Luminescent lanthanide cyclen-based enzymatic assay capable of diagnosing the onset of catheter-associated urinary tract infections both in solution and within polymeric hydrogels

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ABSTRACT: Herein we present a supramolecular (delayed luminescent) Eu(III)-based pH-responsive probe/sensor with the ability to detect the urease-mediated hydrolysis of urea in aqueous solution. A series of photophysical titrations show this Eu(III) chelate behaves as an ‘on-off’ luminescent switching probe, with its luminescence being quenched upon urea being enzymatically converted into ammonia and carbon dioxide. Calculation of the rate constant (k) and activation energy (Ea) for this hydrolysis reaction are detailed; the results demonstrate a direct observation of enzymatic activity in solution by the sensor. The potential application of this probe in detecting the onset of catheter-associated urinary tract infections (CAUTIs) is also demonstrated by incorporating 1.Eu into water-permeable hydrogels that can be utilized as an alternative coating for catheters.

INTRODUCTION

Catheter-associated urinary tract infection (CAUTI) is one of the most common health-care associated infections worldwide, accounting for up to 40% of all nosocomial infections.1,2 Approximately 20% of all hospitalized patients are catheterized.3 Currently, the most commonly deployed prosthetic medical device is the Foley indwelling urethral catheter.4,5 The development of CAUTIs has been directly linked to the duration of catheterization; nearly 100% of patients undergoing long-term catheterization (≥ 28 days) are found to develop catheter-associated bacteriuria (CAB).6 While most cases of CAB are asymptomatic and do not require treatment, some individuals can experience symptomatic episodes of CAUTI, which can result in pyelonephritis, septicemia or endotoxic shock, and ultimately death.7,8 These episodes are triggered by the occurrence of catheter encrustation, where the formation of crystalline polymicrobial-based biofilms on the inner and outer surface of the catheter tube causes the device to become blocked, and as such obstructs urinary flow.9 The bacterial species most associated with catheter encrustation and CAUTI are urease-producing microorganisms; specifically Proteus mirabilis, Proteus vulgaris, and Providencia rettgeri.10 Their ability to secrete urease, an enzyme that catalyzes the hydrolysis of urea (one of the primary constituents within urine) into ammonia and carbon dioxide, results in the pH of the urine being increased towards alkaline, which induces the precipitation of calcium and magnesium phosphate crystals from the urine.4 These crystals accumulate within the bacterial layers of the biofilm, making it highly crystalline with increased resistance to both the hosts immune system and to antibiotic treatment.11,12 Several strategies have been employed to prevent the onset of CAUTI, the majority of which involve coating or impregnating the catheter material (hydrogels and other related soft materials) with antimicrobial agents that will counteract bacterial adhesion.13,14 These agents include silver alloys,6 antibacterials,7 liposomes,8 and urease inhibitors.9 Nevertheless, no single prosthetic medical device currently exists that is able to completely inhibit CAB from developing during catheterization. Consequently, focus has turned to finding ways in which CAUTI can be quickly diagnosed in order to permit early treatment. The primary diagnostic tool currently utilized in the clinic to monitor CAB is the bromothymol
blue colorimetric sensor developed by Stickler, which changes from a yellow (pH 6) to blue (pH 8) color in response to the pH of the urine being elevated by the presence of Proteus bacteria.\textsuperscript{10} Herein we describe an alternative route for diagnosing the onset of CAUTI through the use of a lanthanide-based (Ln(III)) pH-responsive luminescent probe (1.Eu, Scheme 1), which in combination with the enzyme urease, can monitor the hydrolysis of urea (either in solution or within a biocompatible material) in real time.

