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New fluorescence tools for investigating enzyme activity

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Abstract

Novel fluorescence-based enzyme–substrate probes have been fabricated which incorporate a unique utilization of chemically modified micron-sized particles in conjunction with a single-excitation dual-emission wavelength ratio technique. By chemically modifying micron-sized particles with both an enzyme-specific substrate and a reference fluorophore the effects of source intensity fluctuations, fluorophore diffusion, and variances in substrate loading inherent in in situ biological fluorescence assays can be reduced. Thus these probes have the potential to provide more sensitive and less invasive fluorescence detection of enzyme activity in solution, in microorganisms and in single cells. In addition, proper selection of particle size facilitates selective targeting of microorganisms through natural ingestion processes. Examples of source fluctuation and substrate loading corrections are provided for in in vitro experiments with a common esterase species. The in situ application of these probes in individual microorganisms which are used as biosensors is also discussed.

Keywords: Biosensors; Enzymatic methods; Fluorimetry; Microorganisms

1. Introduction

New fluorescence technologies for monitoring enzyme activity in single cells and multicellular microorganisms are required in order to generate more sensitive and less invasive assays. Improvements in the quality of data obtained from single-cell and single-microorganism fluorescence assays will ultimately provide more accurate information regarding cellular processes and allow these processes to be manipulated and better utilized for practical applications.

1.1. Microorganism-based biological sensors

Enzymes play a major role in all living organisms, mediating cellular functions such as metabolism, respiration, and immune system response and perturbations in these functions provide a means for assessing an organism's general health status. Investigating and monitoring these cellular processes in microorganisms can provide the foundation for the development of inexpensive and rapid biological sensors which can be used for chemical toxicity evaluation as well as environmental monitoring.

Detoxification and metabolism-related enzyme levels have been observed to be directly related to

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the type and magnitude of toxicant exposure. Snell and coworkers [1,2] have used separately ingestion of fluorescent micron-sized spheres and enzyme activity to quantitate organism "stress" associated with the exposure to toxicants such as heavy metals and chlorinated organics (e.g. pentachlorophenol). These biological responses were then used as the foundation for rapid environmental monitoring schemes. In both of these experiments, fluorescence image analysis techniques were used for quantitating the number of particles ingested and the extent of enzyme activity in each individual organism. Both spatial and temporal characteristics of enzyme activity in single cells and microorganisms are important since understanding these variances facilitates the development of more reliable sensors.

There are a number of methodological inadequacies that limit the usefulness of enzyme activity measurements obtained from fluorescence images of whole cells and individual microorganisms. Exposure of an organism to soluble enzyme substrates yields fluorescence intensity in numerous locations throughout the organism due to the non-specific transport of enzyme probes across the cellular membranes. Membrane-permeant probes are commonly produced by generation of the acetoxymethyl ester (AM) derivative and although this synthetic modification provides a means for transporting the probes across the cellular membrane [3], which is critical for in situ investigations, it does not usually provide selective targeting of specific locales. This is unfortunate, since in many microorganisms there are regions of increased activity such as the digestive tract which provide important information on health status. Uncharacterized regions of lesser activity complicate image analysis and ultimately the quantitation of fluorescence intensity. Quantitating perturbations in fluorescence when an organism is exposed to a toxicant is difficult since size, uptake, and exact location of the digestive tract are individual specific. Fluorescence intensity generated by enzyme activity also slowly fades as the fluorophore diffuses out of the organism. This method of monitoring enzyme activity not only introduces an uncertainty in the measurement of fluorescence intensity and interpretation of the data, but also limits the investigator to one enzyme assay per individual organism, thus eliminating the possibility of averaging successive exposures and correlating different exposures with the same individual. Fluctuations in source intensity affects the measured fluorescence and provides additional difficulties in quantitating enzyme activity. A slow gradual decline in lamp output and spectral shift (especially with the use of inexpensive mercury-lamp sources) makes daily comparisons of similar biological systems complicated. Heterogeneous cell supports used with inverted microscopes, and changes in cell/organism thickness also add to the variations in delivered and collected radiation. As a result, development of technology that improves quantitation of biological activity by improved probe localization and signal detection is clearly needed.

