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Fluorescence quenching competitive immunoassay in micro droplets

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Abstract

A fluorescence quenching competitive immunoassay in micro droplets was applied to the sensitive detection of the pyrethroid insecticide, esfenvalerate. Laser induced fluorescence from rhodamine dye was used as a marker. The competitive immunoreaction was performed in micro droplets generated by a vibrating orifice aerosol generator system with a 10-μm diameter orifice. Fluorescence that was emitted from the droplets was detected by a 1/8 m imaging spectrograph with a 512 × 512 thermoelectrically cooled, charged-coupled device camera. The conjugate of esfenvalerate with rhodamine exhibited similar fluorescence to that of pure rhodamine 6G. When anti-esfenvalerate antibodies were added to the droplets, the fluorescence decreased. The reduction in emission was due to a strong quenching effect that arises from the interaction between the protein and rhodamine molecules following the antigen–antibody reaction. When a sample of esfenvalerate was added to the droplets, the release of the conjugated rhodamine from the antigen–antibody complex allowed the fluorescence signal to recover. An assay in a picoliter droplet sample was shown to enable detection down to approximately 0.1 nM. A very small mass of analyte could be detected with this method. A sample of river water was used to gauge the impact of matrix effects and was shown to give rise to negligible interference with the immunoassay.

Keywords: Fluorescence; Micro droplet; Quenching; Immunoassay; Esfenvalerate

1. Introduction

Laser induced fluorescence technology, combined with optical microscope systems and sensitive charged-coupled device (CCD) cameras, has become an important tool in biological research (Meixner and Kneppe, 1998; Nie and Zare, 1997; Peck et al., 1997; Powell and Tempst, 2001; Ronai et al., 2001; Tuma et al., 1999). However, background interference is still a major challenge to the more general application of this method, particularly in environmental sciences where the matrix that contains the compound of interest may quench a fluorescence signal, may autofluoresce, or may adversely influence the assay in other ways.

In addition to high-performance optical filters, reduction in the illuminated sample volume is an effective way to overcome background interference in laser induced fluorescence detection. Several schemes have been explored to achieve this goal. Fluorescence detection in hydrodynamically focussed liquid streams has shown significant advantages in reducing background interference to the level at which single molecules can be detected (Nie et al., 1995; Shera et al., 1990; Soper et al., 1992). Capillary electrophoresis has also aided in the detection of single molecules (Castro and Shera, 1995; Chen and Dovichi, 1996).

Other analysis formats can be useful in extending the limits of detection. Micro droplets (droplets $\lesssim$100 μm) can be used to define the interaction volume of laser and analyte very precisely. The sample volumes are typically in the picoliter range. Electrodynamic levitation of picoliter-sized droplets (Barnes et al., 1993; Kung et al., 1998; Lermer et al., 1997) has been used to measure a single droplet for an extended period of time in order to improve the signal to noise ratio (SNR). In addition, micro droplets can remove the possibility of non-specific adsorption on the walls of cuvettes that are used to hold and analyze samples (Welter and Neidhart, 1997). The

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effect of walls can be serious in the analysis of highly lipophilic molecules such as tetrachlorodioxins and pyrethroids, as well as materials that bind to glass such as paraquat. The advantages of micro droplet analyses suggest that they may be useful in the application of immunoassays to the analysis of toxins in environmental samples.

In a quenching competitive immunoassay, a dye molecule, such as rhodamine, is conjugated with the target analyte and the resulting conjugate is used as a marker. Quenching of the fluorescence from the conjugate, or fluorescence resonance energy transfer (FRET) to a donor, have been shown to be useful methods in immunoassays (Matveeva et al., 1996; Wei et al., 1994). Generally, the conjugate is a small molecule. Binding between the analyte and its antibody will modify the local environment of the fluorophore. Therefore, when the conjugate is bound to an antibody, the emitted fluorescence may be quenched. This permits a competitive assay format to be used in which non-conjugated analyte competes with labeled analyte for binding to antibodies. As the amount of analyte in the sample increases, the amount of unbound conjugate increases and hence the fluorescence signal increases.

The target analyte in this study was esfenvalerate, a synthetic pyrethroid widely used for controlling pests on agricultural crops. It is known to be toxic to some nontarget organisms, particularly to fish (Lozano et al., 1992; Tanner and Knuth, 1996; Tomlin, 1997). A need exists for environmental assays for compounds of this type that are rapid, sensitive, and can be performed in real samples that contain a host of biological material that can potentially interfere significantly with the analysis.