As we and others have demonstrated, lanthanide luminescence is a powerful analytical tool that can be exploited for sensing and imaging biological systems.\textsuperscript{11} In particular, we have shown that such luminescent probes can be employed for observing enzymatic reactions in solution in real-time.\textsuperscript{12} This includes the development of glycosylated Ln(III)-based cyclen complexes for monitoring glycosidase enzyme activity and Tb(III)-based cyclen malonamide complexes for monitoring glutathione reductase.\textsuperscript{12a,b} In addition, we have also designed several Ln(III)-based pH-responsive luminescent sensors,\textsuperscript{13} some of which were developed to mimic logic gate operations\textsuperscript{14a,b}, while others were conjugated to gold nanoparticles.\textsuperscript{14c,d}

One example of such a pH responsive design is the Eu(III) cyclen complex 1.Eu: a highly water soluble coordinatively unsaturated octadentate (cationic) complex possessing a single axial metal bound water molecule. This system functions as a reversible luminescent ‘off-on-off’ sensor within the pH range of 2 – 10. The pH-dependent nature of this triacetamide-substituted cyclen derivative arises from the covalently attached 1,10-phenanthroline (phen), which functions as an antenna for populating the 5D\textsubscript{4} excited state of Eu(III).\textsuperscript{15} Specifically, we have demonstrated that the phosphorescent emission of 1.Eu has a bell-shaped pH dependency; corresponding to the pH values of 2 – 4, 5 – 7, and 8 – 10.\textsuperscript{16} We therefore anticipated that 1.Eu could potentially be utilized as a spectroscopic ‘on-off’ sensor/probe to detect the presence of urease-secreting bacteria within urinary catheters. This could be achieved by impregnating water-permeable hydrogels, that could be used as an alternative coating material, with the complex 1.Eu.\textsuperscript{16} Through photophysical analysis, we demonstrated that this was indeed possible; the urease-mediated hydrolysis of urea results in the pH of the system being elevated and as such causes the metal-centered emission of 1.Eu to be quenched. Moreover, we show that the rate of this quenching increases at elevated temperature and upon increasing the quantity of enzyme units (U).

**RESULTS**

**Synthesis and characterization**

Both the synthesis and characterization of ligand 1 and its corresponding Eu(III) complex 1.Eu have been previously reported by us.\textsuperscript{15a} The hydration state (q) was determined by measuring the excited state lifetimes of 1.Eu in H\textsubscript{2}O and D\textsubscript{2}O, with the number of metal bound water molecules in solution being confirmed as one (see Supporting Information, Figure S1).\textsuperscript{15a}

**Luminescent stability of 1.Eu in solution**

Population of the Eu(III) excited state was achieved by indirect excitation of the phen antenna, which was confirmed upon recording the time-delayed emission and excitation spectra of 1.Eu.

The luminescence response of 1.Eu (1 × 10\textsuperscript{-5} M), in the absence and presence of either urea or urease, was monitored in H\textsubscript{2}O over 24 h. Since the normal concentration range of urea in human blood plasma is between 3.0 – 6.5 × 10\textsuperscript{-3} M and urease behaves as a catalyst, concentrations of 2.3 × 10\textsuperscript{-3} M and 0.01 – 0.1 U were utilized, respectively. As expected, five distinct phosphorescence transitions were observed at 580, 593, 615, 654, 683, and 701 nm upon indirect excitation of 1.Eu at 266 nm, which can be assigned to the 5D\textsubscript{4}→7F\textsubscript{i} (i = 0 – 4) transitions of Eu(III), as shown in Figure 1. Indeed, the intensity of all five emission bands was seen to remain constant in aqueous solution at 22 °C, indicating 1.Eu was stable towards Eu(III) dissociation. Similarly, no changes in the luminescent properties of 1.Eu were exhibited when in the presence of urea (2.3 × 10\textsuperscript{-3} M) or urease (0.1 U), with the metal-centered emission remaining ‘switched on’ for up to 24 h (see Supporting Information, Figure S2 – 3). Importantly, repeating these measurements at 37 °C further showed that the luminescent properties of 1.Eu were not temperature dependent. Regardless of urea or urease being present, no...
variation was seen in the emission intensity (see Supporting Information, Figure S6b). These results therefore indicate that the Eu(III) emission is not dependent on the local environment of the pH sensor, which is important to establish with respect to incorporating the sensor into soft polymeric materials (e.g. hydrogels).

