis, the binding of Ru compounds to *P. aeruginosa* PAO1 strain and human fetal lung fibroblasts WI-38 cell line were monitored by measuring the zeta potential values of cell membrane (Table S1).[29] After incubation with **RBNO** and **RB**, the zeta potential value of *P. aeruginosa* PAO1 dramatically increased from -23.55 ± 1.88 mV to -18.75 ± 2.61 mV (**RBNO**) and -17.60 ± 2.48 mV (**RB**), respectively. In contrast, there is just a slight change on the zeta potential value of human fetal lung fibroblast WI-38 cells incubated with **RBNO** and **RB**, increasing from -17.63 ± 1.83 mV to -15.77 ± 2.37 mV (**RBNO**) and -16.10 ± 2.25 mV (**RB**). However, incubation with **RNO** only slightly changed the zeta potential of both baterical and mammalian cells. It is indicated that boronic acid-decoration Ru(II) compounds **RB** and **RBNO** prefer to adhere to bacterial membrane.

To further validate the specific binding of Ru(II) compounds to *P. aeruginosa*, we applied a bacteria-mammalian cell co-culture model to examined the bactericidal effects of the Ru(II) compounds. Human fetal lung fibroblasts WI-38 was seeded into 96-well plate for 24 h to form monolayer cell culture. The monolayer was then infected with *P. aeruginosa* PAO1 at a multiplicity of infection (MOI) of 10 bacteria/mammalian cell. Different Ru(II) compounds with a final concentration of 10 μM were subsequently incubated with the bacteria-mammalian cell co-culture for 30 min with or without photoirradiation. No significant difference was found on the cell viability of both WI-38 fibroblasts and *P. aeruginosa* PAO1 when incubated with the Ru(II) compounds (**RNO**, **RB** and **RBNO**) in the dark. Upon photoirradiation, **RNO** compound caused 50% bacterial death. Boronic acid decorated compounds **RB** and **RBNO** exhibited higher bactericidal activity against *P. aeruginosa* PAO1, leading to approximately 75% and 80% reduction of bacterial viability, respectively. In contrast, all these Ru(II) compounds had no detectable phototoxicity on mammalian cells (Figure S5). The results further demonstrated that boronic acid-decoration facilitates selective targeting of Ru(II) compounds to bacterial membrane.

Synergistic effect of ROS and NO on biofilms eradication.

We investigated the capability of Ru(II) compounds to eradicate planktonic bacteria. *P. aeruginosa* PAO1 was grown to mid-log phase and then resuspended in M9 medium to final bacterial density of $1x10^6$ cells per well in 96-well plate. Subsequently, photosensitizer methylene blue (MB), NO donor diethylamine dinitric oxide (NONOate) and Ru(II) compounds (**RNO**, **RB** and **RBNO**) were added into planktonic bacterial culture with the final concentrations of 10 μM. The control groups were kept in the dark while the experimental groups were irradiated with continuous light (630 nm, 20 $mW{\cdot}cm^{-2}$ for MB; 425 nm, 20 $mW{\cdot}cm^{-2}$ for others) for 20 min. All the compounds exhibits no appreciable toxicity toward planktonic *P. aeruginosa* PAO1 in the dark. In contrast, MB and all three Ru(II) compounds had significantly enhanced bactericidal activities upon photoirradiation. The viability of planktonic *P. aeruginosa* PAO1 in the presence of MB photosensitizer was reduced by approximately 90%, which is consistent with previous studies.[30] **RNO**, **RB** and **RBNO** also attenuated the bacterial viability by 72%, 78% and 92%, respectively (Figure S6). The results indicated that the Ru(II) compounds could efficiently eradicate planktonic *P. aeruginosa* PAO1 upon photoirradiation.