1.2. New enzyme probes

We have fabricated fluorogenic enzyme probes based upon the chemical modification of micron-sized spheres. These chemically modified spheres (CMS) have an impregnated fluorophore as well as a fluorogenic enzyme probe covalently bound to the outside surface of the particle. Enzymatic activity occurring at the particle surface produces a fluorophore that emits in the UV–Vis–NIR region of the spectrum. The impregnated fluorophore has a spectral emission also in the UV–Vis–NIR, but separated from that of the fluorescent enzyme probe. This fluorophore is also unaffected by the chemical nature of the particle's environment. By utilizing the ratio of these two fluorophore emissions generated by a single excitation wavelength, variations in the particle or ingestion rate in individual organisms (which determines the amount of enzyme substrate delivered) and fluctuations in excitation source intensity can be corrected.

These novel enzyme–substrate probes provide less invasive and more sensitive detection of enzyme activity in microorganisms by improving the targeting of the digestive tract, eliminating the leakage of enzyme generated fluorophore, and reducing the effects of instrumental fluctuations. Examples of source intensity fluctuation and substrate concentration loading corrections are provided for in vitro experiments utilizing a common esterase enzyme. In situ application of these probes in microorganisms (rotifers) important in the aquaculture industry and currently proposed as biosensors is also discussed. In

this particular application, it is the physical attributes of the probe (size) that facilitates specific targeting of the enzyme probe in the organisms digestive tract and subsequent excretion of the enzyme probe.

2. Experimental

2.1. Synthesis of esterase–CMS

The synthesis of chemically modified spheres for monitoring esterase activity is diagrammed in Fig. 1. Solid carboxyfluorescein diacetate succinimidyl ester was obtained from Molecular Probes, stored at 0° C, and used without further purification. Dimethylsulfoxide (DMSO) was obtained from Baker and used without additional purification. Fluorescent latex micro-particles, *Fluospheres Nile Red*, were obtained from Molecular Probes and Interfacial Dynamics in the 0.933-μm range. These particles are manufactured by Interfacial Dynamics with amine functional groups (six carbon extensions) and impregnated by Molecular Probes with various fluorophores, in this case Nile Red.

One milligram of carboxyfluorescein diacetate succinimidyl ester in DMSO was placed in a vial with 0.5 ml of (1×10^{10} particles ml^{-1}) 0.933-μm fluospheres impregnated with Nile Red ($\lambda_{ex} = 530$ nm, $\lambda_{em} = 605$ nm). After three hours of incubation, conjugation of carboxyfluorescein diacetate through an amide bond with the particles was obtained. Dialysis against DMSO completed the clean-up. Particles were frozen in DMSO until use.

2.2. In vitro enzymatic assays

Esterase (3.1.1.1) was obtained from Sigma. Salt solutions of these enzymes were obtained in liquid form and stored refrigerated. Carboxyfluorescein diacetate was obtained from Molecular Probes. Tris(hydroxymethyl)aminomethane (THAM) was obtained from Sigma and used without purification. Buffer solutions were prepared with 18.0 MΩ Nanopure water (Barnstead) at 50 mM and pH 8.3.

