C_{60} nanoparticle quenching used as a biological label

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ABSTRACT

Carbon 60 has been used in a functionalized form in a bioassay for a common herbicide, atrazine. It was found that the C_{60} is a very effective quencher of fluorescence from number of common dyes. C_{60} was conjugated to atrazine for use in an immunoassay in which fluorescence from rhodamine was measured. Quenching of the rhodamine emission provided a detection scheme in this assay that yielded very good limit of detection. The C_{60} quenching scheme can be used with a wide variety of fluorescent dyes, permitting the potential use of a range of small, cheap excitation sources.

Keywords: fluorescence, quenching, carbon, fullerene, immunoassay, atrazine

1. INTRODUCTION

Evidence for the photoinduced electron transfer from the excited state of a donor to buckminsterfullerene (C_{60}) has given rise to considerable interest in applications of C_{60} as an optical absorber or fluorescence quencher [1-5]. For example, in semiconducting polymers imbedded with C_{60}, the time scale for photoinduced electron transfer (subpicosecond) is much faster than the radiative or nonradiative decay of the photoexcited state [2, 5, 6], leading to strong modification of the emission.

The usefulness of C_{60} in biological applications arises from its unique behavior. The aromatic behavior of the twenty 6-membered rings and the outwardly-biased π-orbitals give rise to 3.4 nm-size icosahedral clusters of 13 molecules of C_{60} [7] separated by molecules of water [8-10]. The twenty inner water molecules and an outer shell of 60 hydrogen-bound water molecules make the C_{60} clusters water-soluble. In a water soluble form, C_{60} can be used as an efficient quencher of fluorescence in biological applications.

Fluorescence quenching has been used widely as a method for detection in biological assays. Fluorescence resonance energy transfer (FRET) plays a very important role in biological analysis [11-14]. Energy from a "higher energy" dye (donor) can be captured or transferred to a "lower energy" dye (acceptor). The efficiency of the non-radiative energy transfer is highly dependent on the overlap of the emission spectrum of the donor and the excitation spectrum of the acceptor, leading to a narrow range of suitable donor-acceptor pairs. Water soluble C_{60} clusters display advantages over normal fluorescent quenchers and FRET systems in biological applications, with no intrinsic fluorescence emission and the ability to quench a very wide range of fluorescence dyes.

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2. EXPERIMENTAL

2.1 Chemicals

Functional C_{60} (1,2-methanofullerene)-61-carboxylic acid was purchased from Fluka Chemie AG (Milwaukee, WI) and the dye Rhodamine 6G was purchased from Exciton, Inc. (Dayton, OH). Fluorescein and Hoechst 33258 were Sigma products. Functionalized atrazine (Fig. 1) was produced at the University of California, Davis. Magnetic particles coated with goat anti-mouse IgG (5 mg/mL) were purchased from Qiagen, Inc. (Valencia, CA). Atrazine was provided by Ciba-Geigy (Greensboro, NC). The monoclonal anti-atrazine antibody (AM7B.2) was obtained from Dr. A. E. Karu (University of California, Berkeley). Atrazine hapten (2-amino-3-(4-ethylamino-6-isopropylamino-[1,3,5]triazin-2-ylamino)-propionic acid) was prepared at University of California, Davis.

2.2 Equipment

An Ohmicron 60 position Magnetic Rack (Fisher Scientific, Pittsburgh, PA) was used to separate the immune complexes with free C_{60}-atrazine in solution. A UV-VIS spectrometer (Cary 100 Bio) was used to obtain C_{60} absorption spectra. The fluorescence images were taken by using a fluorescence microscope (Nikon Microphot- Applied Scientific Instrumentation, Inc.) and the fluorescence measurements were performed by using an ISA FluroMax 2 Spectrofluorometer.

2.3 Conjugation of C_{60} with atrazine

The usefulness of C_{60} was demonstrated in an immunoassay for atrazine. Atrazine is representative of a class of herbicides that have been widely used, and have given rise to concern over potential human exposure. The conjugate of (1,2-methanofullerene)-61-carboxylic acid with atrazine was prepared as shown in Fig.2. Stock solutions A and B were prepared as follows:

Solution A: 6 mg of 1,2-methanofullerene-61-carboxylic acid was dissolved in 2.4 ml $CHCl_3$ with one drop of N,N-dimethylformamide (DMF) in a 20 ml round bottom flask at 0-4 °C, then 1.5 ml of thionyl chloride was added dropwise over 5 min. The mixture was stirred and heated to 65-75 °C for 1 hour. The flask was put on a rotary evaporator to strip the solvent and excess thionyl chloride ($SOCL_2$) to form the acid chloride.

