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FRET- mediated Quenching of BODIPY Fluorescent Nanoparticles by Methylene Blue and its application to Bacterial imaging

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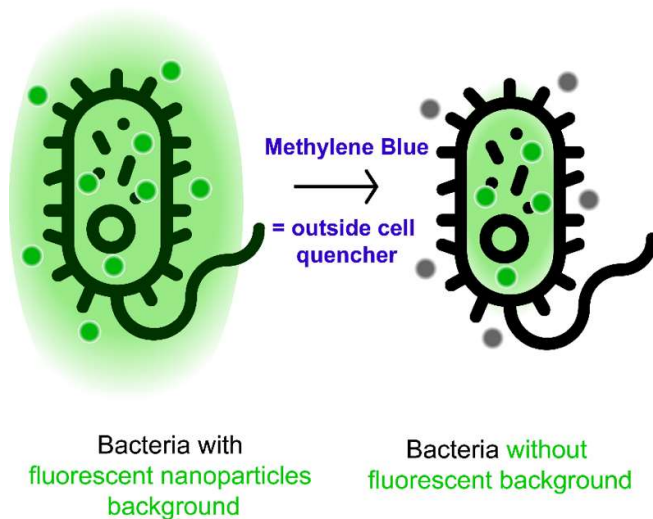
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Abstract

High resolution and good signal to noise ratio is a requirement in cell imaging. However, after labelling with fluorescent entities, and besides several washing steps, there is often an unwanted fluorescent background that reduces the images resolution. For this purpose, we developed an approach to remove the signal from extra-cellular fluorescent nanoparticles (FNPs) during bacteria imaging, without any washing steps. Our idea is to use methylene blue to quench (>90%) the emission of BODIPY-based fluorescent polymer nanoparticle by a FRET process. This “Hide-and-Seek Game” approach offers a novel strategy to apply fluorescence quenching in bioimaging to improve image accuracy.

Graphic abstract



Keywords:

Fluorescent nanoparticles, methylene blue, fluorescence quenching, bioimaging, bacteria

Statements and Declarations

Competing Interests and Funding: There are no competing or conflicting interests to declare.

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1. Introduction

Fluorescence bioimaging allows for the acquisition of images of whole cells or cell compartments in a quick and simple way[1]. For a long time it relied on luminescent molecules that can be tailor-made to target specific organelles, membranes[2,3], or bio macromolecules (e.g. DNA, proteins)[4–7]. However, only a few of these chromophores are approved for use in the human body because of their potential toxicity[8]. They also suffer from many drawbacks including poor water solubility[9], rapid photobleaching[10] or sensitivity of the fluorescence signal to the environment[11].

In this context, fluorescence nanoparticles (FNPs) have emerged as an appealing alternative owing to their excellent optical properties such as high photostability[12,13], brightness[14], and versatility of the emission wavelength[15,16]. The design principle for any doped fluorescent nanoparticle is to gather as many fluorophores as possible together on the same matrix [17]. Unlike single organic dyes, each nanoparticle can thus contain thousands of embedded fluorophores that are either self-aggregated[18] or encapsulated in a matrix (polymer, silica) and protected from its environment[19,20]. Due to the large number of fluorophores embedded in a single particle (up to thousands), FNPs are significantly brighter compared to individual organic dyes [21]. In the meantime, because they are encapsulated, the fluorophores are protected from the outside environment and are therefore more stable, as shown by reduced photobleaching and blinking[22,23].

Usually, for imaging cells are incubated with the fluorescent dyes or nanoparticles, followed by a washing step to remove excess fluorescent material[9,24]. However, because of residual non-specific binding it can be quite difficult to completely eradicate all the fluorescent nanoparticles, causing unwanted signal background and decreased detection accuracy. Alternate or additional strategies to washing can thus be necessary to discriminate the specific fluorescent signal.

