AN ABSTRACT OF THE THESIS OF

Shane W. Monares for the degree of Honors Baccalaureate of Science in Chemistry
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Method for Use in a Microfluidic in-situ Monitoring Device.

Abstract approved: _____

James D. Ingle Jr.

Sulfide is an important species to measure in environmental water samples. The
spectrophotometric detection method for reduced sulfur species (sulfide) based on
generation of methylene blue was adapted to work in an apparatus utilizing microfluidic
PDMS mixing and observation cells with flow channels in the 100 μm range. The
benefits of the system include the use of low volumes of reagents and sample, generation
of low volumes of waste, and the possibility of adaptation to in-situ measurements with a
submergible, microfluidic sensing device. The calibration equation for the detection of
sulfide with this method is [S(-II)] = (Absorbance – 0.0147)/0.0045, where the sulfide
concentration is in micromolar. The detection limit for this method was found to be 1
μM, which is comparable to conventional methods. Topics explored include: sulfide
standard stability and protection from loss due to oxidation and volatilization, preparation
and stability of a single reagent with multiple components, microfluidic apparatus
development, and the calibration of the method for sulfide concentrations.

Key Words: sulfide detection, microfluidics, analytical chemistry, monitoring device,
total analysis system

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Adapting the Methylene Blue Sulfide Detection Method for Use in a Microfluidic in-situ

Monitoring Device

by

Shane W. Monares

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APPROVED:

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Committee Member, representing Chemistry

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Committee Member, representing Chemistry

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Chair, Department of Chemistry

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Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Shane W. Monares, Author

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BACKGROUND

Reduced sulfur (sulfide, or S(-II)) is detectable in environmental samples where sulfur-reducing organisms are present. Sulfide detection can be used to predict the presence of sulfide-reducing species in soil samples and as a measure of environmental redox status. Sulfides are important from an ecological and geological standpoint and are a major component of the sulfur cycle.[1] Furthermore, "sulfide acts as a cellular poison," and can negatively affect aquatic systems near industrial releases of sulfide.[2]

Current methods of sulfide detection in water samples require acquiring the sample often. The samples must be transported for measurement in a laboratory. Sulfide solutions are unstable and loss can occur in the sampling and transport steps. Sulfide standards and samples are often fixed to prevent loss due to oxidation and H_2S (g) volatilization.[2] Means of analytically measuring sulfide include spectrophotometric, electrochemical, chromatographic, and gas analyses.[2] Commercially available methods for detecting sulfide in ground water are reasonably accurate, but inconvenience the user with reagents and the need to collect water samples by hand.[3]

The method presented in this work is based on the classic spectrophotometric technique known commonly as the methylene blue (MB) method.[4] The overall MB reaction can be seen in Scheme 1. According to Kuban, Dasgupta, and Marx, "the organic reagent N,N-dimethyl-p-phenylenediamine (DMPD) is oxidized by iron (III) which reacts with hydrogen sulfide (H_2S) to form methylene blue (MB)."[5] The amount of MB produced is directly related to the concentration of sulfide in the ground water. This MB method is one of the standard methods for sulfide detection.[6]
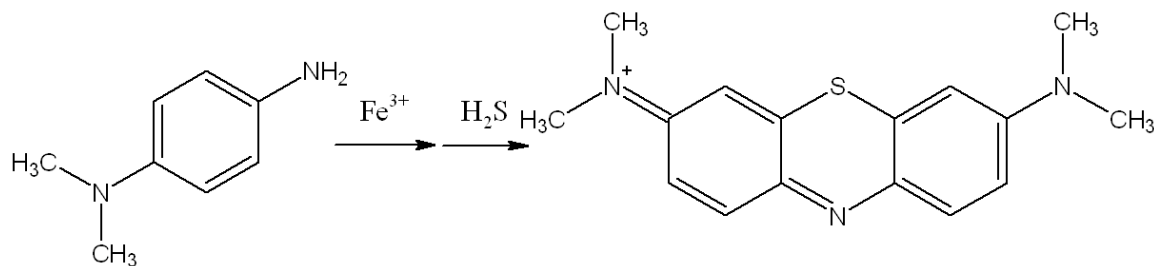
Scheme 1. Basic reaction mechanism for the formation of methylene blue.

Koch has developed and engineered an analytical instrument capable of taking and analyzing environmental samples in-situ by utilizing the physics of mixing solutions in channels in microfluidic chips.[7] It is a microfluidic, total analysis system, also known as the micro water monitoring device (μWMD). The μWMD can be deployed in the field where environmental water samples are desired and programmed to take water samples for virtually any interval of time.[7] The water samples are combined with an onboard colorimetric reagent specific to an analyte of interest that is housed in a refillable bag. When reacted with the analyte, a colored species will be produced, transmittance recorded, and concentration determined.

The work in this thesis is an adaptation of the classic methylene blue sulfide detection for use in a microfluidic device. The reagent concentrations were optimized and the use of the reagent mixture was demonstrated in a benchtop microfluidic mixer and spectrophotometric detection system. Samples are taken and analyzed at the site, which limits the degree of oxidation by air. There is potentially very little day-to-day upkeep on the user's behalf.

EXPERIMENTAL

Reagents

The MB reaction is well known, and the mechanism has been studied in detail by researchers. Kuban, Dasgupta, and Marx (1992) explain that after the formation of the cation radical in the dimethyl-p-phenylenediamine (DMPD) due to oxidation by Fe^{3+} needs to be followed quickly by the H_2S trapping or the diimine will degrade to benzoquinone, leading to small yields.[5] This statement indicates that the DMPD and Fe (III) cannot be mixed and stored together for long periods of time. Furthermore, they report that the highest yields and greatest reaction rates are obtained "when slightly acidic Fe^{3+} is added to strongly acidic DMPD for a period of 5-30 s, followed by the addition of the sample."[5] The MB spectrum is dependent on pH and the maximum absorption band moves to 742 nm at very low pH.

Cline (1969) observed that the DMPD and Fe (III) could be mixed and stored together.[8] The mixed reagent presented in the work by Cline had 22 mM Fe(III), 10 mM DMPD in a solution of 6 M HCl. The final reaction mixture had a mixed reagent:solution dilution of 1:13 and the sulfide concentrations were determined spectrophotometrically using a cuvette.[8]

Koch's design uses two pumps: one for pumping the sample, and another for the reagent.[7] Due to this design, the oxidizing Fe(III) species and the DMPD need to both be housed together for flow through the system by a single pump.

Final Mixed reagent

The final mixed reagent developed contained 22 mM Fe(III), 19 mM DMPD, and 3.88 M or 5.82 M HCl adapted from Cline's method for 0-40 μM sulfide. To prepare, 50 mL of a 50:50 mixture of DI water and concentrated HCl solution (EM Science, 36.5-38.0 %w/w) are placed into the refrigerator overnight to cool.[8]

The HCl concentration in the reagent bottle in Ingle's lab was found to be 7.76 M. A 75% (%v/v) concentrated HCl solution was also used for studies previous to March 23, 2010. A few grams of ferric chloride ($FeCl_3*6H_2O$, Mallinckrodt, AR) were finely ground with a mortar and pestle. Then, 297.3 mg of accurately massed iron chloride powder was added to a 50 mL volumetric flask using a glass funnel. The remaining powder was rinsed into the flask with the cool HCl solution. After all of the iron chloride dissolved, 153.9 mg of N,N-dimethyl-1,4-phenylenediamine oxalate (Aldrich, 98%) was added to the flask, and the remaining volume filled with cool HCl.

The reagent should be kept in a capped flask inside the refrigerator when not in use. The mixed reagent produced reliable and predicted responses when reacted with sulfide standard at least two months after preparation if stored in this manner.

Sulfide standards

A 21 mM stock of sulfide (S(-II)) was prepared and used over the course of a couple of months. A few grams of sodium sulfide nonahydrate (Alfa Aesar, ACS, 98% min.) crystals were rinsed using DI water and patted dry with large Kimwipes as per Standard Methods.[6] To a 50 mL volumetric flask, 253 mg of the sodium sulfide crystals were slowly added and dissolved in deaerated, DI water. When not used, the sulfide stock solution was purged with N_2 gas and placed in the refrigerator to lower oxidation rates.