2. Experiments

2.1. Materials

Esfenvalerate (Fig. 1a) was obtained from Riedel de Haen (Seelze, Germany). Rhodamine 6G was the product of Exciton, Inc. (Dayton, OH). Rhodamine 110 was purchased from Aldrich Chemical Co. (Milwaukee, WI). Esfenvalerate–rhodamine conjugate was synthesized by using the protocol described by Cai et al. (1999); two structures that are consistent with GC/MS analysis are shown in Fig. 1(b). In summary, 3-(Cyano-((*S*)-2-(4-chlorophenyl)-3-methyl-1-oxobutanoxy)methyl) phenoxyacetic acid (40 mg, 0.1 mM) in chloroform (2.4 ml) containing 1 μl of dimethylforamide (DMF) was treated with thionyl chloride (0.25 ml, 3.5 mM) and stirred under N_2 in an oil bath at 65 °C for 1 h (Shan et al., 1999). The mixture was stripped briefly of solvents, hexane (2 ml) added and restripped. The residue was dissolved in DMF (0.5 ml) and was used for the next step. *N*,*N*-Diisopropylethylamine (15 mg, 0.014 mM) was added to a solution of Rhodamine 110 (25 mg, 0.068 mM) dissolved in DMF (1.5 ml) at −60 °C, then the acid chloride (in DMF) was added dropwise. The reaction solution was held at −60 °C for 1 h, and then slowly warmed to room temperature and stirred continuously overnight. The mixture was diluted with 20 ml of ice water and extracted with ethyl acetate (3 × 15 ml). The organic phase was washed with aqueous brine (3 × 10 ml), dried over anhydrous sodium sulfate, and the solvent removed to give a crude product. The product was purified by flash column chromatography (hexane/ethyl acetate: 2/1) to give 4.8 mg (yield 10%) of target conjugate, $R_f = 0.4$ (hexane/ethyl acetate: 2/1). The resulting compound was characterized by LC/MS/MS with $(M+H)^+ = 714$.

Anti-esfenvalerate polyclonal antibody was previously generated at UC Davis (Shan et al., 1999). The crude antibody was purified using Bio-Rad purification column (Econo-Pac Serum IgG purification Kit (732-2026)).

2.2. Apparatus

A TSI Model 3450 vibrating orifice aerosol generator was used to generate the micro drops from a 10 μm diameter orifice (Fig. 2). This instrument used a syringe pump that was mechanically driven with feed back control to deliver a precise, steady flow of liquid to the orifice. The liquid jet that issued from the orifice was perturbed by a piezoceramic device that was excited at frequencies between 20 and 40 kHz. The perturbation of the liquid jet caused breakup of the liquid stream into droplets with a very repeatable and well controlled diameter. The size of the droplets was determined by the liquid flow rate (controlled in turn by the syringe pump), the diameter of the orifice, and the frequency of excitation.

Droplets were illuminated with the focused second harmonic beam of a Nd: YAG laser, $\lambda = 532$ nm, with a pulse repetition rate of 10 Hz and a pulse duration of approximately 7 ns. The fluorescence was collected by a Mitutoyo microscope objective lens (N.A. of 0.55), then focused onto the entrance slit of an imaging spectrometer (SpectraPro-150, Acton Research Corp., MA). Spectra were recorded with a thermoelectrically-cooled camera (TEA/CCD-521-TKMI, Princeton Instruments Inc. Trenton, NJ) that housed a 512 × 512 pixel CCD detector. The camera was controlled by a ST130 controller (Princeton Instruments Inc.). A 532 nm holographic Raman notch filter (Kaiser Optical Systems Inc., MI), was used before the slit of the spectrometer to block elastically scattered laser radiation.