**Detecting the urease-mediated hydrolysis of urea photophysically**

With the luminescent behavior of 1.Eu investigated independently with both urea and urease, we next determined whether 1.Eu could be utilized as a pH-responsive probe to monitor the urease-mediated hydrolysis of urea. This was achieved by recording the photophysical properties of 1.Eu before and 60 min after the addition of urease (0.01 – 0.10 U; increments of 0.01 U) at 22 °C.

The ground state properties were firstly investigated; the UV-vis absorption spectra of 1.Eu was characteristic of substituted phen derivatives, consisting of two main bands centered at 230 and 266 nm, which are assigned to the π-π* intra-ligand transitions of the antenna moiety.15

The addition of urease (0.01 U) to a solution of 1.Eu and urea resulted in the urea being rapidly hydrolyzed. This caused a marked bathochromic shift in the lower energy absorption band of the phen antenna (266→274 nm) and the concomitant appearance of a small shoulder at ca. 312 nm, as shown in Figure 2a, with the appearance of several isosbestic points. This red shift corresponds with the changes exhibited in the pH titration previously reported by Leonard and co-workers, where upon increasing the pH from 5.5 to 9.5 the λmax of the 266 nm band was seen to red shift by ca. 8 nm.19a Moreover, this bathochromic shift was evident regardless of the units of urease added (0.01→0.10 U) to the solution of 1.Eu and urea (see Supporting Information, Figure S6a).

Both the delayed lanthanide luminescence and the phosphorescence spectra were obtained by exciting the same solution at 266 nm. As anticipated, the Eu(III) emission was ‘switched off’ upon the addition of 0.01 U of urease, with ca. 90% quenching being observed after 60 min, as demonstrated in Figure 2b; this being the direct result of the local pH environment of 1.Eu changing. Increasing the concentration of urease (0.01→0.10 U; 0.01 increments) added to 1.Eu resulted in the same amount of quenching in the Eu(III)-centered emission being exhibited (see Supporting Information, Figure S6b). However, the rate at which the emission was ‘switched off’ increased.

The changes observed in the fluorescence emission spectra of 1.Eu were much less dramatic; the intensity of the ligand fluorescence was reduced by ca. 40% and a redshift in the λmax from 400 to 415 nm was observed following the addition of 0.01 U of the enzyme, as shown in Figure 2c. As before, no difference in the extent of quenching was seen upon increasing the quantity of units of urease added to 1.Eu (see Supporting Information, Figure S6c). Excitation spectra were also recorded before and after the addition of enzyme; all the spectra structurally matched those of the absorption spectra (see Supporting Information, Figure S7), and showed that the sensitization process from the antenna to the Ln(III) center was indeed modulated by the addition of enzyme and the resultant change in pH. These results clearly demonstrate that 1.Eu can be used to observe the enzymatic catalyzed hydrolysis of urea in real time, even at micromolar concentrations.

**Determination of the rate constant (k)**

In order to quantify the enzymatic activity of urease in hydrolyzing the urea in solution into ammonia and carbon dioxide, the rate constant (k) of this reaction was next calculated by plotting the quenching in the Eu(III)-centered emission of 1.Eu as a function of time for all the 5D0→7FJ (J = 0 – 4) transitions.

The resulting titration profiles are given in Figure 3 and clearly show that the addition of a higher quantity of urease units results in the Eu(III) emission being quenched to a faster rate (see Supporting Information, Figure S8, for other transition bands). In general, all the Eu(III)-centered emission bands were quenched to the same extent (up to 90%) ca. 60, 20, and 15 min after the addition of 0.01 – 0.02, 0.03 – 0.04, and 0.05 – 0.10 U of urease, respectively. Fitting this luminescent quenching to a monoexponential function allowed for the rate constant value to be determined for each of the 5D0→7FJ transitions within the enzymatic unit range of 0.01 – 0.1 U. As shown in Figure 4, the rate constant was seen to linearly increase as a function of urease activity, with the lowest and highest k values being given for 0.01 and 0.10 U of urease, respectively. This first-order relationship, where the rate of reaction was seen to be directly proportional to the concentration of urease, demonstrated that the higher the quantity of urease units added to the urea and 1.Eu solution,
the greater the decrease exhibited in the activation energy of the reaction, and the faster the rate of hydrolysis.

