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pH-Dependent Si-Fluorescein Hypochlorous Acid Fluorescent Probe: Spirocycle Ring-Opening and Excess Hypochlorous Acid-Induced Chlorination

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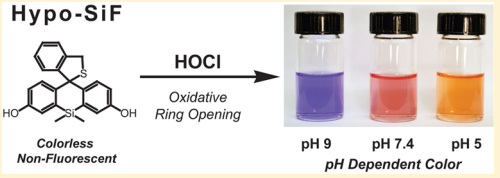
Supporting Information

ABSTRACT: We report the synthesis and characterization of a fluorescent probe (Hypo-SiF) designed for the detection of hypochlorous acid (HOCl) using a silicon analogue of fluorescein (SiF). The probe is regulated in an "off−on" fashion by a highly selective thioether spirocyclic nonfluorescent structure that opens to form a mixture of fluorescent products in the presence of HOCl. Over a range of pH values, the probe reacts with a stoichiometric amount of HOCl, resulting in a mixture of two pH-dependent fluorescent species, a SiF disulfide product and a SiF sulfonate product. The unique colorimetric properties of the individual SiF fluorophores were utilized to perform simultaneous detection of HOCl and pH. When an excess of HOCl is present, the SiF fluorophores become chlorinated, via an intermediate halohydrin, resulting in a more pH independent and red-shifted fluorophore.

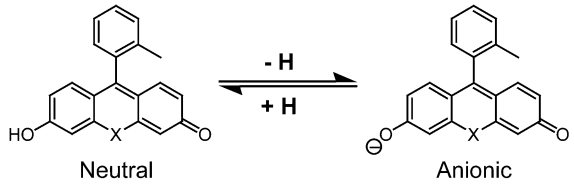


INTRODUCTION

Reactive oxygen species (ROS) are of scientific interest because of their role in biology and their link to a number of diseases. Among the known ROS associated with biological processes, hypochlorous acid (HOCl) has received an increasing amount of attention. Its role in animal immune systems, specifically during phagocytosis, is well documented. However, abnormal production of HOCl has been linked to a variety of diseases, including cystic fibrosis,[1] kidney disease,[2] and certain cancers.[3] Development of imaging techniques for HOCl is therefore of great interest to better our understanding of its role in the immune system and its link to the aforementioned diseases. Fluorescence is widely considered to be an advantageous imaging technique because of its high sensitivity and relatively low technical costs.

A variety of fluorescent probes designed for specific detection of HOCl have been reported. These probes have a HOCl reactive moiety that modulates the fluorescence of the fluorophore, examples of which have included BODIPY,[4] fluorescein,[5] rhodamine,[6] and a silicon analogue of rhodamine (SiR).[7] The SiR fluorophores are an interesting new class of fluorophores because their subtle difference in the heteroatom, i.e., replacing oxygen with silicon, leads to a large red shift (90 nm) in the absorption and emission spectra relative to rhodamine.[7,8] Nagano et al. reported that this approach was also shown to have a comparable red shift (90 nm) on a fluorescein-based analogue.[9] They reported the synthesis of a silicon analogue of their famous Tokyo Green (TG) that was named Tokyo Magenta (TM) because of the significant red shift. In addition to the significant red shift of this fluorophore, a dual-color acid/base property was reported. Like the TG series, TM can exist in the neutral or the anionic form (Scheme 1). However, TM shows a more pronounced change in the absorption spectra as the fluorophore goes from the neutral to the anionic form (110 nm), which gives rise to a clearly distinguishable change in color, i.e., from yellow to magenta.

Scheme 1. Neutral and Anionic Forms of Fluorescein Derivatives Tokyo Green and Tokyo Magenta



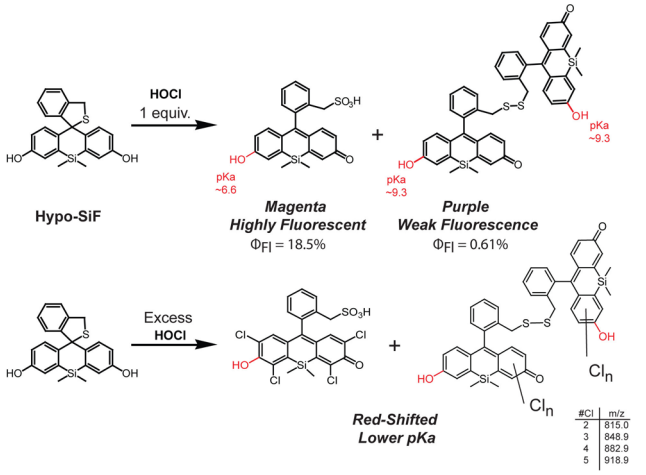
X = O "Tokyo Green"
X = $Si(CH_3)_2$ "Tokyo Magenta"