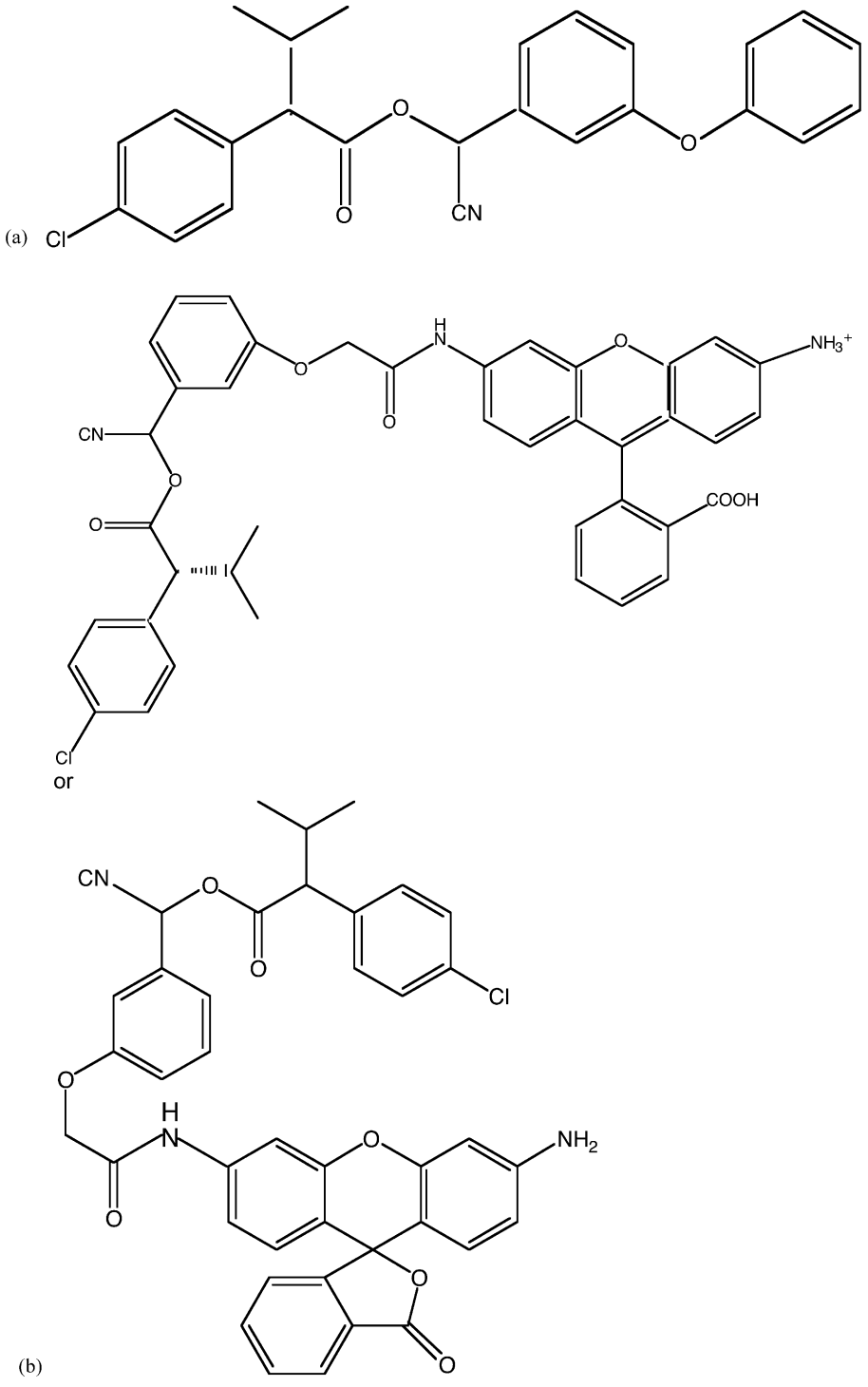

Fig. 1. (a) Structure of esfenvalerate; (b) Possible structures of the esfenvalerate hapten–rhodamine conjugate.

2.3. Methods

A solution of esfenvalerate–rhodamine conjugate was prepared in standard phosphate buffered saline (PBS 0.1 M). Solutions of anti-esfenvalerate antibody of different concentrations were also prepared in PBS. The antibody solution was added to the conjugate solution in a cuvette and binding was allowed to proceed for up to 60 min at room temperature. The kinetics of fluorescence quenching were observed by withdrawing aliquots of the mixture at specific intervals. The influence of non-specific proteins on quenching was investigated by adding bovine serum albumin (BSA) protein to the mixture.

Samples of reacted mixture were added to the syringe pump of the droplet generator. The micro droplets that

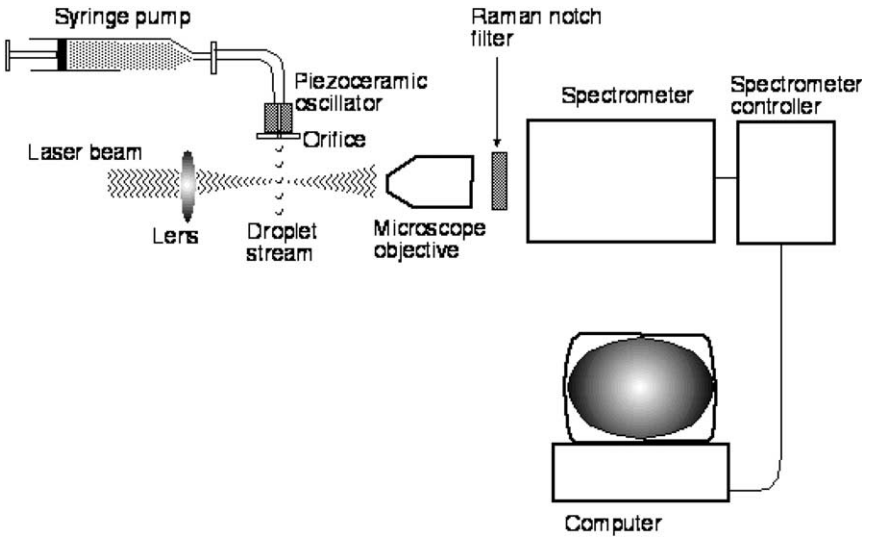

Fig. 2. Experimental apparatus showing piezo based droplet generator, Nd: YAG laser operating at 532 nm wavelength, and detection system. The axis of the detection system is orthogonal to both the laser beam and the direction of travel of the droplet stream.

were generated then passed through the waist of the focused laser beam. Their fluorescent emission was collected by the microscope objective and imaged onto the entrance slit of the spectrometer. The analysis of the fluorescence signal was implemented through the use of a normalized relationship between the concentration of the analyte and the degree of fluorescence quenching, defined as

$$Q = (F_E - F_Q)/(F_{ER} - F_Q) \quad (1)$$

where F_E is the fluorescence of the solution of esfenvalerate–rhodamine conjugate with the esfenvalerate antibody and analyte added; F_Q is the fluorescence of the solution of a fixed amount of esfenvalerate–rhodamine conjugate and its antibody—this quantity indicates the maximum possible extent of quenching; and F_{ER} was the fluorescence of the solution of the esfenvalerate–rhodamine conjugate without antibody or analyte added.