Sulfide standards were protected with a solution of potassium hydroxide. Exactly 94 μL of 2.3 M KOH was pipetted into 25 mL flasks with ~10 mL of deaerated DI water. The 20 μM and 40 μM standards were prepared by pipetting 24 μL and 48 μL into the 25 mL volumetric flasks and filled to the line with more deaerated DI water to yield standards with hydroxide concentration of 0.01 M (pH ~ 10). Chen and Morris (1972) found that sulfide had a lower rate of oxidation in a pH range of about ~9.[9] Standards should be prepared immediately before being run and used no longer than ~5 hr.

Oxidization of the sulfide standards is a constant issue. A number of anti-oxidant methods were explored. A sulfide anti-oxidant buffer solution was prepared in a similar manner to that of Brouwer.[10] A combination of 0.1342 M L-Ascorbic acid (Mallinckrodt), 0.1329 M EDTA (Aldrich), and 2 M KOH solution was prepared and added to certain standards. For other standards the solution was prepared in 0.01 M KOH and 0.01 ascorbic acid solutions.

Methylene blue standard

A 10 μM methylene blue solution was prepared from a 20 μM methylene blue ($C_{16}H_{18}ClN_3S*3H_2O$, Mallinckrodt, OR, 100%) stock solution in water (12.5 mL stock in 25 mL). The stock solution remained usable for this purpose for months if stored in a dark refrigerator. This solution was used to evaluate the yield of the analytical reaction and for adjusting the flow rates in the microfluidic experiment.

Instrumentation

Microfluidic Apparatus

Alitea (model c8/2-xv) peristaltic pump

Peristaltic tubing. PVC flowmeasured pump tubes (0.13 mm ID.)

PEEK or ETFE (EthylTriFluoroEthylene) shut-off valve

Capillary PEEK tubing (100 − 150 μm ID and 360 μm)

2 PDMS cells

Microfluidic serpentine mixing cell 11.7 cm. mixing length (fabricated by Koch)[5]

Microfluidic 0.8 cm pathlength observation cell (fabricated by Koch)[5]

¼-28, LUER, 10-32, short, headless nut and ferrule and other fittings

Connections between cells 1/16 x 0.040" Tefzel tubing

Fiber optics: SMA fiber optic cable to bare fiber SMA adapter

Ocean Optics USB2000 Spectrometer

Ocean Optics LS-1 Tungsten Halogen light source

Neutral-density light filter

Hewlett Packard Omnibook EX2

Procedure

Two methods are compared in this study. The first experiment was a batch method where, using a cuvette, the mixed reagent was mixed with the sulfide standard in a 1 mL:1 mL ratio or in a 0.15 mL:1.85 mL ratio (similar to Cline).[8] The second of the two methods was a microfluidic 1:1 reagent to sulfide standard reaction dilution.

Sulfide Detection – Batch Method

For validation purposes each standard was first reacted with the reagent using a typical cuvette method. A reference was stored with a water blank and then 1 mL of the sulfide standard was added into the 1.2 cm round cuvette. The time acquisition started immediately before the addition of the 1 mL of the mixed reagent. The reaction solution was vigorously mixed using a magnetic spin bar. Typically, the reaction reached completion in 10-15 min.

Normally, while running the cuvette reaction, the microfluidic system can be running to fill the fluidic passages with sulfide standard. After filling the fluidic passage with just sulfide, a reference was taken. Next, the reagent pump was activated. As the reagent reacts with the sulfide in the fluidic passage, the MB signal slowly increases. Depending on flow rate, a steady peak plateau can occur within 15-20 min. As a rule of thumb, the microfludic system was allowed to run until the same corrected absorbance was repeatedly observed for at least 1 min, resulting in a steady plateau.

Microfluidic Method - Flow Rate Equilibration

Two channels were used to pump the standards and mixed reagent into the two input ports of the mixing cell. Channel A (reagent line) and Channel B (standard or sample) were adjusted independently with the tubing pressure control of the individual cartridges on the peristaltic pump. To equalize the flow-rates of the two lines (sample and reagent), the following steps were utilized:

Channel A pumped 10 µM methylene blue and Channel B pumped DI water

Close the shutoff valve for Channel A and remove the cartridge from the pump

Allow flow from Channel B until water flows out of exit port on observation cell

Let light source warm for at least 15 min.

Adjust the integration time to get a maximum signal of ~3000 counts

Store a 100% transmittance

Detach and cover the SMA connector from the light source

Store and subtract the dark current and reattach the SMA connector

Close the shutoff valve for Channel B and remove cartridge from the
        pump and open the valve for Channel A

Switch to Absorbance mode and begin the time acquisition

Allow the methylene blue signal to reach a peak maximum (depending on flow
        rates, this can take upwards of 1.5 hr)

Open the shutoff valve for Channel B and replace cartridge (Channels A and B
        are both connected)

When the peak maximum for the signal has equalized at half the absorbance of
        the methylene blue a 1:1 flow rate has been achieved

Stop the time acquisition

Microfluidic Method - Mixed Reagent and Sulfide Standard Reaction

After the adjustments to the flow rate to a nominal 1:1 reaction dilution within the chip, the calibration of the sulfide standards in the microfluidic cells can be tested. The procedure follows many of the same steps as the Flow Rate Equilibration, but no adjustments should ever be made to the cartridge tightness on the pump, as it will change flow rates.[11] The following steps are used for the determination of sulfide using a mixed reagent in the microfluidic apparatus:

Channel A pumped mixed reagent and Channel B pumped sulfide standard

Close shutoff valve for Channel A and remove the cartridge from the peristaltic pump

Allow flow from Channel B until standard flows out of exit port on observation Cell

Let light source warm for at least 15 min.

Adjust the integration time to get a maximum signal of ~3000 counts

Take a 100% transmittance

Detach and cover the SMA connector from the light source

Store and subtract the dark current and reattach the SMA connector

Switch to Absorbance mode and begin the time acquisition

Reconnect the cartridge to Channel A and open the stop valve (Now both channels are flowing)

Allow the contents of Channels A and B to react until a peak maximum is achieved

Stop and save acquisition

Mixed Reagent Stability in Reagent Bag

A major concern with regards to the stability of the reagent bags was the high acid content of the mixed reagent. Two halves of polyethylene FoodSaver sheets, used to form the reagent bag in the μWMD were separated, completely covered with two separate mixed reagents: 19 mM DMPD/22 mM Fe(III) in either 3.88 M HCl or 7.76 M HCl solutions, and placed in a cupboard for two weeks.

RESULTS AND DISCUSSION

Sulfide Stability

The stability of sulfide solutions is important in any experiment where the sulfide concentration must be known, such as when a calibration curve is needed. Sulfide ion in solution is easily oxidized by O_2 (g) and volatilizes to H_2S (g) when prepared and used. Researchers have studied different means of increasing the stability. Chen and Morris (1972) found that the specific rates of oxidation decreased the most for a pH of ~9.[9]

Sulfide standards (20 µM) protected with an EDTA/anti-oxidant buffer, prepared in the manner of Brouwer,[10] were found to have no reactivity when the mixed reagent was added. The anti-oxidant buffer/sulfide solution was spiked with an additional 84 µL of sulfide stock solution which effectively doubled the concentration and no MB formed. The EDTA in the anti-oxidant buffer is a strong complexant of Fe (III). Because the EDTA was in excess of the sulfide concentration, the free Fe (III) was likely complexed, limiting the possibility of oxidation of the DMPD by Fe (III) and preventing the formation of MB.

Different combinations of base and ascorbic acid were evaluated for protecting the sulfide standards and the results are presented in Figure 1. The slowest oxidation rate of sulfide (lowest slope of -0.016 AU/hr) was observed with solutions protected with a 0.01 M KOH solution. The slopes for unprotected standards (sulfide in deionized water) on July, 29 2010 and July, 31 2010 were -0.036 AU/hr and -0.056 AU/hr, respectively. The MB signal observed immediately after preparation of the standard was as high as any other standard.