We have previously described the development of ultra-bright organic BODIPY fluorescent nanoparticles (FNPs)[25]. In order to study their possible application for microbial imaging, we devised an approach based on cell electroporation to facilitate FNPs penetration in the cells. However, we faced a major issue: excess FNPs could not be completely removed by washing, and it was difficult to assert by conventional microscopy whether the FNPs were indeed internalized by the bacteria or just non-specifically bound on the surface. We thus looked for a strategy to “hide” the FNPs located outside the cell. Si, Y., Gazon, C., Clavier, G. *et al.* FRET-mediated quenching of BODIPY fluorescent nanoparticles by methylene blue and its application to bacterial imaging. *Photochem Photobiol Sci* (2022). <https://doi.org/10.1007/s43630-022-00215-1>

cells based on fluorescence quenching. Boland *et al.* found out that the fluorescence of the FITC-labeled nanoparticles outside eukaryotic cells (NCI-H292 cell line) is quenched when incubated with 0.11% trypan blue for 1 min. This technique was used to quench the signal from nanoparticles adsorbed to the cell surface for flow cytometry analysis[26]. However, the quenching mechanism and the bioimaging application were not studied, and this technique doesn't allow for the analysis of fixed cells. Other examples of such an approach include the reversal of biotin–methylviologen quenching by avidin in aqueous solutions[27] or the intramolecular quenching of a fluorophore (2,3-diazobicyclo [(2.2.2)]oct-2-ene)-labeled oligomer by a terminal guanine residue in water[28]. However, until now, few applications of energy transfer have been developed for biological applications especially for microorganism bioimaging.

Herein, we present a novel approach applying the fluorescence quenching process between BODIPY-based fluorescent nanoparticles (FNPs) and methylene blue (MB) to improve the imaging of the interaction between the FNPs and bacterial cells. Methylene Blue was chosen as a cationic blue dye since it is easily accessible, cheap and FDA approved[29]. We conducted a photophysical study to reveal that the mechanisms behind the very high quenching (>90%) of FNPs by methylene blue is due to Förster Resonance Energy Transfer (FRET). Methylene blue was therefore applied in bioimaging approach of *E. coli* electroporated with FNPs to “hide” the excess nanoparticles that cannot be removed by the common washing protocol. Thanks to the improved resolution and accuracy of detection, this “Hide-and-Seek Game” approach offers a novel and promising strategy to apply fluorescence quenching to bioimaging, especially to precious samples where cells can be easily lost during the washing steps.

2. Results and discussions

The fluorescent nanoparticles (FNPs) were synthesized as previously described[25] (details in ESI). The FNPs are composed of a hydrophobic core composed of poly(styrene-*co*-BD π) and a hydrophilic shell of poly(ethylene oxide)-*block*-poly(acrylic acid) that is negatively charged. The FNPs have a hydrodynamic diameter of 65nm, emit at 543nm with a quantum yield of 36% (Fig. S1). Thanks to the high number of BODIPY copolymerized in a single FNPs (n=2975), the overall brightness of the FNPs is $7.8 \times 10^7 \text{ M}^{-1} \text{ cm}^{-1}$. Methylene blue (MB) is a well-known cationic biocompatible dye [30], with a strong absorption band around 660 nm ($\epsilon=95000 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$)[31]. We hypothesized that the overlap between the emission spectrum of FNPs and the absorption spectrum of MB can induce energy transfer

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(ET) where FNP are quenched by MB (Fig. 1a). Moreover the cationic dye shall interact electrostatically with the anionic FNP's shell thus respecting the ET distance criterion. Finally MB is known to have quite a low luminescence efficiency (22%)[32], thus if sensitization by FRET occurs introduction of additional fluorescence signal will be limited. We made titration experiments to characterize the quenching process of FNP by MB. We put a FNP solution with a concentration of 0.5nM inside a cuvette and then added methylene blue solution gradually. Next, we recorded emission spectra after each addition of MB until the emission intensity of FNP reach a plateau (Fig. 1b). After the addition of MB, we found out that the fluorescence intensity of FNP decreases continually, at the same time, the fluorescence intensity of MB increases slightly. In the same conditions but without FNP, MB does not exhibit fluorescence emission (Fig. S3). Thus the decrease in the fluorescence intensity of FNP is correlated with the increase of the emission of MB indicative of an energy transfer from FNP to MB. If photoinduced electron transfer (PET) was active, BODIPY would be quenched but MB emission would not be detected since MB would be reduced to its colorless reduced form, leukomethylene blue[33].