Subsequently, we investigated whether these Ru(II) compounds could induce *P. aeruginosa* biofilm dispersal. Since extracellular polysaccharides are one of the main components of bacterial biofilm, we envisioned that boronic acid-decorated Ru(II) compounds should bind tightly to the biofilm. Therefore, *P. aeruginosa* PAO1 biofilms were developed and incubated with 20 μM Ru(II) compounds for 20 min in the dark. The amount of ruthenium bound to the biofilm was quantified by inductively coupled plasma-mass spectrometry (ICP-MS). Interestingly the amount of Ru bound to the biofilm after treatment with **RB** and **RBNO** was ca. 3x that for **RNO**, which lacks the boronic acid group (Figure S7). Notably, upon photoirradiation. **RBNO** is the most potent, eliminating 90% of bacteria in biofilm as well as 80% of the biofilm biomass (Figure S8). The biofilm eradication capability of **RNBO** is also remarkably higher than that of MB and NONOate. Importantly, further investigation demonstrated that **RBNO** was also effective in eradicating biofilms formed by three clinical isolated antibiotic-resistant *P. aeruginosa* strains (Figure S9). Moreover, we also examined the biofilm eradication potency of Ru compounds against Gram-positive bacteria methicillin-resistant *Staphylococcus aureus* (MRSA). As shown in the Figure. S10, **RBNO** could also eliminate the MRSA biofilm. However, MRSA is more resistance to RBNO eradication compared to *P. aeruginosa* PAO1.

Antibiotic tobramycin is clinically used to treat *P. aeruginosa* infections of cystic fibrosis patients and colistin is considered as a last-resort for multidrug-resistance Gram-negative bacterial infections.[31-33] To further investigate the anti-biofilm property of **RBNO**, we compared the dose-dependent biofilm eradication capability of **RBNO** and four clinical used antibiotics, including tobramycin, gentamicin, ceftazidime and colistin. As shown in Figure 2a and 2b, **RBNO** and all antibiotics could reduce bacterial viability and biofilm biomass in a dose-dependent manner. Typically, **RBNO** (with photoirradiation) and colistin are both highly potent, which could almost eradicate all the bacteria and biomass in biofilm at concentrations of 40 μM and 20 μM, respectively. In contrast, tobramycin, gentamicin and ceftazidime are much less effective, even high-concentration treatment of antibiotics (400 μM) could only partially eradicate the biofilm.

Confocal laser scanning microscopy (CLSM) was also applied to examine the biofilms dispersal effect of Ru(II) compounds.[34] In brief, the mature biofilm was stained with SYTO9 (green) to label all bacterial cell and propidium iodide (PI, red) was used to label dead bacteria after Ru(II) compounds treatment. As shown in Figure 2c, the intact biofilm before treatment has average thickness of 25.31 ± 2.32 μm and bacterial viability of higher than 95%. **RNO, RB** and **RBNO** treatment with photoirradiation caused biofilm thickness decreased to 18.83 ± 2.09, 11.29 ± 0.86 and 3.89 ± 0.51 μm, respectively. The bacterial viability was also declined to 68.3%, 31.4% and 7.3% (Table S2). The morphology of remained biofilms was further analyzed by scanning electron microscopy (SEM). The biofilms showed varying degree of destruction by Ru(II) compounds (Figure 2d). In consistent with the CLSM results, the decoration of boronic acid and the NO-releasing function remarkably enhanced the biofilm eradication potency of Ru(II) compounds. Especially, no embedded bacterial cells were observed after treatment with and photoirradiation of **RBNO**, indicative of complete disruption of the biofilm. Taken together, the results demonstrated that **RBNO** is a highly effective Ru(II) compound for eradicating *P. aeruginosa* biofilms.

To further confirm that photo-triggered released NO enhanced the biofilm eradication property of **RBNO**, the NO scavenger PTIO was applied in combination with **RBNO** to remove the released NO in biofilm dispersal experiments. Indeed, addition of PTIO significantly decreased the potency of NO donor NONOate and **RBNO** to eradicate biofilm (Figure 2e). Particularly, the biofilm removal potency of **RBNO** was alleviated to the same level of **RB** in the presence of PTIO, indicative of the synergistic effect of released NO and Ru(II)-based photosensitizer on biofilm eradication.