The effect of temperature on the rate of hydrolysis was also studied by repeating the above measurements at both 30 and 37 °C (see Supporting Information, Figure S9–10). At elevated temperatures the Eu(III)-centered emission became 'switched off' much more rapidly. More than 90% quenching in the phosphorescence of 1.Eu occurred 45, 20, 10, and 5 min after the addition of 0.01, 0.02, 0.03 – 0.05, and 0.06 – 0.08 U of urease, respectively, when at 37 °C (see Supporting Information, Figure S10). Indeed, increasing either the temperature or the quantity of enzyme units added to the solution of 1.Eu and urea gave rise to a higher rate constant, whereby \( k_{37°C} > k_{30°C} > k_{22°C} \) and \( k_{0.1U} > k_{0.01U} \) of enzyme (see Supporting Information, Figure S11). This can be explained by the fact that the enzymatic activity of urease increases as a function of temperature, with the optimal temperature of urease being known to be 60 °C.17 As such, the conversion of urea into ammonia and carbon dioxide occurs at a quicker rate at higher temperatures, and in turn causes the pH of the system to also change at a much faster rate, which is observed by the Eu(III) probe. Hence, 1.Eu in combination with urea, can be employed in a luminescent assay to monitor enzymatic hydrolysis reactions in real time in solution.

Determination of the activation energy (Ea) from the Arrhenius Equation

Having established a relationship between the rate of hydrolysis of urea and temperature, the activation energy (Ea) of this reaction was next calculated by utilizing the Arrhenius equation \( \ln(k) = \ln(A) - \frac{Ea}{RT} \).19 Plotting the rate constant as a function of \( 1/T \) for all the \( ^5D_{0} \rightarrow ^7F_{J} \) (\( J = 0 \) – 4) transitions resulted in a negative linear correlation being evidenced in all four of the Arrhenius plots, with \( \ln(k) \) decreasing as the quantity of urease was increased by 0.01 U intervals between 0.02 – 0.05 U (see Supporting Information, Figure S12). The average values obtained for \( E_a \) (activation energy) and \( A \) (pre-exponential factor) are presented in Table 1 for each enzymatic unit. Indeed, both the mean \( E_a \) and \( A \) for the urease-mediated hydrolysis

![Figure 3](image-url)

**Figure 3.** Changes in the delayed-Eu(III) emission of 1.Eu \( (1 \times 10^{-5} \text{ M}) \) as a function of time in an aqueous solution of urea \( (2.3 \times 10^{-3} \text{ M}) \) at 295 K \( (\lambda_{exc} = 266 \text{ nm}) \), measured at a) 580 nm, b) 593 nm, c) 615 nm, and d) 701 nm upon the addition of urease \( (0.01 – 0.10 \text{ U}) \).

![Figure 4](image-url)

**Figure 4.** Changes in the rate of hydrolysis of urea as a function of urease units at 295 K, calculated from the quenching in Eu(III)-emission of 1.Eu (shown in Figure 3), with all linear fits obtained having a \( R^2 \geq 0.98 \).
reaction of urea were found to correlate well with reported literature, with an average value of 44.9 kJ mol\(^{-1}\) and 1.4 \(\times 10^7\) min\(^{-1}\) being calculated for each, respectively.\(^{20}\) This relatively low value for the \(E_a\) indicates that the hydrolysis of urea proceeds at a rapid rate in solution. This means the pH of the solution changes at a fast rate and as such explains the dramatic quenching exhibited for \(1.\text{Eu}\).

IC\(_{50}\) toxicity studies of \(1.\text{Eu}\) and urea

With the intention of incorporating this Eu(III)-based pH-responsive probe into biomaterials that are currently utilized for catheters and the attached urinary collection bags, we next investigated the cytotoxicity properties of \(1.\text{Eu in vitro}\). Both the complex \(1.\text{Eu}\) and urea were found to be relatively low in toxicity and potency, with an IC\(_{50}\) value of 200 \(\mu\text{M}\) and 10 mM being given for each, respectively (see Supporting Information, Figure S13). These high IC\(_{50}\) values suggest that prosthetic medical devices, such as catheters, impregnated with \(1.\text{Eu}\) would not be expected to have any major negative impact on a patients’ health or body.