In summary, the fluorescence intensity of FNP decreases dramatically up to a loss of 90% upon addition of 2.6 nM to 1 μ M of MB. The decrease in fluorescence reaches a plateau from 6 to 20 μ M MB to reach 98% of quenching (Fig. S4a).

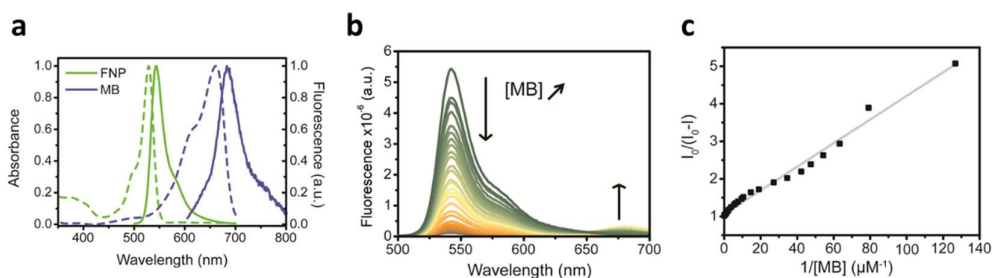


Fig. 1: a. Absorption (dashed lines) and emission (continuous lines; $\lambda_{exc}=495$ nm) spectra of FNP (green) and MB (blue) in water. b. Fluorescence emission spectra ($\lambda_{exc}= 495$ nm) of a solution of FNP at 0.5 nM upon titration of MB from 2.63 nM to 21 μ M in Milli-Q water at room temperature. c. Relative fluorescence intensity of a solution of FNP [FNP]=0.5nM with increasing MB concentration: modified Stern-Volmer plot and linear fit (y-intercept = 1.07; slope = 0.031 μ M).

To better understand the mechanism of the quenching process, the data are represented as Stern-Volmer plots (Fig. S4b). Generally speaking, a linear Stern-Volmer indicates a single class of dyes, all accessible to the quencher equally [34,35]. In our case, because of the Stern-Volmer plot negatively deviates from linearity toward the x-axis, therefore, there are two or even more populations of

fluorophores presented, one of which is buried that could not be accessible to the quencher. We thus decided to analyze the data with a modified Stern-Volmer plot (Fig. 1c) according to equation S3[34,36]. The plot is close to linearity, and the part of the initial fluorescence of FNP accessible to MB quencher is 0.94, meaning that 94% of the initial FNP fluorescence is quenched by MB.

To confirm this high quenching efficiency and obtain insight into the nature of the ET, we performed time-resolved fluorescence measurements of FNPs ([FNPs] = 0.5 nM in Milli-Q water) with increasing concentration of MB up to 2.63 μ M (Fig. 2a). Upon addition of MB to FNPs, we can see a clear shortening of the fluorescence lifetimes, indicative of a possible Förster Resonance Energy Transfer (FRET) involving FNPs as the donor and MB as the acceptor. Fluorescence decays were fitted by a sum of 3 exponentials to extract the average lifetime and thus calculate the FRET efficiency (E) (Fig. 2b, Equation S6). The FRET efficiency is very large and equals to 96% at its maximum, meaning that up to 96% of the FNP fluorescence can be quenched due to MB in solution. This result corroborates our initial observation with static fluorescence quenching.