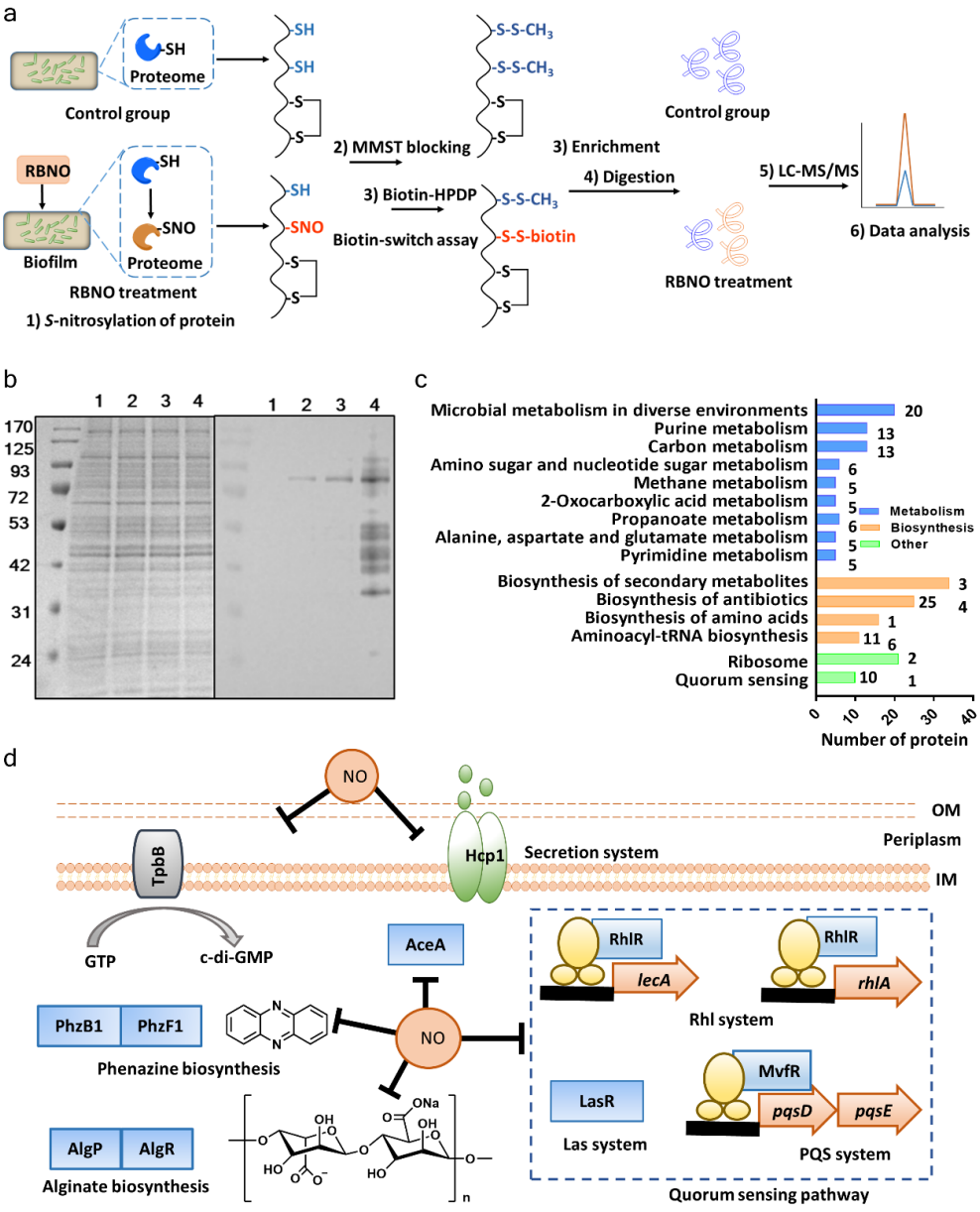


Figure 3. Identification of *S*-nitrosylated proteins from *P. aeruginosa* PAO1 proteome. (a) Flowchart of biotin-switch assay coupled to mass spectrometry to identify S-nitrosylated proteins from biofilm bacteria. (b) Detection and visualization of *S*-nitrosylated protein from *P. aeruginosa*. The total bacterial proteins were visualized by Coomassie blue staining (left) and the S-nitrosylated proteins were blotted using HRP-streptavidin (right). The numbers indicate the protein with different treatments. 1: control; 2: bacteria treated with biotin-switch assay alone; 3: bacteria pre-incubated with 20 μM **RB** followed by biotin-switch assay; 4: bacteria pre-incubated with 20 μM **RBNO** followed by biotin-switch assay. (c) KEGG pathway analysis of identified S-nitrosylated proteins from *P. aeruginosa* PAO1. (d) Mechanism of NO mediated *P. aeruginosa* biofilms dispersal. The scheme shows the interferes of NO-mediated S-nitrosylation on different regulatory pathways for biofilm formation

NO mediated S-nitrosylation of protein on biofilm signaling pathway.

It is well-documented that NO functions as a signaling molecule that causes *P. aeruginosa* biofilm dispersal.[13] Recently, a heme-binding protein Nosp has been identified to be a novel NO sensor in *P. aeruginosa*. Nosp is able to regulate the phosphorelay activity of a hybrid histidine kinase that is involved in biofilm regulation.[35] Besides direct binding to the sensor protein, it is also reported that NO could regulate protein function via S-nitrosylation of cysteine residues.[36] The high efficacy of **RBNO** to eradicate biofilms prompted us to further investigate the impact of released NO on *P. aeruginosa* proteome. We employed a biotin-switch assay to examine the NO-mediated *S*-nitrosylation of protein in *P. aeruginosa* PAO1 (Figure 3a).