MICROTITER PLATE BASED ASSAY FOR CHARACTERIZATION OF DEHALOGENATION ACTIVITY OF NZVI UTILIZING HALOGENATED PHENOLS AS SUBSTRATE

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ABSTRACT

Nanoscale zero valent iron (nZVI) has been applied to the removal of a wide variety of pollutants, by way of reductive reaction mechanisms; targeting, in particular, halogenated organics. However, the evaluation of reactivity of nZVI towards dehalogenation has not yet been standardized in previous studies. Even though there are advanced chromatographic techniques, which are powerful tools for determining the mother compound and subsequent degradation products, they generally require a long analysis time and complicated set-up. In this light, it is necessary to develop a simple colorimetric assay for the versatile use in many standard laboratories, using only a spectrophotometer for color intensity measurement.

The modified indophenol reaction that is originally used for ammonia detection was suggested for the detection of phenol. Since the para position of phenol, which is the site of the indophenol reaction, is occupied by halogen atoms, e.g. Cl, Br, and I, para-halogenated phenols are expected to be undetectable by the indophenol reaction. As a result, the suggested color assay showed superior selectivity towards phenol, in the presence of a halogenated phenol. The developed color assay was miniaturized with a 96-well microtiter plate. Reagent concentration and reaction time were further optimized, and detection and quantification limits were obtained. The sensitivity of the color assay in the microtiter plate was comparable, with respect to a normal cuvette; therefore, application of a microtiter plate can increase the simplicity and applicability of the suggested method for testing of nZVI reactivity.

In this study, three types of nZVI, bare Fe, Ni/Fe, and Cu/Fe, were applied as a reductant for halogenated phenols. The dehalogenation activity of nZVI varied significantly, depending on which halogen atom is at para-position. Therefore, the different halogenated phenols can be an indicator for comparing reactivity of nZVIs; and, it will be further compared with other halogenated organics, i.e. PCE or TCE. Finally, sample pre-treatment, using $NaCO_3$ solution, was suggested in order to minimize the effect of residual iron on the color assay. The residual iron in the sample could be successfully removed by precipitation in 30 minutes, and a stable spectra baseline could be obtained.

Keywords: Nanoscale zero valent iron; Indophenol reaction; Dehalogenation reactivity; Colorimetric assay; Multiwell microplate

1. Introduction

Nanoscale zero valent iron (nZVI) has been intensively studied for environmental remediation due to its strong reductive power and reactivity, which is derived from its small size and high specific surface area (Fu *et al.* 2014). The removal mechanism by nZVI concerns the direct transfer of electrons to the contaminants transforms into non-toxic or less toxic species (Tosco *et al.* 2014). A wide variety of pollutants, including halogenated organics, nitro aromatic compounds, heavy metals, and anions, has been tried to remediate by utilizing the reduction reaction mechanism (Crane & Scott, 2012).

Depending on an aim of research and laboratory environment, a number of different nZVIs were prepared and compared. The synthesis conditions of nZVI brought considerably different nZVI properties (Hwang *et al.* 2011). Moreover, surface modification of nZVI has been intentionally performed to enhance nZVI properties desirable for environmental remediation (Sakulchaicharoen *et al.* 2010). Additionally, bimetallic particles with deposition of a thin layer of transition metals have been applied to enhance the reactivity, especially for the reductive dehalogenation (Barnes *et al.* 2010).

However, the evaluation of nZVI reactivity toward dehalogenation has not yet been standardized throughout previous studies. Advanced chromatographic techniques are powerful tools to analyze the compounds both qualitatively and quantitatively, but it generally requires long analysis times and complicated set-ups. In this light, a simple color assay for use in laboratories, requiring only a basic spectrophotometer, for determining color intensity should be developed. In our previous study, the modified indophenol method for detecting phenol, which is product from dehalogenation of 4-chlorophenol, was suggested to determine nZVI reactivity (Hwang *et al.* 2015).

