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Hydrolysis of 3-carboxy-6,8-difluoro-7-hydroxycoumarin (Pacific Blue™) succinimidyl ester under acidic and basic conditions

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ABSTRACT

The highly sensitive technique of microchip capillary electrophoresis (μCE) with laser-induced fluorescence (LIF) detection is under development for future in situ spaceflight missions to search for the organic chemical signatures of life. One fluorescent probe that enables this technology for amine, amino acid, and dipeptide analysis is 3-carboxy-6,8-difluoro-7-hydroxycoumarin (Pacific Blue™, PB) succinimidyl ester. Of particular importance is the hydrolysis of PB succinimidyl ester, which precludes long-term aqueous storage during spaceflight and therefore has a significant impact on instrument design and operation. As such, it is important to characterize the chemical stability of this dye to hydrolysis prior to spaceflight. Here, we study the hydrolysis kinetics of the PB succinimidyl ester at pH values between 3 and 10.5 using μCE-LIF. The PB succinimidyl ester has the longest lifetime at pH 4 (7.3 ± 0.1 h), with dramatically shorter half-lives in the basic pH regime. This work represents a first step in the full characterization of this fluorescent probe for spaceflight applications.

1. Introduction

One approach in the robotic in situ search for extraterrestrial life is to perform quantitative compositional analysis on mixtures of organic compounds on alien worlds. Because we cannot assume extraterrestrial life chose the same set of essential organic molecules or the same chirality found on Earth, detection of extraterrestrial life will rely upon techniques capable of quantitative analyses of a broad range of organic molecules. Broad compound class coverage yields valuable information about a sample because abiotic processes yield racemic mixtures of a statistical distribution of organic molecules, while biotic processes yield homochiral mixtures enriched with organic compounds essential for life [1]. Extraterrestrial targets potentially suitable for life in the past or present include Mars, Enceladus, and Europa [2]. These targets exhibit indications of an oxidizing chemistry [3] which would limit quantitative organic chemical compositional analyses using flight-heritage pyrolysis followed by gas chromatography (GC) coupled to mass spectrometry (MS). This is due to the degradation of complex organics during pyrolysis under oxidizing conditions [4]. In contrast, liquid-based methods for extraction and analysis of organic molecules are more robust to oxidizing chemical environments. Additionally, complex organic molecules that could be indicative of life may be present in such low quantities on extraterrestrial targets that methods with pM sensitivity or lower may be required for unambiguous detection of extraterrestrial life or key molecular intermediaries required for the emergence of life.

Many analytical methods have been used or proposed for spaceflight applications. The most commonly implemented technique on space probes has been pyrolysis followed by GC–MS. However, liquid-based μCE-LIF analysis, due to its high sensitivity and low sample, reagent, buffer, and power consumption [6], is of particular interest for future missions. The fluorescent probe 3-carboxy-6,8-difluoro-7-hydroxycoumarin (Pacific Blue™, PB) succinimidyl ester [7] has enabled highly sensitive (pM, sub pptr) analyses of amines, amino acids [5,6] and dipeptides [8] in astrobiologically relevant samples including the Murchison meteorite and regolith collected from the Atacama Desert [6d]. However, the chemical stability of the fluorescent probe used in μCE-LIF must be characterized before it can be considered sufficiently robust for spaceflight.

Abbreviations: PB, 3-carboxy-6,8-difluoro-7-hydroxycoumarin (Pacific Blue™); μCE, microchip capillary electrophoresis; LIF, laser-induced fluorescence.

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Of particular importance is the hydrolysis reaction of PB succinimidyl ester, which precludes long-term aqueous storage during spaceflight. Because this reagent must be stored dry and rehydrated in situ, it has a significant impact on instrument design and operation. The complexity of the microchip layout and therefore instrument design increases with each additional reservoir of dehydrated dye. To simplify the instrument design, it is of extreme importance to minimize dehydrated reservoirs. This can be accomplished by increasing the number of experiments that can be performed between rehydration and extensive dye hydrolysis. Therefore, it is critical to explore chemical ways to increase the longevity of PB succinimidyl ester and other hydrolysis-prone aqueous reagents. Here, we characterize the hydrolysis kinetics of PB succinimidyl ester in aqueous solution under acidic and basic conditions using microchip capillary electrophoresis (μCE) with laser-induced fluorescence detection (LIF). This work furthers the chemical characterization of this fluorescent probe for spaceflight and is essential for future experiment and instrument design.