3. Results and discussion

3.1. Fluorescence of esfenvalerate–rhodamine conjugate

The wavelength at the maximum emission intensity of the esfenvalerate–rhodamine conjugate appeared at about 550 nm. The spectra of the fluorescence emission of the conjugate and of pure rhodamine are shown in Fig. 3(a) where it is apparent that a slight reduction in the emission intensity could be attributed to the conjugation process. The fluorescence intensity of the conjugate exhibited a linear dependence on conjugate concentration (Fig. 3b). Photobleaching was avoided by limiting the laser energy to 8 mJ per pulse for all the experiments.

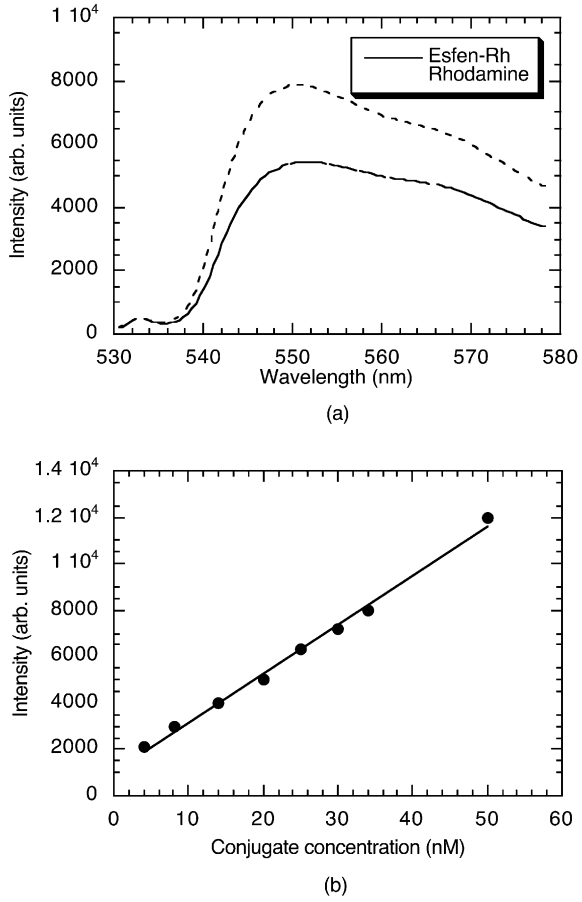

Fig. 3. (a) Fluorescence curves of rhodamine 6G (2 nM) and esfenvalerate–rhodamine conjugate in 0.1 M PBS (20 nM); (b) Fluorescence intensity vs concentration of esfenvalerate–rhodamine conjugate in 0.1 M PBS, slope of line was 211 nM^{-1} with $r = 0.997$.

3.2. Shape and size of micro droplets

The drop shape and size can affect the measured signal; these parameters can be varied by changing the piezo frequency and liquid flow rate, given a fixed diameter of the orifice. In general, use of a micro droplet confers certain advantages on the analysis. The shape of the droplet causes internal focusing of the input laser beam and leads to an intense internal radiation field that can lead to strong emissions when resonances are present. This optical phenomenon was not analyzed in the present study. The absence of surfaces that are present in cuvettes helps to minimize the elastic scattering of laser light. Futhermore, the small size of the micro droplets ensures that all the fluorophores that are present in a sample are interrogated by passage through the measurement volume of the laser.

Images of the drop fluorescence were used to determine whether satisfactory droplet formation had been achieved. Best results were obtained for a piezo driving-frequency of 25 kHz. The liquid flow rate through the orifice was also important. A flow rate of 0.38 ml min^{-1} was found to lead to satisfactory droplet formation, given the orifice that was used. The droplet diameter could be accurately estimated from the simple relationship between the drop size (d, diameter), piezo frequency (f) and flow rate (Q):

$$d = \left(\frac{6Q}{\pi f}\right)^{1/3} \quad (2)$$

The diameter of the droplets in the experiments was approximately 39 μm.

3.3. Antibody quenching

It was found that the fluorescence of the conjugate was quenched after the esfenvalerate and its antibody formed an Ag–Ab complex. Fig. 4(a; a–d) show fluorescence images of the conjugate in a micro droplet with varying amounts of esfenvalerate antibody added to the samples. The volume of liquid that was imaged contained approximately 4.96×10^{-18} mol of esfenvalerate–rhodamine conjugate. After the addition of the anti-esfenvalerate antibody, it is readily apparent from Fig. 4(a) that the intensity of the fluorescence image decreased.

