Light Emission from Ruthenium-Labeled Penicillins Signaling Their Hydrolysis by β-Lactamase

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Bacterial resistance to β-lactam antibiotics (including the penicillins and cephalosporins) is a major and growing health problem.[1] Resistant bacteria usually secrete enzymes called β-lactamases that efficiently hydrolyze the β-lactam ring of offending antibiotics, causing the antibiotics to become inactive. In this paper we report the sensitive detection of β-lactamases using ruthenium-labeled penicillin substrates. The electrochemiluminescence (ECL) properties of ruthenated penicillins change dramatically upon hydrolysis by β-lactamases. The high sensitivity of the method results from a mechanism involving intramolecular electron transfer from the hydrolyzed penicillin to ruthenium, resulting in light emission.

In ECL reactions, an electrode oxidizes or reduces stable compounds to form less stable species that chemically react, resulting in light emission.[2] A well-known ECL reaction involves the co-oxidation of an amine and ruthenium(II) tris(bipyridine) ($Ru(bpy)_3^{2+}$) to form reactive species, culminating in the emission of a photon (620 nm) from ruthenium.[2b,3] This reaction has been exploited in immunoassays and DNA probe assays using antibody−$Ru(bpy)_3^{2+}$ and oligonucleotide−$Ru(bpy)_3^{2+}$ conjugates as sensitive reporter labels.[4] In these applications, excess tripropylamine is used as the amine coreactant.

Recently, it was discovered that β-lactam antibiotics can act as the amine component in the ECL reaction.[5] Fortuitously, the abilities of penicillins to act as ECL coreactants dramatically increase when they are hydrolyzed by β-lactamase, thus allowing the detection of this clinically important enzyme.[1] As shown in eqs 1−5, the mechanism of the ECL reaction of hydrolyzed penicillins (penicilloates) and $Ru(bpy)_3^{2+}$ is believed to involve a series of steps including electrode oxidation of both the penicilloate and $Ru(bpy)_3^{2+}$, spontaneous deprotonation of the penicilloate, penicilloate-to-ruthenium one-electron transfer to form a ruthenium excited state, and finally light emission as ruthenium decomposes to the ground state.[3b]

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^- \quad \text{electrode oxidation} \quad (1)$$

$$\text{penicilloate} \rightarrow \text{penicilloate}^{+\bullet} + e^- \quad \text{electrode oxidation} \quad (2)$$

$$\text{penicilloate}^{+\bullet} \rightarrow \text{penicilloate}^{\bullet} + H^+ \quad \text{deprotonation} \quad (3)$$

$$\text{penicilloate}^{\bullet} + Ru(bpy)_3^{3+} \rightarrow \text{penicilloate fragments} + Ru(bpy)_3^{2+*} \quad \text{electron transfer} \quad (4)$$

$$Ru(bpy)_3^{2+*} \rightarrow Ru(bpy)_3^{2+} + h\nu\ (\lambda_{max} = 620\ \text{nm}) \quad \text{luminescence} \quad (5)$$

The step shown in eq 4 is critical because the penicilloate-derived radical species is very unstable. This highly reducing species must diffuse through aqueous solution to transfer an electron to $Ru(bpy)_3^{3+}$. We hypothesized that the efficiency of this step, and overall light emission, could be increased by antibiotic−$Ru(bpy)_3^{2+}$ conjugation. As a result, diffusion would not be required and amine-to-ruthenium electron transfer would be intramolecularly facilitated by the covalent approximation of the two reacting species.[6] Studies of facile long-range intramolecular electron transfer in ruthenium-labeled small biomolecules[7] and proteins[8] demonstrated the feasibility of the concept.

$Ru(bpy)_3^{2+}$-labeled derivatives of ampicillin (Ru-Amp) and 6-aminopenicillanic acid (Ru-APA) were prepared by reacting the primary amines of antibiotics with an *N*-hydroxysuccinimide derivative of $Ru(bpy)_3^{2+}$ (IGEN, Inc., Gaithersburg, MD) (Figure 1). Both conjugates were excellent substrates of *Bacillus cereus* β-lactamase I with k_{cat}/K_m values of $3.9 \times 10^8\ s^{-1}\ M^{-1}$ for Ru-Amp and $9.8 \times 10^7\ s^{-1}\ M^{-1}$ for Ru-APA. As seen[5] with unconjugated mixtures of free penicillins and $Ru(bpy)_3^{2+}$, both Ru-Amp and Ru-APA were weakly electrochemiluminescent, but became highly electrochemiluminescent when the β-lactam component was hydrolyzed by β-lactamase (Figure 2). However, when hydrolyzed, both conjugates displayed much greater ECL increases than did comparable equimolar mixtures of $Ru(bpy)_3^{2+}$ and ampicillin (Amp) or 6-aminopenicillanic acid (6-APA), indicating that intramolecular electron transfer between the β-lactam and ruthenium indeed occurred. Similar results were seen when the β-lactams were hydrolyzed with dilute NaOH. The standard curves in Figure 3 show that β-lactamase-catalyzed hydrolysis of 50 nM Ru-APA can be detected by ECL. Although the nonlinearity of Figure 3 appears to argue against a light-enhancing intramolecular intermediate step, factors beyond the mechanism in eqs 1−5 can cause nonlinearity. For example, in an ECL instrument the electrode surface that electrogenerates the reactive species is physically separated from the photomultiplier tube (PMT). Thus, in a fast reaction, the amount of light actually being recorded depends on diffusion and is proportional to the number of emitting molecules times the distance squared from the PMT.