2. Materials and methods

2.1. Materials

3-carboxy-6,8-difluoro-7-hydroxycoumarin (Pacific Blue™, PB) succinimidyl ester was purchased from Invitrogen Corporation (Carlsbad, CA). Sodium tetraborate ($Na_2B_4O_7 \bullet 10H_2O$) was purchased from Fisher Scientific (Fair Lawn, NJ) and used to prepare 100 mM aqueous solutions with 18 MΩ•cm water. The pH was adjusted using either 1 M NaOH or 1 M HCl (Sigma–Aldrich, St. Louis, MO) and measured using a glass electrode and a digital pH meter (Orion 290A, Thermo; Waltham, MA).

2.2. μCE analysis

MicroCE-LIF was conducted utilizing commercial microdevices from Micralyne (Edmonton, Canada) and external supporting hardware that has been described previously [6c]. Briefly, a 405 nm diode laser (CVI Melles Griot, Carlsbad, CA) was used for excitation in laser-induced fluorescence detection. Fluorescence detection optics consisted of a commercial Nikon Eclipse TE2000-U inverted microscope system with a CCD camera (Cascade 650, Photometrics). A LabSmith HVS448 High Voltage Sequencer (Livermore, CA) was used to drive electrophoresis. Separations were conducted using 100 mM borate buffer (pH 9.2) in the separation channel with previously published injection and separation potentials [6c]. Data was processed using PeakFit (Systat Software Inc., San Jose CA). Electropherograms were baseline-corrected and filtered using a 0.2% Loess function. Peak migration times were corrected using Origin 8.1 (OriginLab Corporation, Northampton, MA) to account for run-to-run variations.

2.3. Hydrolysis rate determination

The hydrolysis kinetics of 3-carboxy-6,8-difluoro-7-hydroxycoumarin (PB) succinimidyl ester at different pH values and room temperature (~25 °C) were determined by μCE-LIF analysis. PB succinimidyl ester was first dissolved to 20 mM in DMF. It was subsequently dissolved in 100 mM aqueous borate solutions at various pH values for each hydrolysis experiment. Fresh PB succinimidyl ester solutions were prepared for each experiment. At high pH where hydrolysis was empirically determined to be rapid, the hydrolysis solution was directly pipetted into the sample well and sampled every few minutes via repeated electrophoretic injections. At lower pH where hydrolysis occurred on longer time-scales, the solutions were stored in capped Eppendorf tubes between electrophoretic analyses. Although many of these pH values were out of the buffering regime of borate, it was used over the entire pH range to maintain consistent electrolyte composition. Due to the very different rates of reaction at different pHs, 20 μM PB succinimidyl ester was chosen for pH < 10 and 100 μM PB was chosen at pH >10. Hydrolysis half-lives were determined by monitoring the decline in peak area of the un-hydrolyzed dye peak.

3. Results

The primary hydrolysis reaction of PB succinimidyl ester is the hydrolysis of the succinimidyl ester moiety (Fig. 1). This hydrolysis was studied using microchip capillary electrophoresis (μCE) with laser-induced fluorescence (LIF) detection; an example electropherogram is shown in Fig. 2. Because optimal separation of these species has been demonstrated at pH 9.5, this pH was chosen for the separation buffer. Therefore, the hydrolysis rates shown at low pH are slightly higher than their true value due to the ~2 min spent in the separation column. Three peaks are observed in the electropherogram: the first peak (~35 s) corresponds to the PB succinimidyl ester and the later peaks to hydrolysis products. Due to the formation of multiple hydrolysis products under basic conditions, the hydrolysis rate was quantified by the rate of disappearance of the PB succinimidyl ester peak as a function of time. An example of this plot is given for pH 8 in Fig. 3. Pseudo-1st order kinetics are expected given the high concentration of the reactant water (~55 M) compared to the reactant PB succinimidyl ester (100 μM). This hypothesis was experimentally confirmed by the fact that data follows an exponential decay function ($R^2 > 0.98$) as expected for first order kinetics (see supporting information). This enabled the calculation of a half-life of PB succinimidyl ester using a pseudo-1st order approximation.

