The Effect of the Oxygen Scavenging System on the pH of Buffered Sample Solutions: in the Context of Single-molecule Fluorescence Measurements†

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In single-molecule fluorescence experiment, the oxygen scavenging system is indispensable for avoiding photo-bleaching of fluorescent dyes. Here we report that the gloxy-based oxygen scavenging system commonly used in single molecule fluorescence experiments can disturb the solution pH considerably. To track in situ pH change, we utilized the pH-sensitive conformational transition of an i-motif and examined the transition with ensemble and single-molecule FRET measurements. Based on our results, we also suggested several practical remedies for the stability of the solution pH.

Key Words: Oxygen scavenging system, pH, Single-molecule fluorescence, i-motif, Gloxy

Introduction

Single molecule fluorescence becomes one of the most powerful analytical and quantitative techniques to investigate biological problems.1 By acquiring a variety of information from individual biomolecules, the technique enables us to capture mechanistic details which were not previously available and to overcome the limitations set by traditional ensemble measurements.2 To measure fluorescence from a single dye molecule is not an easy task because the signal is weak and the dye has a finite fluorescence lifetime.3 When a dye molecule is excited by an excitation light source such as a laser, it becomes more vulnerable to oxidation.4 Attacked by oxygen species, the dye molecule is oxidized and subsequently becomes non-fluorescent. In this state, the dye molecule is called photo-bleached. One well-known and effective way to avoid or delay photo-bleaching is to use so-called oxygen scavenging systems (OSS).5 The best known and most popular system is a mixture of glucose, glucose oxidase, and catalase (the mixture of the enzymes are called “gloxy”).6 The first is the substrate of the second in the oxygen capturing reaction and the third recovers water from hydrogen peroxide as summarized below. By consuming two glucose molecules, one oxygen molecule is eliminated from the solution after one enzymatic cycle.

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\begin{align*}
\text{glucose} + \text{O}_2 + \text{H}_2\text{O} & \overset{\text{glucose oxidase}}{\longrightarrow} \text{gluconic acid} + \text{H}_2\text{O}_2 \\
2 \text{H}_2\text{O}_2 & \overset{\text{catalase}}{\longrightarrow} 2 \text{H}_2\text{O} + \text{O}_2
\end{align*}
\]

As the reaction proceeds, the concentration of oxygen will decrease, which makes photo-bleaching of fluorescent dyes less severe. As a by-product, gluconic acid is formed. Since it is acid, it may change the pH of the solution. Since pH is a critical parameter in biological phenomena, the stability of pH in biology experiment is desired to obtain reliable results and avoid any undesirable artifacts. Rather surprisingly, this issue has never been documented before.

In this report we would address this issue and characterize the stability of pH of the solution containing the gloxy-based OSS. To monitor the pH of the solution, we utilized a pH-sensitive conformational transition of a cytosine-rich oligonucleotide. In acidic conditions (pH < 6.5), the single-stranded DNA can form a folded structure called i-motif (Fig. 1).7 The folding-unfolding conformational transition can be directly monitored by Fluorescence Resonance Energy Transfer (FRET),1,8 which is able to probe distance change occurring at the nanometer scale which is the relevant length scale for formation of the i-motif structure.

Here we characterized how the pH of the solution with OSS changed as the oxygen scavenging process proceeded in three different approaches: direct measurement of the pH of the solution by a commercial pH meter, ensemble FRET measurements from a donor- and acceptor-labeled cytosine-rich oligonucleotide, and single-molecule FRET measurements of the same molecule. The last represents single molecule fluorescence measurements of our particular interest in this report.

From this work, we discovered that even within a typical FRET measurement time (< 1 hr), the pH of the solution with OSS changed quite considerably in the range of δpH ~ 0.3 to 1 depending on the experimental conditions. This finding alerts that if anyone wants to be sure about the pH of the solution in single-molecule fluorescence measurements, one has to be careful to avoid significant change in pH.

