Article

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Single-Bead Fluorescence Microspectroscopy: Detection of Self-Quenching in Fluorescence-Labeled Resin Beads

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We have characterized two sets of fluorescence-labeled beads with single-bead fluorescence microspectroscopy. Our results demonstrate the possible self-quenching effect on the fluorescence in the resin bead and, therefore, call for a careful selection of fluorophores and a rigorous control of the labeling reaction yield in order to generate labeled beads for combinatorial chemistry and other disciplines.

It is natural to think that when a fluorophore is attached to a resin bead, the bead will fluoresce and the intensity of the fluorescence will be proportional to the fluorophore concentration. However, in this article, we present experimental evidence that shows that this assumption does not always hold true. Since the technique of labeling beads with fluorophores has begun to play an important role in fields such as combinatorial chemistry,[1] the spectral properties of fluorescent beads deserve analysis.

Split-and-pool technology[2] in combinatorial chemistry assembles one-bead-one-compound libraries. It is a method used to make a huge number of diverse compounds rapidly. Associated with this approach, fluorophores have been used to tag beads in order to record synthesis history.[3] To identify the active compounds on beads, fluorescence-labeled proteins were used to bind ligands that are attached to beads. The newly fluorescent beads are then selected, yielding lead compounds in the search for enzyme inhibitors,[4] carbohydrate ligands,[5] and binding site information.[6] For the same purpose, fluorescence activated cell sorting (FACS) technique has been used to identify protein binding beads. Similar approaches have also been used to develop a novel class of optical sensors.[7]

Bead fluorescence has been studied by the microscopy method, e.g., to identify fluorescence-stained beads.[4b] However, there is, with the exception of a confocal microscopy study of the single-bead fluorescence,[3] a lack of understanding of the spectroscopic properties of fluorescent beads. Scott and Balasubramanian[12] reported the fluorescence property of resin bead suspensions. Since for one-bead-one-compound libraries a single bead is the ultimate reactor and assay unit, we reported here the fluorescence results on single resin bead. We have characterized two sets of fluorescence-labeled beads with single-bead fluorescence microspectroscopy. Our results demonstrate a self-quenching effect on the fluorescence in the resin bead and, therefore, call for a careful selection of fluorophores and a rigorous control of the labeling reaction yield in order to generate labeled beads for combinatorial chemistry and other disciplines.

Results and Discussion

1. Single-Bead Fluorescence: Self-Quenching. Wang resin **1** (1.0 mmol/g) reacted with a fluorescent molecule 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid (NBD-X) to form **2** as shown in Scheme 1. The time course of this reaction was monitored by the single-bead FTIR microspectroscopy.[8] The relative peak areas at 1732 cm^{-1} indicate the relative yields of the product **2**. The reaction between the fluorescence dye and the Wang resin proceeded by 10% and 20% at 5 and 10 min, respectively. The reaction reached completion in 1.5 h as shown in Figure 1A. These beads contained a loading of the fluorescent molecule NBD-X at various levels and were subjected to the single-bead fluorescence measurements.

All single-bead fluorescence spectra were collected on a SEE 1000 microspectrometer equipped for transmission, reflectance, and fluorescence spectroscopy. Fluorescence measurements utilized a filtered 100 W mercury lamp for incident illumination. Exciting light was obtained at 436 nm for NBD-X-labeled bead and 345 nm for dansylhydrazine-labeled bead.

The fluorescence spectra were obtained from beads that were washed free of any starting materials and solvent. Beads were placed on a glass slide and measured directly. The spectral measurement area was a square with 12.5 μm on a side. In all cases, 10 scans were averaged. Beads were first located utilizing the transmission light source of the microscope. The transmission light source was then blocked and the fluorescence lamp activated. The bead with 10% loading (5 min reaction) showed strongest fluorescence (Figure 1B) while the bead with 20% loading (10 min reaction) exhibited only $^1/_5$ the intensity. The beads of the 60 min reaction sample were nearly completely quenched (only 2% fluorescence intensity).

