Volume 6 | Number 6 | 21 March 2014 | Pages 1611–1978

Analytical Methods

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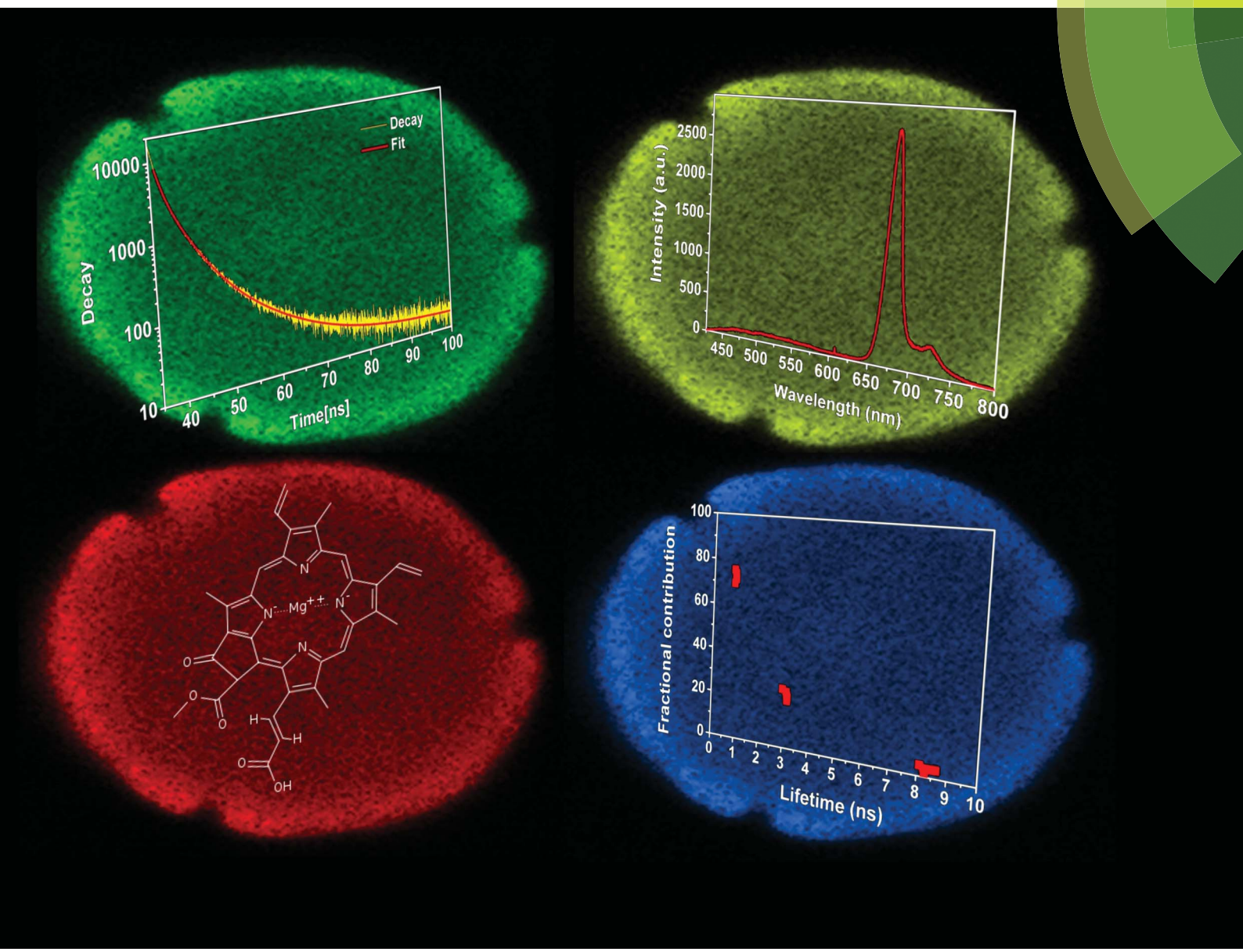

ISSN 1759-9660

ROYAL SOCIETY OF CHEMISTRY

PAPER

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Using spectral analysis and fluorescence lifetimes to discriminate between grass and tree pollen for aerobiological applications

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Using spectral analysis and fluorescence lifetimes to discriminate between grass and tree pollen for aerobiological applications

Cite this: *Anal. Methods*, 2014, **6**, 1633

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Received 3rd July 2013
Accepted 5th December 2013

DOI: 10.1039/c3ay41093e

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A study has been performed that provides the first fluorescence lifetime results on the intrinsic fluorescence monitored for specific *in situ* biochemical components of individual pollen grains. The results obtained show that such measurements can provide a basis for analytical discrimination between a variety of airborne grass and tree pollen.