For the two standard solutions that were protected with ascorbic acid and base, the initial absorbance signal was much lower than that of the control standard run on the same day. The absorbance of MB was reasonably stable, indicating low oxidation of sulfide, but in error because the MB signal is reduced and the attenuation is inconsistent. Ascorbic acid by itself protects the standard solution, but the MB absorbance signal is between 0.05 and 0.12 AU less than the standards with no oxidation protection (not pictured in Figure 1). For the solution protected with only 0.01 M KOH (C in Figure 1), the slope was lower than the slopes of the controls. Based on the findings from the stability studies, the KOH-protected sulfide standards were used for further experiments.
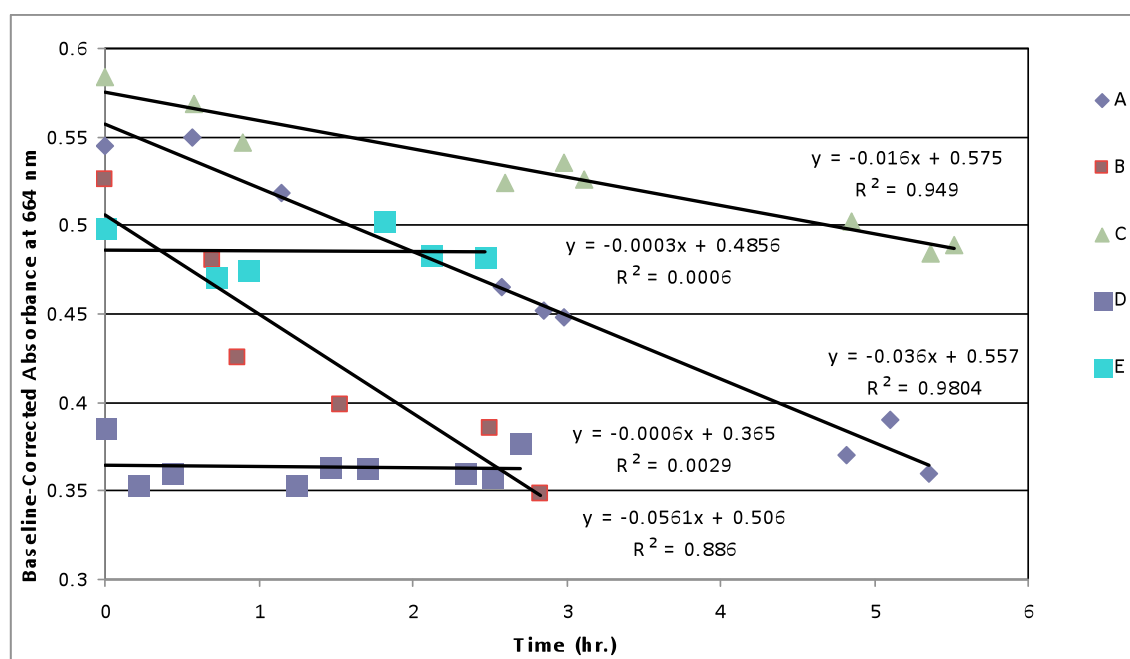
Figure 1. Loss of MB reaction signal due to oxidation and different reagent media. [A] Water only (July 29), [B] Water only (July 31), [C] 0.01 M KOH, [D] 0.01 M ascorbic acid and 0.01 M KOH (July 30), [E] 0.01 M ascorbic acid and 0.01 M KOH (July 31). The X-scale is time from the first run of the series soon after the standard was prepared.

Mixed Reagent Reactivity

When adapting the original mixed reagent to work in a 1:1 reaction dilution, a number of factors were considered: the mixed reagent needed to have a suitable concentration of both Fe (III) and DMPD, react quickly with an equal volume of standard, and remain reactive during storage for long periods of time. The original mixed reagent was highly acidic, but because it was mixed with the standard in a 1:12.3 ratio, the final acidity of the reaction mixture was considerably less and the dominant peak was still at 664 nm.[8]

The main goal was to find a final reaction mixture where the dominant peak was a MB peak. In less acidic media, such as in the experiments done by Cline, the dominant MB peak is at 664 nm. Cline's reagent was first diluted so that when mixed with the sample in a 1:1 ratio, the final pH was ~0.35, the same calculated pH of the original Cline reagent mixed with the sample in a 1:13 ratio.[8] This diluted reagent had a light pink color and was unreactive. At the higher pH, there seems to be a higher susceptibility of oxidation by the DMPD, rendering it useless. The chemistry of the MB formation reaction is discussed in detail by Kuban, Dasgupta, and Marx.[5] The very high acidity of the modified mixed reagent (3.88 or 5.80 M HCl) kept it stable during storage, up until its use in a reaction, and helped to prevent this oxidation.

Dilutions of reagents and acid were performed on Cline's mixed reagent to determine the conditions providing the highest yield of MB. Three reagent concentrations were studied for stability and reactivity with both a 1:12.3 and the 1:1 dilution: ½x, 1x, and 2x, where 1x is defined as 3.88 M HCl/17.8 M DMPD/22.0 mM Fe (III). With all three reagents, strong signals were observed at 664 nm in the 1:12.3 dilution. Figure 2

shows that the peak at 742 nm (doubly-protonated form of MB) becomes more dominant as the acid and reagent concentrations increase. For the 1:1 dilution, the size of the methylene blue bands at 664 and 742 are small in the 0.5x, and 1x standard reagent concentrations, but much larger for the 2x standard reagent concentrations.

Figure 2. Reaction spectra for the three standard reagent concentrations using the 1:12.3 and 1:1 reaction dilutions. As the reagent (and acid) concentrations increased, the absorbance signal response in the 1:1 reaction dilution at the 742 nm wavelength increased.
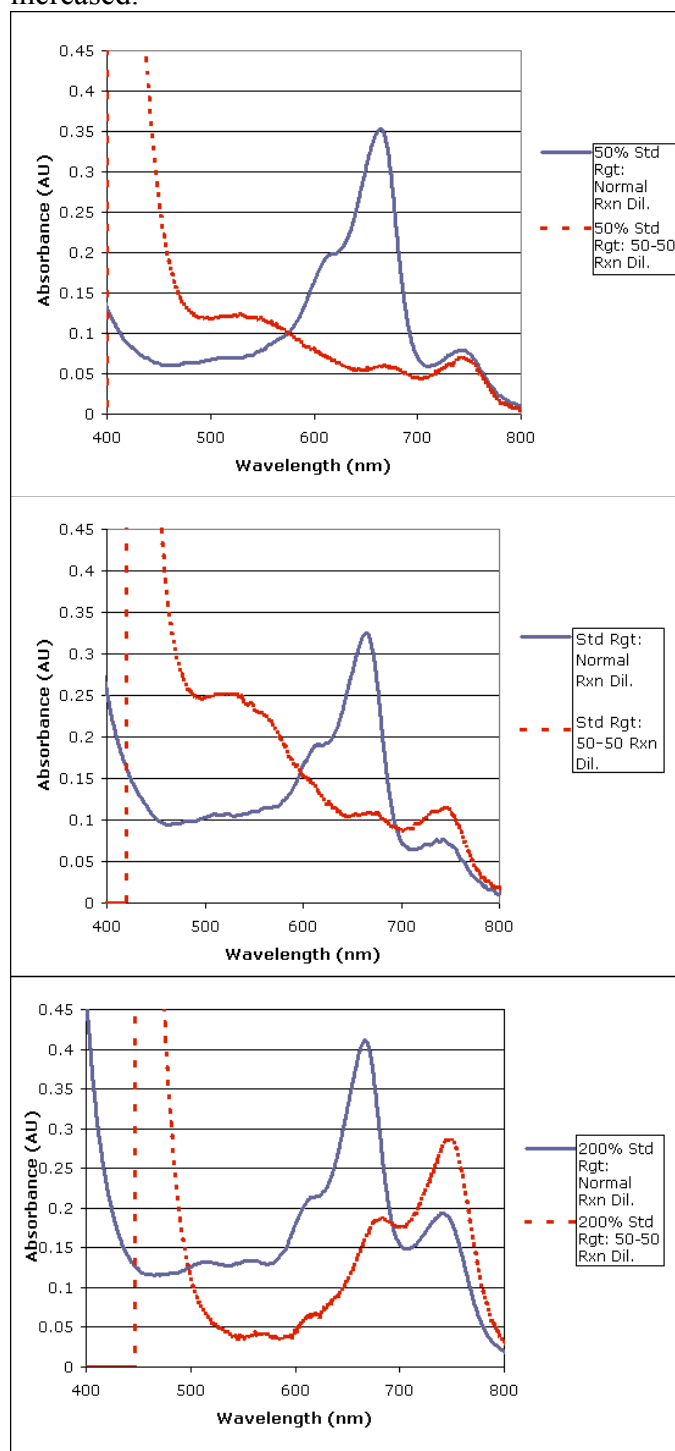
Because it was clear that a very high acid concentration mixed reagent solution was necessary for stability, the analysis wavelength was changed from 664 nm to 742 nm for which the band is higher. The reason for the shift in the maximum absorbance is due to the second protonation of MB. There is a pH-dependant equilibrium between the two peaks, in which the 742 nm peak is dominant at very low pH. According to Kuban, Dasgupta, and Marx, "With increasing acidity, the MBH^{2+} cation (pK estimated to be -0.8) with a characteristic absorption at 745 nm is formed."[5] At the relatively high acid concentration used in the mixed reagent, almost all MB should be in the MBH^{2+} form.