The mechanism of hydrolysis (Figures S01 and S02 in the supporting information) suggests that it should be both acid- and base-catalyzed. In order to explore aqueous conditions enabling a longer PB succinimidyl ester half-life, we monitored the decay of aqueous PB succinimidyl ester over the pH range of 3–10.5 (Fig. 4). At all pH values the data follows an exponential decay function ($R^2 > 0.97$), as pseudo-1st order kinetics are expected due to the high concentration of the reactant water and the catalytic nature of OH^- and H^+. We found the longest half-life to be 7.3 ± 0.1 h at pH 4 with a nearly-linear decline ($R^2 = 0.97$) to 9 ± 2 min at pH 9. Half-lives were <10 min at pH > 9, indicating that aqueous storage of PB succinimidyl ester at optimal amino acid labeling conditions (pH 9.5) [6c] would be unacceptable. However, short-term storage at pH 4 would be feasible.

4. Discussion

Microchip capillary electrophoresis (μCE), a relatively recently developed analytical method, was chosen for this study over other

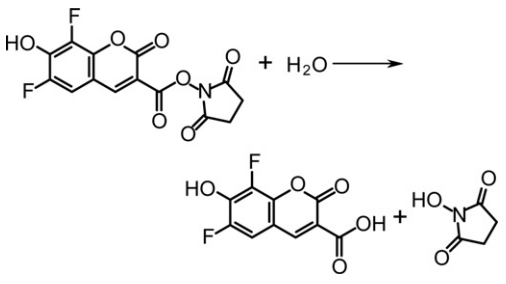

Fig. 1. The hydrolysis reaction of 3-carboxy-6,8-difluoro-7-hydroxycoumarin (Pacific Blue™, PB) succinimidyl ester with water.

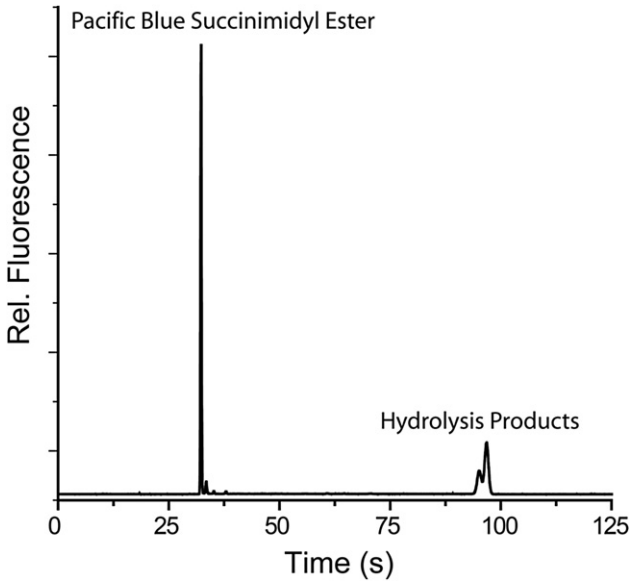

Fig. 2. Electropherogram of the separation of a 20 μM solution PB succinimidyl ester (pH 9) that has partially hydrolyzed.

techniques for a number of reasons. CE, whether conducted in traditional capillaries or in a chip-based microchannel, requires small sample volumes (~1–10 μL). When coupled to laser-induced fluorescence detection, sensitivities in the pM regime can be achieved [5,6c–d]. These factors combined enabled us to use a mere 5.2 mg of the relatively expensive PB succhinimidyl ester (~$44/mg) for the entire μCE study presented here. Other analytical techniques are not only generally less sensitive, thus requiring higher concentrations of analyte, but also require larger sample volumes. For example, 5 mg was required for each attempted NMR experiment, leading to a single experiment reagent cost approximately

