A reassessment of the association between azulene and [60]fullerene. Possible pitfalls in the determination of binding constants through fluorescence spectroscopy†

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We show here that the recently reported surprisingly large association constant ($K = 7.6 \times 10^4$ M^{-1}) between azulene and [60]fullerene is due to experimental artifacts, pointing out potential errors in the characterization of association equilibria by fluorescence spectroscopy, and suggesting the best experimental practices.

Since the discovery of fullerenes the design and synthesis of molecular receptors able to form stable complexes with these species has attracted increasing interest due to their importance in materials chemistry[1] and in the separation of fullerenes.[2] The interaction of the curved conjugated carbon networks of fullerenes with molecular receptors characterized by the presence of properly assembled recognizing units has been suggested as a significant motif for the formation of inclusion complexes. Accordingly, host molecules with concave shapes such as calixarenes,[1] resorcinarenes,[1] buckybowls,[3] and π-extended tetrathiafulvalene (TTF) derivatives[4] have appeared to be ideal candidates for molecular receptors with the ability to recognize fullerenes through concave–convex π–π interactions. The role of these interactions has been recently pointed out,[3,5] even though it has been recognized that they are not always necessary to achieve a highly stabilized complex.[6]

Very recently, Komatsu and coworkers reported, by means of fluorescence quenching experiments, *unexpectedly* large binding constant between azulene and fullerenes (C_{60} and C_{70}) in toluene solution ($K = 7.6 \times 10^4$ and 7.9×10^4 M^{-1}, respectively), results that were interpreted in terms of a strong flat-π–curved-π interaction where the dipolar character of azulene plays an important role. Quite interestingly, the binding constants were observed to be almost independent of the particular azulene derivative employed.[7]

On the basis of these observations, it seemed particularly interesting to exploit the binding properties of azulene and its derivatives for the development of molecular receptors for fullerenes characterized by the presence of two or more azulene moieties as recognizing subunits. However, quite surprisingly, no evidence of association between azulene and C_{60} was obtained employing either UV-Vis absorption or fluorescence spectroscopy, in sharp contrast with the previous report.[7] We show in this communication that the high affinity reported for azulene and C_{60} is artifactual, and use this example to indicate the possible pitfalls in the determination of association constants by fluorescence spectroscopy, and to suggest the correct experimental practices.

The azulene spectra reported in ref. 7 had peaks at about 750 nm. However, azulene has no emission in this spectral region and its main fluorescence maximum is at about 375 nm.[8] The spectra reported are therefore an artifact, due to a second-order transmission of diffraction grating monochromators, which, when set to select a given wavelength λ, also partially transmit light with wavelength $\lambda/2$.[9] For this reason, the real azulene fluorescence, with a peak at about 375 nm, also produces an artifactual peak at around 750 nm (Fig. 1).

Second-order artifacts can be easily eliminated by using a cut-off filter in the emission channel, which absorbs all radiation with wavelengths below the cut-off value. Therefore, these filters allow accurate measurements of sample emission in the spectral region *above* the cut-off value, without interference from second-order artifacts of wavelengths *below* the cut-off, which are completely blocked. This is clearly illustrated by Fig. 1, showing the effect of a 590 nm cut-off filter: not only is the real azulene emission (in the range 325–425 nm) removed, because of filter absorption, but also the apparent emission