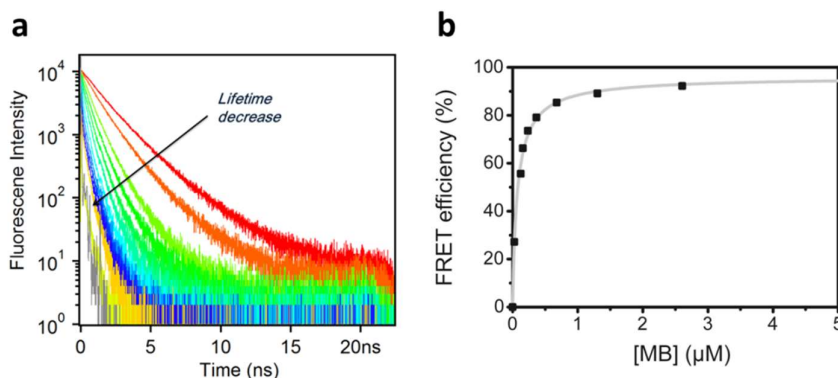


Fig. 2: a. Fluorescence decays ($\lambda_{exc}=495$ nm, $\lambda_{F}=542$ nm) of FNPs ([FNPs]=0.5 nM in Milli-Q water) upon sequential addition of MB (up to 2.63 μ M). b. FRET efficiency (black squares) calculated using lifetime measurements of FNPs upon sequential addition of MB. The continuous grey line is the fit of the experimental data by a Hill function.

There are two possible hypothesis for this efficient quenching: i) On one hand, methylene blue is a small, positively charged molecule and the shell of the FNPs contains acrylic acid units that are negatively charged at pH 7 (Fig.S1 cd). There might be an electrostatic interaction between the carboxylates moieties and the cationic dye, locating MB preferentially near the FNPs shell rather than in a homogenous distribution in solution. ii) On the other hand, the BODIPY fluorophores inside the particles are in distinct environments due to the core of the particle is composed of poly(styrene-*co*-BD π).

We previously demonstrated[37] that these FNPs are constituted of two BD π populations : a small percentage of isolated BD π , mainly located deep in the core of the FNPs, and the majority of the BD π forming aggregates and preferentially located on the outer part of the hydrophobic core (*i.e.* close to the shell / water). Knowing this non homogeneous repartition of BD π within FNPs, we assume that methylene blue can only quench the aggregated BD π (majority) in the outer part of the core but not the ones deeply buried in the particle. Moreover, we know that the energy transfer between closely spaced BD π is very efficient and fast. We thus believe that a single MB acceptor can quench multiple BD π donors. Moreover, we observed a slight blue shift of the emission of the FNPs when the MB concentrations increased during the titration.(Fig. 1b). This is probably because of the selective quenching of the exposed BD π versus the buried ones. These data reveal that BD π aggregates radiating at longer wavelengths are more readily quenched than BD π emitting at shorter wavelengths. These fluorescent spectra demonstrate that quenched BD π (preferentially localized on the shell) present an emission maximum at 543 nm, while protected BD π (embedded in the hydrophobic core) emit at 540 nm[23].

The hydrodynamic diameter of the FNPs (~65nm, Fig. S1b) is too large to allow their transfer inside the bacteria by passive transport[9]. We therefore used electroporation for the internalization of FNPs into bacterial cells, by creating holes in their membranes. Fig. 3 shows how FNPs interacts with the bacteria after electroporation: Fig. 3a is the phase contrast image and Fig. 3b is the fluorescence image of the same bacterium. We observed green spots (FNPs) on the fluorescence image of this bacterium. To obtain a more clear visualization, we have overlapped image a and b(Fig. 3c), one can notice that all the particles are associated with the bacterial cell.