2. Single-Bead Fluorescence without Self-Quenching. The reaction in Scheme 2 was carried out as described

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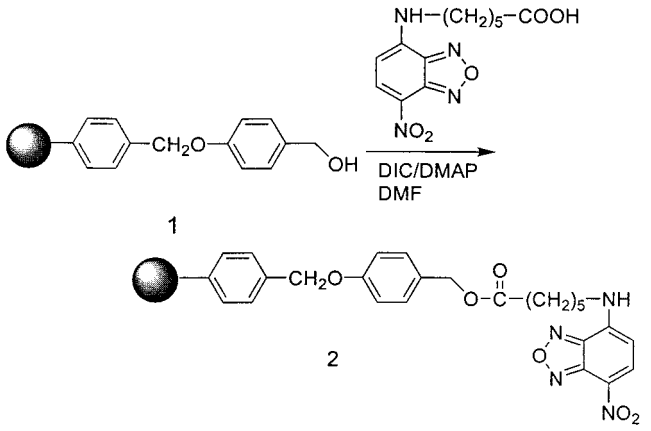
Scheme 1



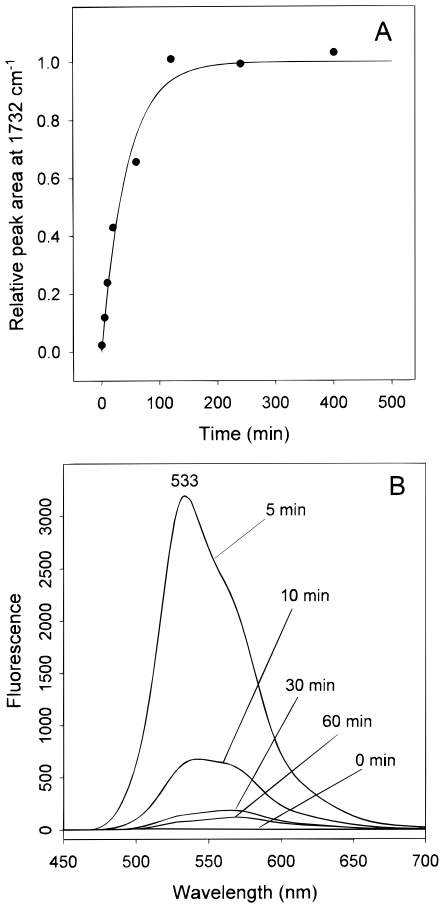
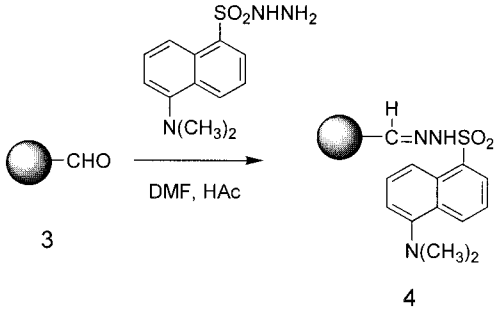


Figure 1. (A) Time course of the reaction in Scheme 1. Integrations of an IR band of the product at 1732 cm^{-1} were plotted against time. (B) Single-bead fluorescence spectra of samples after reacting for various times. The loading of each sample can be estimated from A.

previously.[9] Formylpolystyrene resin **3** (0.61 mmol/g) reacted with dansylhydrazine to form the resin-bound compound **4**. The increase in the fluorophore loading with the reaction time is shown in Figure 2A. The relative peak areas at 2790 cm^{-1} (hydrazone band) indicate the relative yields of the product **4**. Single-bead fluorescence spectra of beads with different loadings of dansylhydrazone are shown in Figure 2B. No quenching is observed. The fluorescence intensity of beads increases with time. The trend parallels the increase of dansylhydrazone loading on bead.