Using excitation at 405 nm, the most striking differential spectral observations were determined for the individual grass pollen, all of which gave rise to bands with wavelength maxima at 675 nm and 725 nm. The feature is readily attributable to chlorophyll-a and is absent from the tree pollen counterpart spectra. The fluorescence lifetime experiments provided unambiguous evidence to show that chlorophyll-a was located in a region resembling a "free" solution environment. Furthermore the results strongly indicate that a portion of the chlorophyll-a found in grass pollen is also bound to a protein. The fluorescence lifetime data also provide evidence for both the grass and tree pollen to contain fluorescing metabolites such as flavin adenine mononucleotide (FMN) and flavin adenine dinucleotide (FAD), likely bound to protein. The work is relevant to the study of atmospheric dispersions of Primary Biological Aerosol Particles (PBAP) because the discriminatory lifetime fluorescence parameters obtained might be utilized for their real-time detection if suitable technical adaptions to current analytical instrumentation can be made. Furthermore the fact that distinctive fluorescence spectra for pollen can be measured using visible excitation wavelengths ($\lambda > 400$ nm) may provide the basis for future instrumentation to be developed that can likely reduce or eliminate many potential interferences from chemical species such as certain Polycyclic Aromatic Hydrocarbons (PAHs).

Introduction

Primary biological aerosol particles (PBAP) are ubiquitous in the atmosphere and include a range of differing types including pollen, fungal spores, bacteria and viruses.[1–3] The main function of pollen is to transport DNA/genetic material in plants but their dimensions and (bio)-chemical compositions have led to many studies on their potential health effects.[4–6] For example contents such as nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidases cause oxidant stress in the lung epithelium and boost allergic lung inflammation while the presence of adenosine represents a potent immunoregulatory substance.[7] Thus allergens can get trapped in the nasopharynx and trachea leading to adverse reactions such as pollinosis (hay fever), diseases such as asthma and even mortality.[8,9] In this regard it has been reported that, for studies performed in the Netherlands, a strong association exists between the day-to-day variation in pollen concentrations with death due to cardiovascular disease and chronic obstructive pulmonary disease (COPD).[10] Additionally there is evidence that under wet conditions or during thunderstorms, pollen grains may release part of their content thereby inducing asthmatic reactions in patients suffering from pollinosis.[11]

There are of course linkages between the above health issues and climate change because increased air temperatures significantly influence pollen production and their airborne concentrations. Longer pollination seasons are also likely to increase the duration of allergic reactions in sensitized subjects.[12] Hence the real-time monitoring of airborne pollen and other PBAP using a variety of spectroscopic and light scattering techniques represents an area of growing development and consequence.[13–22]

PBAP contain a variety of fluorescent materials as discussed in detail in several publications. They include: "Sporopollenin", which is a complex biopolymer exine layer;[23–25] amino acid related, like tryptophan and DNA;[26] Reduced Nicotinamide Adenine Dinucleotides, NADH/NAD(P)H, which are the primary end-products of photosynthesis and are often bound to proteins;[27,28] flavonoids/riboflavin and flavo-proteins, such as flavin adenine mononucleotide or dinucleotide (FMN or

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FAD);[29–31] chlorophyll, thought mainly to be associated with plant debris;[32] azulenes;[26] melanine/eumelanin in fungal spores.[31] The light absorbing chromophores associated with the groupings especially the amino acids and NAD(P)H, have become established targets for the detection and quantification of PBAP using the real-time, fluorescence techniques referenced above. However it is still not clear which of the fluorescence parameters are most relevant for discriminating between the various types of atmospheric PBAP.[33]

Previous experimental studies on the UV/Visible absorption spectra of PBAP and the fluorescence characteristics of pollen and other secretary cells have been published.[34–38] Furthermore some recent laboratory investigations by O'Connor *et al.* on the fluorescence spectra obtained from a variety of PBAP, as dry, solid powders have shown that distinguishing spectroscopic signatures could possibly be linked to individual botanical divisions orders and families. Importantly, the grass pollen samples investigated uniquely and in clear contrast to the other pollen types registered a sharp fluorescence band at 675–680 nm.[39] This observation clearly indicates the little reported phenomenon of chlorophyll-a presence within grass pollen.