The optimum acid and reagent concentration was determined by measuring the average corrected absorbance of the standard mixed reagent in 2x acid, in 1.5x acid, and with 2x all reagents were 0.346, 0.452, 0.432 AU respectively. The data in Table 1 indicate that the reagent with 5.8 M HCl produced the highest average corrected absorbance. Cline's reagent used 6 M HCl for the mixed reagent,[8] and it was found that ¾x of the HCl stock in Gilbert 254 equals 5.8 M. For the majority of the experimentation, the standard reagent was 22 mM Fe (III) and 19 mM DMPD in 5.8 M HCl.

Table 1. Testing different reagent and acid concentrations to determine the most reactive species.

2-Sep-09		Absorbance (AU)		
Reagent	Trial	742 nm	804 nm	742 nm - 804 nm
Std Rgt: 7.76 M HCl	1	0.386	0.019	0.367
	2	0.377	0.024	0.353
	3	0.341	0.023	0.318
			AVG	**0.346**
			STDEV	0.025
Std Rgt: 5.8 M HCl	1	0.499	0.031	0.468
	2	0.501	0.029	0.472
	3	0.438	0.019	0.419
			AVG	**0.453**
			STDEV	0.030
2x Std Rgt: 7.76 M HCl	1	0.474	0.032	0.442
	2	0.485	0.033	0.452
	3	0.436	0.034	0.402
			AVG	**0.432**
			STDEV	0.027

With a 1:1 standard to reagent volume in a round, 1.2 cm pathlength cuvette, a calibration curve was obtained for 0-40 μM sulfide solutions with a mixed reagent of 5.8 M HCl/19 mM DMPD/22 mM Fe (III). Figure 3 shows the calibration curve. The dominant absorption band in the reaction solution has a maximum at ~742 nm. The absorbance signal was baseline corrected with the absorbance in the flat part of the spectrum at 804 nm. Visually, the reaction solution is yellow-green. The green could be the formed MB "mixing" with the excess Fe (III) in solution. This calibration curve provides good evidence to support that sulfide could be detected using the 1:1 reagent/sample dilution necessary for use in a microfluidic chip.
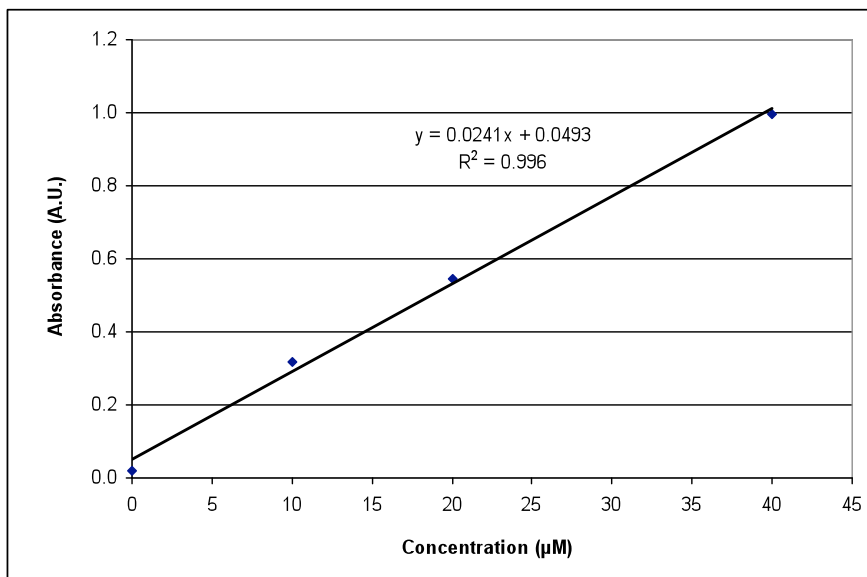
Figure 3. Calibration curve for the MB formation using 1:1 reaction dilution in a cuvette. The correct absorbance is plotted and is the absorbance at 742 nm minus the absorbance at 804 nm where the spectrum is flat.

The starting and ending reagent concentrations can be seen in Table 2. The final concentrations of Fe (III) and DMPD in the reaction mixture for this work is ~10 times greater than the final reaction solution for Cline's method and the acid concentration is ~4 times larger. The mixed reagent used in the study is stable for at least 3 months. Comparatively, CHEMetrics test kits, with reagents stored separately, expire within 2 years.[3]

Table 2. Reagent concentrations before the reaction and after dilution for Cline's method, and this work.

	Mixed Reagent Concentrations		
	HCl (M)	Fe (III) (mM)	DMPD (mM)
Cline's method	6	22.2	10.8
This Work	3.88	22	19
	Final Reagent Concentrations (After Dilution)		
	HCl (M)	Fe (III) (mM)	DMPD (mM)
Cline Method (4 mL Rgt + 50 mL Sample)	0.44	1.64	0.80
This Work (1 mL Rgt + 1 mL Sample)	1.94	11	9.50

Mixed Reagent Stability

For practical use with a microfluidic device in a benchtop apparatus, the mixed reagent must be stable for a reasonable amount of time, such as at least a day. For a remote application with a submergible device,[7] the reagent must be stable for a week or more, and storable in a reagent bag held with the microfluidic device. The reagent bags used by Koch are formed using FoodSaver polyethylene sheets.[7]

After rinsing with water, it was found that the coarse side of the polyethylene half covered with the 3.88 M HCl mixed reagent was more flimsy than the control and had white staining. The smooth half of the polyethylene sheet felt like the control and also had white staining. Both halves of the sheets exposed to the 7.76 M HCl mixed reagent were more flimsy than the control, and had yellow and white staining. The smooth half of the 7.76 M HCl sample was starting to bifurcate at one of the corners.

A few milliliters of the 5.80 M HCl mixed reagent were injected into one of the fully formed reagent bags (sealed and with PEEK tubing inserted) on September 19, 2009. A few months later, the reagent was removed and the reagent bag was intact, with no observed damage to the structure of the bag.

Detecting Sulfide with a 1:1 Mixed Reagent:Sulfide Ratio

Introduction

The reaction of sulfide and the mixed reagent has been shown to produce a strong, reproducible band at 742 nm. A 1:1 volume ratio of the reagent to the sample or standard with sulfide is needed for the microfluidic mixer based on a two pumps and a 2-channel confluence point. Koch demonstrated that good mixing only occurs when the ratio of the two input flow rates to the mixer is near 1.[7] Obtaining a calibration curve for the detection of sulfide standards using a microfluidic apparatus was a main goal of this project.