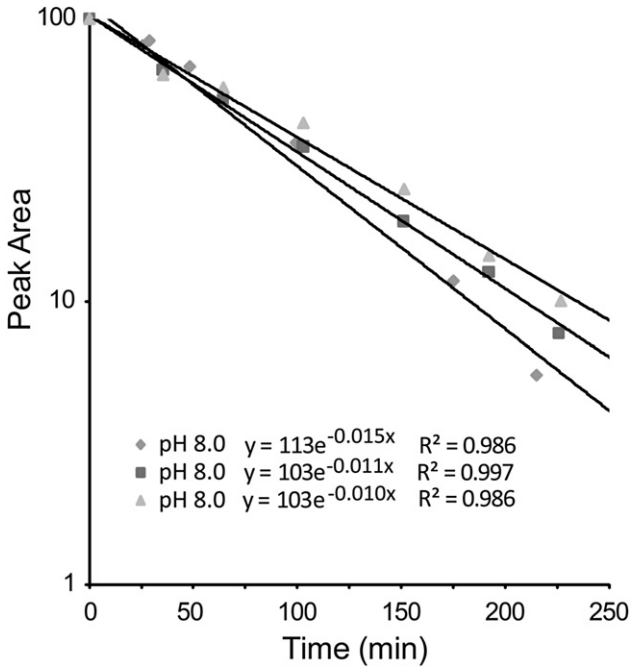

Fig. 3. The decline in PB succinimidyl ester μCE peak area over time at pH 8. Peak areas were normalized to the initial data-collection peak area. Data collected using 100 mM borate, pH 9.5 in separation channel and pH 8 in the sample. Each data point represents one experiment.

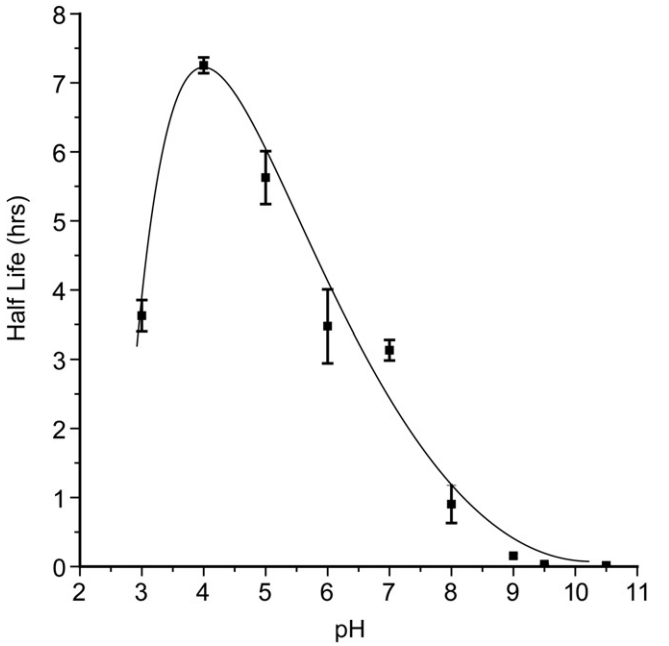

Fig. 4. Half-lives of PB succinimidyl ester (PBSE) at various pH. Data was obtained using μCE with 100 mM borate buffer, pH 9.5, in the separation column and the indicated pH in the sample. Initial PBSE concentrations were 20 μM for all pH except those >10, where 100 μM was used. Pseudo-1st order kinetics models fit the hydrolysis data ($R^2 > 0.97$) at all pH values examined. Error bars are calculated from the standard deviation of 3 independent kinetics experiments. The line "fit" is meant to guide the eye and can be misleading at higher pH.

equal to the total reagent cost for our entire microchip CE study ($234). Additionally, microchip CE is capable of achieving separation of unreacted PB succinimidyl ester from its hydrolysis products with analysis times of less than 2 min, while traditional CE using long (>20 cm) capillaries can require >10× analysis times. At higher pH where the dye has an aqueous half-life on the order of minutes, traditional CE may be a less feasible technique to study this reaction.