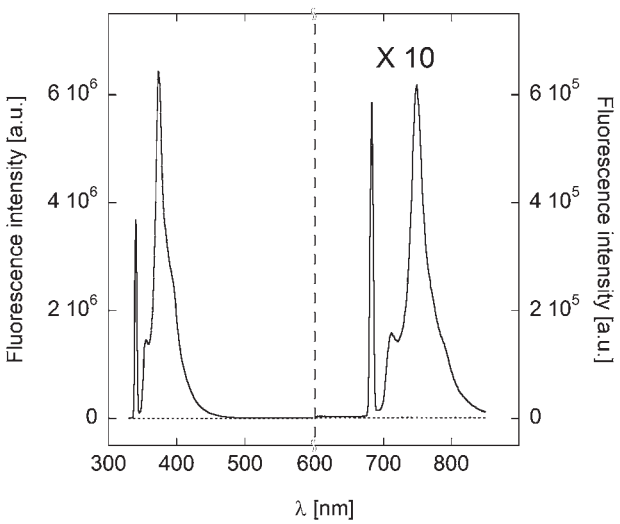

Fig. 1 Emission spectra azulene in toluene (84.6 μM). $\lambda_{exc} = 341$ nm. Solid line: no filter. Dotted line: 590 nm cut-off emission filter.

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† Dedicated to Prof B. Pispisa, on the occasion of his retirement.

band due to second-order transmission in the 650–825 nm range disappears completely. Since the filter employed has no significant absorption in the latter spectral range, this clearly demonstrates that the apparent emission in that region is actually due to detection of light of shorter wavelength.

Another possible source of artifactual peaks in the emission spectrum is scattering of the excitation light. The most intense of these peaks is due to Rayleigh or elastic scattering, and it is therefore at the same wavelength as the excitation light. Other minor peaks are observed at higher wavelengths, and are due to inelastic (or Raman) scattering.[9] The peak at 341 nm in Fig. 1, and its second-order transmission replica at 682 nm (also present in the spectra of ref. 7) are actually due to Rayleigh scattering of the excitation light. This type of artifact can be easily spotted by acquiring a spectrum of the solvent alone, since scattering peaks are obviously present also in the absence of a fluorophore.

The second-order transmission is a fixed fraction of the actual intensity, and therefore observation of azulene emission at the wrong wavelengths should have not affected the quenching measurements performed in ref. 7. However, those measurements showed that the peak at 682 nm (second-order transmission of scattered excitation light) was reduced by fullerene titration exactly in the same way as the peak at 750 nm (second-order transmission of azulene fluorescence). Since the scattered excitation light cannot be affected significantly by intermolecular association, this is a clear indication that the observed fluorescence quenching is due to another artifact, as explained below.

Quenching of azulene fluorescence caused by titration with C_{60} was employed in ref. 7 to determine the association

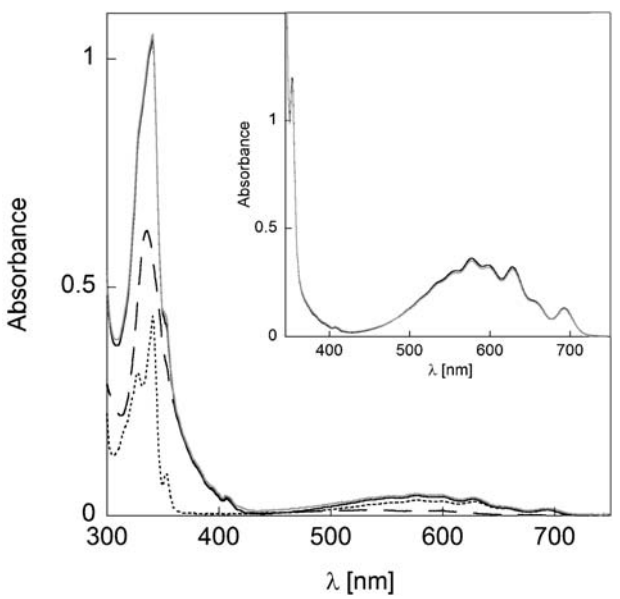

Fig. 2 Absorption spectra of toluene solutions of azulene (1 mM, dotted line), C_{60} (0.1 mM, dashed line) and azulene and C_{60} together (same concentrations as above, black solid line). The gray solid line represents the sum of the absorption spectra of the pure azulene and C_{60} solutions. Inset: comparison between the spectrum of a 10 mM azulene and 0.1 mM C_{60} solution and the sum spectrum in a restricted wavelength region. Optical path: 1 mm.