Relative to the antibody, the conjugate is a small molecule. The impact on fluorescence emission can be interpreted in the following manner—interaction between the antigen (esfenvalerate) and the antibody draws the fluorophore into the folded structure of the antibody, leading to quenching of the fluorescence. The specific nature of the quenching by the antibody was confirmed by replacing the esfenvalerate selective antibody with increasing concentrations of BSA. The BSA was found to have no influence on the intensity of the conjugate fluorescence at concentrations far higher than the typical antibody concentrations.

3.4. Fluorescence recovery by the addition of esfenvalerate

The impact of fluorescence quenching can be diminished by adding esfenvalerate to the sample. The equilibrium between the esfenvalerate–rhodamine conjugate and esfenvalerate antibody was altered by the addition of esfenvalerate itself, establishing a new equilibrium among esfenvalerate, conjugate, and the antibody. This competitive reaction can provide a mechanism for carrying out an esfenvalerate assay, with the attractive property that the signal of interest increases with an increasing concentration of the analyte.

The establishment of an altered equilibrium state in the mixture in a cuvette requires time. The effect of finite rate kinetics is evident in Fig. 4(b–c). The fluorescence spectrum of the pure conjugate is compared in Fig. 4(b) with the spectrum obtained by the addition of the antibody at various reactions times. It can be seen that the binding reaction is essentially complete after 40 min. Fig. 4(c) shows that the time to reach quasi equilibrium is approximately independent of antibody concentration. All further experimental results were obtained by allowing the mixture to react for 40 min prior to interrogation in the droplet apparatus.

Fig. 5 illustrates the impact of adding esfenvalerate antibody. Fig. 5(a) shows the fluorescence intensity of the conjugate, and the quenching that was obtained by adding esfenvalerate antibody. The fluorescence intensity of the conjugate decreased from 3000 to 440 counts on the CCD detector (Fig. 5(a)) 40 min after the addition of the antibody. Following the addition of esfenvalerate, the conjugate was released from the immune complex and the fluorescence intensity increased, following the log-linear relationship shown in Fig. 5(b). It is apparent that this scheme can be effective for the detection of esfenvalerate, at least with the pure reagents that were used in this test.

The interference of the matrix in an environmental assay is always a concern. The natural fluorescence from biological materials in a sample can pose a serious problem because of their strong background emission. Although the esfenvalerate–rhodamine assay had shown promise as a sensitive method, it was not certain that it could be used in realistic environmental samples. Consequently, a sample of river water was obtained from a local source (Putah Creek, Solano County CA) for a test of the practical efficacy of this approach. Although the water was not characterized chemically, significant amounts of organic matter were present, including suspended material. The turbidity of the water