A series of different microfluidic configurations were used. The mixer and observation cell can be on one chip or on separated chips that are connected by Tefzel tubing. The most convenient apparatus utilized a combination mixing and observation chip (seen in Figure 4). Initially, a single PDMS chip was used and is particularly useful because it mimics the combination chip within the μWMD.[7] Solutions can move through the mixing channels to the observation channel relatively quickly because there is a small dead volume in the channel connecting the mixer outlet to the observation cell input. It was observed that if the combination cell was not thoroughly cleaned after each use, there was a high tendency for clogs to form in the microfluidic channels.
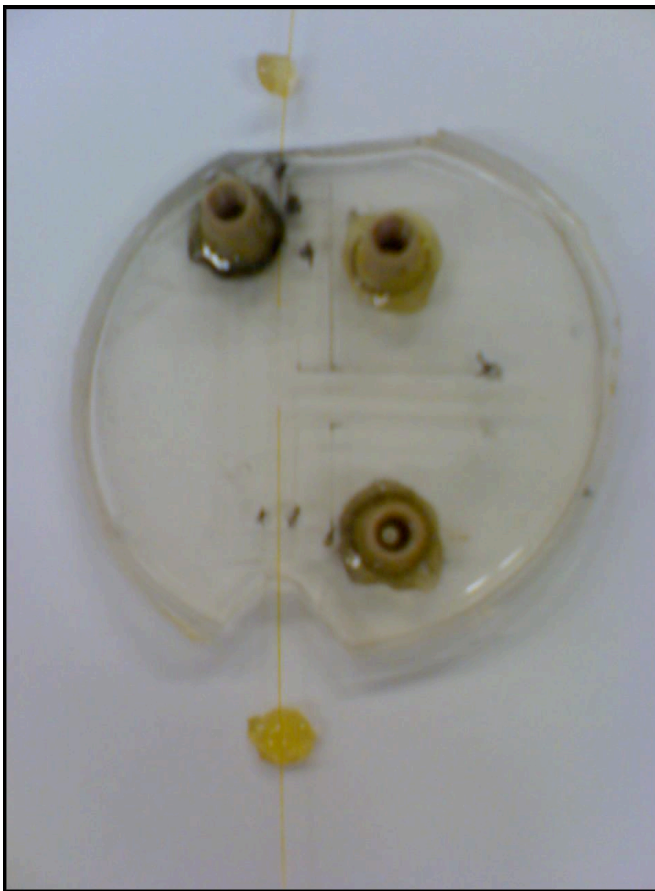
Figure 4. Microfluidic combination mixing/observation chip. This cell was used for early experiments. Its benefits are a small dead volume and similarity to the µWMD chip, but it has a tendency to clog and rupture if not cleaned thoroughly.

Microfluidic Chip Clogging and Rupturing

On September 17, 2009, a test run was conducted with a 20 μM sulfide sample

and the mixed reagent. On October 10, 2009 there was no flow through the cell. The cell

was viewed under a microscope and there was a black substance clogging a couple of the

channels in the chip (Figures 5a and 5b). These clogs completely blocked flow of reagent

and standard.
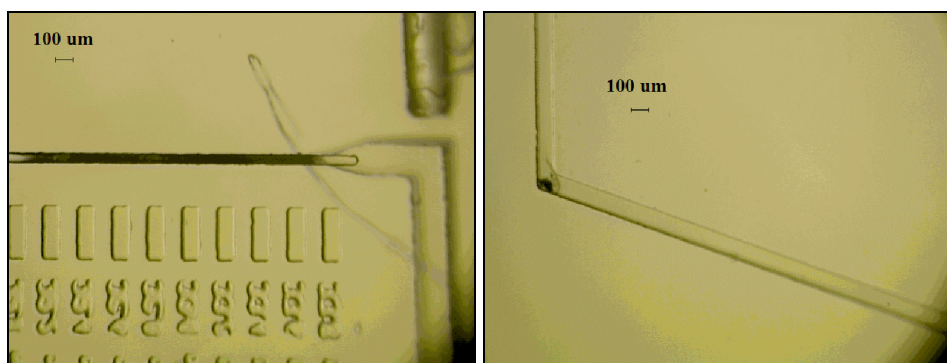
Figures 5a and 5b. Clogs in the combination cell before cleaning procedures.

Koch was able to clean the clog in the cell using three methods in succession: 1)

placing the chip in a sonicating bath of DI water, 2) massaging and pinching the clogged

area while pumping water through the cell, and 3) massaging the top of the chip while the

bottom of the chip was pressed against the bottom of the sonicating bath.[11] The results of

the cleaning can be seen in Figures 6a, 6b, and 6c. After sonicating and pumping water

through the cell for an additional 50 min. to the work by Koch, the clog was completely

removed (Figures 7a, 7b, and 7c).
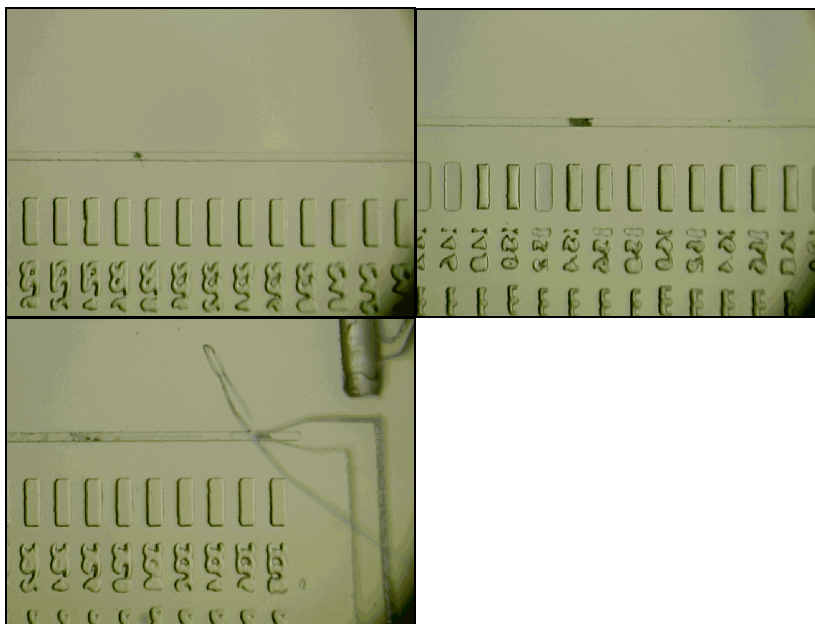
Figures 6a, 6b, and 6c. Mixing channel clogs after the initial cleaning.[11]
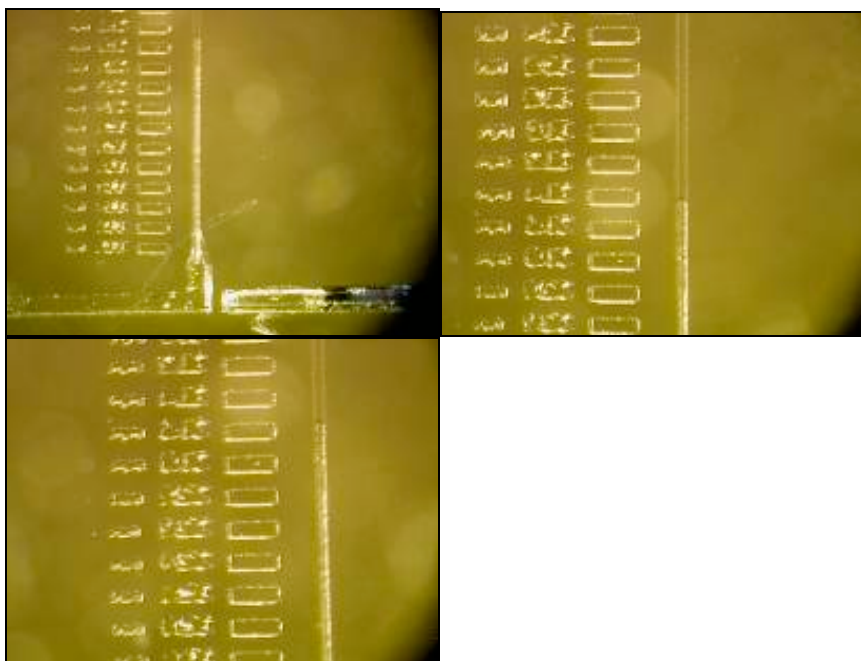
Figures 7a, 7b, and 7c. Mixing channel clogs after further cleaning. The cells were sonicated in a DI water bath and water was pumped through the cell for an additional 50 min. after Koch's initial cleaning.