constant between these two molecules. However, as the authors state in a final note, "the largest absorption of azulene at around 340 nm also contains absorption from the fullerene", as clearly shown in Fig. 2. Absorption of the molecule added during a fluorescence titration experiment at the wavelength of excitation or at the wavelengths used to follow emission is a common source of artifacts caused by the so-called "inner-filter effect", which results in a spurious decrease in the observed fluorescence intensity.[9] The geometry used in most commercial fluorimeters is right-angle observation of the *center* of a *centrally* illuminated cuvette. This means that the excitation beam has to travel about half of the cuvette optical path before reaching the cuvette center, where it excites the molecules whose fluorescence will be detected. In the same way, the emitted light has to travel through approximately half the cuvette thickness before reaching the detector. Any molecule added to the sample which can absorb significantly either the excitation or the emission light will cause a significant decrease of the measured intensity. The extent of this effect can be roughly estimated with the following formula:

$$I_{\text{real}} = I_{\text{measured}} \times 10^{A(\lambda_{\text{exc}})/2} \times 10^{A(\lambda_{\text{em}})/2} \qquad (1)$$

where the measured intensity I_{measured} is corrected for the effects due to the absorption of the titrating molecule at the excitation and emission wavelengths. Absorption values are divided by 2 since the observation volume is located approximately in the cuvette center. In the case of a 8.23×10^{-6} M fullerene concentration, in a 1 cm cuvette (the conditions used in ref. 7) this correction could account for the whole apparent quenching effect.

False quenching effects caused by inner-filter artifacts can be easily detected by changing the optical path of the cuvette. Any real quenching effect will not depend on the size of the cuvette employed, while the inner-filter effect will decrease drastically with shorter optical paths (according to eqn (1)). The quenching efficiency measured for a 84.6 μM azulene, 5 μM C_{60} solution in toluene (λ_{exc} = 341 nm, λ_{em} = 376.5), in 10 × 10 mm cuvette was 0.32, but it decreased to 0.21 in a 5 × 5 mm cuvette and to 0.14 in a 3 × 3 mm cell. The strong dependence on cell size demonstrates the artifactual nature of the quenching effect. Another way to reduce inner-filter effects is to choose excitation and emission wavelengths where absorption from the titrating molecule is minimal. Even though this is not possible in the case of azulene and C_{60}, due to the high overlap of their absorption spectra (Fig. 2), when using λ_{exc} = 353 nm and λ_{em} = 392 nm (where C_{60} absorption is significantly reduced) the quenching efficiency is further reduced, down to 0.09.

Fig. 3 compares the results previously reported[7] with those obtained under optimized experimental conditions (3 × 3 mm cuvette, λ_{exc} = 353 nm, λ_{em} = 392 nm). Obviously the apparent association constant is extremely reduced by using these conditions. However, even in this case some inner-filter effect is still present, and it can be approximately corrected by using eqn (1). By considering this effect, quenching by C_{60} is practically nonexistent (Fig. 3). Considering the approximate nature of the inner-filter correction factors (which depend strongly on the exact position inside the cuvette of the focus