### Table 1. Summary of the activation energy and pre-exponential factor for the urease-mediated hydrolysis of urea in aqueous solution, determined from the quenching in Eu(III) emission of \(1.\text{Eu}\).

<table>
<thead>
<tr>
<th>Urease / U</th>
<th>(E_a) / kJ mol(^{-1})</th>
<th>(A) / min(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>46.7 ± 2.3</td>
<td>3.2 (\times 10^7) ± 140</td>
</tr>
<tr>
<td>0.03</td>
<td>42.9 ± 2.8</td>
<td>5.2 (\times 10^6) ± 190</td>
</tr>
<tr>
<td>0.04</td>
<td>43.1 ± 2.9</td>
<td>4.5 (\times 10^6) ± 180</td>
</tr>
<tr>
<td>0.05</td>
<td>46.8 ± 3.4</td>
<td>1.3 (\times 10^7) ± 220</td>
</tr>
</tbody>
</table>

Water-permeable hydrogels impregnated with \(1.\text{Eu}\)

Having studied the photophysical properties of \(1.\text{Eu}\) in solution, we next turned our attention towards forming soft luminescent biomaterials; the intention being that they could be utilized as diagnostic materials to detect the onset of CAUTIs.

With this in mind, the complex \(1.\text{Eu}\) was non-covalently impregnated within water-permeable hydrogels using a poly(HEMA) matrix, which was prepared according to our previously published methodology.\(^{14a,16}\) A homogenous solution of HEMA, EGDMA, and \(1.\text{Eu}\) were polymerized under free-radical polymerization using AIBN to afford a hard brittle acrylic monolith with the complex encapsulated (0.033% w/w). The monolith was then swelled in deionized water to yield soft polymer hydrogel materials of \(1.\text{Eu}\), which were transparent to the naked eye under ambient light and red emissive upon irradiation at 254 nm, as is evident from Figure 5.

As anticipated, the characteristic spectroscopic properties of complex \(1.\text{Eu}\) were retained upon encapsulation within the hydrogel. The Eu(III)-centered emission spectra, which was obtained by suspending the swelled gel (30 mm \(\times 10\) mm \(\times 1.2\) mm) in a stirred solution of Millipore H\(_2\)O, matched exactly with that observed for \(1.\text{Eu}\) in solution (Figure 6; c.f \(1.\text{Eu}\) (Hydrogel)); this suggests that the coordination environment of the Eu(III) ion was not substantially changed by the polymeric matrix. Furthermore,
emission was quenched by over again red shifted by from Figure 7. Repetition of these measurements using a response was also visible to the naked eye under UV light, prominently bright red (where by the gel was seen to change from being predominant the solution studies, the photophysical parameters of the of urease (0.5 U) at 22 °C, using urea as the supernatant. Both the UV-vis absorption and luminescence spectra coincided with that observed in solution for 1.Eu (Hydrogel)).