During a run on October 16, 2009 with standard reagent and sulfide standard, the flow appeared to be blocked. To unblock the channel, the flow rate was doubled, but the cell began leaking from the bottom. Koch observed the cell under the microscope and located a tear.[11] According to Koch, "the PDMS separated from each other, but not fully. Then, the pressure ruptured the PDMS"[11] The tear can be seen as a green line on Figure 8. A clog near the confluence point caused a buildup of pressure within the cell that caused the rupture.
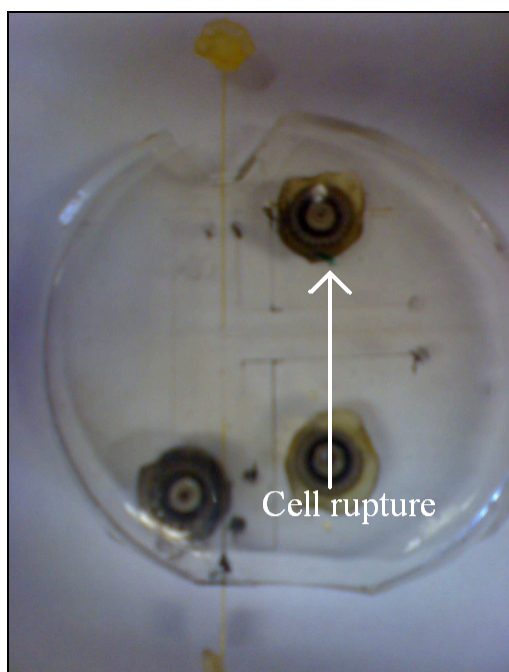
Figure 8. Tear in the combination cell. This tear was most likely caused by a backup of pressure due to a clog near the confluence point.[11]
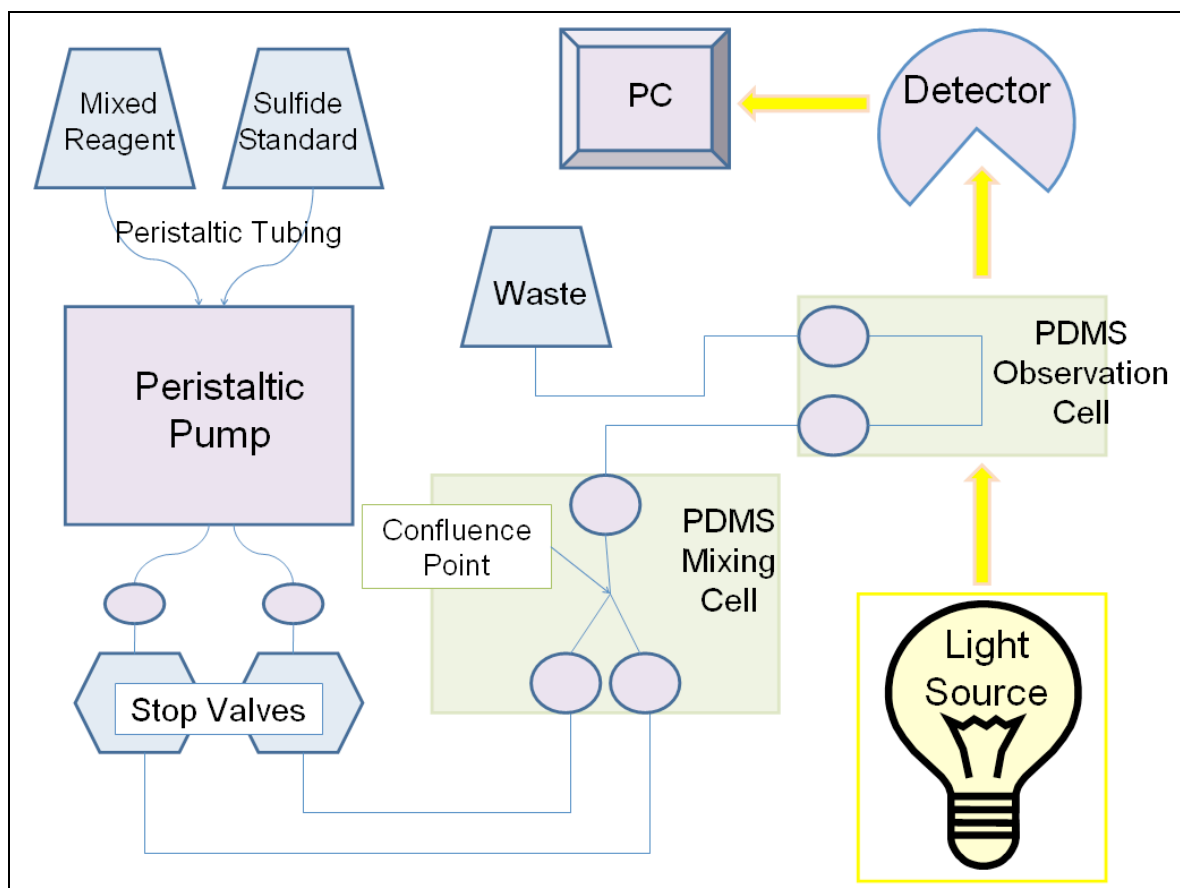
Adjustments to the Microfluidic Apparatus

The reagent oxidizes metal surfaces because of the high acid concentration. In the original apparatus design the junction between the peristaltic tubing and the rest of the line was a PrecisionGlide needle (23G1). Even when the line was rinsed with water after experimentation, oxidation of the needle point was observed on October 22, 2009. It was theorized that the black precipitate causing the clogs was related to the oxidation of the metal surfaces. For this reason, the needle point junction was replaced on both lines by capillary tubing and a capillary junction (Upchurch P-770 and P-882 MicroTight ZDV Adapters). It was later determined that the black precipitate is formed only when the reaction solution or the standard reagent evaporates, despite the presence of the needle junction.

The type and configuration of components went through a number of changes. To eliminate the sulfide standards from flowing back into the reagent line before the mixing channel, a backflow regulator (1/4-28 Female to 10-32 Male, Upchurch CV-3336) was tested. The backflow regulator was not helpful in this application because it, at times, completely stopped the flow and had a relatively high dead volume. Clear, plastic observation z-cells (not microfluidic) were tested, but it was too difficult to keep bubbles out of the light path. The plastic z-cells had an input and exit port on the top and bottom respectively, and SMA adapters on either of the sides. The z-cells functioned as a cuvette as the reaction solution filled the cell. The z-cells also had higher dead volumes compared to the observation cell in the PDMS chips.

Many microfluidic observation and mixing cells were evaluated for mixing and flow. Because the combined mixer and flow chip developed the rupture, a separate

mixing chip was connected to a microfluidic observation cell.[11] The two cells were connected with Tefzel tubing (1/16" OD, 0.040" ID). The dead volume due to the interface between the two cells is about ~259 μL. With a peristaltic pump flow of 10-12% RPM (~22 μL/min) it took ~15 min for flow from the mixer output into the inlet channel of the observation cell.

The final apparatus as described in the Experimental Section and Figure 9 provides several benefits including a low number of bubbles, fewer sharp corners, and microfluidic elements that mimic the combination cell. The drawbacks of the final apparatus are a higher total flow volume, long times for calibration and equilibration, and a larger number of elements to maintain.

**Figure 9**. Flow schematic of the final microfluidic apparatus. Benefits of the apparatus include few bubbles, few sharp corners, and all microfluidic mixing and observation elements. The pathlength for the PDMS observation cell was 0.8 cm. Alitea (model c8/2-xv) peristaltic pump, running at 10-12% RPM, Ocean Optics USB2000 Spectrometer, and an Ocean Optics LS-1 Tungsten Halogen light source were used.