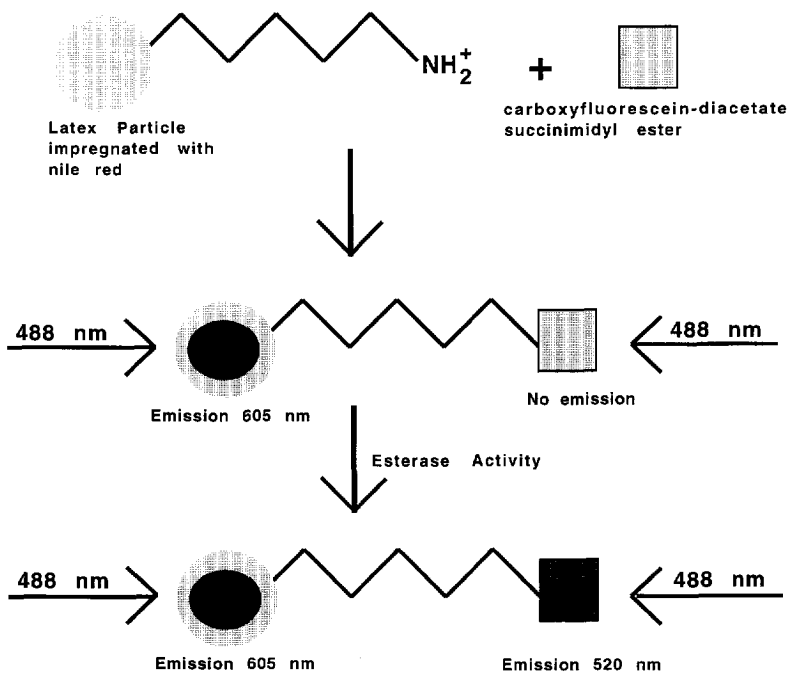

Fig. 1. Esterase–CMS probe design. A one-step reaction is used in conjunction with a dialysis step, for reaction clean-up, to produce an enzyme probe that targets esterase species. A Nile Red impregnated fluorescent micron-sized particle ($\lambda_{ex} = 530$ nm, $\lambda_{em} = 605$ nm) is used for the substrate carrier and carboxyfluorescein diacetate ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 520$ nm) is used as the enzyme substrate.

2.3. Emission spectra and in vitro enzyme assays

A multichannel fluorimeter was utilized to collect emission spectra of the chemically modified spheres and to conduct the enzymatic assays. The fluorimeter consisted of an argon-ion laser (Coherent) for excitation at 488 nm, $f/3$ collection optics, a Spex CP200 spectrograph incorporating a single holographic grating (200 grooves mm^{-1}) with a 200–850 nm spectral range, and a Princeton Instruments charge coupled device (CCD) detector. The CCD detector incorporated an EEV 1152×298 1-inch chip and was liquid nitrogen cooled. Software for instrument control and data analysis was written in house with Labview (National Instruments).

2.4. Fluorescence analysis of microorganisms

Rotifers of the species *Brachionis calyciflorus* were obtained from Professor T.W. Snell, School of Biology, Georgia Institute of Technology. Organisms were hatched from cysts by overnight incubation in a standard freshwater medium. The organisms were allowed to ingest the CMS particles for 15–30 min and were then immediately anesthetized with tricaine methylsulfonate. White light/fluorescence images were obtained with an inverted microscope (Olympus, IMT-2) in an epi-fluorescence geometry utilizing a 500 nm dichroic mirror (Olympus) and a 515 nm long-pass filter (Schott Glass). Isolated measurements of enzyme activity and particle fluorescence utilized 520 ± 10 nm and 600 ± 25 nm bandpass filters, respectively. A Javelin 2/3 inch CCD TV-rate camera was used for imaging in conjunction with a video capture board (Perspectics) and a Macintosh IIci. IPLAB was used to control data acquisition as well as to quantitate fluorescence intensity. An argon-ion laser (Coherent) was used for excitation.

3. Results and discussions

Chemically modified spheres (CMS) targeting esterase-related enzymes were constructed as an initial