To quantitate the intramolecular effect in Ru-APA, we related the ECL of the conjugate to that of various unconjugated mixtures of 6-APA and $Ru(bpy)_3^{2+}$. The ECL of 10 μM Ru-APA was compared with that of mixtures of 10 μM $Ru(bpy)_3^{2+}$ and various concentrations of 6-APA (Figure 4). The ECL increase when 10 μM Ru-APA was hydrolyzed was found by extrapolation to be equivalent to that of 10 μM $Ru(bpy)_3^{2+}$ mixed with 1.25 mM 6-APA. This suggests that intramolecular electron transfer in hydrolyzed Ru-APA resulted in a 125-fold increase in the effective 6-APA concentration.

In general, long-range intramolecular electron transfer can occur through space (by direct or solvent-mediated electron transfer), through bonds (covalent or hydrogen bonds or van der Waals interactions),[9] or through some combination of the two.[10] It is unclear from our ECL data which pathway is operative in penicillin-to-ruthenium electron transfer. The mode

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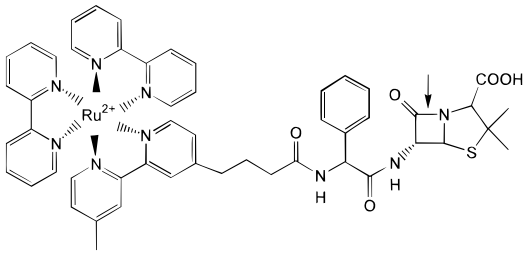
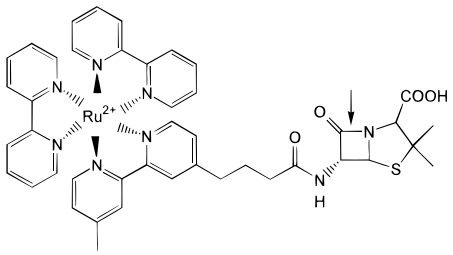


$Ru(bpy)_3^{2+}$-Labeled Ampicillin (Ru-Amp)



$Ru(bpy)_3^{2+}$-Labeled 6-Aminopenicillanic Acid (Ru-APA)

Figure 1. Chemical structures of $Ru(bpy)_3^{2+}$-labeled ampicillin (Ru-Amp) and $Ru(bpy)_3^{2+}$-labeled 6-aminopenicillanic acid (Ru-APA). β-Lactamase hydrolyzes the amide bonds indicated by arrows.

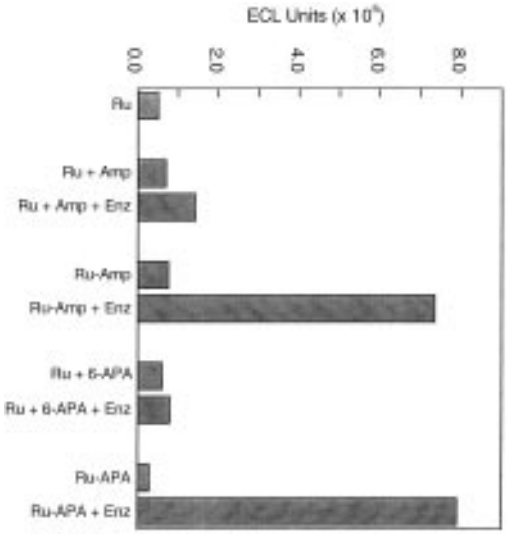


Figure 2. ECL effects of penicillins before and after β-lactamase treatment. The bar denoted by Ru shows the ECL of 30 μM $Ru(bpy)_3^{2+}$ in the absence of any amine. Other concentrations used were the following: $Ru(bpy)_3^{2+}$ + Amp (25 μM of each) ± Enz (441 nM β-lactamase I from *B. cereus*), Ru-Amp (25 μM) ± Enz (441 nM), $Ru(bpy)_3^{2+}$ + 6-APA (23 μM of each) ± Enz (347 nM), and Ru-APA (25 μM) ± Enz (347 nM). Samples in 0.1 M sodium phosphate, pH 7.5, were incubated at room temperature for 60 min, then ECL was generated and read using an ORIGEN Analyzer (IGEN, Inc., Gaithersburg, MD). The instrument uses a personal computer to integrate a potentiostat, an electrochemical flow cell, and a luminometer.