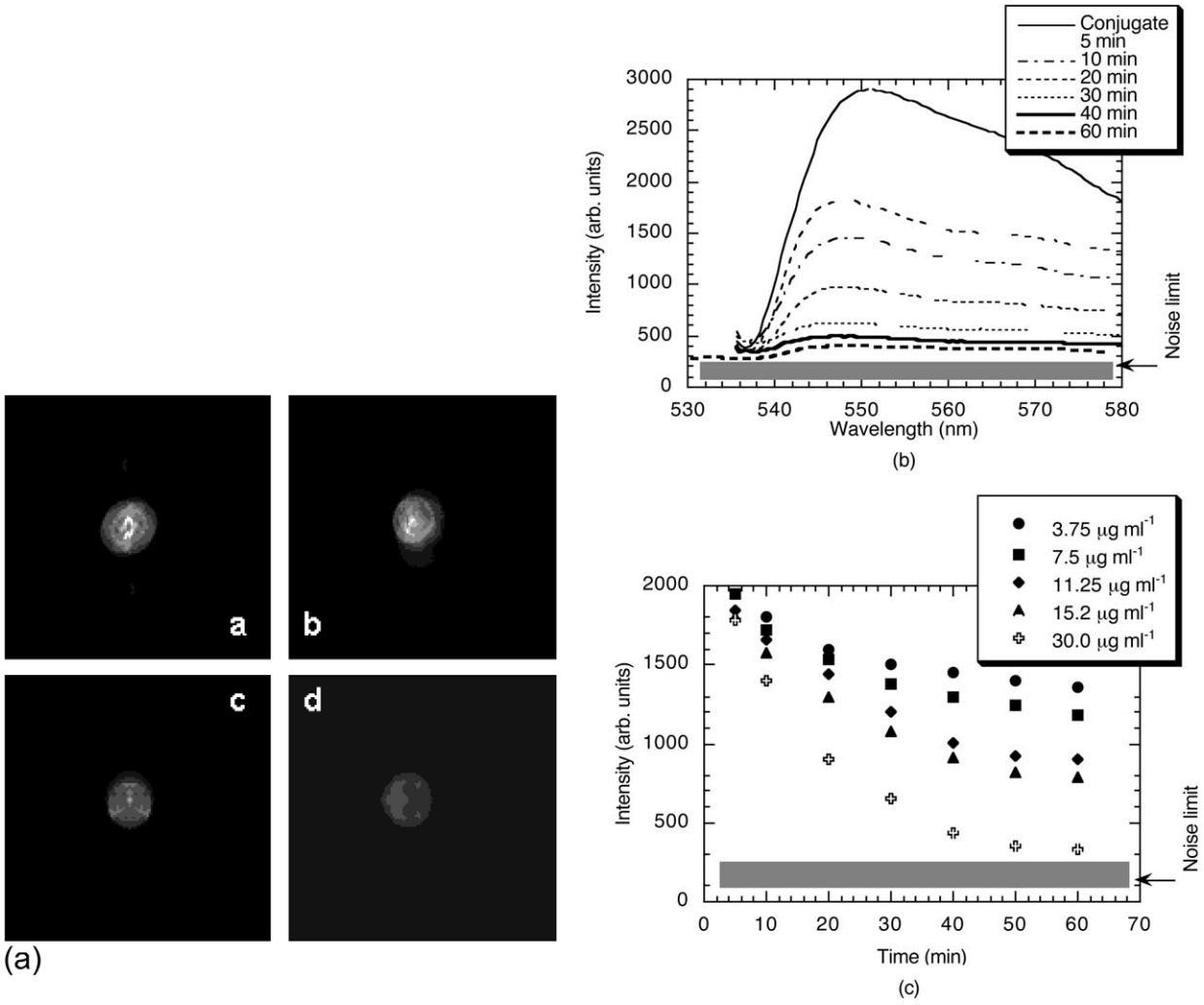

Fig. 4. (a): Fluorescence images of micro droplets with 8.1 nM of conjugate and esfenvalerate antibody concentrations of (a) 0; (b) 3.75; (c) 11.2; (d) 30 μg ml^{-1} respectively; (b): Fluorescence emission spectrum from 8.1 nM of conjugate with 30 μg ml^{-1} of the anti-esfenvalerate antibody for reaction times of 5, 10, 20, 30, 40, 50, 60 min; (c): Time-course of fluorescence quenching by anti-esfenvalerate antibody for antibody concentrations of 3.75, 7.5, 11.25, 15.2 and 30 μg ml^{-1}.

was higher than normally encountered in typical environmental samples.

The assay was carried out in this system-the results are shown in Fig. 6. Fig. 6(a) indicates that the river water matrix had a small effect on the emission from the conjugate, compared to conjugate in pure water, and a small effect on the emission when the antibody was present. The responses of the fluorescence emission to the concentration of esfenvalerate in river water and pure water samples are shown in Fig. 6(b). The trends in fluorescence emissions are similar in both cases, although the slopes and y-axis intercepts of the curves are different. The increased y-intercept in river water at zero added esfenvalerate concentration suggests the presence of compounds in the river water that mimic the binding behavior of esfenvalerate, or suggests the presence of some esfenvalerate itself in the sample. The reduced slope that is observed in the river water assay is indicative of non-specific binding of the antibody.

Fig. 6(a) reveals an effect of the matrix that leads to a typical change of about 25–30% in the fluorescence emission. This was about the same magnitude as that observed in a standard ELISA protocol run in microtiter plates. The effects appear to be lie with the assay and not with the apparatus. This problem could be addressed in several ways when running real samples, and we have used each of these methods in previous studies. One could run the standard curve in the presence of matrix, one could dilute the sample with PBS to reduce interference with a decrease in sensitivity, or one could do a preassay solid phase extraction or other purification–concentration step to remove matrix

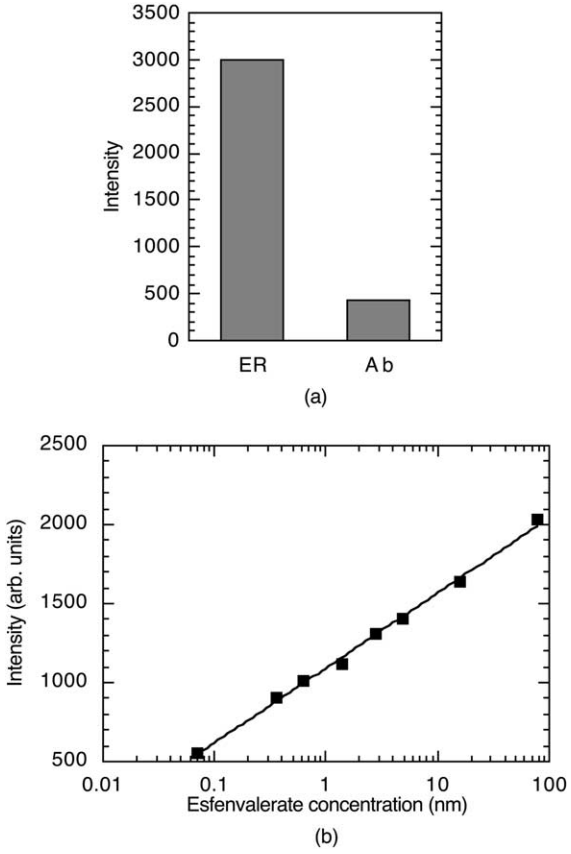

Fig. 5. Fluorescence recovery from the addition of esfenvalerate (a) Comparison of emission from esfenvalerate–rhodamine conjugate only (ER) with emission from conjugate plus antibody (Ab). The conjugate and the antibody concentration were 8.1 nM and 30 μg ml^{-1}, reacted for 40 min (b) Plot of measured emission intensity vs esfenvalerate concentration.