Scheme 2



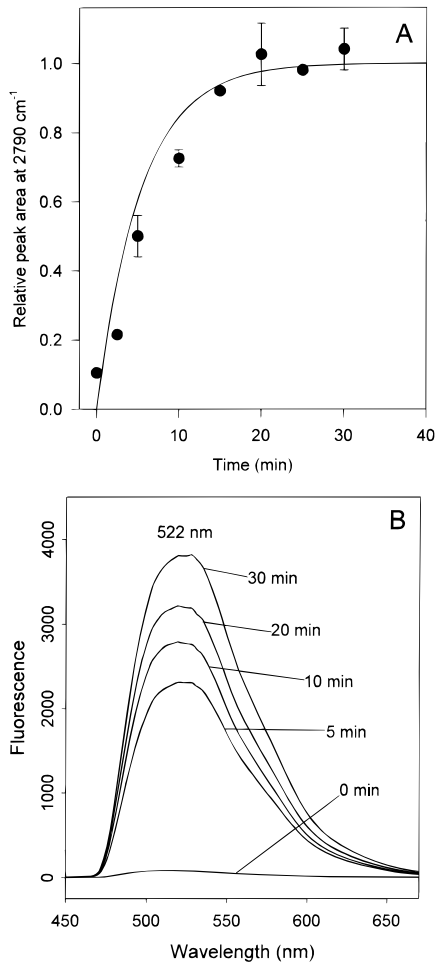


Figure 2. (A) Time course of the reaction in Scheme 2. Integrations of an IR band of the product at 2790 cm^{-1} were plotted against time (average of three measurements). (B) Single-bead fluorescence spectra of samples after reacting for various times. The loading of each sample can be estimated from A.

3. Mechanism of the Self-Quenching. Self-quenching can occur in high-concentration solutions of fluorophores.[10] Two determining factors are the interfluorophore distance and the spectral overlap between the absorption spectrum and the emission spectrum of the sample. There appears to be no simple and reliable method to precisely quantify and correct the self-quenching effect. An empirical way is to use fluorophore with a large Stokes' shift and use a dilute solution. The dependence of energy transfer on the physical distance between the donor and the acceptor has been demonstrated.[11] The efficiency of energy transfer is 100% at a distance of 1.2 nm and 16% at 4.6 nm.[11]

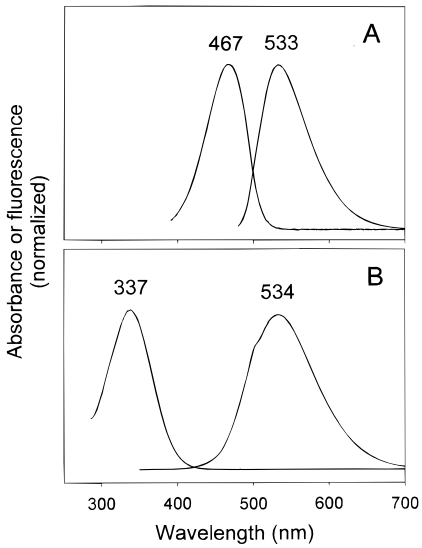


Figure 3. (A) UV−vis absorption and fluorescence emission spectra of NBD-X. (B) UV−vis absorption and fluorescence emission spectra of dansylhydrazine. The UV−visible and fluorescence spectra of dyes were measured in ethanol. The concentrations of dyes are ~10 μM for the UV−visible measurement and ~1 μM for the fluorescence measurement. All spectra were normalized to just compare their absorption or emission maxima. The excitation light for fluorescence is at 436 nm for NBD-X and 345 nm for dansylhydrazine.

The distance between fluorophore molecules on a resin bead can be estimated by noting that the average bead diameter for the resins we used was 88 μm and there are 2.1×10^6 beads in 1 g of resin beads. The volume of a single bead is 3.6×10^{-13} m^3. The average single-bead loading is 480 pmol, assuming the loading is 1.0 mmol/g which corresponds to 2.9×10^{14} sites on a single bead. The distance between molecules, assuming that a site exists as a small sphere within the bead, is therefore 1.34 nm when the bead is fully loaded. Even without considering the bead swelling effect, the distance between fluorophore molecules is already too small to avoid fluorescence resonance energy transfer. Bead swelling will dramatically reduce the dynamic distance between sites and, therefore, increase energy transfer. Site isolation is only partially achieved within polystyrene resins[12] and is not possible within PS−PEG resins.