Experimental Section

Samples. For FRET measurements, we used a cytosine-rich oligonucleotide which can form the i-motif structure in acidic conditions. The molecular module we used was formed by hybridizing two oligomers: 5′-GGC GGC TGG CGA CGG CAG CGA GGC (Cy5) TCC CTA ACC CTA ACC

†This paper is to commemorate Professor Kook Joe Shin's honourable retirement.
CTA ACC CT (Cy3) -3’ and 5’-GCC TCG CTG CCG TCG CCA GCC GCC (Bio)-3’. We purchased those oligonucleotides from Integrated DNA Technologies, Inc (Coralville, USA). In the first strand, the first 24 nucleotides are complementary to the second strand to form a duplex stem. A donor (Cy3) and an acceptor (Cy5) dye were terminally and internally labeled, respectively, on the first strand for FRET measurements. Biotin (Bio) was labeled in the second strand to attach the whole module in the sample chamber.

The oxygen scavenging system based on gloxy was prepared according to the standard protocol reported previously. We usually prepare 400 μL of the gloxy solution stock (100 x) which contains 0.04 g of glucose oxidase (Sigma-Aldrich), 127 μL of catalase (Sigma-Aldrich), and 260 μL of T50 (10 mM Tris-HCl pH 8.0 and 50 mM NaCl). 4 μL of gloxy from the stock was diluted 100 times to have 400 μL of the buffer mixture (10, 50, or 100 mM MES, 50 mM K+, and 2 mM Trolox) containing 16 μL of 10% w/v D-(+)-glucose (Sigma-Aldrich).

Direct pH Measurements by a pH Meter. We used a commercial pH meter (Mettler Toledo) for pH measurements. We used MES buffers at three different pH values: pH = 5.6, 6.0 and 6.6. In this experiment, we scaled up the volume of the sample solution to a few mL for reliability. After adding gloxy and glucose, we measured the pH of the solution at various time points as shown in our data (Fig. 2). To see how the buffering capacity is important, we used MES buffers with three different concentrations (10 mM, 50 mM, and 100 mM).

Ensemble FRET Measurement. We measured fluorescence spectra of i-motif samples (DNA: 5 nM) in 10 mM MES buffer with various pH values using a commercial fluorescence spectrometer (Scinco, Korea). This buffer also contained 50 mM K+ and 2 mM Trolox. The samples were excited at 532 nm and their emission spectra were measured from 550 nm to 730 nm. As a control, fluorescence was measured from i-motif samples stored in gloxy-free buffers with pre-defined pH values (5.5, 6.0, 6.15, 6.25, 6.45, and 6.6). For pH = 7.5, we used 10 mM HEPES instead of MES. In order to monitor time-dependent pH variation in the solution with OSS (initial pH = 6.45), fluorescence from i-motif samples was measured at different time points (10 min, 20 min, 30 min, 45 min, and 1 hr) after adding gloxy and glucose to the solution.

Figure 1. Schematic cartoon of the folding-unfolding conformational transition of i-motif. (a) Folded structure of i-motif. Colored, dotted lines indicate base pairing via hydrogen bonds. (b) Base pair between cytosine and protonated cytosine in i-motif. Dotted lines indicate hydrogen bonds. (c) Folding-unfolding transition of i-motif.

Figure 2. Direct pH measurement of gloxy-containing MES buffer with a commercial pH meter. Small symbols indicate data points and lines are visual guides. (a) Time-lapse measurements of the pH of gloxy-containing 10 mM MES buffers with different initial pH values (black square: pH = 5.6, red circle: pH = 6.0, blue triangle: pH = 6.6). (b) Time-lapse measurements of the pH of gloxy-containing MES buffers initially at pH ~ 5.5 with different MES concentrations (black square: 10 mM, green circle: 50 mM, and purple triangle: 100 mM). The black curves in (a) and (b) represent identical data.