On the basis of this colorimetric property of TM, we chose to couple the silicon analogue of fluorescein (SiF) fluorescent core with a spirocyclized moiety to control the fluorescence. We used a spirothiophene moiety, which was shown to have a selective response to HOCl in the rhodamine[6] and SiR[7] systems. Over a range of pH values, the probe reacts with a stochiometric amount of HOCl, resulting in a mixture of two pH-dependent fluorescent species, a SiF disulfide (**8**) product and a SiF sulfonate (SiF-SO_3H) product (Scheme 2). The unique colorimetric properties of the individual SiF fluorophores were utilized to perform simultaneous detection of HOCl and pH. When an excess of HOCl is present, the SiF fluorophores undergo chlorination, resulting in a red-shifted, more pH independent fluorophore (Scheme 2) and allowing for the detection of a larger concentration range (0−80 equiv) of HOCl compared to similar probe systems.[6a,7] Herein, we

Received: February 15, 2013

   dx.doi.org/10.1021/ja401426s | J. Am. Chem. Soc. XXXX, XXX, XXX−XXX

Scheme 2. Oxidative Ring-Opening of Hypo-SiF with Stoichiometric and Excess Amounts of HOCl

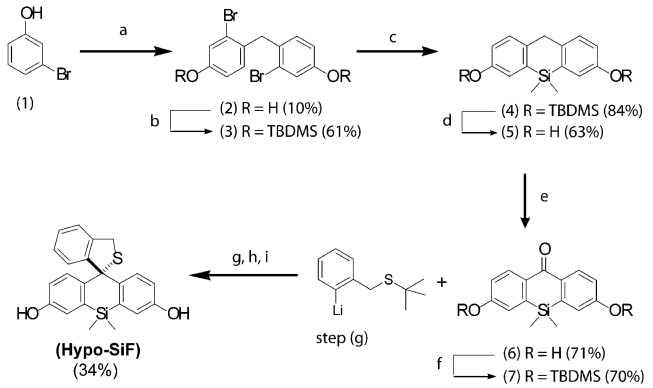


present our synthetic approach to a spirocyclized SiF probe and its fluorescence and colorimetric response to HOCl and pH.

RESULTS AND DISCUSSION

Synthesis of HOCl Probe. The Hypo-SiF probe was synthesized starting from 3-bromophenol (Scheme 3). The

Scheme 3. Synthesis of Hypo-SiF[a]



[a]Reagents and conditions: (a) H_2CO, hydrochloric acid (HCl)/methanol, 50 °C; (b) *tert*-butyldimethylsilyl chloride ((TBDMS)Cl), imidazole, DMF, rt; (c) *n*-BuLi, dimethylsilyl chloride, tetrahydrofuran (THF), −78 °C to rt; (d) tetrabutylammonium fluoride (TBAF), THF, rt; (e) DDQ, methanol, rt; (f) (TBDMS)Cl, imidazole, DMF, rt; (g) 1-bromo-2-[(*tert*-butylsulfanyl)methyl]benzene, *n*-BuLi, THF, −78 °C to rt; (h) TBAF, THF, dilute HCl, rt; (i) TFA, reflux.

condensation of 3-bromophenol with formaldehyde in an acidic medium yielded **2**, which was then protected with the *tert*-butyldimethylsilyl (TBDMS) protecting group on the phenolic oxygens to give compound **3**. The next step was lithiation of compound **3** to provide a nucleophilic species that was trapped with dimethyldichlorosilane, forming the cyclized product **4**, which after deprotection of the TBDMS groups gave compound **5**. Compound **5** was then oxidized to afford the ketone intermediate **6**. Unfortunately, oxidation of the protected cyclized product with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) gave a very low yield, so it was necessary to remove the TBDMS group before the oxidation step. Reprotection of the phenolic alcohols on compound **6** with the TBDMS group furnished compound **7** in good yields. Lithiation of (2-bromobenzyl)(*tert*-butyl)sulfane with *n*-butyllithium (*n*-BuLi) formed the lithiated nucleophile, which was used to react with compound **7**. The alcohol species, resulting from the nucleophilic attack, simultaneously underwent dehydration during deprotection of the TBDMS group. Further treatment of the mixture with trifluoroacetic acid (TFA) removed the *tert*-butyl group, leading to the spirocyclized product Hypo-SiF.