and efficiency of electron transfer depends on various factors including conformational flexibility, steric hindrance, and the nature of the bridging material.[7b,c,9] Because the ECL differences between Ru-Amp and Ru-APA are minor, apparently neither the greater number of covalent bonds (3) in the Ru-Amp bridging linker (affecting a through bond mechanism) nor the bulky ampicillin phenyl side chain (possibly effecting a

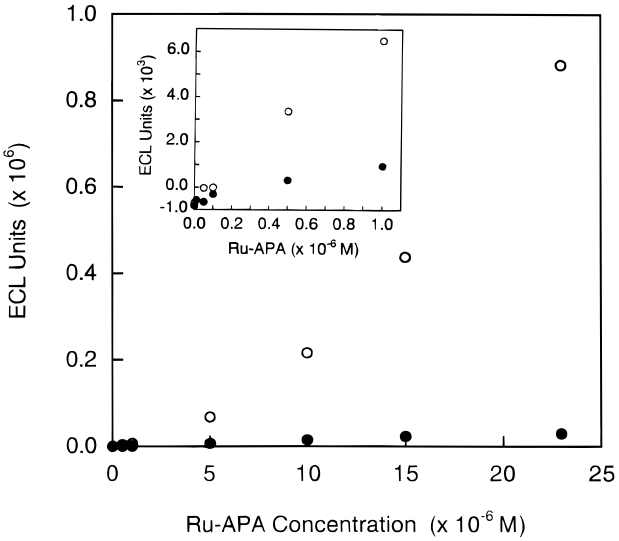


Figure 3. Standard curves of Ru-APA concentration versus ECL before (closed circles) and after (open circles) total hydrolysis by *B. cereus* β-lactamase I. The inset shows an expanded scale (0−1 μM Ru-APA). The experiment was carried out essentially as described in Figure 2.

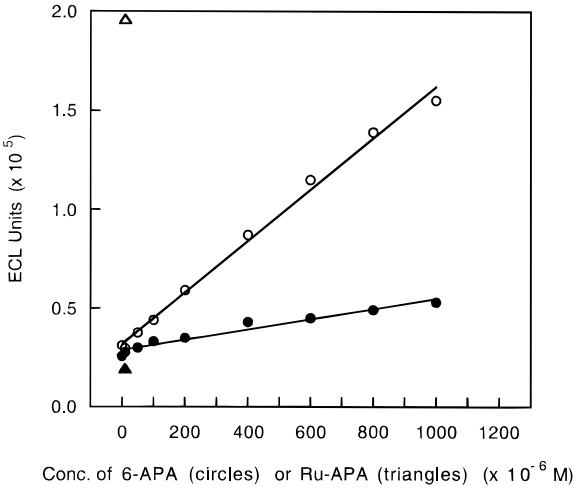


Figure 4. Quantitation of the intramolecular effect in the ECL of Ru-APA. The standard curves relate the concentration of intact (open circles) and enzyme-hydrolyzed (closed circles) 6-APA to ECL intensity. In both curves, the concentration of $Ru(bpy)_3^{2+}$ was held constant at 10 μM. For comparison, the ECL of 10 μM Ru-APA is shown before (closed triangles) and after (open triangles) enzyme hydrolysis. The experiment was carried out essentially as described in Figure 2.

through space mechanism) greatly affect electron transfer. Because the alkyl linker between the antibiotics and $Ru(bpy)_3^{2+}$ is conformationally flexible, electron transfer may have occurred by direct contact (through space).

In conclusion, this work suggests that a critical intermediate step in penicilloate-promoted ECL is a one-electron transfer reaction from a highly unstable penicilloate-derived radical to $Ru(bpy)_3^{3+}$. By covalently linking the two reactants to form a single compound, efficiency of electron transfer was intramolecularly enhanced resulting in increased light emission. Ruthenium-labeled β-lactam antibiotics may become valuable tools for the clinical detection of resistance-causing β-lactamases.

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Supporting Information Available: Experimental details for preparation and enzymatic analysis of Ru-Amp and Ru-APA and ^{1}H NMR and mass spectra of Ru-Amp (4 pages). See any current masthead page for ordering and Internet access instructions.

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