Solution B: 17 mg of atrazine was dissolved in 1.5 ml DMF and 5 mg of N,N-diisopropylethylamine was then added.

The stock solutions A and B were mixed in a round bottom flask at –20 °C by using iced water with sodium chloride, stirred overnight, and gradually allowed to warm to room temperature. The flask was then put onto the rotary evaporator to strip the solvent. The remaining solution was washed three times by centrifuging with ethanol at 4°C and 10000 g for 5 min.

2.4 Atrazine immunoassay

Monoclonal anti-atrazine antibody AM7B.2 solutions were diluted in PBSB (0.1 M PBS containing different concentrations of atrazine antibody with 0.2 % of bovine serum albumin). A series of concentrations of atrazine-C_{60} conjugate were prepared as follows: 100 μL of AM7B.2 and 100 μL of different dilutions of atrazine-C_{60} were mixed in 12 × 75 mm borosilicate glass test tubes containing 750 μL of 0.1 M PBS and incubated at room temperature for 60 minutes. Magnetic particles were treated with 0.2 % BSA along with goat anti-mouse IgG; 50 μL of the treated beads was added to each of the test tubes and incubated at room temperature for 60 minutes with shaking. The magnetic particles were separated from the solution by placing the tubes into an Ohmicron 60 position Magnetic Rack. The particles were washed with 1 ml of PBS. The supernatants were pooled together and mixed with 1 ml of 10^{-8} M Rhodamine 6G solution.

Competitive immunoassays of atrazine were carried out as follows: 100 μL of the atrazine–C_{60} conjugate (30 μg/mL) in 0.1 M PBS, 100 μL of AM7B.2 and 100 μL of different dilutions of unlabeled atrazine were mixed in 12 x 75 mm borosilicate glass test tubes containing 650 μL of PBS and incubated for 60 minutes at room temperature. Following the incubation, 50 μL of magnetic particles coated with goat anti-mouse lgG was added to each test tube and the tubes were incubated at room temperature for 60 minutes at room temperature with shaking. The immune complex then was separated from the supernatant by placing the tubes into an Ohmicron 60 position Magnetic Rack. The fluorescence intensity was measured by using a fluorospectrometer (Instruments S.A., Inc., Edison, NJ) with excitation at 525 nm and emission at 548 nm. Three replicates were taken for standard deviation that was found around 5%.

3. RESULTS AND DISCUSSIONS

3.1 Quenching

We chose Rhodamine 6G (a red dye), Fluorescein (a green dye) and Hoechst 33258 (a blue dye) to demonstrate the quenching effect of C_{60} particles. The results in Fig. 1 show that C_{60} leads to a surprisingly strong quenching effect from blue to red wavelengths. For the red dye, 40 nmol ml^{-1} of C_{60} quenched Rhodamine emission by more than 75%; for the blue dye, about 10 nmol ml^{-1} of C_{60} quenched the fluorescence by more than 90%. The difference in quenching efficiency between the two dyes may be related to the absorption spectrum of C_{60} (Fig. 2). C_{60} has a large absorption range, extending from 200 nm to 800 nm. The absorption peaks around 300 nm and diminishes at longer wavelength.

3.2 Quenching by Carbon 60

Figure 3 shows the effect of C_{60} on Rhodamine fluorescence in two different size cuvettes with the same concentrations of Rhodamine and C_{60}. The fluorescent emission from the Rhodamine does not depend on the optical path length through the solution, indicating that extinction of the fluorescence emission by C_{60} (by scattering or absorption) is not the dominant effect. The reduction in emission intensity in the presence of C_{60} is due to fluorescence quenching.

The fluorescence data were plotted in Stern-Volmer form [15]

$$I_0/I = 1 + k_q \tau_0 [Q] \quad (1)$$

where the quantities I_0 and I represent the fluorescence intensities in the absence and in the presence of C_{60} respectively, k_q is the quenching constant, and τ_0 is the life time of the fluorophore in the absence of the quencher. The linear relationship is described by the correlation of equation (2)

$$I_0/I = 0.97 + 19870\,[Q] \quad (2)$$

The lifetime of the Rhodamine molecule is approximately 4×10^{-9} s [16, 17]. The slope of the Stern-Volmer plot then leads to an estimated quenching coefficient of about $k_q = 5 \times 10^{12}$ M^{-1} s^{-1}.