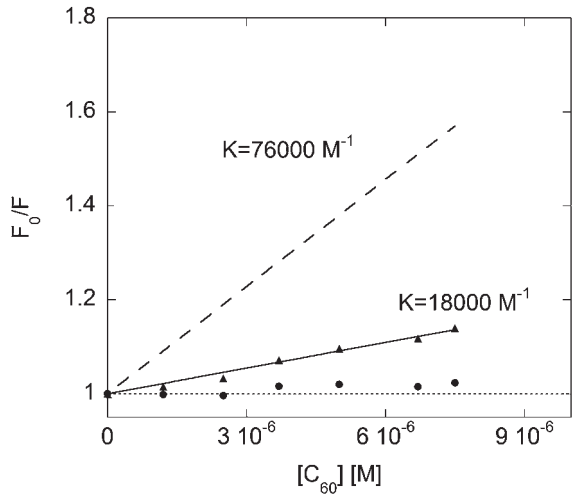

Fig. 3 Stern–Volmer quenching curves obtained for the azulene–C_{60} system under different experimental conditions. The dashed line represents the result reported in ref. 7, triangles are the experimental points obtained with a 3 × 3 mm cuvette, λ_{exc} = 353 nm, λ_{em} = 392 nm, while circles are the same experimental data approximately corrected for residual inner-filter effects. Azulene concentration in toluene 84.6 μM. F_0 and F represent the azulene fluorescence intensities measured in the absence and in the presence of C_{60}, repectively.

of the excitation beam), these data are consistent with the absence of any complexation at all between the two molecules.

The high overlap between azulene and C_{60} absorption spectra prevents fluorescence measurements at higher concentrations, where the inner-filter effect would be too high, even using the correct experimental conditions. However, ground-state complexation between two chromophores usually also causes significant perturbation in their absorption spectra, which therefore can be used to study the association process. Fig. 2 shows the absorption spectra of toluene solutions of azulene (1 mM), C_{60} (0.1 mM) and of both species. The absorption spectrum of the solution containing both chromophores is identical to the sum of the spectra of the two isolated molecules, indicating the lack of any interaction even at the relatively high concentration used, which, for fullerene, is close to its solubility limit.[10] Even when increasing the azulene concentration to 10 mM, no additional bands were observed (Fig. 2, inset). Considering also that no significant peak shifts were observed in the ^{1}H and ^{13}C NMR spectra, even under low-temperature conditions,[7] we can definitely conclude that in toluene solution the association constant between azulene and C_{60} is negligibly small, if not zero.‡

Before concluding, it is worth mentioning a few other factors that, while not present in the case of azulene and C_{60}, might affect significantly the determination of association processes by fluorescence spectroscopy measurements. For instance, even in cases where a real quenching effect of steady-state fluorescence intensity is actually present, this does not automatically demonstrate intermolecular association, since several excited-state quenching processes which take place at a distance (such as energy or electron transfer) are possible, without a real binding between fluorophore and quencher.[9] This possibility should be discarded by conducting time-resolved fluorescence experiments before concluding that an association is taking place. The time-decay of the excited fluorophore is only affected by excited-state processes, since association in the ground-state usually results in the formation of non-fluorescent complexes, which cause a decrease in the overall steady-state fluorescence intensity, but do not affect time-resolved measurements, since they do not contribute to the emitted light. In the case of azulene and fullerene no excited-state quenching was observed (data not shown).

Finally, extreme care should be taken to rule out photobleaching phenomena. Some fluorophores are not photostable, and, under the intense illumination of the fluorimeter excitation beam, they can undergo significant photochemical degradation, giving rise to an apparent decrease of their emission intensity. The presence of these phenomena can be verified by measuring the emission intensity as a function of illumination time. In the case of azulene no significant photobleaching was apparent under the experimental conditions employed.

Fluorescence spectroscopy is a very powerful and sensitive technique to study intermolecular association processes. However, care should be taken to avoid several trivial artifacts, such as second-order transmission, scattering, inner-filter effects, excited-state quenching processes, and photobleaching. When these effects were properly taken into account in the case of azulene complexation with C_{60} in a toluene solution, the binding constant was too small to be measured in the concentration range determined by the solubility of C_{60}, and was definitely lower than 10 M^{-1}.‡ This upper limit is in line with the binding constants reported previously for the association of C_{60} with polynuclear aromatic hydrocarbons,[11] clearly indicating that azulene does not display any *unusual* binding behavior towards fullerenes.

Notes and references

‡ Considering that no additional bands appear in the absorption spectrum of the solution containing both chromophores, we can conservatively assume that the percentage of C_{60} molecules bound to azulene is <10%, if association occurs at all. Since the azulene concentration was increased up to 10 mM, this places an upper limit of 10 M^{-1} on the association constant.

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