Within plants it is well-known that chlorophyll-a and chlorophyll-b are synthesized initially from glutamic acid *via* a number of enzymatic steps that lead to its precursor, protochlorophyllide. However the inclusion of chlorophyll-a by grass pollen grains in addition to DNA/genetic material remains little explored. This is not surprising because *mature* pollen grains in grass contain plastids but not chloroplastids and therefore the presence of chloropyll is unexpected. The questions then naturally arise as to whether the chlorophyll-a found within grass pollen is "free" or associated with any protein at all and/or with its photosynthetic chloroplast end-product, NAD(P)H, a biochemical that is present in all living systems. In principle, dual fluorescence spectra and lifetime measurements can provide useful information about this possibility, the other biocatalysts that might be present and also the surroundings in which they are found.[40] In fact the measurement of fluorescence decay profiles has not been used hitherto in the study of PBAP as an aid to their discrimination, in spite of the well-known dependence of emission lifetime on localised environment.

Therefore in this paper we have measured the UV/Visible absorption spectra of three types of grass pollen (*Dactylis glomerata*, (DG) *Lolium perenne* (LP) and *Anthoxanthum odoratum* (AO)) and, for contrast, three tree pollen (*Fagus sylvatica* (FS), *Quercus robur* (QR) and *Quercus ilex* (QI)). Their intrinsic fluorescence was monitored by measurement of spectra and lifetimes for individual grains using fluorescence lifetime imaging microscopy (FLIM), a technique that has been widely applied to the study of various biological systems including *stained* pollen grains.[40–46]

Results and discussion

UV/Visible absorption spectra

UV/Visible absorption spectra were obtained for the six types of pollen listed above and are shown in Fig. 1.

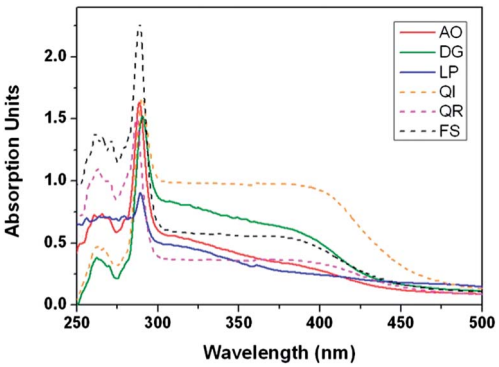

Fig. 1 UV/Visible absorption spectra: *Anthoxanthum odoratum* (AO) *Dactylis glomerata* (DG) *Fagus sylvatica* (FS) *Lolium perenne* (LP), *Quercus robur* (QR) and *Quercus ilex* (QI).

All of the pollen display similar spectra especially with regard to the structured feature centred ~260 nm, a sharper feature in the 280–290 nm region and finally a long tail from about 300 nm to 450 nm. It should be noted that the relative absorption values of the various pollen shown in Fig. 1 may be affected by scatter in the UV region. However the spectra obtained are entirely consistent with the variety of biochemical components known to be present in pollen and absorb light in the 250–500 nm spectral region. These materials include polymers such as lignin, amino acids like tyrosine, phenylalanine, tryptophan, DNA, NAD^+, NAD(P)H, flavonoids/riboflavin, flavo-proteins, azulene and chlorophylls. To further complicate any definite interpretation of the absorption spectra, NAD(P)H/$NAD(P)^+$ can bind to flavo-proteins such as Ferredoxin and Rubisco.[47,48] In addition chlorophyll-a, if present, may also become protein bound.[47]

Nonetheless several of the observed spectral features can be associated with particular chromophores associated with pollen. Thus the oxidized form of the coenzyme, NAD^+, in solution, shows an absorption maximum at 260 nm (cut-off ~390 nm) due to the Adenine group while its NAD(P)H reduced counterpart absorbs at about 340 nm (cut-off ~390 nm) due to the nicotinamide group.[49,50] When in the form, ferredoxin–$NADP^+$ reductase (FNR), the spectrum displays a shift to longer wavelengths because of the flavin component and the material absorbs light out to at least 450 nm.[51]