[37] In brief, only S-nitrosylated cysteines are converted to biotinylated cysteines without disturbing the free thiols and pre-existed disulfide bond. As shown in Figure 3b, large-scale and robust S-nitrosylation of *P. aeruginosa* proteome were observed after **RBNO** treatment. In contrast, only a single weak S-nitrosylation protein was observed after **RB** treatment. Totally 149 S-nitrosylated *P. aeruginosa* proteins were identified by mass spectrometry (Table S3). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis demonstrated that an enrichment of proteins involved in various KEGG pathways (Figure 3c and Table S4). It is implied that the *P. aeruginosa* proteome could be dramatically altered by NO-mediated S-nitrosylation.

Intriguingly, several proteins related to biofilm formation and dispersal were also identified to be S-nitrosylated by NO, such as essential transcriptional regulators and enzymes in quorum sensing (QS) system (LasR, RhlR, MvfR, PqsD, PqsE) (Figure 3d). It should be noted that the identified proteins PqsD and PqsE are responsible for biosynthesis of pseudomonas quinolone signal (PQS) 2-heptyl-3,4-dihydroxyquinoline. It is reported that the PSQ is essential for biofilm development.[38] Moreover, some of the identified proteins are responsible for biosynthesis of the main components of biofilm, for examples rhamnolipids (by RhlA), alginate (by AlgR and AlgP) and lectin (LecA).[39-41] In particular, a diguanylate cyclase (TpbB) was also found to be S-nitrosylated by NO. The enzyme is involved in biosynthesis of cyclic diguanylate (c-di-GMP).[42] S-nitrosylation of TpbB would probably interfere with the enzyme activity and resulted in the decline of intracellular c-di-GMP level, which subsequently lead to the dispersal of biofilms.[43]

The proteomic results indicated that NO-induced S-nitrosylation would probably interfere with the biofilm regulatory network in *P. aeruginosa* (Figure 3d). However, how S-nitrosylation affects the physiological function of these proteins warrant further investigation. Taken together, our results revealed that the NO-induced biofilm dispersal were probably associated with the *S*-nitrosylation of multiple protein targets involved in the biofilm-formation regulatory network.