HOCl Titrations and Probe Specificity. Phosphate buffer solutions containing 5 μM Hypo-SiF and 0.5% dimethylformamide (DMF) for dissolution were prepared in various pH (4–9) solutions. These solutions, in the absence of HOCl, are colorless and nonfluorescent because the probe maintains its spirocyclic structure. At pH 7.4, the addition of HOCl results in a magenta-colored solution. The UV–vis spectrum of this solution shows a λ_{abs} at 586 nm (Figure 1A). Excitation at this

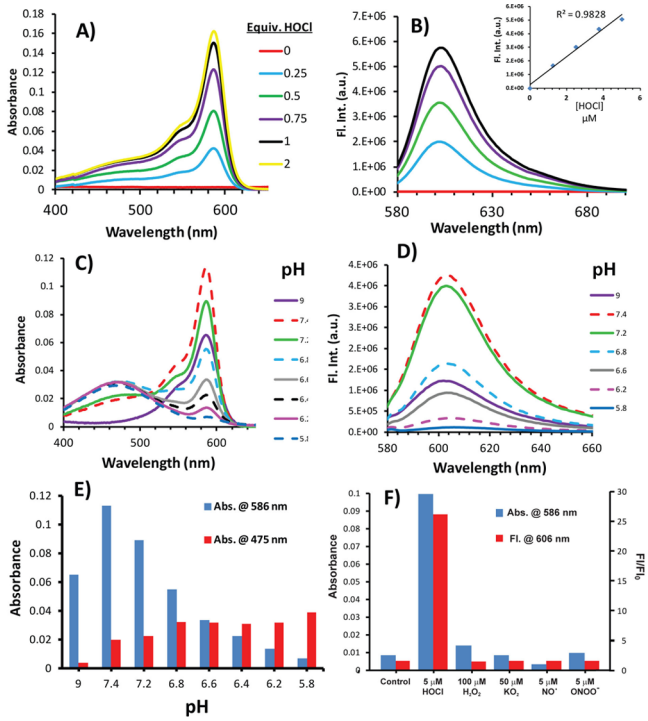


Figure 1. Response of a 5 μM solution of Hypo-SiF with HOCl in pH 7.4 phosphate buffer solution and 0.5% DMF. (A) Absorption spectra and (B) emission spectra with excitation at 570 nm (1 equiv of HOCl added to a 5 μM concentration of the probe in different pH buffer solutions). (C) Absorption spectra and (D) emission spectra with excitation at 570 nm. (E) λ_{586} and λ_{475} absorption. (F) Specificity of the probe under similar conditions with various ROS.

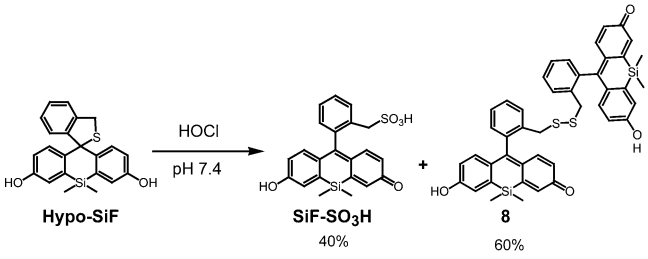
wavelength results in a strong fluorescence band centered around 606 nm (Figure 1B). The intensities of these bands increase linearly with additional amounts of HOCl, leading to a stoichiometric amount. When 1 equiv of HOCl is added to acidic solutions containing Hypo-SiF, a pH-dependent trend in the UV–vis and fluorescence response is observed. Specifically, the λ_{586} band begins to decrease, while a new relatively broad band at 475 nm increases as the solutions containing HOCl become more acidic (Figure 1C). The corresponding fluorescence spectra of these solutions show that the probe's fluorescence intensity decreases under increasingly acidic conditions (Figure 1D). The anomalously low response from

dx.doi.org/10.1021/ja401426s | J. Am. Chem. Soc. XXXX, XXX, XXX–XXX

pH 9 indicates that the probe is responsive toward HOCl and not the hypochlorite anion, which has a pK_a = 7.54. The ratio between the λ_{586} and the λ_{475} bands remains constant as the concentration of HOCl increases to 1 equiv. Therefore, the fluorescence and UV−vis intensities and the ratio between the λ_{586} and the λ_{475} bands can be used to indicate the concentration of HOCl and the pH of the solution simultaneously (Figure 1E). When the probe was tested against other biologically relevant ROS, it was found to have a high degree of specificity for HOCl. Hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$), and peroxynitrite ($ONOO^-$) were all found to have little or no enhancement in the absorbance and fluorescence of the probe (Figure 1F). The selectivity observed in this system is believed to be due to the higher oxidative strength of HOCl compared to other ROS and the stability of the thioether probe, which is consistent with similar probe systems.[6a,7]