The amino acids tryptophan and tyrosine also absorb light in the 260–290 nm region with a spectral structure very similar to that observed in Fig. 1. The spectrum of DNA is the average of its four component bases showing a maximum absorbance between 250 and 260 and a cut-off at ~320 nm.[52] Riboflavin and related species such as quercetin show this band but also absorb light up to ~500 nm.[53–56] The cellular cofactor, FAD absorbs light from 300 to 500 nm with distinct absorbance maxima at ~375 nm and ~450 nm.[30] UV spectra of the outer layer of pollen and spore walls (exine/exosporium) have been measured previously using a microspectrograph and indicate one main feature between 250 and 310 nm.[34] By contrast chlorophyll-a and chlorophyll-b show distinctive absorptions in the 350–450 nm and also sharp features in the 620–680 nm range.[54,57]

The above spectroscopic data indicate that only the chlorophylls and the flavin-related such as FMN, FAD, FNR or quercetin would absorb light at the excitation wavelength, 405 nm, used in the fluorescence experiments described below.

Fluorescence spectra of individual pollen grains

The spectra obtained for several individual grains of the three grass pollen are shown in Fig. 2. Although the spectra are qualitatively similar there is a degree of variability between individual pollen grains with respect to relative intensities of the 450–600 nm and 650–750 nm features. This observation is possibly linked to variations in the biochemical contents. It should be noted that the very sharp lines observed at ~610 nm in all of the fluorescence spectra obtained are due to an optical artefact.

The fluorescence spectra obtained for three of the tree pollen investigated are shown in Fig. 3. A number of individual grains were again probed for each case.

The most striking differential observation is found for the spectra of the individual grass pollen shown in Fig. 2 where the longer wavelength feature with fluorescence maxima at 675 nm and 725 nm is readily attributable to chlorophyll-a. The bands are absent from the tree pollen counterpart spectra given in Fig. 3. For all samples, a broader emission is present in the 450–650 nm range with a common wavelength maximum at ~500 nm. There are clear differences in intensity ratio between the chlorophyll-a bands and the shorter wavelength feature for the grasses. Thus the sharp chlorophyll-a band is dominant for *Dactylis glomerata* and of less relative intensity with respect to the broad feature for both the *Lolium perenne* and *Anthoxanthum odoratum* samples.

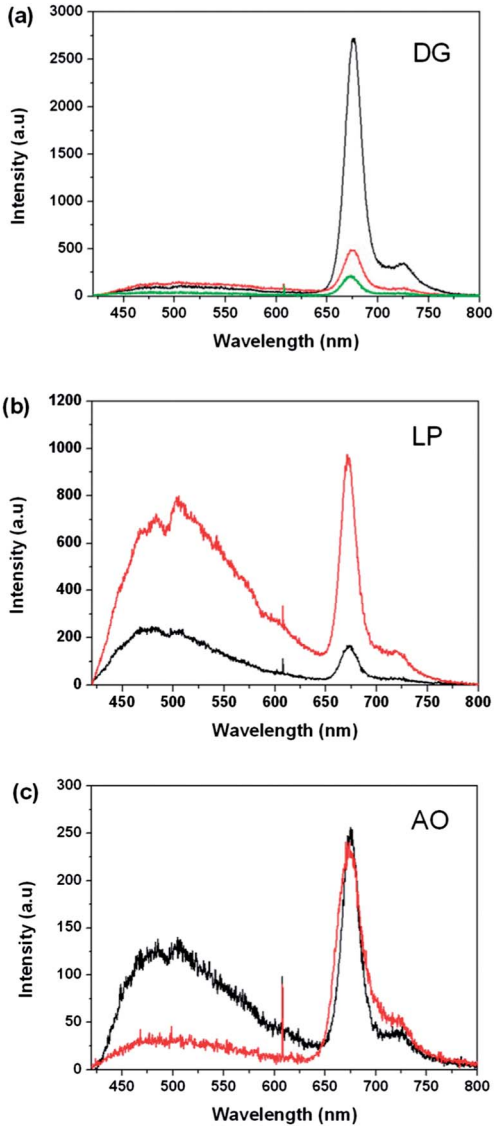

Fig. 2 Fluorescence spectra (λ_{ex}: 405 nm) of individual grass pollen grains of: (a) *Dactylis glomerata* (DG), (b) *Lolium perenne* (LP) and (c) *Anthoxanthum odoratum* (AO).