Single Molecule FRET Measurement. Single-molecule FRET experiments were carried out in an object-type TIRF (Total Internal Reflection Fluorescence) microscope (Nikon ECLIPSE Ti-U). The details of the experimental procedure can be found elsewhere. The i-motif sample was contained in 10 mM MES buffers at pH = 6.6. In the time-lapse
measurement, gloxy and glucose were added to the reaction mixture immediately before injecting the sample solution to the chamber (< 1 min) and the sample chamber was sealed with epoxy to avoid any evaporation or further oxygen dissolution.

Results and Discussions

In order to test quickly whether the oxygen scavenging system indeed changes the pH of the buffered solution, we first measured the pH of such a solution directly using a commercial pH meter. We tested three solutions with different initial pH's. We used MES buffer as it is good for weak acidic conditions (pH ~ 5.6-6.7). As shown in Figure 2(a), the pH of those buffered solutions dropped rapidly and significantly. Over half an hour, the pH value dropped by more than 1. This was more than what we expected to see, especially as we used well-established buffer solutions. To check whether the buffering capacity was sufficient for typical buffer conditions, we tested the pH change for three different concentrations of MES. As seen in Figure 2(b), the pH value dropped much less for 100 mM MES buffer. Even at that highest buffer concentration tested, the pH still changed somewhat, that is, by ~0.3 over one hour. To rule out the possibility that the observed pH drop was due to dissolution of carbon dioxide, that is, formation of carbonic acid, we also measured the pH of the solution without gloxy and observed no change in pH within a few days, indicating the buffers we used are good enough to accommodate to such a minor environmental perturbation (data not shown).

In order to confirm the gloxy-induced pH drop alternatively, we utilized the pH-dependent conformational transition of a cytosine-rich oligonucleotide. When a single-stranded oligonucleotide is rich in cytosine, it can adopt the i-motif structure in acidic conditions because the protonation of a cytosine permits hydrogen bonding with another cytosine as depicted in Figure 1(b). Since this folding transition is sensitive to pH, such a molecule has been suggested as a molecular pH sensor.

This folding-unfolding transition involves a nanometer-scale structural change, which can be readily detected or identified via FRET measurements. In order to monitor the transition by FRET, we designed a cytosine-rich molecule with a donor dye at one end of the foldable sequence and an acceptor dye at the other end of the foldable sequence. If the sequence folds to an i-motif structure, the two dyes will be in close proximity and the energy transfer from the donor to the acceptor will be facilitated. Then, the fluorescence from the sample will undergo a spectral shift from greenish-yellow (560 nm) to red (660 nm). By measuring the height of the peak wavelength, \( \lambda_{em} \sim 660 \text{ nm} \), we can estimate the extent of the folding transition. As a control, we first measured fluorescence spectra of the i-motif sample at pre-defined pH values (See Fig. 3(a)). In this experiment, the sample solution did not contain gloxy. As expected, the lower the pH of the solution was, the higher the peak at 660 nm was. According to these calibration spectra, we can judge the pH of the solution at the moment of measurement. To maximize the FRET change caused by pH change, we only used a weak acidic pH value (pH ~ 6.45) as the initial pH to make sure that a small pH drop is sufficient to trigger the confor-
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mational transition and consequently to display a measurable change in FRET signal. Figure 3(b) shows the fluorescence spectra of the i-motif sample with OSS at various time points. The peak at 660 nm rose as the time passed and in other words, the reaction of oxygen scavenging proceeded. After 60 minutes of incubation, the pH of the solution appeared to be pH ~ 6.15 according to the control measurement. This is not a small change. This result is qualitatively consistent with the results from direct pH measurements. We also observed smaller spectral changes when we used buffers with higher concentrations, which is also consistent with the results obtained in direct pH measurements.

We then asked whether exposure to atmospheric oxygen makes any difference (that is, the rate and extent of pH change), we carried out similar measurements with the sample cuvette open to air. As expected, the peak at 660 nm rose more rapidly presumably because more oxygen was dissolved and became available to the aforementioned chemical reaction to produce more gluconic acid (Fig. 3(c)).