Isolation and Characterization of SiF Chromophores. On the basis of the complex response that was observed, we decided to isolate the highly fluorescent products to identify the species involved in the ratiometric response. This was achieved by oxidizing Hypo-SiF with HOCl on a bulk scale at pH 7.4, followed by immediate extraction with EtOAc, which allowed for an 86% recovery by mass of a brightly colored mixture. Upon further separation of the extracted fraction by chromatography, it was shown to contain two compounds, SiF sulfonate species (SiF-SO_3H) and a SiF disulfide (**8**), as shown Scheme 4. Both species are consistent with previous studies on the oxidation of thiols by HOCl.[6a,7,10]

Scheme 4. Oxidation of Hypo-SiF in pH 7.4 Buffer Solution Forming SiF-SO_3H and Disulfide 8[a]



[a]Percent composition determined by NMR.

We then investigated the pH-dependent photophysical properties of the isolated species. Individually, these chromophores' spectra (Figure 2 A−D) resemble the response observed from the Hypo-SiF probe (Figure 2 A−D). Both compounds have λ_{586} and λ_{475} absorption bands that vary depending on the pH (Figure 2 A,B). However, differences in the phenolic proton pK_a between the two chromophores were observed.

The pH dependence of the absorption spectra from **8** was found to differ slightly from that of SiF-SO_3H. The absorption spectra of **8** show a more sudden decrease in the 586 nm band accompanied by an increase in the 475 nm band as the pH is lowered from 10 to 7.4 (Figure 2B). These differences in the spectral response of **8** relative to SiF-SO_3H indicate that SiF-SO_3H has a lower pK_a than **8**. From the UV−vis pH titration curve of SiF-SO_3H, a single inflection point is observed corresponding to a pK_a ≈ 6.6 for phenolic proton (Figure 2E), defined as half the maximum. Compound **8** is a diprotic system;

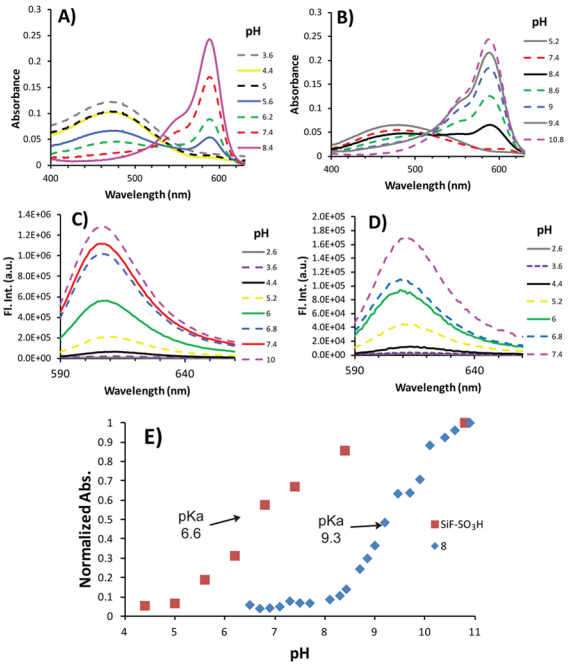


Figure 2. Photophysical properties of 5 μM SiF-SO_3H (A, C) and 5 μM **8** (B, D) buffer solutions with 0.5% DMF. Fluorescence spectra were excited at 570 nm. Titration curves (E) are from λ_{max} at 586 nm.

however, the individual phenolic protons appear to have similar pK_a values, i.e., pK_a ≈ 9.3 (Figure 2E).

The fluorescence spectra of **8** and SiF-SO_3H show a similar pH dependence, which correlates well with their absorption spectra (Figure 2C,D).

