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Fluorescence quenching and ligand binding: A critical discussion of a popular methodology

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ABSTRACT

In recent years fluorescence quenching has become a popular tool to investigate various aspects of ligand binding. Unfortunately, various pitfalls are often overlooked in a large number of papers, published in many different journals. In this criticism we discuss a number of possible mistakes and show how they may affect the data and their analysis. Moreover, we point to problems in the understanding of the fundamentals of fluorescence quenching, and show direct contradictions within many of these papers. This review hopefully contributes to a re-appraisal of the published literature and to a more appropriate use of fluorescence quenching to study ligand binding.

1. Introduction

Recent years have seen an enormous popularity of using fluorescence quenching methodology to study ligand binding to a variety of (usually biological) molecules. In many cases these papers follow a common blueprint:

a. A ligand is proposed to bind to a fluorescent (macro)molecule, the latter usually a protein;
b. Addition of that ligand is shown to quench the emission of the fluorophore;
c. Using the Stern–Volmer equation it is shown that the bimolecular quenching rate constant is too large for a collisional quenching mechanism. This suggests ligand binding as the cause for quenching;
d. Binding constant (and often stoichiometry) are then determined;
e. Many papers also determine the distance between fluorophore and quencher using Förster Resonance Energy Transfer (FRET) as the proposed quenching mechanism.

Unfortunately, the apparent simplicity of the methodology has led to the introduction of a number of errors in the data interpretation.

Recently, we independently criticised in short communications the methodology used in many articles using fluorescence quenching to study ligand binding [1,2]. However, the incorrect use of this experimental approach is extremely widespread, with papers published in at least 25 different journals and total papers numbering well over 100. We thus believe it is necessary to reiterate our criticism in a more expanded form in this journal. In this paper we have tried to explain, as simply as possible, the most important fluorescence- and ligand binding-related complications in this methodology. For illustrative purposes we have included mathematical modelling of theoretical data to illustrate the impact of some of the pitfalls.

The criticism is general, but at various points we discuss specific references from this or other journals for illustrative purposes. Moreover, we will focus mainly on ligand binding to proteins, with the fluorophore generally being the tryptophan residue(s), as this situation applies to the vast majority of the articles using this methodology. However, many elements of the criticism apply to any fluorophore-ligand binding analysed using fluorescence quenching. Henceforward we will refer to the articles we criticise as The Articles or Articles.

2. The methodology and its pitfalls

2.1. Why does the fluorescence change?

Addition of a compound to a solution containing a fluorescent macromolecule may change its emission through different

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mechanisms. The first important question to answer is "why does this addition change the fluorescence?" There are several possible answers:

1. The inner-filter effect.
2. Collisional quenching.
3. Binding-related changes in fluorescence.
   a. Ground-state complex formation between the ligand and the fluorophore(s) in the macromolecule.
   b. Excited-state quenching in the complex (e.g. energy transfer).
   c. Binding-induced structural changes of the protein around the fluorophore(s).

Of course, only changes in fluorescence associated to binding (point 3) can be used to follow association phenomena, and therefore effects 1 and 2 must be demonstrated to be negligible or appropriately corrected for before any data analysis is even attempted.

2.2. The inner-filter effect

The inner-filter effect refers to the absorbance (or optical dispersion) of light at the excitation or emission wavelength by the compounds present in the solution. Usually, the optics of commercial fluorimeters focus the exciting light and collect the emission from the centre of the cuvette. Therefore, when absorption at the excitation wavelength is significant, less light reaches the centre of the sample and thus the fluorescence of the fluorophore is reduced, while absorption at the emission wavelength reduces the emitted light that reaches the detector. This is a problem whenever the ligand used in a titration absorbs at the excitation and/or emission wavelengths. Also any dilution of the fluorophore upon ligand titration needs to be corrected. Due to the non-linear nature of the inner-filter effect this may require special attention.

If the geometry of the instrument is such that the collected intensity comes exactly from the centre of the cuvette, the inner filter effect can be estimated from:

$$F_{obs} = F_{corr} * 10^{-\frac{A_{ex}*d_{ex}}{2} - \frac{A_{em}*d_{em}}{2}} \quad (1)$$

where F_{obs} is the measured fluorescence, F_{corr} the correct fluorescence intensity that would be measured in the absence of inner-filter effects, d_{ex} and d_{em} the cuvette pathlength in the excitation and emission direction (in cm), respectively, and A_{ex} and A_{em} the measured *change* in absorbance value at the excitation and emission wavelength, respectively, caused by ligand addition (in a 1 cm pathlength cuvette) [3].