Conclusion

In summary, we have designed a boronic acid-functionalized Ru(II)-based photosensitizer **RBNO**. Positive-charge and boronic acid facilitate the compound to adhere to the bacterial membrane and biofilms. This novel Ru(II) compound takes the advantage of both photodynamic antimicrobial therapy and photorelease antimicrobial therapy. Light-triggered released NO from the compound stimulates biofilm dispersal and liberates *P. aeruginosa* bacteria from the biofilms. Without the protection of biofilms, the free-floating bacteria were eliminated by ROS generated from the Ru(II) PS. Therefore, this dual-functional compound could readily eradicate mature biofilms at low concentration, which reduced the cytotoxicity to mammalian cells. Furthermore, proteomic analysis identified that series of proteins involved in quorum sensing and biofilm formation could be S-nitrosylated after **RBNO** treatment, suggesting the molecular basis of NO-regulated biofilm dispersal. All these results revealed the potential application of the novel Ru(II) compound to combat biofilm infections caused by human pathogenic bacteria.

Experimental Section

***In vitro* detection of NO release from Ru(II) compounds upon photoirradiation:**

In vitro NO release from Ru(II) compounds with photoirradiation was detected using the Griess assay as described previously.[44] 40 μM Ru(II) compounds dissolved in PBS buffer was irradiated with light (425 nm, 20 $mW \cdot cm^{-2}$) for 20 min. At each time interval, 50 μL of solution was transferred into a 96-well plate. 50 μL Griess I and 50 μL Griess II reagent were added into each well. The absorbance at 540 nm was subsequently measured on a Cytation 3 microplate reader (BioTek).

For EPR detection of NO release from Ru(II) compounds. Ru(II) compound (20 μM) was incubated with 40 μM 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) in PBS and subjected to photoirradiation (425 nm, 20 $mW \cdot cm^{-2}$) for 20 min as described previously.[45] The sample was subsequently loaded into a 0.5 mm capillary tube. The EPR spectra were collected on an EPR X-band spectrometer (Bruker A300). The EPR instrument settings were as follows: microwave frequency, 9.85 GHz; microwave power, 10 mW; modulation frequency, 100 KHz; modulation amplitude, 1 G; number of scans, 3.

Biofilm binding capabilities and eradication activities of Ru(II) compounds

Overnight cultures of *P. aeruginosa* PAO1 or clinical isolates were diluted with fresh M9 medium (0.8% glucose) to 5×10^6 CFU/mL. Diluted bacterial cultures were then added into a 24-well flat-bottom plate (2 mL/well) and incubated at 37 °C to allow bacterial biofilm formation. For MRSA biofilm, the MRSA were cultured in the Luria-Bertani (LB) broth (containing 0.5% glucose). After 48 h, medium was discarded and planktonic cells were removed by washing the biofilms with PBS twice.

To examine the binding capabilities of Ru(II) compounds, 20 μM Ru(II) compound solution was added into each well and incubated at 37 °C for 20 min in the dark. Then the solution was removed and the biofilms were subsequently scraped and treated with 70% HNO_3 at 60 °C overnight. The ruthenium contents of biofilms were further analyzed by inductively coupled plasma mass spectrometry (ICP-MS).

To investigate the biofilms eradication activities of Ru(II) compounds, 1 mL of 20 μM Ru(II) compound solution was incubated with the biofilm for 30 min. The biofilms were then treated with or without 20 min photoirradiation (425 nm, 20 $mW \cdot cm^{-2}$). The biofilms were further incubated at 37 °C in the dark for another 70 min. The bacterial cell viabilities in the biofilms were determined by MTT assay and the remained biofilms were quantified using the crystal violet dye.

Confocal laser scanning microscopy and scanning electron microscopy analysis of *P. aeruginosa* biofilm

The analysis of bacterial biofilms by confocal laser scanning microscopy was performed as described previously.[46] The biofilms were stained with LIVE/DEAD™ *Bac*Light™ Bacterial Viability Kit (Thermo)

according to the manufacturer's protocol. The green-fluorescent SYTO 9 dye stain all the bacterial cells in biofilms and the red- fluorescent propidium iodide (PI) stain the dead bacterial cells. After staining, the biofilms were washed by PBS and analyzed on a laser scanning confocal microscopy (LSM 710, Carl Zeiss, Germany). The images were collected at: SYTO 9 ($\lambda_{ex}/\lambda_{em}$ =488/520 ± 20 nm), PI ($\lambda_{ex}/\lambda_{em}$ =538/617 ± 20 nm). The biofilm coverage, thickness and bacterial cell viability values were analyzed by Carl Zeiss ZEN microscope software.