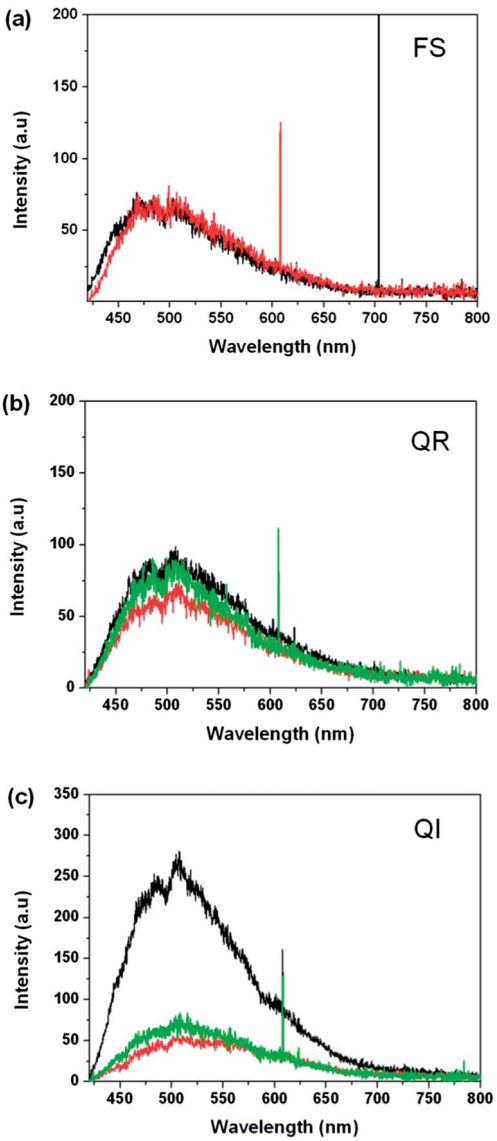

Fig. 3 Fluorescence spectra (λ_{ex}: 405 nm) of individual non-grass pollen grains of: (a) *Fagus sylvatica* (FS), (b) *Quercus robur* (QR) and (c) *Quercus ilex* (QI).

In addition to chlorophyll, protein bound nicotinamide adenine dinucleotide phosphate, such as ferrodoxin–NAD(P)H, NAD(P)H–FMN or NAD(P)H–FAD, could absorb light at the 405 nm excitation wavelength used in the experiments. Riboflavin and quercetin would also be excited leading to a very broad emission that extends from 470–700 nm with a maximum at ~520 to 550 nm. Other flavin-related biochemicals are also known to emit at wavelengths greater than 450 nm. For example the broad fluorescence spectra of FAD (500–600 nm) protein bound and in solution using FLIM (Fluorescence Lifetime Imaging Microscopy) following excitation at 450 nm has recently been published.[30]

Hence, in agreement with the absorption spectra results, the appearances of the fluorescence spectra shown in Fig. 2 and 3 can be explained by the involvement of just two or three light absorbing chromophore types. However to provide more quantitative information about this suggestion, fluorescence lifetime measurements are required in order to compare with related studies of "free" and protein bound chlorophyll-a and NAD(P)H, as well as FMN and FNR.[40,47,50]

Fluorescence lifetimes of individual pollen grains

The fluorescence lifetime data (mean values with Pre-Exponential Factors α_1, α_2 and α_3, PEF) obtained for the three grass pollen are summarised in Table 1. The fluorescence spectra clearly indicate two major emission components as shown in Fig. 2. Therefore to reduce complications from analysing the $\lambda >$ 625 nm (LongPath, LP) and $\lambda <$ 625 nm (ShortPath, SP) regions together, optical filters were applied in order to probe the chlorophyll-a fluorescence behaviour in isolation. For comparison the lifetimes of four non-grass pollen samples were also investigated over their whole emission range because they did not fluoresce to any great extent >625 nm. Whole individual grains were probed in each case by the FLIM technique and the lifetime distributions were found to be homogeneous.

Fig. 4 shows comparative data obtained for the grass DG (SP and LP) alongside that obtained for the tree pollen, FS. From inspection of the residuals using an IRF (Instrumental Response Function) treatment, the fluorescence lifetime data obtained could be satisfactorily fit, $\chi^2 < 1.3$, to all the decays. No constraints were applied to fix any lifetime to a pre-determined value for any pollen studied.