Flow Rate Adjustment

The flow rates for the two channels were adjusted to be approximately equal a number of times. First, the absorbance of a 10 μM MB solution was measured, and then the tightness of the peristaltic tubing cartridge for the other channel with water was adjusted until absorbance was about half that of 10 μM methylene blue. The time dependence of the process is in Figure 10. There are up and down variations in the absorbance until about the 5000 s mark when it was decided the maximum absorbance was reached. The unsteady climb to the maximum absorbance is possibly due to the flushing of dead volumes and elements in the channel with methylene blue. Figure 11 shows the MB spectra with and without dilution. The true ratio of flow rates is found by dividing the absorbance with dilution by the absorbance without dilution. The ratio was 0.292 AU/ 0.625 AU = 0.467, which means there is approximately a 53:47 water:MB flow rate ratio.
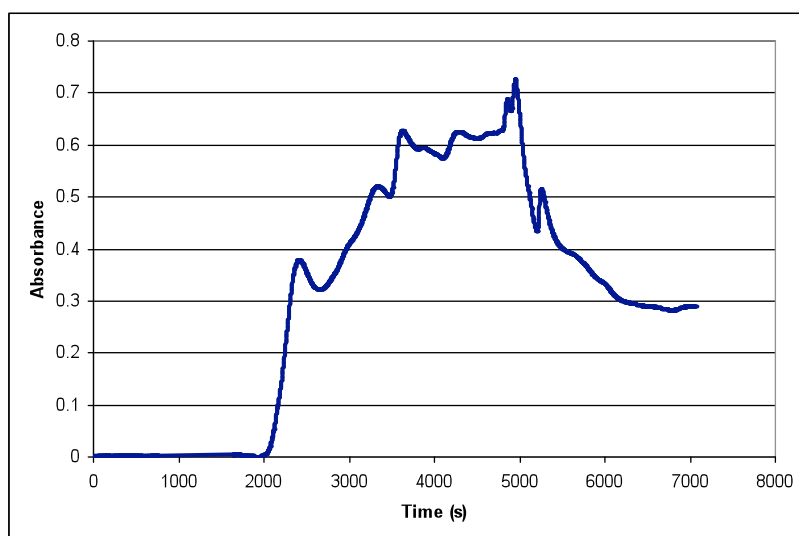
Figure 10. Absorbance during adjustment of dilution factor with microfludic mixer. With only MB, the absorbance reached a maximum of 0.625 AU. The flow rate of the second channel (water) was adjusted to an absorbance of 0.292.
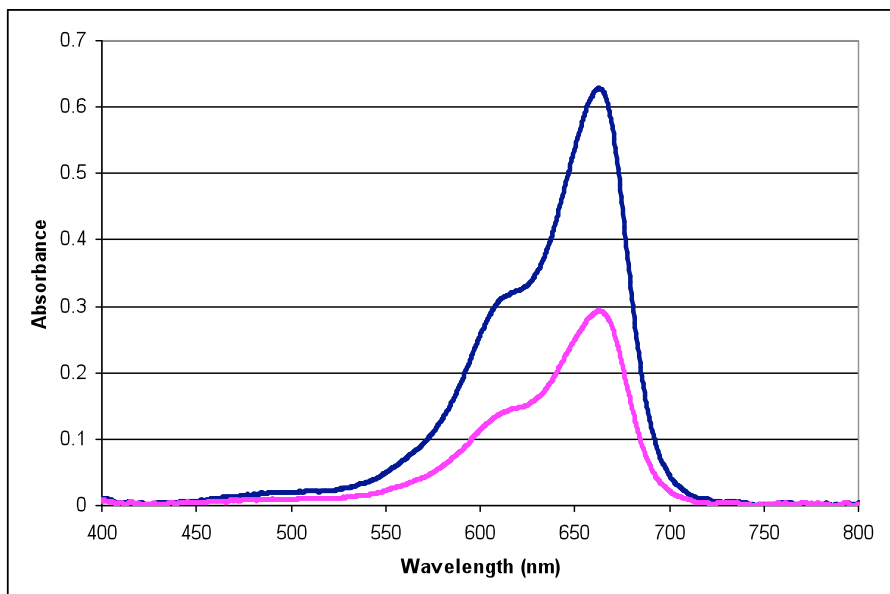
Figure 11. MB spectra of undiluted 10 µM MB (blue line) and after the addition of the water and flow rate adjustment (pink line).

Detecting Sulfide with a 1:1 Mixed Reagent:Sulfide Ratio Using Microfluidics

The calibration data obtained on April 15, 2010 are seen in Figure 12. For each of the two sulfide concentrations, 3 standards were prepared and run. The calibration curve shown is not valid for the standards because the blank data were obtained on April 1, 2010 for a detection limit study.

There is a reasonably positive, linear correlation between the absorbance and sulfide concentration. The ratio between the blank-corrected 40 µM and 20 µM standard is 5.35, which is more than double the expected ratio of 2. There was still evidence to say that this use of the methylene blue method to detect sulfide is viable, but another trial was needed to improve the linearity
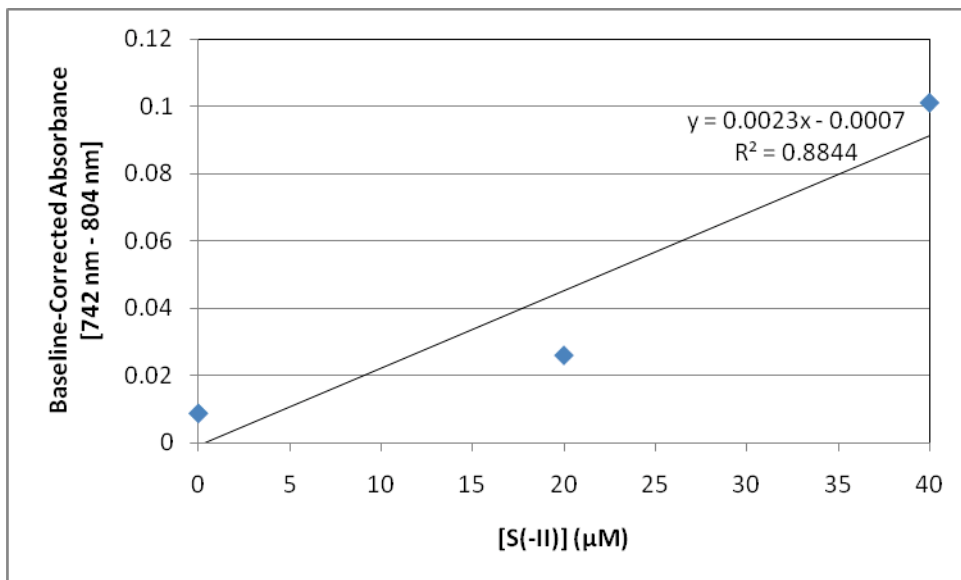
Figure 12. Calibration curve of the average baseline-corrected absorbances for the MB reaction in the microfluidic chip. The blank data used for the 0 µM point was taken on April 1, 2010 and the two standards were run on April 15, 2010.

Absorbance data gathered on April 29, 2010 are summarized in Table 3. The data were obtained on a single day and only one standard was prepared for each concentration for both the standard cuvette apparatus and the microfluidic apparatus. For the microfluidic apparatus, the absorbance is presented as an average because an absorbance spectrum was saved every 5 min until seven readings were collected for both the 20 μM and 40 μM solutions.

The cuvette-based absorbances are still higher than the microfluidic-based absorbances. The absorbance is expected to be 1.5x greater for the cuvette method because the cell pathlength is 1.2 cm compared to microfluidic cell pathlength of 0.8 cm. The 0.401 AU for the 40 μM standard divided by the 1.5 pathlength ratio, and then multiplied by the 47/53 ratio for the flow rate equilibration gives an absorbance of 0.237 AU. There is a percent difference from the expected chip absorbance (adjusted above) and the observed chip absorbance of 0.187 AU of 21%.

The calibration curves created out of the baseline-corrected absorbance data for the cuvette and microfluidic chip are seen in Figure 13. Note that the cuvette absorbance for the 20 μM standard is not half the absorbance of the 40 μM standard even when the blank is subtracted. The blank-subtracted absorbance ratio of the 40 μM to 20 μM sulfide standards in the cuvette is 1.81 compared to the 1.68 ratio seen in the blank-subtracted data for the same standards in the microfluidic cells apparatus. This suggests that the mixing ratio of the mixed reagent and the sulfide is different from the ratio of MB and water.