To prepare the biofilm samples for scanning electronic microscope (SEM) analysis, the remained biofilms after Ru(II) compound treatment were rinsed with PBS and then fixed with 2.5% glutaraldehyde. The fixed biofilm samples were further dehydrated with a graded ethanol series (30%, 50%, 70%, 90% and 100%) and transferred to *t*-butyl alcohol. The specimens were then freeze-dried and coated with gold by sputtering with a plasma multicoater.

Detection of S-nitrosylated proteins from P. aeruginosa PAO1.

The NO-mediated *S*-nitrosylated proteome were converted to biotinylated proteome using the biotin switch method.[37] The mature *P. aeruginosa* biofilms were treated with Ru(II) compounds with photoirradiation for 20 min. After irradiation, the biofilms were transferred into tubes and washed with PBS twice. The bacterial cells in biofilms were lysed by Branson digital sonifier. The supernatant of cell lysate was collected by centrifugation (16,000 rpm, 10 min, 4°C). The biotin switch assay was performed according to previous study with a few modifications. In brief, 1 mg of bacterial cell lysate (quantitated by BCA assay) was diluted in 4 mL pre-chilled acetone to remove the excess amount of Ru(II) compounds. The solution was incubated at −20°C for 20 min and then subjected to centrifugation (2000 *g*) at 4°C for 10 min. The collected pellet was resuspended in HENS buffer supplemented with 4 mM MMTS to block the free thiols without disturbing the S-nitrosothiols or pre-existing disulfide bonds. The excess MMTS was removed by adding 4 volumes of pre-chilled acetone. The precipitated protein in cell lysate was collected by centrifugation and resuspended in HENS buffer. Streptavidin agarose beads were added to remove the endogenous biotinylated proteins. The supernatant solution was collected by centrifugation and transferred into a new vial. Subsequently, biotin-HPDP (final concentration: 0.5 mM) and ascorbate (final concentration: 1 mM) were added in the vial and incubated at RT for 1 h to allow the conversion of the *S*-nitrosylated proteins into biotinylated proteins. The solution was diluted with pre-chilled acetone to precipitate protein and remove the excess labeling reagents. The precipitated pellets were mixed with non-reducing SDS-PAGE loading buffer and directly subjected to SDS-PAGE without boiling. For western-blot analysis the *S*-nitrosylated proteins, the protein bands in SDS-PAGE gel were transferred to a PVDF film and blotted with streptavidin-HRP.

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Keywords: ruthenium compound • photodynamic therapy • photorelease • nitric oxide • bacterial biofilm

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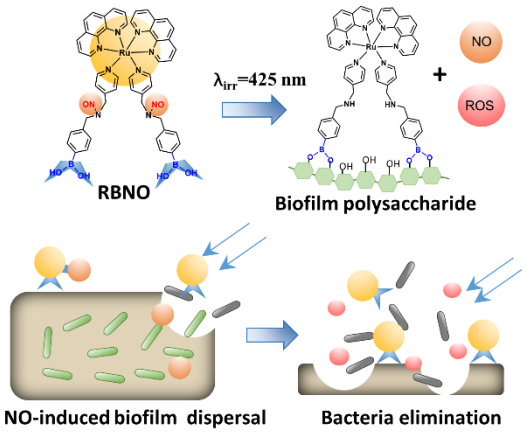

Light-triggered Nitric Oxide Release Photosensitizer to Combat *Pseudomonas aeruginosa* Biofilm Infections

Z. Zhao, H. Li, X. Tao, Y. Xie, L. Yang, Z.W. Mao* and W. Xia*

A boronic acid-decorated ruthenium compound **RBNO** functions as both a photosensitizer (PS) and a nitric oxide (NO) releasing agent. The compound **RBNO** is capable of binding to bacterial cell envelope and the biofilm matrix. Upon photoirradiation, released NO from the compound can induce bacterial biofilm dispersal. And the exposed bacteria are eradicated by the reactive oxygen species (ROS) generated from PS.