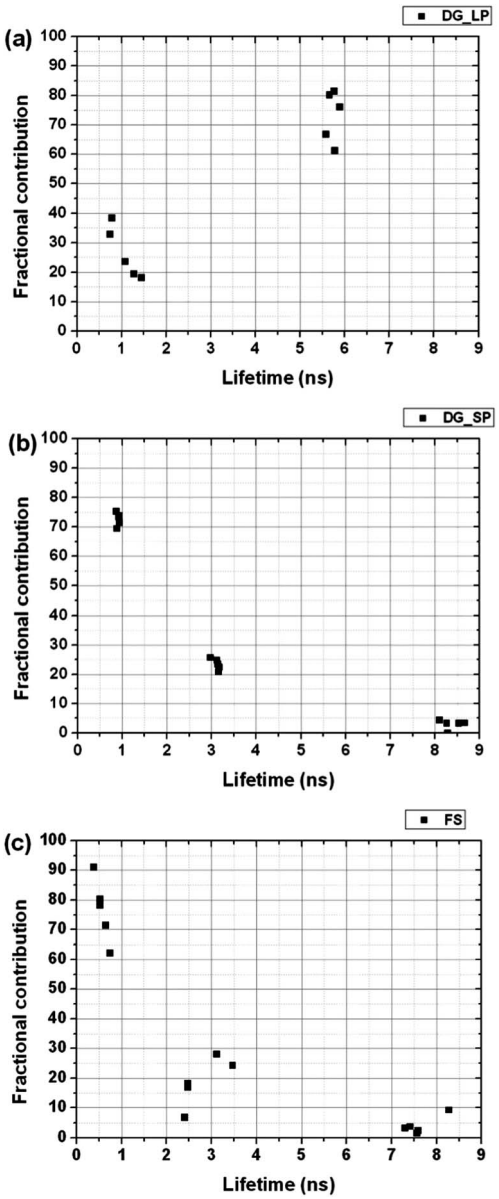

Fig. 4 Fluorescence lifetime data obtained for individual grass pollen grains: (a) *Dactylis glomerata* (DG) with Long Path filter, LP; (b) *Dactylis glomerata* (DG) with Short Path filter, SP; (c) *Fagus sylvatica* (FS).

Table 1 Fluorescence lifetime data for the pollen studied including results using SP, short-path and LP, long-path optical filters. The τ values are mean lifetimes and the PEFs (Pre-Exponential Factors- α_1, α_2 and α_3) are percentage contributions. Mean Chi-Squared values of the fitting are also reported

Pollen	τ_1/ns (α_1)	τ_2/ns (α_2)	τ_3/ns (α_3)	χ^2
Dactylis glomerata (LP)	0.95 (26.7)	5.6 (73.3)	—	1.14
Anthoxanthum odoratum (LP)	1.63 (45.2)	5.7 (54.8)	—	1.09
Lolium perenne (LP)	2.16 (25.3)	5.2 (74.7)	—	1.18
Dactylis glomerata (SP)	0.89 (72.8)	3.1 (23.6)	8.3 (3.6)	1.15
Anthoxanthum odoratum (SP)	0.93 (70.5)	3.2 (26.2)	9.1 (3.34)	1.09
Lolium perenne (SP)	1.01 (65.5)	3.2 (27)	8.5 (4.5)	1.30
Tilia cordata	0.96 (69)	3.0 (26.5)	8.7 (4.4)	1.13
Fagus sylvatica	0.56 (76.8)	2.9 (19)	7.6 (4.2)	1.09
Quercus robur	0.51 (80.7)	2.4 (16.9)	7.2 (2.3)	1.25
Quercus ilex	0.49 (84.6)	2.2 (13.6)	6.7 (1.9)	1.02

The LP data for all grass pollen could be fitted well to bi-exponential traces, whereas all other data sets required tri-exponential fittings to obtain acceptable χ^2 values. For contrast, one tree pollen was investigated using the LP filter but it displayed little intrinsic fluorescence above 625 nm and no reliable, repeatable lifetime results could be obtained.