C_{60} in water is an electronegative molecule showing some aromatic behavior in the twenty 6-membered (but not the twelve 5-membered) rings with the π-orbitals biased outwards [8]. An icosahedral water shell surrounds a single C_{60} molecule and links the inner water molecules by hydrogen bonds. In addition, the C_{60} molecules have a tendency to conjugate or coagulate into a nano-size particle that has 13 C_{60} molecules separated by water molecules [7, 8].

An estimate of the collision rate in solution between C_{60} clusters and rhodamine molecules can be derived if the diffusivity of the large C_{60} cluster is taken to be zero. Then the Smoluchowski equation indicates that the collision rate, assuming a diameter of 3.4 nm for the C_{60} cluster [8], is approximately $k_d = 2 \times 10^{11}$ M^{-1} s^{-1}. The order of magnitude discrepancy between the measured quenching coefficient and the collision rate coefficient is not accounted for simply by uncertainties in the estimates of properties – fullerene is apparently more effective in quenching rhodamine fluorescence than a simple collisional mechanism would suggest. The linearity of the Stern-Volmer plot indicates that only one quenching mechanism is at work. This could be either dynamic or static. Preliminary results show that the quenching

constant increases with solution temperature, providing strong evidence that the quenching is not static in nature since complexes of dye and C_{6d} would be less stable at higher temperatures and the quenching would be reduced. If the quenching were dynamic in nature, the effect must be augmented by some characteristic of the C_{60} molecule or its cluster.

The photoinduced charge transfer from the excited fluorescent dye to C_{6o} fullerene occurs on a picosecond time scale, more than 10^3 times faster than the fluorescence decay rate of the dye [2, 6]. This observation leads to the suggestion that the C_{60} structure may be a very effective fluorescence quencher through multiple electron transfer reactions from multiple fluorophores to a single C_{60} molecule. Sariciftci et al. [2] found that each C_{60} molecule can accept up to 6 electrons. It is very likely that the charge on the resulting anion promotes interactions over a sphere of action that is effectively greater than the physical size of the cluster. Electrostatic interactions may account for the highly effective nature of C_{60} quenching.

3.3 Immunoassay

An immunoassay for the herbicide atrazine was chosen to demonstrate the use of C_{60} as an effective fluorescence quencher. We used 1 μg ml^{-1} of the monoclonal anti-atrazine antibody (AM7B.2) and 30 μg ml^{-1} of C_{60}-atrazine in a competitive immunoassay for atrazine. A separation procedure was adopted for the assay by using magnetic beads covered with a second antibody. Magnetic beads removed the immune complexes of C_{60}-atrazine with atrazine antibody, and the free C_{60}-atrazine remained in the solution, acting as a quencher of rhodamine fluorescence. For a given amount of the antibody, as the C_{60}-atrazine concentration increased in solution, the quenching effect increased. For a given amount of C_{60}-atrazine in solution, as the antibody concentration increased, the quenching effect decreased. The results of the assay are shown in Fig. 4. The detection limit (LOD) in this experiment was 2.5 ng ml^{-1}, which compares to an LOD of about 0.1 ng ml^{-1} in an ELISA for atrazine [18]. Although the C_{60} label does not offer sensitivity equal to the ELISA, it does offer some advantages. A broad range of dyes can be used in the assay if they fall within the absorption range of C_{60}, which is quite broad. A dye can be chosen that matches a particular excitation source, such as a small diode laser. Hence, this approach lends itself well to miniaturization.

4. CONCLUSIONS

Carbon $_{60}$ is available in a functionalized form that makes it useful in immunoassays. The quenching of rhodamine fluorescence was found to take place at a rate in excess of that indicated by an estimate of collision rates in solution. It is possible that charge transfer from excited dye molecules to electron-deficient C_{60} can account for the quenching, with electrostatic interactions serving to accelerate the interactions between the fluorophore and the quencher. The application of the C_{60} – rhodamine system to an immunoassay for a common herbicide, atrazine, showed that it can be a very useful approach to achieving reduced limits of detection.