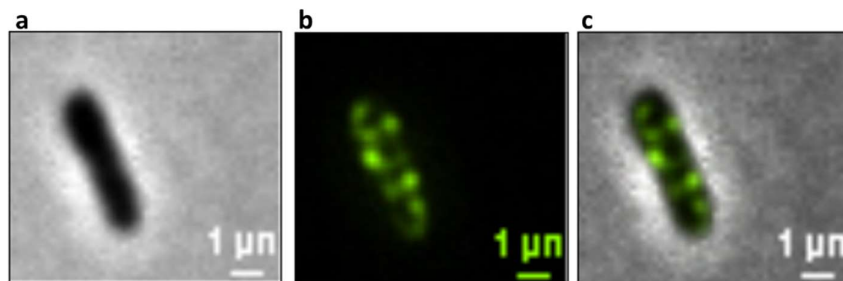


Fig. 3: Bioimaging of one *E. coli* cell interacting with FNPs after electroporation. a: phase contrast image, b: fluorescence image, c: overlap of images A and B (scale bar = 1 μ m)[23].

Six Rounds of washing of the cells with PBS did not remove all the non-specifically bound FNPs. Therefore, in order to distinguish whether the FNPs had entered the bacterial cells or were sitting on its surface, we have used methylene blue when bioimaging the *E.coli* electroporated with FNPs to quench the fluorescence of the nanoparticles outside the cells. It has been reported that the outer membrane in gram negative bacteria such as *E.coli* avoids or slows down the uptake of MB in the cells[38]. Therefore, *E.coli* cells that were electroporated with FNPs were placed inside an Eppendorf tube, MB was added and quickly mixed, and samples were immediately prepared for microscope analysis by placing them on a microscope slide.

Before the addition of methylene blue an excess of free nanoparticles could still be seen on the image even after six washing steps with PBS (Fig. 4ac). After the addition of MB, the free FNPs outside the bacteria were quenched, therefore could not be detected by the fluorescence microscope. Particles internalized by the bacteria were protected by the cell membrane and were not quenched by the MB, thus remaining fluorescent and visible under the microscope indicating a successful selective quenching process (Fig. 4bc). Using this quenching approach, we have successfully hidden the free FNPs on the fluorescence image, giving a clear background. The particles located inside the bacteria are thus easily distinguished (Fig. 4d), with an increase in the resolution and accuracy of detection as compared to the picture without MB quenching (Fig. 4c).

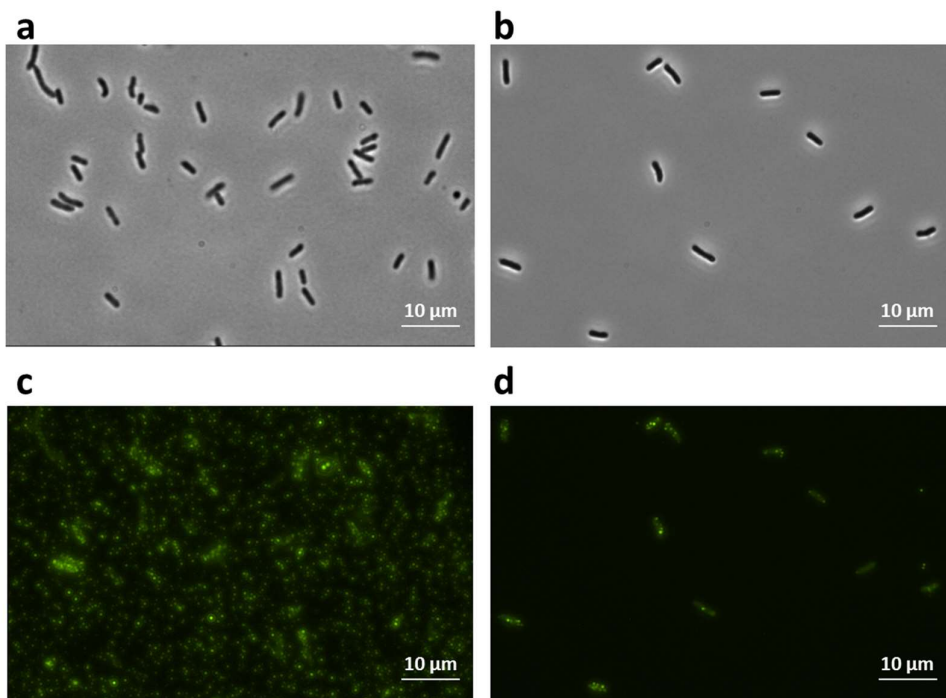


Fig. 4: Electroporated *E. coli* interacting with FNPs before (a, c) and after (b, d) quenching with MB (a, b: phase contrast images; c, d: fluorescence images).