It must be noted that at least two distinct fluorescent hydrolysis products are observed in the electropherograms (Fig. 2). In an attempt to determine the identity of these products, we conducted a series of UV–Vis spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry (MS) experiments that varied both hydrolysis time and pH (supporting information). Because of the ability of these techniques to detect a vast array of chemical species, we observed that many other species were formed in addition to the two distinct fluorescent species. The multitude of chemical species formed made it impossible to determine the exact identity of the two fluorescent species, and highlight the usefulness of a separation-based analytical technique, such as microchip CE, when undertaking a study examining only one component of a complex mixture.

Optimal amino acid labeling and separation of labeled amino acids both occur at approximately pH 9.5 [6c], while we have demonstrated that optimal short-term aqueous storage occurs at pH 4. This indicates that a future mission using PB succinimidyl ester could carry the dehydrated dye, pH 4 buffer, and a pH 9.5 buffer. With the appropriate combination of a weak pH 4 buffer system and a strong pH 9.5 buffer system, the mission could be capable of storing PB succinimidyl ester at pH 4 and adjusting to the appropriate labeling and separation pH of 9.5 for discrete aliquots of the aqueous dye.

The temperature regimes of potential in situ missions could range from as high as 60 °C for a fly-by mission to collect samples from the plume of Enceladus to below 0 °C for landed missions to

Mars, Europa, Enceladus, or Titan. However, the instrument would be housed in or near a temperature-controlled electronics "warm box," and thus would not experience ambient conditions at any landed location. Here, we studied hydrolysis at room temperature (~25 °C) due to the complexity of maintaining consistent reduced or elevated temperatures throughout both hydrolysis and analysis. We are developing capabilities to conduct temperature-dependent studies in future work. While it must be noted that hydrolysis rates will increase at higher temperatures and decrease at lower temperatures, the data presented here provides a baseline model both for researchers concerned with Earth-bound studies (potentially using other succinimidyl ester-based dyes) and also for our future work exploring various temperature regimes expected on future planetary missions.

The dye characterized here is unique in its aqueous reaction efficiency, high extinction coefficient and quantum yield, making it ideal in the search for extraterrestrial life by enabling ultra-highly sensitive amine and amino acid analyses. However, other fluorescent probes even more robust to hydrolysis, including those capable of labeling additional compound classes, would be extremely valuable for future missions of planetary exploration. These probes would enable broader coverage of the entire library of possible organic chemicals, increasing the chance that potential future μCE in situ instrumentation will be able to unambiguously fingerprint extraterrestrial life in our Solar System.

5. Conclusion

This work provides a much-needed step in the full characterization of the amine-reactive probe PB succinimidyl ester for both terrestrial and spaceflight applications. The lifetime of this probe in aqueous conditions dictates how the dye should be stored and handled and consequently impacts the design of the spaceflight instrument. Based on the longest half-life of PB succinimidyl ester in water (7 h, pH 4), if the dye is stored dry and rehydrated on-location prior to analysis, a pH 4 buffering system must be incorporated to preserve the longevity of the dye after rehydration. Additionally, if the dye is rehydrated on-location to 100 mM at pH 4, it will be useable for over 91 h (3.8 days) before the concentration drops below 10 μM. Therefore, any instrument using aqueous solutions to store this reagent will need to carry a new aliquot of dehydrated dye for roughly every four Earth days of operation. Due to the inherently low reagent sample volumes and concentrations needed for μCE analysis, a spaceflight system carrying a mere 5 mg ($221 reagent cost) would be capable of conducting over 100 analyses. This work represents the first step forward in fully characterizing PB succinimidyl ester for spaceflight applications and will have a major impact in the design of μCE-LIF instrumentation for future missions.

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Appendix A. Supplementary data

Supplementary data related to this article can be found in the online version at doi:10.1016/j.dyepig.2012.08.005.

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