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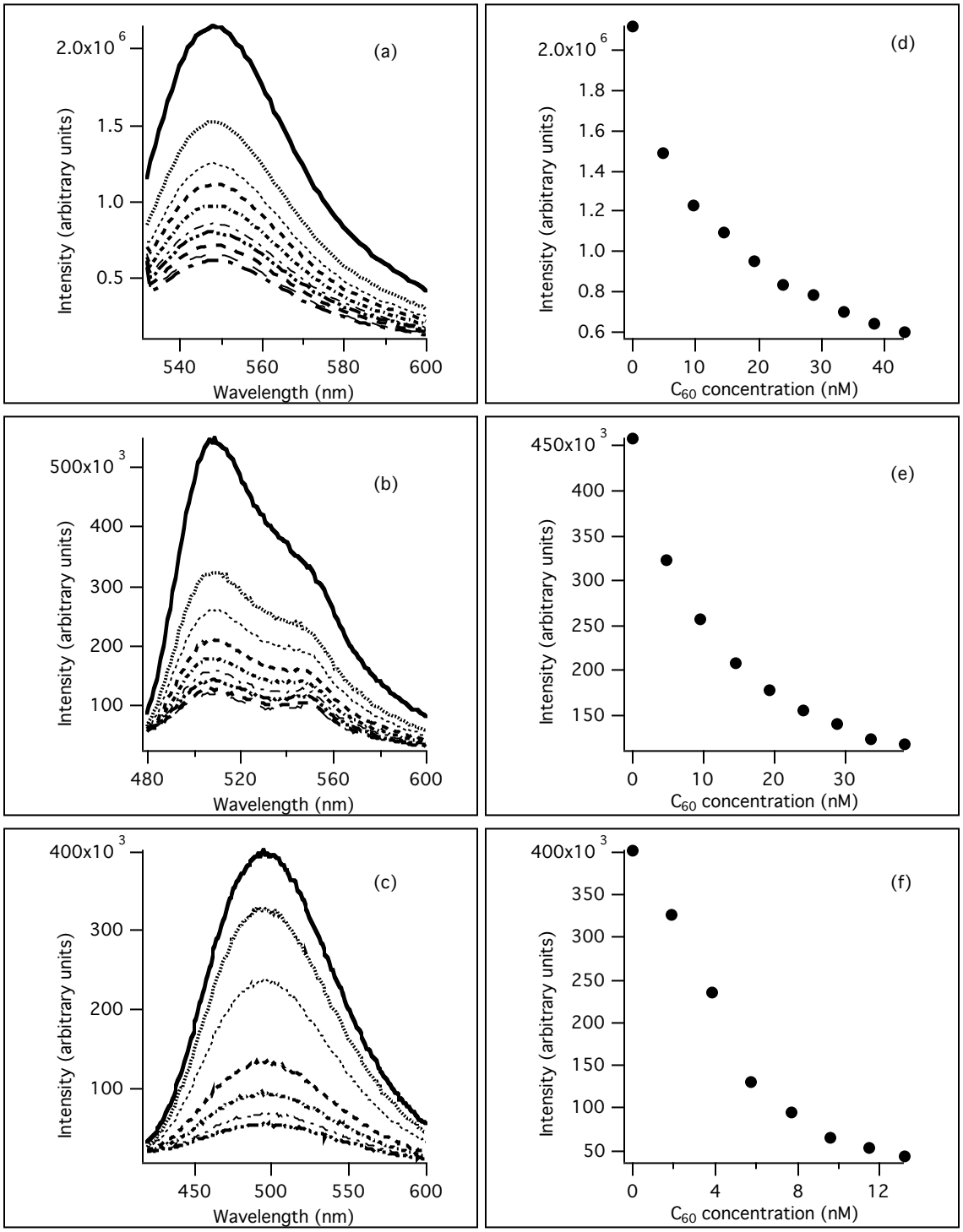

Fig. 1 C_{60} quenching the emission of fluorescence dye rhodamine 6G (1 □ 10^{-8} M, A and D), fluorescein (1 □ 10^{-8} M, B and E) and Hoechst 33258 (1 □ 10^{-8} M, C and F). In A, B, C, the curves from bottom to top represent the quenching effect by C_{60} from high concentration to low concentration. The corresponding concentrations are shown in D,E, F respectively.