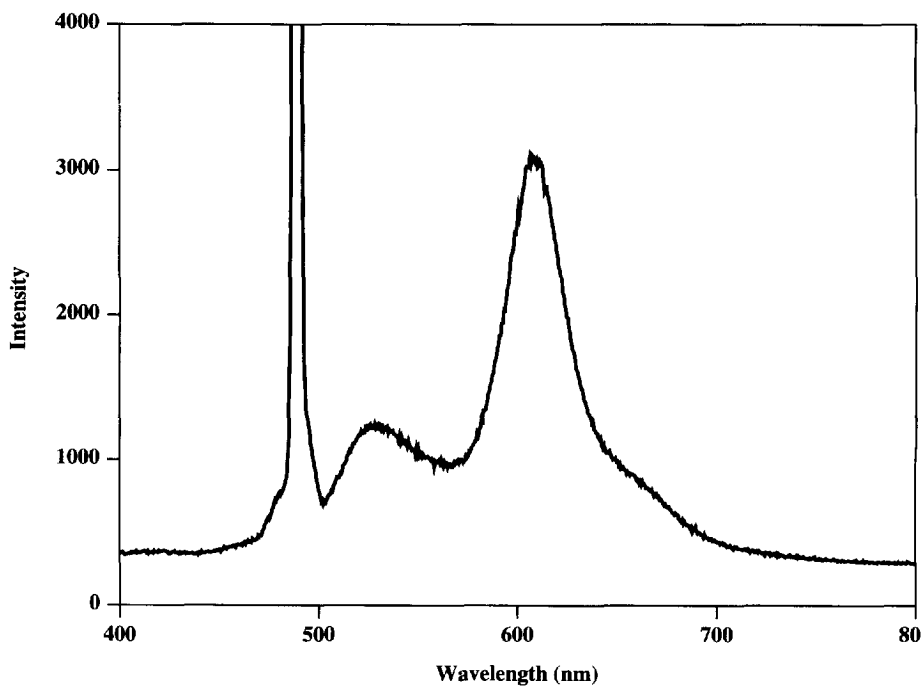

Fig. 2. Emission spectra from a solution of esterase–CMS which have been partially hydrolyzed by an esterase enzyme. The emission peak at 520 nm is due to fluorescein generated by enzymatic activity on the particle surface. The peak at 605 nm is due to the fluorophore Nile Red impregnated in the latex particle. The excitation source Raleigh line (488 nm argon-ion laser) is included as a reference.

demonstration of this enzyme probe technology. This esterase probe incorporated the non-fluorescent molecule carboxyfluorescein diacetate covalently bound to the surface of a one-micron latex sphere impregnated with the fluorophore Nile Red. Coupling of this enzyme substrate to the particle is completed in a single one-step reaction by utilizing an amine functionality on the particle and a succinimidyl ester functionality on the enzyme substrate (carboxyfluorescein diacetate). The need for traditional chromatographic steps for probe "clean-up" is eliminated since micron-sized particles can be separated from the chemical reagents by a quick dialysis protocol. Enzymatic cleavage of the acetoxymethyl esters yields the highly fluorescent fluorescein molecule, which has an emission maximum near 520 nm when excited with 488 nm radiation. Emission from the particle, which is excited simultaneously with the enzyme-generated fluorophore, is at approximately 605 nm. A pictorial description of the esterase probe system is provided in Fig. 1, and the emission spectrum of partially hydrolyzed esterase–CMS probes is provided in Fig. 2.

3.1. Ratio-based spectroscopic measurements

The use of a reference signal in the field of analytical spectroscopy is well ingrained and is utilized extensively to correct for perturbations in instrumental characteristics. An extension of this basic idea is used to reduce the effects of variations in the amount of substrate delivered in situ and source/pathlength fluctuations in the quantitation of fluorescence signals generated from cells and microorganisms. Obtaining this correction capability has traditionally focused on three molecular level strategies, and has been used extensively with ion probes [3,4]. All three strategies rely on a ratio of signals generated from the location of the fluorescent probe. In the first strategy, molecules that yield a change in absorbance profile after binding of an ion are used. A single emission wavelength is monitored and the ratio of this signal at two excitation wavelengths is used. This type of experiment can easily be remembered by the acronym DESERT (dual-excitation single-emission ratio technique). Extensive spectroscopic and synthesis effort has been directed in this area, with the most well-known probes being used for intracellular calcium determination [3–5]. Unfortunately, few molecules have isosbestic points which provide one of the two excitation points. In the second strategy, molecules that undergo emission shifts upon binding of an ion are used. In this case a single excitation wavelength is used and two appropriate emission filters or monochromator settings are used [3,4]. This technique can easily be remembered with the acronym SEDERT (single-excitation dual-emission ratio technique).

The third strategy, also a SEDERT type, does not focus on spectroscopic changes in a single species but attempts to place both a probe molecule and a reference molecule in the same general spatial location. This requires simultaneous delivery of both species to the target location by either linking them to the same carrier or by mixing separate carriers. Successful demonstrations of these probe systems have been made by Molecular Probes [6] as well as Bronk et al. [7]. In the Molecular Probes system, both biological probe (ion) and reference fluorophore are conjugated to high molecular weight linear chains of dextran. With respect to the work conducted by Bronk et al., two independent dextran chains were used, with one serving as a carrier of the biological probe and one carrying the reference fluorophore. These two different dextran species were then mixed before use.