Herein, the colorimetric assay to determine nZVI reactivity was further developed and optimized through multiwell microplate based analysis. Firstly, previously developed phenol detection method was further miniaturized and optimized for quantification in 96 wells microplate. The expended range of target compounds, e.g. nitrate, nitrobenzene, and para-positioned halogenated phenols, which can be analyzed by same reagent to create color, were applied. The range of target compounds provided a graduated characterization of reducing activity. Based on the overall results, a simple and effective assay to determine nZVI reactivity is suggested.

2. Methods
2.1. Miniaturization and optimization of color assay for analyzing reaction products
In this study, same concept of reagents was applied to create color product. The use of similar reagents gives simplicity and versatility on analysing several reaction products in the same time. Moreover, the use of multiwell microplate offers the advantage of reading multiple samples at the same time and small sample volume for quantification.

Ammonia analysis was conducted according to standard method with slight modification (4500-NH3-D) (APHA *et al.* 2012). Basically, ammonia measurement use ammonia as a limiting reagent and excess amounts of phenol and hypochlorite were applied to ensure all ammonium forms indophenol, catalyzed by sodium nitroprusside. Reagent A was prepared by diluting 1 mL of 10% hypochlorite solution and 66mL of 0.5M NaOH solution to 100 mL with DIW. Reagent B was prepared as 26 g/L of phenol and 0.3 g/L of sodium nitroprusside in DIW. Sample of 230 µL is transferred to a 96-well microplate and 10 µL of each reagent are added to the sample. The mixture allowed to react in the dark for 2 hours and subsequently quantified with a microplate reader at 630 nm.

In our previous study, the phenol detection by the indophenol reaction was successfully developed by simply substation of phenol as limiting substrate in entire reaction (Hwang *et al.* 2015). Reagent C was prepared as ammonium chloride and sodium nitroprusside in DIW. Equal volumes of reagents A' and C were pre-mixed to make the reagent AC. Then, 20 µL of reagent A'C is added to the sample. The maximum absorbance was recorded at 680 nm. Same reagents A' and C were used for aniline analysis. In short, 10 µL of reagents A' was added to the sample, and subsequently 10 µL of reagent C was added after 15 minutes. The maximum absorbance was recorded at 690 nm.

In order to evaluate the optimal conditions for the reagents we tested the color assay for different concentration levels of hypochlorite for reagent A' (0.1-4 mL of 10% hypochlorite solution/100 mL reagent A') and ammonium chloride for reagent C (50-5000 mg N/L). The reaction time was also further optimized through obtaining the calibration curve at different time intervals up to 24 hours.

2.2. Reduction of compounds by nZVIs

In this study, the mild chemical reduction of iron salts was used to prepare nZVI, as stated in previous research (Hwang *et al*, 2014). In short, $FeSO_4 \cdot 7H_2O$ solution was reduced by $NaBH_4$ solution introduced via peristaltic pump. The black precipitate was collected and washed via centrifugation. The washed nZVI particles were subsequently dried in vacuum at 60 $^{\circ}$C for 4 hours. Bimetallic particles were prepared via spontaneous reduction of second metal ion (Chen *et al*. 2011). After 10 min of redox reaction between the second metal and nZVI, the resulting bimetallic particles were collected and washed.

Batch tests for nZVI reactivity were conducted in 50-mL glass serum vials. The desired amount of nZVI was weighed in reaction vials inside of anaerobic chamber. Then, reaction vial was filled with 39 mL of DIW and securely capped. Each stock solution of 1 mL was injected through septum to initiate the reaction. The nominal concentrations of nitrobenzene, nitrate, para-halogenated phenols and nZVI were 500 µM, 25 mg-N/L, 500 µM, and 2 g/L, respectively. Samples were taken periodically and filtered through 0.45 µm syringe filters. The concentration of reaction product in the sample was measured as described in 2.1. The reaction kinetic was further interpreted by pseudo first order kinetic.