To ascertain whether these luminescent hydrogels were also pH-responsive, their ability to monitor the hydrolysis of urea into carbamic acid and sense the associated change in pH from acidic to basic, was studied. As with the solution studies, the photophysical parameters of the gel were recorded both before and 200 min after the addition of urease (0.5 U) to 22 °C, using urea as the supernatant. The UV-vis absorption and luminescence spectra were recorded both before and in 200 min after the addition of urease (0.5 U) to 22 °C, using urea as the supernatant. Both the UV-vis absorption and luminescence spectra coincided with that observed in solution for 1.Eu, as shown in Figure 6; the π-π* band in the UV-vis was once again red shifted by ca. 10 nm, while the Eu(III)-centered emission was quenched by over 85%. This 'on-off' response was also visible to the naked eye under UV light, where by the gel was seen to change from being predominantly bright red (on) to a faint pink/purple (off) color upon hydrolysis of the urea in solution, which is evident from Figure 7. Repetition of these measurements using a higher concentration of enzyme (1.0 U) and an elevated temperature (37°C) resulted in exactly the same spectroscopic trends being evidenced (see Supporting Information, Figure S14 – 19). However, the rate at which the Eu(III) emission was 'switched off' varied. Increasing both the enzyme (0.5 → 1.0 U) and the substrate concentration (2.3 × 10⁻³ → 10 × 10⁻³ M of urea) gave rise to a faster rate of hydrolysis, and consequently increased the rate of luminescent decay exhibited for the hydrogel by 1.5 fold (see Figure 8). Furthermore, raising the temperature of the system to physiological temperature (37 °C) also caused the rate of quenching to substantially increase, with the 'on-off' response being observed in less than 60 min (see Supporting Information, Figure S20). Unlike the solution studies, which are instantaneous, the response time of the hydrogels are diffusion controlled. As such, diffusion of the substrate and enzyme into the hydrogel matrix depends on the ambient conditions, as well as both the gels thickness (diffusion length) and surface area (accessible pores). While this response time is slower than that exhibited in solution, it is still relatively fast, which signifies the potential of this design for clinical use.

To ensure that these hydrogels were robust and that the complex did not leech out of the polymeric matrix, we also recorded the emission spectra of the supernatant solution over a 2 week period. Only a negligible amount of leeching of 1.Eu from the cross-linked matrix occurred at room temperature (see Supporting Information, Figure S21 – 22), indicating that the complex was indeed retained.

![Figure 7](image_url)

**Figure 7.** Photographs of the a) 'on' and b) 'off' state of the hydrogel, when irradiated at λmax = 254 nm. Left image was acquired before the addition of urease and the right image 200 min after.

![Figure 8](image_url)

**Figure 8.** Changes in the delayed-Eu(III) emission of the swelled 1.Eu-based hydrogel as a function of time in an aqueous solution of urea at 295 K (λexc = 266 nm), measured at 580 nm, 593 nm, 615 nm, 654 nm, 683 nm, and 701 nm upon the addition of urease. Concentrations of urea and urease utilized were a) 2.3 × 10⁻³ M, 0.5 U, b) 2.3 × 10⁻³ M, 1.0 U, c) 10 × 10⁻³ M, 0.5 U, and d) 10 × 10⁻³ M, 1.0 U.
within the hydrogel. However, under physiological conditions (37 °C) the complex slowly leached out into the supernatant solution; a gradual, but minimal increase in the metal-centered emission of the supernatant was observed for 7 days, after which the intensity plateaued (see Supporting Information, Figure S23 – 24).

CONCLUSIONS

In this article, we report on the development and application of a pH-responsive luminescent probe that can be impregnated within soft polymer-based hydrogels to signal both biofilm formation and encrustation within catheters in the clinic. Specifically, we have demonstrated that this Eu(III)-based macrocyclic probe, 

1. Eu, is capable of detecting the pH changes associated with the enzymatic hydrolysis of urea both in solution and when encapsulated within hydrogels. In particular, this system has been shown to behave in an ‘on-off’ responsive manner; as the urea is hydrolyzed to ammonia, the pH of the system changes from being mildly acidic to neutral/slightly alkaline, and hence results in the luminescence of 1. Eu being quenched. Although the gel studies were only conducted for 7 days, after which the intensity plateaued (see Supporting Information, Figure S23 – 24).

ASSOCIATED CONTENT

Synthetic details, characterization, additional spectroscopic data for both the in solution and hydrogel studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript. / Notes
Any additional relevant notes should be placed here.

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ABBREVIATIONS

CAUTI, catheter-associated urinary tract infection; CABB, catheter-associated bacteriuria; phen, 1,10-phenanthroline; Ln(III), lanthanide; Ea, Activation energy; A, pre-exponential factor; HEMA, 2-hydroxyethyl methacrylate; EGDMA, Ethylene glycol dimethacrylate; AIBN, azobisisobutyronitrile.

REFERENCES


18. Owing to the rapid rate of hydrolysis, it was not feasible to monitor the changes in phosphorescence at 37 °C for quantities of enzyme > 0.08 U.