Fabrication of enzyme probes with micron-sized spherical particles incorporating a reference fluorophore in addition to a fluorogenic enzyme probe covalently attached to the surface allows the single-excitation dual-emission ratio technique to be exploited and provides exciting new possibilities for investigating enzyme activity in situ. This probe design has been characterized by in vitro as well as in vivo experiments and the results are discussed below.

3.2. In vitro source corrections with chemically modified spheres

Quantitation of the source fluctuation correction capability due to the utilization of the single-excitation dual-emission ratio capability designed into the CMS probe system was conducted. One-micron-sized particles containing Nile Red as a reference fluorophore and covalently attached carboxyfluorescein

diacetate as an enzyme substrate were investigated in vitro.

Correcting enzyme-generated fluorescence intensity measurements for source intensity fluctuations was demonstrated with esterase–CMS particles that were partially hydrolyzed due to exposure to a methanol–carbonate solution. Argon-ion laser excitation at 488 nm was used in conjunction with neutral density filters (Melles Griot) to systematically alter the excitation power illuminating the cuvette containing this suspension of partially hydrolyzed particles. Intensity measurements were taken (single-point) at the emission maximum of each fluorophore and the intensity ratio of these two signals was calculated (see Fig. 2). Integration times were adjusted to maintain an adequate signal-to-noise ratio. The results of this simple demonstration for an average of ten trials (data not shown) provided a relative standard deviation (R.S.D.) of 3% for the ratio (520 nm/605 nm) over the approximately three orders of magnitude range of excitation powers (1 mW–1 W) used. This range of powers is certainly extreme for short-term source fluctuations, but is not unrealistic for the magnitude of change that can occur over many months with mercury and xenon lamps, and demonstrates the capability of the CMS probes to correct for daily changes in source output.

3.3. In vitro substrate concentration corrections with chemically modified spheres

The precision of enzyme assays is affected by variations in the concentrations of enzyme and substrate used in an assay. Changes in substrate concentration, when a constant enzyme concentration is used, directly affects the observed fluorescence signal when working in the first-order region of the rate versus substrate concentration curve. Although substrate concentrations in in vitro assays are easily controlled in the laboratory, the precision of in situ measurements is relatively poor due to the difficulties in routinely delivering the same concentration of substrate to the targeted location. As an example, when targeting the digestive tract of a microorgan-

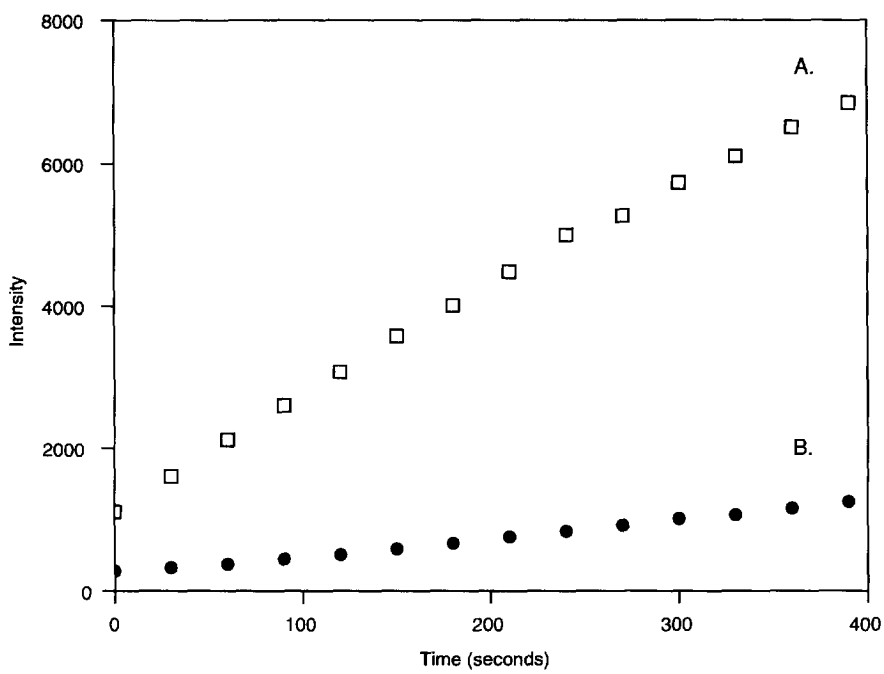

Fig. 3. Relative hydrolysis rates for (A) esterase–CMS (3.4 μM) and (B) "free" carboxyfluorescein diacetate (3.5 μM). Identical enzyme concentrations were used.

ism with CMS probes any variations in feeding rate (particle uptake) will influence the concentration of enzyme substrate that is delivered to this locale.