3.   Results and discussion
3.1. Optimized color assay for analyzing reaction products in multiwell microplate
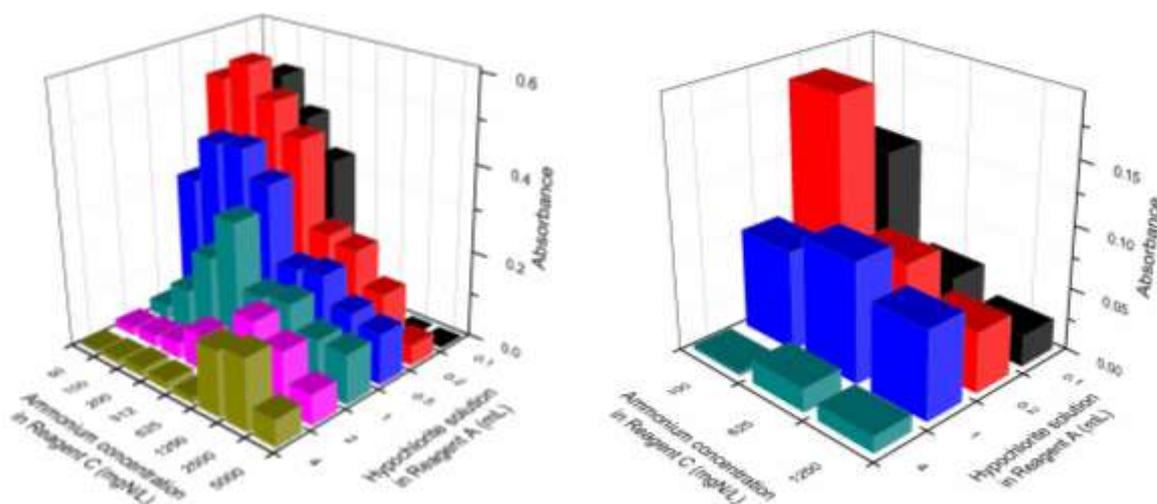
Figure 1: Optimization of reagent concentration on color development for (a) phenol, and (b) aniline. Note that 50 µM of each product were applied and absorbance was taken after 2 hours.

In case of ammonia analysis, further optimization on reagent concentration and time was not performed since it is an already standardized method. The values of the LOD and LOQ in 2 h were 2.9 and 9.6 µM, respectively. Effect of reagents concentration on phenol detection was investigated as presented in Figure 1 (a). The maximum absorbance was obtained in the case of 0.2 mL of 10% hypochlorite in reagent A' and 100 mg-N/L of ammonia in reagent C. Moreover, the optimal reaction time can be determined as 2 hours since it showed comparably high absorbance and linearity, as well as shorted reaction time within stable time range, 2-4 hours. The values of the LOD and LOQ were 0.3 and 3.3 µM, respectively. The reagent for phenol detection was tested for detection of both aniline and nitrobenzene in the concentration 50 µM. Aniline produces a clear peak at 690 nm of wavelength regardless presence of nitrobenzene. Thus it was obvious that the selectivity of the color assay adequately distinguished aniline. Figure 1 (b) demonstrates that the optimal reagents concentrations for aniline detection are the same as the phenol assay's. Its LOD and LOQ are slightly higher as LOD 0.5 and LOQ 5 µM.