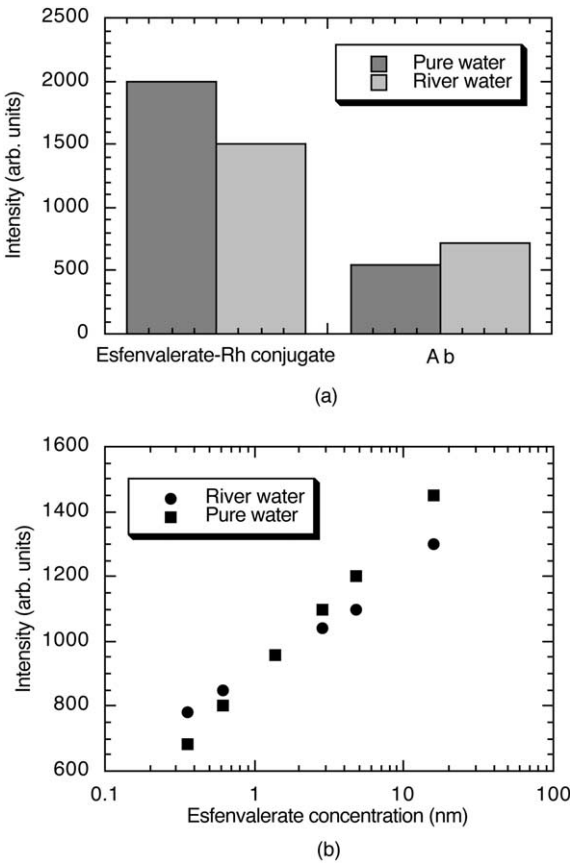

Fig. 6. Comparison of fluorescence emission from samples prepared with nanopure water and with typical river water that contains significant amounts of organic material (Putah Creek, Solano County CA., pH 7.2). The conjugate and the antibody concentrations were 8.1 nM and 30 μg ml^{-1} with 40 min of reaction time (a) comparison of emission from esfenvalerate–rhodamine conjugate in pure water and river water, and a comparison of the conjugate plus antibody (Ab) in pure water and river water; (b) emission intensities as a function of esfenvalerate concentration using pure water and river water as matrices.

effects and increase sensitivity further. This is a most stringent test of the assay, and despite some evident matrix effects, the results indicate the potential for this analysis in realistic environmental samples.

According to the definition described in Eq. (1), we can find the relationship between Q and the concentration of esfenvalerate. The results that are shown in Fig. 7 show that the addition of analyte caused the fluorescence signal to increase. At a concentration of about 71 nM of esfenvalerate, further release of the esfenvalerate–Rh conjugate ceased, leading to a maximum Q of about 0.62-the addition of further esfenvalerate had no effect on the signal. Below 0.07 nM of esfenvalerate, fluorescence recovery was not apparent above the background noise level. Based on the relationship between $(F_E - F_Q)/(F_{ER} - F_Q)$ and the concentration of esfenvalerate, the IC_{50} was 1.91 nM with a detection limit of about 0.1 nM.

The volume of each droplet was 0.27 nl; approximately 30 droplets were detected in each 1 s measurement interval. Therefore, the mass of esfenvalerate that

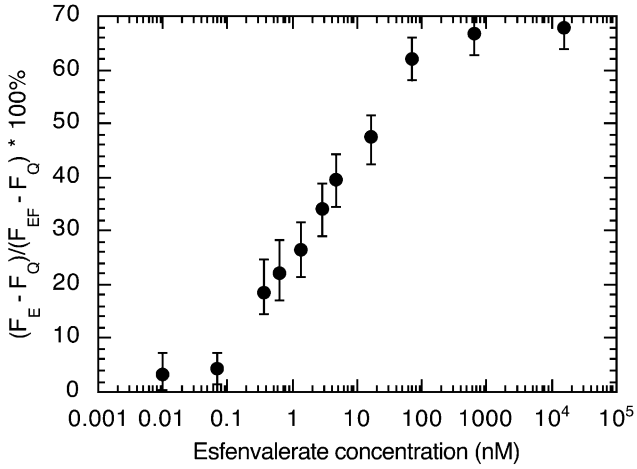

Fig. 7. Relationship between $(F_E - F_Q)/(F_{ER} - F_Q)$ and concentration of esfenvalerate. The IC_{50}, was 1.91 nM. The bars show the standard deviation of curves run on different days.

corresponded to the IC_{50} was a remarkably small 6.5×10^{-6} ng; the mass corresponding to the detection limit was 3.4×10^{-7} ng. For comparison, measurements of esfenvalerate using a 96 well plate ELISA with a UV–vis fluorospectrometer (Instruments S.A. Inc, Edison, NJ) indicated an IC_{50} of 62.5 nM with a detection limit of 6.5 nM (Shan et al., 1999). The corresponding masses of esfenvalerate in 1 well were 2.6 and 0.27 ng respectively.