By utilizing the single-excitation dual-emission ratio capability incorporated into the esterase–CMS probe, corrections for the measured fluorescence signal due to variations in substrate concentration when a fixed enzyme concentration is used in vitro was quantitated. By holding the enzyme concentration constant and varying the particles per ml (substrate concentration) kinetic curves of intensity versus time were obtained (data not shown). As the particle density is increased, the concentration of enzyme substrate in the cuvette increases accordingly and the fluorescence signal increases proportionally. After correction of the intensity versus time data for background noise, the fluorescein/Nile Red (520 nm/605 nm) intensity ratio was calculated. Twelve time points between 10 and 20 min were used to calculate a ratio for each individual substrate concentration (particle density). In theory, the same fluorescein/Nile Red ratio (520 nm/605 nm) should be obtained if the substrate concentrations are located in the first-order region of the rate versus substrate concentration curve. The experimentally determined average of this ratio was 0.65 with an R.S.D. of 2.3%. These results are very reasonable for the magnitude and number of dilutions required in this demonstration.

3.4. In vitro comparison of free and bound enzyme substrate kinetics

A comparison of the pseudo-first order enzyme kinetics for free carboxyfluorescein diacetate and the esterase–CMS was conducted. The results of the intensity versus time plots are provided in Fig. 3. In these in vitro experiments the enzyme concentration was held constant and similar concentrations of either carboxyfluorescein diacetate covalently attached to one-micron particles or free in solution were used. The manufacturers data was used to estimate the substrate concentration carried by the particles. A coupling efficiency of 100% was assumed, providing a conservative estimate of the total substrate bound to the particles. The results of these experiments show that a significant increase in rate exists between carboxyfluorescein diacetate bound to latex particles when compared to a solution containing free enzyme substrate. Although a change in the enzyme–substrate binding due to the immobilization is possible, it is also possible that substrate immobilization on micron-sized particles provides an increase in the collision probability for the system and therefore an effectively higher substrate concentration is realized. This phenomenon is currently being explored further, as considerable benefits for investigating very dilute solutions of enzymes could result.

3.5. Investigating enzyme activity in microorganisms

The use of microorganisms as biosensors for environmental monitoring has shown the potential for providing inexpensive rapid sensors and thus research in this area has become significant. One species that has received intense study as an environmental biosensor is the rotifer *Brachionus calyciflorus*. Rotifers are of global importance in fresh and marine waters, in the food chain of estuaries, and are a major food source in aquaculture. They are also significant grazers of phytoplankton and significantly influence water quality.

The digestive tract is an important locale for monitoring changes in metabolism induced by perturbations in water quality. Perturbations in enzyme activity in rotifers, specifically in the digestive tract, are sensitive and rapid indicators of environmental stress associated with exposure to organic and inorganic compounds. Researchers screening field sites have used soluble enzyme substrates to probe enzyme activity and perturbations in these levels in the rotifers digestive tract after exposure to contaminated water samples [8]. Difficulties inherent in these assays include the significant variability in rotifer size and substrate uptake. These variations between individuals present difficulties when interpreting fluorescence images since the size, shape, and location of the fluorescence image to be quantitated is chosen arbitrarily.

Many aquatic microorganisms, including rotifers, identify food by size and thus the correct choice of particle size allows selective targeting of the digestive tract of different organisms as well as providing a means of selectively targeting specific organisms in a heterogeneous mixture based on factors such as size and age. Particles in the micron size range are ingested naturally by filter feeders and have been

shown to localize in the rotifers digestive tract [9]. Proper sizing of the enzyme probe therefore facilitates targeting the digestive tract of aquatic organisms by "natural" ingestion mechanisms.

In an initial demonstration of the CMS probe technology rotifers were investigated with esterase–CMS and the R.S.D. in the mean enzyme activity compared to the standard assay.