3.2. Graduated characterization of reducing activity by applying a range of reducible compounds

Range of reducible compounds was tested to characterize a graduated reducing activity. In this study, nitrate, nitrobenzene, and para-positioned halogenated phenols, i.e. 4-chlorophenol, 4-bromophenol, 4-iodophenol, were applied. The Figure 2 (a) presents reactivity of nZVI toward different reducible compounds. As a result of nitrate and nitrobenzene reduction by nZVI, ammonia and aniline could be produced. Aniline production was the fastest and rate constant were calculated as 17.9 /hour, which is 6.75 time faster than nitrate. On the other hands, phenol production was not observed in case of all halogenated phenols.
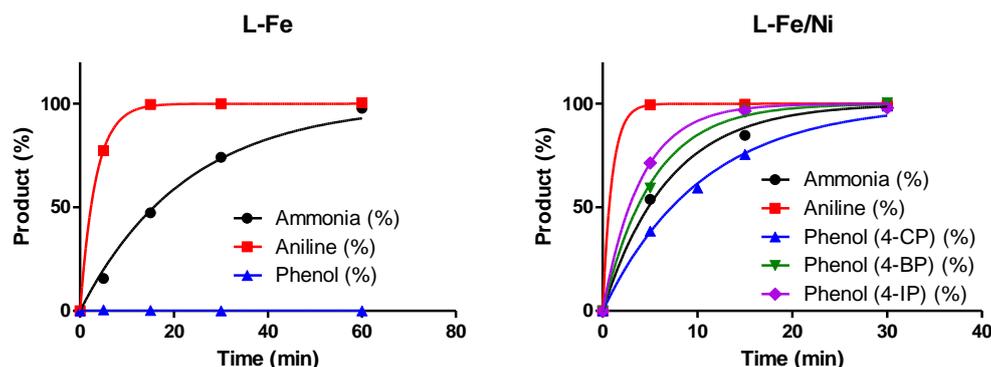
Figure 2: Generation of reaction products by reaction with (a) monometallic nZVI, and (b) bimetallic Fe/Ni (5%). Note that iron concentration in all cases was 2 g/L, and curve was fitted by pseudo first order kinetic.

The overall reactions were much accelerated by adding second metal as catalyst as presented in Figure 2(b). The 5% Fe/Ni bimetallic particles showed 3.9-5.2 times higher reaction rate constants than nZVI particles. It indicates that addition of second metal on the nZVI surface can promote iron corrosion due to galvanic effects between nickel and iron. In addition, catalytic reactivity of second metal, nickel was also confirmed by para-positioned halogenated phenols. The clear difference caused by existence of nickel explains a reduction mechanism, which is a formation of reactive hydrogen on the second metal particle surface followed by dehalogenation and hydrogenation by the reactive hydrogen. A significant difference of reaction kinetic was also obtained among para-positioned halogenated phenol depending on substituted halogen atoms. The reaction was faster in the order of 4-iodophenol, 4-bromophenol, and 4-chlorophenol. This order is well agreed with average bond enthalpies of chemical bonding. This close relationship explains a reason of different reaction kinetics with halogen atoms.

In summary, nitrate and nitrobenzene can be used to determine general reduction reactivity of nZVI while catalytic reactivity of second metal can be determined by para-positioned halogenated phenols. The graduated reducing reactivity toward different reducible compounds is able to be an indicator for determining and estimating difficulties of reaction.

4. Conclusions

The modified indophenol assay for determining reductive reactivity of nZVI has been suggested. The color reaction for three different reaction products, ammonia phenol, and aniline are simple and versatile since same types of reagents are able to be applied for all reactions. All color reactions were miniaturized into multiwell microplate, which provide convenient and efficient assay platform. Reaction conditions, i.e. reaction time and reagent concentrations, were further optimized. Small quantity of sample, 230 µL, is enough to quantify reaction products within 2 hours of reaction time. The three groups of compounds showed graduated reactivity and were possible to distinguish a reaction mechanism between normal reduction and catalytic behavior of second metal. Reaction kinetic was fast enough to complete reaction within 1 hour. The preliminary results of nZVI reactivity obtained by suggested assay would be useful to determine

suitable reaction condition for remediation work and estimate performance and required time. Therefore, suggested reactivity test with multiwell microplate based color assay will be useful and simple tool in various nZVI related research topics.

ACKNOWLEDGEMENT

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