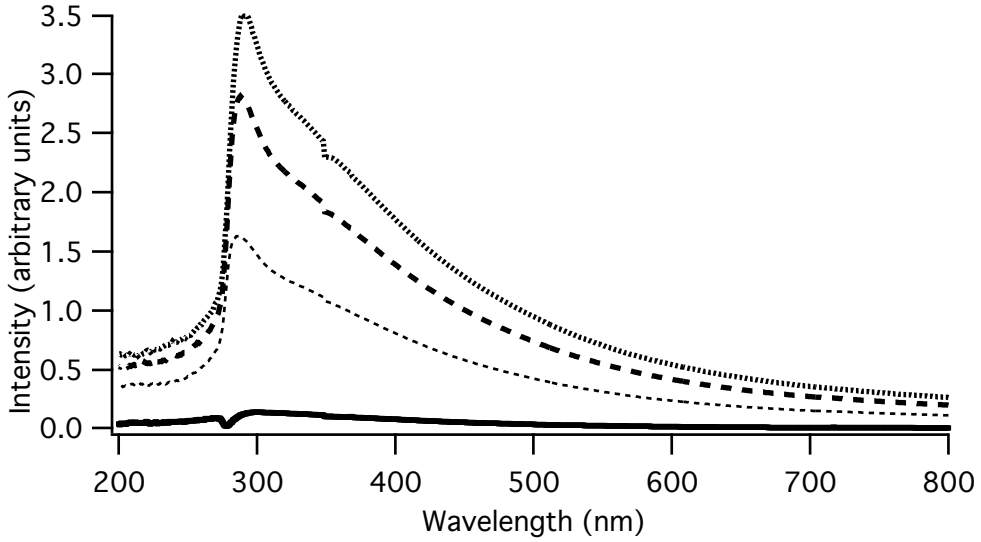

Fig. 2 Absorption spectra of C_{60} – atrazine conjugate. From bottom to top, the concentrations of C_{60} atrazine conjugate are 0.19, 0.76, 2.68, 4.59 mM in ethanol.

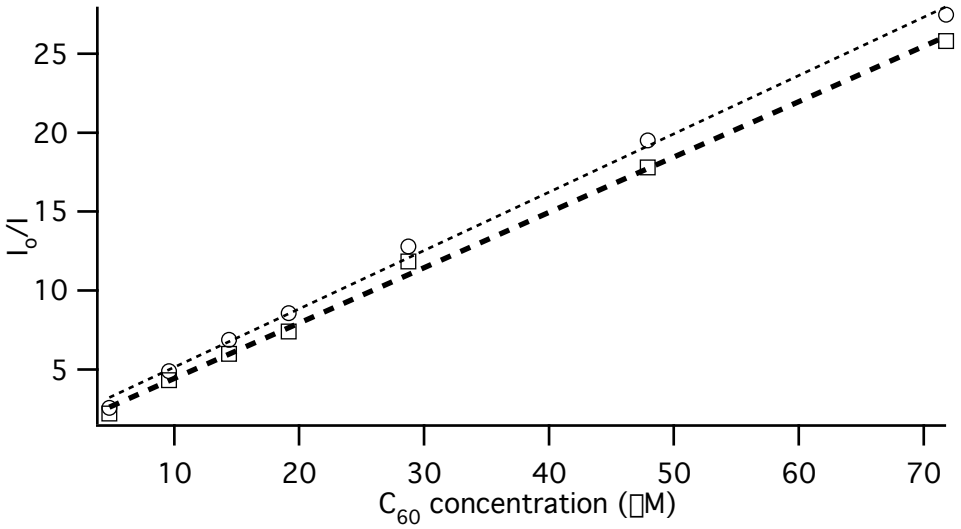

Fig. 3 C_{60} quenching of rhodamine 6G ($2 \square 10^{-8}$M) fluorescence in large cuvette and small cuvette.

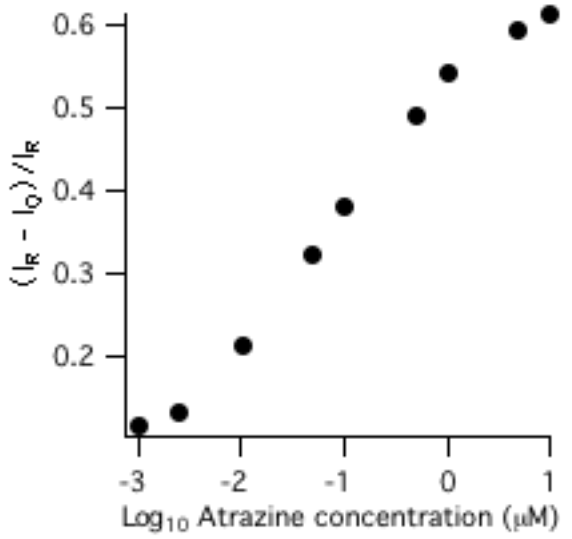

Fig. 4 Competitive immunoassay of atrazine using C_{60}-atrazine quenching of the fluorescence of rhodamine 6G (1×10^{-8}M). A series of glass tubes were prepared as follows: atrazine antibody concentration of 1μg ml^{-1} with 3%BAS, C_{60}-atrazine concentration of 30 μg ml^{-1} and a series of different concentrations of sample atrazine. After 40 min of the reaction, 50 μl of BioMag bead with goat anti-mouse lgG antibody was added to each of the tubes. After another 40 mins, the magnetic beads were taken out. Then standard rhodamine 6G solution was added. Detection limit: 2.5 ng L^{-1} and IC_{50}: 50ngL^{-1}.