In addition to differences in the pH-dependent photophysical properties of the two fluorescent species, the fluorescent intensity was also notably lower for the disulfide species. The sulfonate product SiF-SO_3H displays a bright fluorescence with a quantum yield of 18.5%, whereas the disulfide shows a lower quantum yield and molar absorptivity (Table 1). The relatively

Table 1. Photochemical Properties of Hypo-SiF, Compound 8, and SiF-SO_3H[a]

	λ_{abs} (nm)	molar exctinction coeff ($cm^{-1}M^{-1}$)	λ_{Fl} (nm)	quantum yield (Φ_{Fl})[b]
Hypo-SiF	nd	nd	nd	nd
8	586	69000	606	0.0061
SiF-SO_3H	586	102000	606	0.185

[a]The photochemical properties of Hypo-SiF, compound **8**, and SiF-SO_3H were measured in 0.1 M NaOH/H_2O solution. [b]Determined by using an average from rhodamine B and rhodamine 6G in ethanol as the standard reference.

low fluorescence from **8** may be due to quenching from intramolecular fluorescence resonance energy transfer (FRET), which has been reported in a variety of other homodimers.[11]

Colorimetric Detection of HOCl and pH. The pH-dependent response of Hypo-SiF is potentially useful in environmental analysis of HOCl and could also benefit from the colorimetric response provided by Hypo-SiF at different pH values. For example, maintenance of a swimming pool requires one to monitor HOCl and pH levels. Commercially available colorimetric indicator solutions for pH and HOCl are typically

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sold together as a kit for proper pool maintenance. Orthotolidine provides varying shades of yellow, allowing for detection of 0.5−10 ppm HOCl in solution, while a separate solution with phenol red displays a colorimetric response indicating the pH. However, the probe Hypo-SiF provides a colorimetric response, indicating both pH and HOCl concentration, by generating a distinct color for different pH values with HOCl (Figure 3). The varying degrees of these

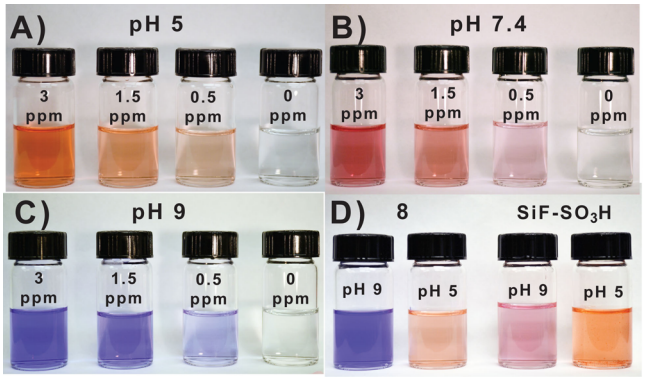

Figure 3. Colorimetric response of Hypo-SiF to increasing concentrations of HOCl at pH 5 (A), pH 7.4 (B), and pH 9 (C). Simultaneous colorimetric detection for different concentrations of HOCl and pH values was obtained by adding 800 μL from a 0.5 μM stock solution containing Hypo-SiF in DMF to a 10 mL buffer solution containing HOCl. Color from the isolated species **8** and SiF-HySO$_3$H in either pH 9 or pH 5 buffer solution (D).

distinct colors can be used to determine relative levels of HOCl by the "naked eye" (Figure 3A−C). The multicolor response at different pH values is due to the presence of the two main products, **8** and SiF-SO$_3$H, which differ in color (Figure 3D). At low pH, both compounds are orange. However, at high pH, SiF-SO$_3$H appears magenta, while compound **8** has a darker purple color. The combination of these colors as the pH changes leads to different shades of magenta, purple, and orange.

HOCl-Induced Chlorination. The response of Hypo-SiF toward 1 equiv of HOCl produces a linear and well-defined ratiometric absorption spectrum. However, as HOCl is titrated above a stoichiometric amount, a different response is observed. At pH 7.4, aliquots between 10 and 80 equiv of HOCl cause the λ_{586} band to begin to red shift stepwise. This bathochromic shift was also observed in the fluorescence spectra until finally λ_{612} and λ_{624} bands were observed for the absorption and fluorescence spectra, respectively (Figure 4).