White light/fluorescence images of the rotifer *B. calyciflorus* exposed (15 min) to esterase–CMS are provided in Figs. 4 and 5. The one-micron-sized particles are clearly localized in the digestive tract with the majority present in the lower regions. Fig. 4 was obtained with a long-pass 515 nm filter (Schott) which allows both the esterase (fluorescein) and particle signals (Nile Red) to be measured. Regions of red intensity denote particle location while locales exhibiting orange and yellow colors are combinations of fluorescein and Nile Red emissions. In Fig. 5, a 520 ± 10 nm bandpass filter was used to separate the enzyme signal (fluorescein) from the particle emission. Clearly the geometry of the fluorescent region matches that observed in Fig. 4. Quantitation of esterase activity with 50 individual rotifers provided a mean intensity measurement with an R.S.D. of approximately 30%. A similar R.S.D. was obtained when the Nile Red signal was quantitated with a 600 ± 25 nm bandpass filter. The novelty of the CMS probes in this situation is that both ingestion rate (Nile Red signal) and enzyme activity (fluorescein signal) are determined in the same experiment. This reduces the time and cost associated with conducting complimentary toxicity assays. The similarity in the R.S.D.s of these two biological processes provides a measure of the variation between individual organisms. In this investigation the rotifers ingested a significant number of CMS probes and thus the enzyme assay was conducted under

Fig. 4. Fluorescence image (100 ×) of the rotifer *B. calyciflorus* exposed to esterase–CMS. Particles are localized in the digestive tract. The image was collected with a Schott 515 long-pass filter. Red regions of the organism denote the presence of a CMS particle and insignificant enzyme activity. Regions of yellow and orange denote both fluorescein and Nile Red emission indicating enzymatic activity.

Fig. 5. Fluorescence image (100 ×) of the rotifer *B. calyciflorus* exposed to esterase–CMS. Particles are localized in the digestive tract. This image is of the same organism pictured in Fig. 4 but imaged with a 520 ± 10 nm bandpass filter. Fluorescein emission indicating enzyme activity is observed in the same spatial location as the orange/yellow regions shown in Fig. 4.

pseudo-zero order conditions and there is no need to correct for the ingestion rate of each individual. Quantitation of enzyme activity with the standard fluorescence methodology yields R.S.D. levels between 20 and 100%. Further investigation of the effects of exposure time to these probes and age of the organisms is ongoing.

Advantages in the use of this type of enzyme probe include the fact that the fluorophore generated from enzymatic activity is bound to the particle. The particles are excreted by the organisms after several minutes facilitating repeated analysis with a similar probe or with others. This situation also greatly reduces unwanted localization in cellular compartments and membranes since the particle cannot cross the membrane of cell organelles. An increase in assay sensitivity is realized since leakage of the fluorescent probe from the organism is eliminated as a result of the covalent attachment of the fluorophore to the particle. Additional advantages include: (1) the latex particles have a low probability of metabolic degradation, (2) there is a fixed spatial geometry for the enzyme probe and reference fluorophore, thus reducing undesirable energy transfer which complicates quantitation of enzyme activity, and (3) the reference signal is shielded from environmental conditions (pH, oxidation, etc.) that can alter this signal.

4. Summary

Covalent binding of fluorogenic enzyme substrates to micron-sized particles provides many advantages for quantitating fluorescence intensity obtained from whole microorganism images and generates many interesting possibilities for in vitro and in vivo analysis of enzyme activity. This biological probe concept may be used to design probes to target various biological systems and biochemical pathways since it is based upon a generic carrier system.

Probe design possibilities include the following: (1) the carrier particle can be constructed with organic or inorganic materials, (2) the internal reference signal can be generated by organic or inorganic species such as rare-earths and chelates of rare-earths, (3) particle size can range from angstroms to microns with commercially available particles in this range, and (4) the coupling chemistry between the probe and the carrier can utilize standard protein coupling chemistry as well as current peptide synthesis schemes. The major advantage of this technology results from the use of a single excitation source in combination with a covalently bound enzyme substrate and a reference signal which is generated with an additional fluorophore incorporated into the carrier particle. Fluctuations in source intensity and in the amount of enzyme substrate delivered internally to the organisms are corrected for by using the ratio of the enzyme generated signal and the reference fluorophore. Numerous applications for solution, single cell, and single organism analysis are being investigated.

Acknowledgements

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