The fluorescence lifetime of chlorophyll-a in a variety of environments has been extensively studied because of its key role in photosynthesis. In chloroplasts under physiological conditions, the fluorescence lifetime of chlorophyll-a depends upon the activity of the PS II reaction centres. If the active

centres are closed (*i.e.* no photosynthetic reactions occur) the measured values reach ~3 ns but when the centres are opened to allow the non-radiative photosynthesis process, they decrease to 170–300 ps.[58] In contrast the fluorescence lifetimes for chlorophyll-a in deoxygenated and oxygenated polar solvents such as methanol and ethanol, respectively, have been measured to be ~5.5 ns.[59] From Table 1 it is clear that all of the grass pollen, DG, LP and AO, exhibit a major lifetime component at 5.2–5.7 ns for fluorescence collection at wavelengths >625 nm. In addition, all three show lifetime decays at ~1 to 2 ns as more minor contributions. The longer measured lifetime values are fully consistent with the presence of "free" chlorophyll-a in grass pollen whereas the shorter lifetimes are consistent with the binding of chlorophyll-a to proteins, previously measured as 0.7–1.7 ns in aggregates (2.8–3.4 ns in monomeric forms).[60] However, as noted above, it is recognised that grass pollen do not contain chloroplasts although flavoproteins are known to be present and this aspect will be discussed further below.

An alternative explanation for the shorter lifetime component is that the enzyme product of chlorophyll-mediated photosynthesis, NAD(P)H, in some form might also be expected to be present for the grass pollen investigated here. The intensity and the lifetime of NAD(P)H has been found to strongly depend on the microenvironment in which it is located. In fact NAD(P)H has a mean fluorescence lifetime between 1 and 5 ns when bound to proteins.[40,47] The lifetime is much shorter at ~0.1 to 0.4 ns, presenting as at least a biexponential decay, when in its "free" form. It has also been shown that there can be two fluorescence lifetime pools for NAD(P)H bound to proteins, one shorter at ~1 to 2 ns and the other at ~3 to 4 ns. For example when bound FNR is excited at 450 nm two fluorescence lifetime decay components are measurable: 1.4 ns (12%) and 3.9 ns (88%).[51] Therefore the fluorescence lifetime measurements reported here are also potentially consistent with the grass pollen fluorescence originating from chlorophyll-a as well as NAD(P)H bound to a protein *e.g.* FNR.

Thus in order to distinguish between these possibilities, the LP data for the grasses were compared and contrasted with their SP decays and also the tree pollen results. The latter would, of course, be expected to be more relevant to any NAD(P)H and flavo-protein emissions. The measurements were repeated several times over the full pollen surfaces and some of these data are shown in Fig. 4. Over the whole data set for all the pollen studied (Table 1) the fluorescence dynamic response for the non-grass pollen and the grass SP required three components for good fitting: short (0.5–1.0 ns) as a major contribution; intermediate (2.0–3.0 ns) and a very minor component classified as long (7–9 ns).

These findings provide strong evidence that both "free" and flavo-protein bound chlorophyll-a are responsible for the two lifetime components of the grass pollen using the Long Path filter, rather than NAD(P)H involvement. It is of further note that the measured (tri-exponential) lifetimes and PEF obtained for the 450–600 nm (SP) wavelength contribution to the grass pollen emissions are very similar to those obtained for the tree pollen.

From the data in Table 1, in terms of potential discrimination between the tree pollen, it may prove to be of utility that the two Oak pollen, QR and QI, show almost identical lifetime behaviours that are different from the Beech and Lime examples (FS and TC respectively). As discussed above, it has been shown that differing orders and botanical families show somewhat differing fluorescence spectral properties. However many more examples would need to be studied to determine whether lifetime behaviours offer a similar discriminatory tool for distinguishing between, say, botanical orders or pollen delivery by catkin and other flowering mechanisms.[39]

Although the fluorescence lifetime behaviour of quercetin has not been reported, bound and "free" FMN has been previously studied; the latter form exhibits a lifetime component at about 4.6–4.7 ns with the bound monomer form being weaker in intensity and showing a further, minor lifetime component at ~1.0 ns.[29] However it should also be noted that a further important lifetime component of FMN aggregate bound to protein has also been reported to take a value of 7.6 ns. The long-lived lifetime value was explained by suggesting that no quenching groups, such as cysteine, were present at the binding site.[61] By contrast FAD is often taken to emit much weaker fluorescence[31] although some very recent work performed on FAD in both solution and biological cells shows that its fluorescence lifetime behaviour can be monitored.[30] The data given in that report could be best fit to four exponentials. These comprised a very short lifetime feature (0.08 ns), a short component (0.7 ns with 20% error) a longer one (~3 ns with 10% error) and finally a very long-lived lifetime (9–10 ns with 30% error), when FAD was *bound* to cells. The relative contributions to the multi-exponential profile for the very short, short, long and very long components bound within cells were (60%), (25%), (13%) and (2%) respectively. These data contrasted with the solution ("free") lifetime results for FAD with $\tau_1 \sim 7$ ps (66%), $\tau_2 \sim 0.2$ ns (3%), $\tau_3 \sim 2$ ns (17%) and $\tau_4 \sim 4$ ns (14%) contributions at all pH studied.