Regardless of the extremely short distances between reactive sites and the solvent swelling effect, the second bead series does not show a fluorescence quenching even though the fluorophores are as highly concentrated as the fluorophores in the first bead series. The cause was elucidated by examining the overlap of the absorption and emission spectra of the fluorophore.

The absorption and fluorescence emission spectra of the two fluorophores used above are compared in Figure 3. The absorption and fluorescence spectra of NBD-X showed an overlap. This means that the excitation-induced emission from one molecule can be absorbed by an adjacent molecule. In contrast, the absorption and fluorescence spectra of dansylhydrazine exhibit a larger Stokes' shift and very little overlap. This spectral separation guarantees the absence of self-quenching in the second bead series.

In a study of bead suspension,[13] self-quenching was also observed at higher resin loading. Although suspension study does not distinguish the site−site or bead−bead quenching, single-bead study does. Therefore, the self-quenching effect is confirmed in both macro- and microformat. Attention must be paid to selecting the fluorophore when a high-affinity lead (expected high fluorescence labeling, therefore, may be high self-quenching) is to be selected.

In summary, the structure of the resin beads and a high fluorophore concentration on bead will always cause self-quenching if there can be energy transfer between fluorophores. The examination of the overlap between the absorption and fluorescence spectra of the fluorophore is crucial to avoid the self-quenching effect.

Experimental Section

Materials. All resins used in this study were purchased from NovaBiochem (San Diego, CA). Resins are based on 1% cross-linked divinylbenzene−styrene copolymer. They are 100−200 mesh. All reagents, if not specified, were purchased from Aldrich (Milwaukee, WI).

The Single-Bead FTIR Method. All spectra were collected on a Nicolet Magna 550 FTIR spectrophotometer coupled with a NicPlan microscope. The microscope is equipped with a 15× Cassegrain objective and a liquid nitrogen cooled mercury−cadmium−telluride (MCT) detector. General procedure for IR measurement is as in ref 8. Flattened bead was used throughout the IR experiments.[8b]

Data Treatment and Analysis. IR spectra were normalized by making the intensity of a polystyrene band at 1947 cm^{-1} equal. The areas under the typical bands of the starting material or the product were integrated. The values of integration were then plotted against time. These data points were fitted to a pseudo-first-order rate equation by using a nonlinear regression program—SigmaPlot for Windows (Jandel Scientific, San Rafael, CA) on a personal computer.

Single-Bead Fluorescence. All single-bead fluorescence spectra were collected on a SEE 1000 microspectrometer equipped for transmission, reflectance, and fluorescence spectroscopy. Fluorescence measurements utilized a filtered 100 W mercury lamp for incident illumination. Exciting light was obtained at 436 nm. The filter set to isolate the excitation line consisted of three filters contained in a cube: a narrow band-pass filter isolated the appropriate mercury line; a dichroic filter acted to reflect the exciting light onto the sample and to transmit any fluorescence light to the detector; a final long-pass filter further isolated the emitted light from the excitation source.

The optical bench of the SEE 1000 microspectrometer was fitted with 5×, 20×, 50×, and 100× objectives for imaging the sample. The spectrograph was equipped with a single 600 grooves/mm grating blazed at 500 nm, and the detection system was a 1024 element linear CCD array.

The fluorescence spectra were obtained from beads, washed free of any starting materials, and placed on a glass slide. No further sample preparation was necessary nor were the beads flattened. All spectra were taken using the 20× objective which yielded a total magnification of 200× with

a field of view of 1100 μm in diameter. The spectral measurement area was a square with 12.5 μm on a side. In all cases, 10 scans were averaged. Beads were first located utilizing the transmission light source of the microscope. The transmission light source was then blocked and the fluorescence lamp activated.

Dye UV−Visible and Fluorescence Measurement. The UV−visible and fluorescence spectra of dyes were measured in ethanol. The concentrations of dyes were ∼10 μM for the UV−visible measurement and ∼1 μM for fluorescence measurement. All spectra were normalized to just compare their absorption or emission maxima. The excitation light for fluorescence is at 436 nm for NBD-X and 345 nm for dansylhydrazine.

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