The limit of detection of the assay is determined by both system noise and inherent limitations of the particular bioassay (non-specific binding for example). The nature of the bioassay itself is not readily changed. However, gains in detectability could be achieved by optimizing the SNR. Noise in CCD cameras arises from several sources. Thermal noise gives rise to a random dark current that is generated on each pixel at a level of typically 1 electron/pixel/s. This source of noise is minimized by cooling the detector, in this case thermo-electrically. The on-board amplifiers and associated electronics also give rise to a source of noise, so-called read noise. The magnitude of read noise is constant, regardless of exposure time, and is typically about 10 electrons/pixel. The illumination of the CCD can also contribute to the noise. Background light from stray scattering or laser line light that leaks through the Raman notch filter adds to the noise, although this is not a random contribution. However, at low light levels, random photon shot noise may be a significant contribution to the overall noise of the system, particularly for longer exposure times. The overall SNR can be written as

$$\mathrm{SNR} = \frac{I\eta t}{\sqrt{(I + I_b)t + I_d t + N_r^2}} \quad (3)$$

where I is the photon flux incident on the CCD detector in photons/pixel/s, η is the quantum efficiency of the pixels, t is the exposure time, I_b is the background light level, I_d is the dark current (electrons/pixel/s), and N_r, is the read noise (rms electrons/pixel). For the 1 s exposure time and the characteristics of the CCD camera, SNR for our lower light levels was about 10. Eq. (3) indicated that the primary noise source was photon noise under these conditions. This could be minimized by using a longer exposure time at the expense of observing larger volumes of sample. On-chip binding (the addition of signals from neighboring pixels prior to read out) of the CCD could also be useful in this regard.

The micro droplet approach that has been discussed could be applied to the improvement of classical fluorescence-based immunoassays. For example, use of compounds with more intense fluorescence or better fluorescence properties (quantum yield or Stokes shift) will improve the assay sensitivity. Given the fact that the implementation of the micro droplet assay in this work is photon noise limited, the improvement of the quantum yield of the fluorophore, and resistance to photobleaching, offer the greatest promise for improved sensitivity. Quantum dots offer considerable promise in this regard.

Furthermore, coupling materials to the antibody that enhance the quenching, could expand the linear range of the assay.

Imunoassays can be formatted and implemented in many ways. The micro droplet method could be applied to classical microwell systems with a ‘slurper’ system commonly available on research spectrometers. It is possible that the simplest application would be as a detector on a continuous flow or FIA-like system. Such approaches are particularly attractive for on-line or sequential monitoring. In addition, the entire system lends itself to microengineering where very small reaction volumes can lead to the conservation of expensive reactants.

4. Conclusions

Carrying out chemical analyses in very small volumes confers significant benefits. Micro droplets are particularly useful because they accurately define the volume of sample that is interrogated by a focused laser beam. In addition, scattering from the matrix of the sample is minimized, as is elastic surface scattering. Micro droplets are ideally suited for environmental analyses using a competitive, homogeneous immunoassay. Measurements of esfenvalerate concentration have shown that the quenching of fluorescence from an esfenvalerate–rhodamine conjugate via binding with a specific antibody provides a sensitive assay for esfenvalerate itself. The analyte displaces the conjugate from the binding site on the antibody, allowing the conjugate fluorescence to increase. Analyte concentrations of about 0.1 nM can be measured with this method, a significant improvement over a typical commercial instrument. In addition, the approach is sufficiently robust to permit measurements to be obtained in realistic, environmental samples of water.

The general techniques demonstrated here with immunoassays also have potential for application to highly sensitive detection systems in biochemistry and analytical chemistry. For example, fluorescence quenching and FRET can be readily applied to receptor binding assays for high throughput drug screening, or for rapid or stop flow enzyme kinetics. In addition, the micro droplet method may be well suited for application to high performance liquid chromatography. Post column break-up of the liquid flow into droplets could be used to conduct this form of sensitive detection.

For most analytical applications, sensitivity is discussed in terms of concentration. However, in some cases sample size is severely limited, for example in

neonatal testing, where an increasing number of assays are being run on very small samples. A very high sensitivity in terms of mass is apparent with the micro droplet assay in which it is found that only 6.5×10^{-6} ng of esfenvalerate are needed to inhibit 50% of the fluorescence. In terms of mass, the limit of detection was 3.4×10^{-7} ng.

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