Next, in order to evaluate the reliability of typical single molecule fluorescence measurements, we monitored FRET change from individual i-motif molecules. Figure 4 showed how single-molecule FRET histograms changed as the reaction by OSS proceeded. As a control, we measured FRET

![Figure 4](image-url)

**Figure 4.** Indirect pH measurement of gloxy-containing MES buffers through the formation of the i-motif structure by single FRET measurements. (a-d) FRET efficiency histograms of the cytosine-rich oligonucleotide in 10 mM MES buffer at pre-defined pH values ((a) 6.0, (b) 6.15, (c) 6.25, and (d) 6.6). (e-f) FRET efficiency histograms of the cytosine-rich oligonucleotide in 10 mM MES buffer with the initial pH of 6.6 after different incubation times ((e) histograms taken from 55 min to 75 min since gloxy was introduced, (f) histograms taken from 105 min to 125 min since gloxy was introduced.). (g) FRET efficiency histograms of the same sample as in (d, e, and f) after incubating it in 10 mM gloxy-free MES at pH = 6.6 for one hour.
histograms from the same i-motif samples at pre-defined pH values (Fig. 4(a-d)). We could clearly identify two peaks, one at high FRET (> 0.9) corresponding to the folded state existing at low pH and the other at low FRET (> 0.3) corresponding to unfolded states existing at high pH. The peak at zero FRET corresponds to donor-only molecules. We injected the sample solution to the chamber immediately after adding gloxy and glucose to the reaction mixture in 10 mM MES at pH = 6.6 (see Fig. 4(d) and at this moment, the time is set to be zero). Initially, there was only one FRET efficiency peak around 0.3 besides the donor-only peak at the time = 0. After one hour, the emerging peak at FE ~ 0.9 became comparable to the low FE peak. According to the control FRET measurements, the FRET after 1 hour is equivalent to the FRET measured at pH ~ 6.3. Thus the pH of the solution after 1 hour is likely to be 6.3. To rule out the possibility that the change in FRET efficiency histograms was not due to actual change in pH in the chamber but due to slow folding kinetics, we restored a gloxy-free MES buffer (10 mM, pH = 6.6), waited for one hour, washed the chamber with the same MES buffer (10 mM, pH = 6.6) containing fresh gloxy, and performed FRET measurements immediately. The reason for one hour incubation is that our i-motif molecule became less sensitive and thus slowly adopted itself to environmental pH change once folded. As expected, the FRET histogram obtained as such is almost the same as the one obtained at pH = 6.6. In other words, the histogram change shown in Figure 4(e, f) must be due to actual change in pH in the chamber.

This indicates that despite the small decrease in pH compared to the two bulk tests done in the open-air environment, there was a still sizable drop in pH. This decrease in pH should contribute to the error of the solution pH. Without properly dealing with the pH drop, biological experiments sensitive to the environmental pH may be spoiled by undesirable artifacts. Aware of this problem, we would like to suggest several remedies: (i) one has to inject the buffer to the chamber immediately after preparation, (ii) one has to finish experiments within the time limit set by pH tolerance, (iii) if one has to do experiments over a long period of time, the sample chamber has to be periodically rinsed with a buffer containing fresh gloxy (in other words, a buffer mixed with gloxy shortly before use), (iv) in any event, whenever permitted, one should use buffers with high concentrations (> 50 mM) and (v) avoid any unnecessary exposure to air (oxygen) by sealing.

Conclusions

Here we reported that the gloxy-based oxygen scavenging system commonly used in single molecule fluorescence experiments can disturb the solution pH considerably. Due to the acidic by-product (gluconic acid) of the oxygen scavenging reaction, the solution becomes progressively more and more acidic which will cause pH-sensitive measurements to be problematic. Three independent tests all supported this conclusion although air-sealed cases such as single-molecule assay were less troublesome. To avoid or lessen this trouble, we suggested several remedies given in this report.

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References