3. Conclusions

The fluorescent nanoparticles (FNPs) described here have a great potential for bioimaging applications thanks to a set of beneficial properties: large number of copolymerized BODIPY fluorophores (≈ 3000) in a hydrophobic environment, a hydrophilic and biocompatible shell and high brightness (approximately 1000 times larger than traditional organic dyes). However, after incubation with cells, extensive washing is not sufficient to remove excess FNPs in the surrounding media and may preclude accurate detection. We thus devised an original approach to “hide” the excess FNPs by selective fluorescence quenching of those found outside the cells and facilitate the observation of the ones that have entered the cells.

Methylene blue was selected for its cationic nature, its reduced permeability to *E. coli*, its blue color (can act as an energy acceptor based on its good spectral overlap with BODIPY emission). MB thus acted as a quencher and its (limited) luminescence could be sensitized as demonstrated by titration studies. They revealed that the FNPs emission is reduced by $\sim 98\%$, demonstrating a very effective quenching process of FNPs by methylene blue due to FRET. There are two complementary explanations for this

very efficient quenching: electrostatic interaction between the cationic MB and FNPs carboxylates that preferentially locates the MB in the FNPs shell and a dense population of BDPs close to the core-shell interface that is accessible to MB.

FNPs were inserted in the bacteria by electroporation. Subsequently, quick addition of MB allowed to visualize only the internalized FNPs by fluorescence microscopy while “hiding” those left outside in the media or adsorbed non-specifically on the surface of the bacteria that were not removed by washing the samples with PBS. Particles within the bacteria were protected by the cell membrane and could not be quenched by MB, thus remaining fluorescent and visible by optical microscopy.

The FRET process is broadly used in bioimaging[39] or biosensing[40], especially to track the binding of proteins or DNA to specific ligands[41]. However, there are fewer examples of the use of energy transfer to improve the contrast in bioimaging. Therefore, this “hiding” process is a novel and promising strategy to apply fluorescence quenching to bioimaging in bacteria, especially to precious samples where cells can be easily lost during the washing steps, which to our knowledge, has not been applied before. With the improved resolution and accuracy of detection, this “Hide-and-Seek” approach extends the applicability of our FNPs that could be further functionalized on their surface with labels that could target specific areas in cells such as mitochondria or specific proteins. The approach may also be easily extended to a broader range of luminescent nanoparticles e.g. in single-particle tracking application to study the dynamics in different biological systems such as cells, tissues, organisms, bacteria and other living systems in the future. The possibility to use this “hide-and-seeK” approach with extension of BODIPY conjugation (red-shifted absorption and emission) is currently underway in our group. The pairing quencher shall have the following requirements: be cationic, be an energy acceptor (absorption spectrum overlapping red-BODIPY emission), be a limited cell permeant, have a limited luminescence efficiency (in order to avoid introducing additional fluorescent signal). Due to its wide absorption band, MB acceptor can be paired to a BODIPY donor with an emission up to 650 nm. On the other side, other membrane-impermeant dyes like cationic cyanine dimers (i.e.TOTO-1, JOJO-1...) could be envisioned as bright energy acceptors with such fluorescent nanoparticles donors.

Supplementary Information

The online version contains supplementary material available.

Author Contributions

Yang Si: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing - original draft

Chloé Grazon: Investigation; Methodology; Writing - review & editing

Gilles Clavier: Funding acquisition; Conceptualization; Supervision; Writing - review & editing

Jean-Frédéric Audibert: Investigation; Methodology

Bianca Scravi: Funding acquisition; Conceptualization; Project administration; Supervision; Validation; Writing - review & editing

Rachel Méallet-Renault: Funding acquisition; Conceptualization; Project administration; Supervision; Validation; Writing - review & editing

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