When an excess amount of HOCl is added under more acidic conditions, a larger change in the absorption and fluorescence is observed. At pH 6.2, the response from 1 equiv of HOCl shows

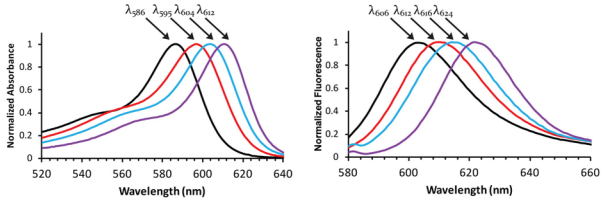

Figure 4. Bathochromic shift in UV−vis (A) and fluorescence (B) with additions of 1, 20, 40, and 80 equiv of HOCl to a 5 μM solution of Hypo-SiF in pH 7.4 buffer solutions.

mostly absorption around the λ_{475} band and a low amount of fluorescence from the λ_{606} band. However, around 5 equiv, the absorption λ_{586} band and the fluorescence λ_{606} band begin to red shift and grow in intensity. By 40 equiv of HOCl, the peaks have reached full intensity and have shifted to 612 and 624 nm for the absorption and fluorescence spectra, respectively (Figure 5). A similar response was also found at pH values of

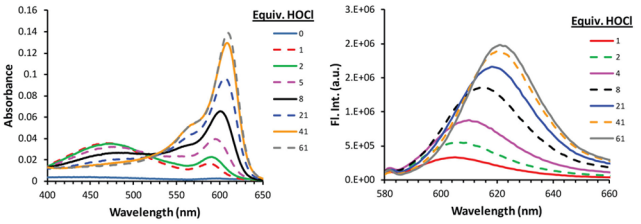

Figure 5. Absorbance (A) and fluorescence (B) titrations of Hypo-SiF with excess HOCl in pH 6.2. Fluorescence spectra were excited at 590 nm.

6.8 and 5 (Supporting Information). These results indicate that the probe can show a different response toward HOCl depending on the relative concentrations of the probe and HOCl. If knowing the pH and concentration of HOCl in a solution is desired, then maintaining a higher concentration of the probe should be applied. If a relatively pH insensitive fluorescence response is desired, then a lower concentration of the probe can be applied. Furthermore, the chlorination effect on the probe allows for the detection of a larger concentration range (0−80 equiv) of HOCl compared to similar probe systems.[6a,7]

To further characterize this system, we isolated the fluorescent products generated when an excess of HOCl was used by adding 80 equiv of HOCl to Hypo-SiF in a pH 7.4 buffer solution. The products were extracted using methylene chloride after the solution was acidified to pH 3. The extracted products were found to have the same λ_{612} band in the absorption spectrum as that observed from excess HOCl titrations. The NMR of the resulting crude product was complex and difficult to interpret. Purification was similarly problematic, and no meaningful separation was achieved. However, liquid chromatography−mass spectrometry (LC−MS) was helpful in characterizing the products in hand (Figure 6). A mixture of various chlorinated forms of the disulfide was prominent in the LC−MS chromatogram. Molecular ions

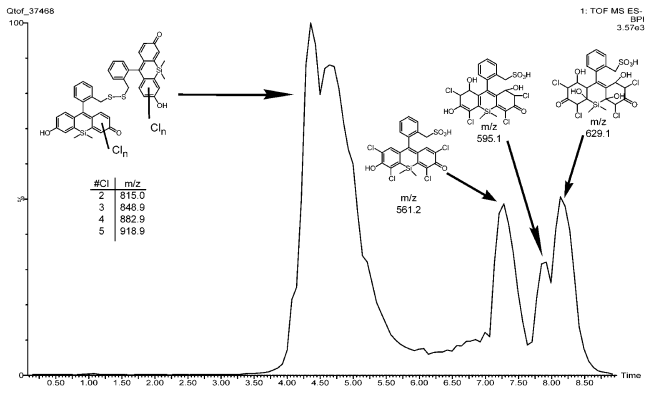

Figure 6. LC−MS chromatogram of the isolated mixture after treatment of Hypo-SiF with 80 equiv of HOCl.

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corresponding to m/z of chlorinated isomers containing two, three, four, and five chlorine atoms on the xanthene core were detected. The exact structure and location of the chlorine atoms could not be determined. Additionally, a tetrachloro-SiF fluorophore containing a sulfonate moiety was also identified in the LC−MS chromatogram. Chlorohydrins were also found and are believed to be intermediates leading to the SiF-fluorophore product.