It is important to realise that Eq. (1) assumes that the fluorescence comes exactly from the middle of the cuvette. This may be the case for some spectrometers, but certainly not all. A recent paper by Gu and Kenny [4] discusses several correction methods and potentially relevant instrument-specific factors. As discussed in that paper, deviations, even with more advanced correction methods, will occur in particular at high absorbance values. Thus, rather than correcting the inner-filter effect, it may be more appropriate to minimize it by simple practical considerations, such as reducing cuvette thickness, or selecting excitation and emission wavelengths that minimize ligand absorption. However, this may not always be practically possible, and in those cases the correction method may be used, but with the caveat that this does not necessarily fully correct the impact of the inner-filter effect. Alternatively, the more sophisticated correction approaches described by Gu and Kenny may be used [4].

But what increase is significant enough to warrant attention? From Eq. (1) it is easy to show that a change in absorbance equal to 0.03 already corresponds to a 3% reduction in fluorescence intensity. To more clearly show the potential impact of the inner-filter effect, we performed mathematical modelling on a simple fluorophore in a solution with increasing absorbance upon addition of a non-binding compound. Fig. 1A shows the effect of titration with different compounds, which, at the maximal added concentration, have an absorbance at the excitation wavelength ranging from 0.1 to 4. Even with the lowest increase to 0.1 at the highest added concentration, we can see a drop of approximately 10% in observed fluorescence. That is, the inner-filter effect has a measurable impact even at very small changes in absorbance. One may even construct Stern–Volmer plots (see further below) that appear meaningful solely from inner-filter induced quenching (Fig. 1B). In the particular example in Fig. 1B the highest added concentration of 50 μM has an absorbance of either 0.1 or 0.4 in a 1×1 cm cuvette. If this data is analysed using the Stern–Volmer equation (Eq. (2)), a binding constant of ca. 2400 M^{-1} (final absorbance of 0.1) or 11,000 M^{-1} (final absorbance of 0.4) would be deduced, even though the observed quenching is not due to ligand binding at all. A real experimental example is discussed in Stella et al. [1]. Their reanalysis shows that the binding constant of 76,000 M^{-1} for the azulene–fullerene system reported by Rahman et al. [5] is essentially entirely caused by a failure to correct for the inner-filter effect.

Several Articles show, or can be calculated to show, increases in absorbance at excitation and/or emission wavelength that are (much) larger than 0.1 at the highest concentrations added (cf. [6,7]), sometimes easily reaching an increase of 1 [8]. Thus, the inner-filter effect must be considered a confounding factor in the observed quenching in these Articles.

It is important to note that an inner-filter effect can be caused also by non-absorbing ligands in case they induce significant light scattering, as in the case of protein-membrane interaction studies, in which titration is performed with a suspension of liposomes. In

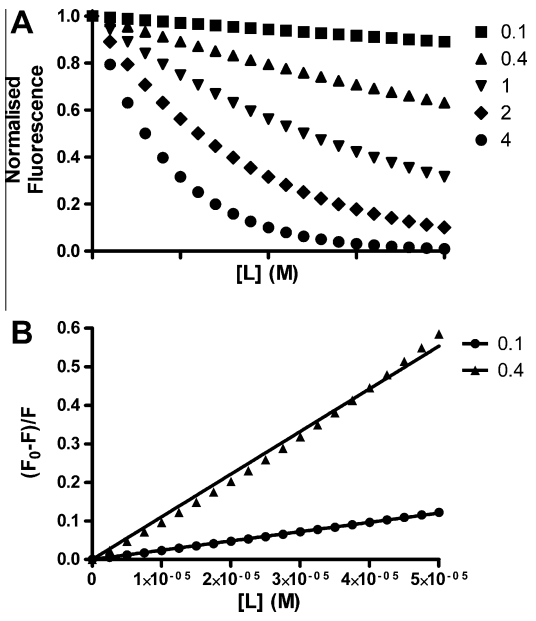

Fig. 1. (A) Effect of the inner-filter effect on the normalized fluorescence of a fluorophore in a 1×1 cm cuvette. Absorbance values of the highest added concentrations are 0.1, 0.4, 1, 2, and 4, respectively. Data points calculated using Eq. (1), with the absorbance increased in 20 equal steps to the indicated highest absorbance value. For practical purposes we only assumed an inner-filter effect at the excitation wavelength; (B) Stern–Volmer plots (Eq. (2)) created for the apparent quenching of a fluorophore by addition of a light-absorbing non-binding compound with an absorbance (excitation wavelength) at the highest added concentrations of 0.1 or 0.4, respectively. The lines depict linear regression curves that were forced through the origin.