Table 3. Absorbance data for April 29, 2010 cuvette and microfludic chip calibrations with sulfide concentrations in the range of 0-40 μM.[a]

Cuvette	Absorbance				
[S(-II)] (μM)	742 nm	804 nm	742 - 804 nm		Standard - Blank
0	0.046	0.028	**0.019**		-
20	0.311	0.082	**0.230**		0.211
40	0.453	0.052	**0.401**		0.382
Microfluidic Chip	Absorbance				
[S(-II)] (μM)	742 nm	804 nm	742 - 804 nm	St. Dev Corrected	Standard - Blank
0	0.026	0.017	**0.009**	-	-
20	0.126	0.011	**0.115**	0.003	0.106
40	0.204	0.017	**0.187**	0.003	0.178

[a] Spectral data was obtained using a 1.2 cm pathlength cuvette and a 0.8 cm pathlength microfluidic observation chip. Single runs were used for the cuvette and the 0 μM standard for the chip.
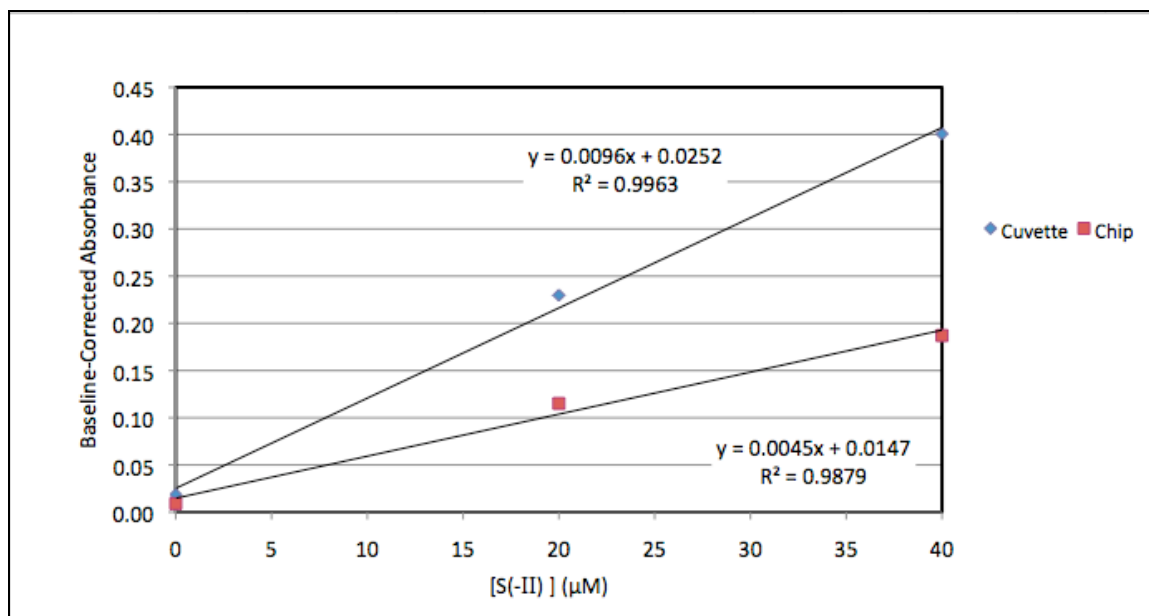
Figure 13. Calibration curves for the cuvette and the microfludic cells. The same solutions were used in the chip and the cuvette validation techniques. For the 20 μM and 40 μM chip data points, 7 data points were collected over 30 min. with a continuous flow of one solution.

From the chip calibration curve, the following equation that could potentially be used in the analysis of real, environmental samples:

[S(II-)] = (Abs − 0.0147)/0.0045, where [S(II-)] is the sulfide concentration of the sample (µM), and Absorbance = $Abs_{742\ nm}$ − $Abs_{804\ nm}$.
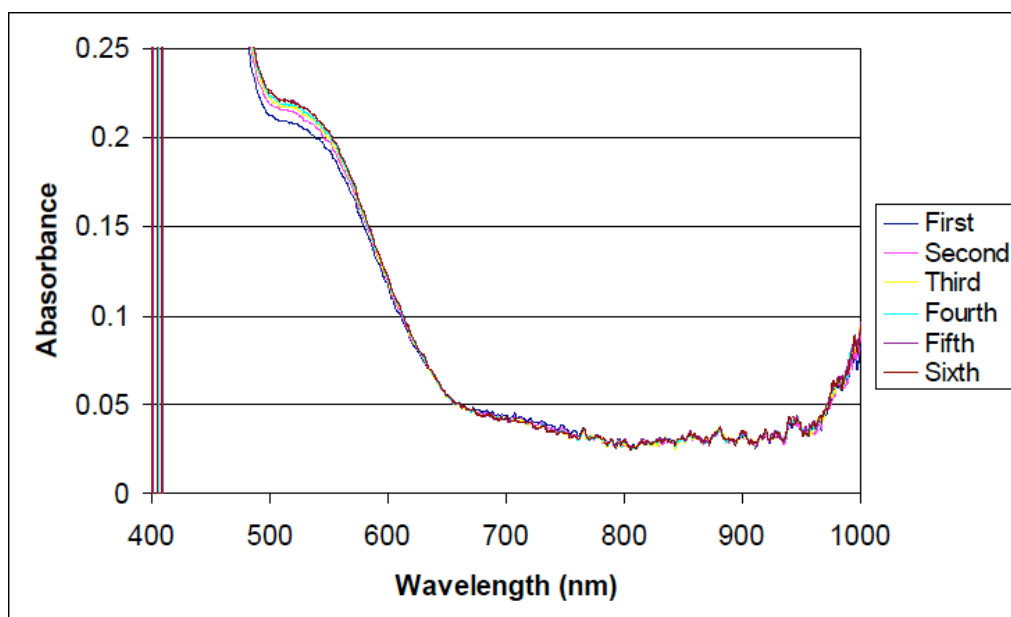
Detection Limit

      The detection limit for this method was found using blank reaction data collected on April 1, 2010. The data presented in Table 4 were collected from the reaction of DI water with the mixed reagent in 5-min. intervals for half of an hour. The blank spectra were reproducible and can be seen below in Figure 14. The slight absorbance in the blank reaction solution is most likely part of the mixed reagent spectrum and not the product of sulfide contamination. The detection was calculated by dividing 3 blank standard deviations by the slope of the calibration curve and found to be 0.7 µM at the low end, which is comparable to other sulfide molecular absorption detection methods.[2]

Table 4. Data collected for the determination of the detection limit.[a]

Absorbance		
742 nm	804 nm	742 nm - 804 nm
0.036	0.027	0.010
0.035	0.026	0.010
0.034	0.025	0.009
0.034	0.026	0.009
0.034	0.027	0.007
0.034	0.026	0.009
	Average	0.009
	Std. Dev.	0.001
	3 * (Std. Dev.)	0.003

[a] Detection limit data found by reacting the standard reagent with DI H_2O.

Figure 14. Spectra of the blank reaction data used for the detection limit. The mixed reagent was reacted with DI water in 5-min intervals for half an hour. DI water was used instead of 0.01 M KOH to simulate a more realistic environmental pH.

CONCLUSIONS

Sulfide standards have been successfully detected using a microfludic mixing and flow cell by adapting the reagent conditions of the classic methylene blue method. The development of this method provides a clear pathway for its use in a submergible, micro total analysis device. Differences in the microfludic method compared to classical, benchtop methods include: a single reagent mixture with three components, the measurement of absorbance for the band at 742 nm, and sample to reagent ratio or dilution of 1:1. Similar to previous studies, sulfide standards in 0.01 M KOH were more stable than unprotected standards, while it was determined that ascorbic acid-protected standards generated a smaller absorbance..

With a peristaltic pump, flow rates were roughly equalized to a 47:53 standard: reagent dilution ratio. A calibration curve based on two standards and a blank was prepared, and the equation for the determination of sulfide concentrations in the microfluidic device was $[S(-II)] = (Abs – 0.0147)/0.0045$, where $[S(II-)]$ is the sulfide concentration of the sample (μM), and Absorbance $= Abs_{742\ nm} – Abs_{804\ nm}$. The detection limit for sulfide with this method was 0.7 μM. The method has been reproducible with the benchtop microfluidic apparatus. The reagent was shown to be stable in sealed reagent bags prepared by Koch for use in a submergible device. It is, therefore, hypothesized that this method would work in a real world setting at detecting sulfide at a level between 1-40 μM.

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