Although direct analysis of the individual products was not possible because of difficulties in purification, the mixture's pH-dependent properties were investigated. The mixture characterized to contain the chlorinated species (ChloroMix) was titrated to examine its pH dependence (Figure 7). We found

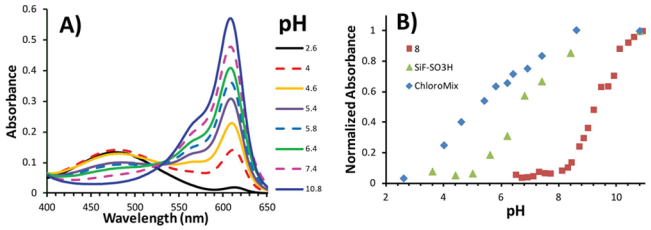

Figure 7. pH-dependent absorbance spectra of ChloroMix (A) and titration curve of λ_{max} for ChloroMix, **8**, and SiF-SO_3H (B).

that ChloroMix is fluorescent at lower pH, whereas compound **8** and SiF-SO_3H are relatively nonfluorescent under acidic conditions. We believe this result is due to the increased acidity of the phenolic alcohol caused by the electronegative chlorine groups on the fluorophore. This correlates well with previous studies on the pH dependence of chlorinated forms of fluorescein, where the addition of chlorine to the xanthene core is known to lower the pK_a of the phenolic proton.[12]

Progress toward the formation of the new red-shifted fluorescent species can be monitored through the shift in λ_{max} (Figure 8). The reaction proceeds more readily under acidic

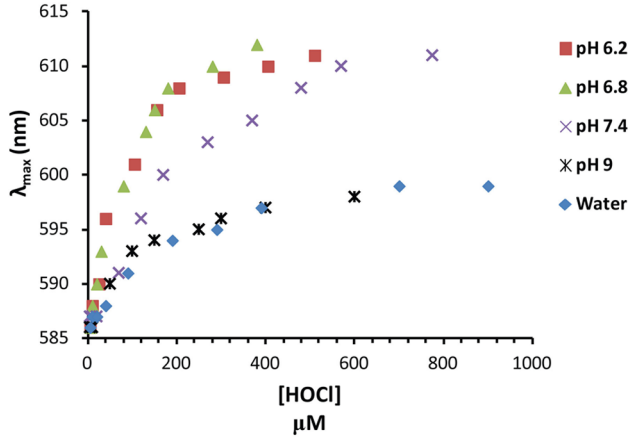

Figure 8. Change in λ_{max} from the response of 5 μM solutions of Hypo-SiF with increasing concentrations of HOCl for solutions in water or buffered solutions at pH 9, 7.4, 6.8, and 6.2.

conditions relative to neutral or basic conditions. By 30 equiv of HOCl, solutions buffered at pH 6.2 and 6.8 have fully shifted to the λ_{612} band, whereas pH 7.4 requires approximately 90 equiv of HOCl before the λ_{612} species are observed. Under basic conditions (pH 9), the λ_{586} species are not observed and only a red-shifted λ_{max} at 595 nm is observed. We tested the effect that the buffer has on the reaction. In the absence of phosphate buffer, the probe was found to only produce the λ_{586} species.

The reaction kinetics for the oxidation at the sulfur center and the chlorination of the Si-xanthene core were explored by monitoring the fluorescence (Figure 9). The addition of 1 equiv

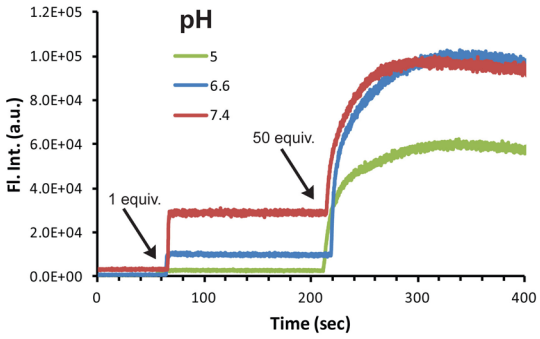

Figure 9. Kinetic study for the reaction of 5 μM solutions of Hypo-SiF with aliquots containing 1 and 50 equiv of HOCl. The fluorescence intensity was monitored at 608 nm.

of HOCl results in a rapid rise in fluorescence intensity at pH corresponding to the formation of a mixture of SiF-SO_3H and **8**. The rise in fluorescence occurs rapidly and remains stable until additional HOCl is added. Upon addition of 50 equiv of HOCl, a relatively slower rise in fluorescence is observed, where maximum fluorescence is achieved over the course of ∼80 s. It is interesting to note that when excess HOCl is added to Hypo-SiF, a similar fluorescence intensity is obtained for pH 7.4 and 6.6. Therefore, under these conditions the fluorescence intensities of the chlorinated SiF products are less dependent on pH compared to those of SiF-SO_3H and **8**. These results also indicate that both the oxidative ring-opening and the HOCl-induced chlorination take place on a relatively fast time scale, making both processes suitable for various applications. The reaction mechanism is presumably similar to that of previous studies on the HOCl-induced chlorination of fluorescein.[12]

Detection of Myeloperoxidase Activity. An exciting potential application of this probe system is determination of ROS in a biological system. However, the primary biological application of this probe is in fluorescence imaging of the cellular environment, where the colorimetric properties may not be relevant. Therefore, we opted to evaluate a scenario where the probe responds to an excess amount of HOCl to see if we get the expected, more fluorescent, chlorinated product. We examined the response of Hypo-SiF to the biological agent myeloperoxidase (MPO). Neutrophils utilize the enzyme (MPO) to generate HOCl as a defensive response to a multitude of pathogens. In the presence of H_2O_2, MPO actively converts Cl^- to produce HOCl over a broad pH range. We prepared solutions containing MPO (extracted from human neutrophils), sodium chloride, and the fluorescent probe. Aliquots of H_2O_2 were added, and the fluorescence was measured. Leading to 2 equiv of H_2O_2, the probe forms the characteristic fluorescent λ_{606} band (Figure 10A) from a mixture of **8** and SiF-SO_3H, where a relatively low fluorescence is observed at lower pH compared with pH 7.4 (Figure 10B). However, when excess H_2O_2 is added, the fluorescence λ_{max} begins to shift and the fluorescence intensity increase signifies the formation of the more pH independent chlorinated products. By 40 equiv of H_2O_2, a bright relatively stable

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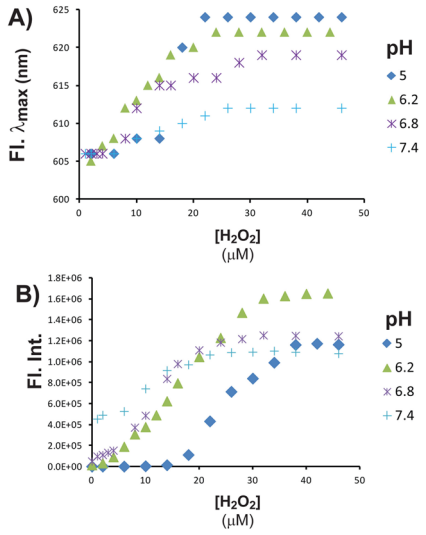

Figure 10. A 2.5 mL solution containing 50 nM MPO, 1 μM Hypo-SiF, and 150 mM NaCl was prepared at various pH values. These solutions were then titrated with aliquots of H_2O_2 and allowed to stir for 5 min before the fluorescence was measured. (A) Fluorescence λ_{max}. (B) Fluorescence intensity at the respective λ_{max}.

fluorescence is observed at each pH. Interestingly, subtle differences between the rates of HOCl-induced chlorination of the fluorophore were observed at the different pH values. By monitoring the shift in the fluorescence λ_{max}, the degree of chlorination on the SiF fluorophore can be assumed (Figure 10B). At lower pH values (5–6.2), a more chlorinated product is observed with λ_{624} and λ_{622} for pH 5 and 6.2, respectively. Under more alkaline conditions (pH 6.8–7.4), a less chlorinated product is observed. On the basis of a comparison of the MPO results with those from the HOCl titrations (Figure 8), a likely explanation for this observation is that MPO is more active in a more acidic environment. This observation correlates well with previous studies on the pH-dependent behavior of MPO.[13,14]

CONCLUSIONS

We have shown that the fluorescent properties of a silicon analogue of fluorescein can be regulated by a spirocyclic structure. The probe Hypo-SiF displays a distinct "off–on" fluorescence-based response when reacted with HOCl. The HOCl reaction with the probe generates a mixture of two dominant fluorescent species, SiF-SO_3H and **8**, that were characterized at various pH values. When an excess of HOCl is present, the SiF fluorophores undergo chlorination, via an intermediate halohydrin, resulting in a red-shifted more pH independent fluorophore. The fluorescence from HOCl-induced chlorination of the SiF fluorophore was utilized to monitor the activity of MPO at low pH. Biological imaging using Hypo-SiF is ongoing and will be reported in a future paper.

ASSOCIATED CONTENT

Supporting Information

Experimental details, synthetic procedures, and compound characterization. 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.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (Grant NIGMS-1R15GM080721) and the National Science Foundation (Grant CHE-0719185). Additional support was provided by Southern Illinois University in the form of startup (C.N.S.). This manuscript is dedicated to the memory of Professor Daniel J. Dyer.

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