Hence from the lifetime and absorption results obtained for the grass (SP) and tree pollen investigated in this study it is suggested that FAD and/or more likely FMN likely bound within cells, are present. Both materials are known products of cellular metabolism and FMN emission from the bacterium *Vibrio fischeri* has been measured showing similar fluorescence dynamic responses to those reported here.[61]

Materials and methods

The pollen samples were purchased from Allergon AB, Sweden. All of them were kept refrigerated at 2 °C and checked by optical microscopy before use in order to determine whether any foreign bodies were present. Samples were all found to be pure and appeared as dry, solid powders and were not chemically or physically altered before experimentation. Therefore they can be considered representative of a portion of ambient, airborne PBAP released by their host organism before any degradation, due to aging, physical or chemical processes in the atmosphere, (*e.g.* oxidation by ozone), could occur.

Absorption spectra were acquired using a Shimadzu UV PC-2401 double beam spectrophotometer equipped with a 60 mm integrating sphere. Samples were held between two pieces of fused silica and immersion oil of refractive index of 1.52. A sample holder containing no sample was used as a blank for the measurements.

Single pollen imaging and spectroscopic characterisation was performed using a time-resolved confocal fluorescence microscope (MicroTime 200, PicoQuant).

The pollen grains were deposited onto a glass coverslip and the sample was mounted up-side down with the pollen grain facing the objective. The output of a 405 nm, ~70 ps pulsed laser (LDH-P-C-405B, 40 MHz) was coupled with the main confocal unit *via* a polarisation-maintaining, single mode optical fibre. In a typical experiment, the excitation power used was kept below 100 nW (*ca.* 80 nW). Emission spectra were recorded by directing the sample luminescence onto the entrance slit of a monochromator equipped with a 300 g mm^{-1} grating (SP2356, Acton Research) and a thermoelectrically cooled, back illuminated CCD (Spec10:100B, Princeton Instruments). In a typical experiment, PL spectra were recorded with an input slit width of 50 μm and an integration time of 30 seconds. For the SP/LP spectral discrimination experiments, a 03SWP410 filter (Melles Griot) was used for the acquisition of fluorescence images/spectra below 625 nm (SP) and 10 LWF-550 and 10-LWF-650 filters (Newport) were employed for acquisition of light above 625 nm (LP).

The intensity decays were analyzed in terms of a multi-exponential model using SymPhoTime v. 4.7 software (PicoQuant, GmbH). The instrument response function of the system was reconstructed by the software and its contribution removed from the collected data. The time-resolved spectra were then reconstructed from the decay curves using a sum of exponentials:

$$I(t) = \sum_i \alpha_i e^{\frac{-t}{\tau_i}}$$

where α_i and τ_i are pre-exponential factors and fluorescence lifetimes, respectively. The fits were evaluated by their residuals (random deviation between measured and fitted data and χ_R^2 factor inferior to 1.3). Satisfactory fitting was achieved using 2 exponentials for chlorophyll-a emissions while 3 exponentials were necessary for the other measurements. Fluorescence lifetime images were also plotted using the amplitude average lifetime calculated as $\overline{\tau} = \sum_i \alpha_i \tau_i$.

Conclusions

Although the chemical composition of pollen has been studied by many plant physiologists and biochemists, the main contents established have provided no quantitative basis for analytical distinction between species. Such results indicate mainly DNA-related/amino acids/allergenic materials, carbohydrates and lipids in the interior and "sporopollenin" as the exterior.[62] Although the use of fluorescence *spectra* has been found previously to be relevant to the detection of certain biocatalytic components of PBAP such as chlorophyll-a and NAD(P)H, the fluorescence lifetime study reported here represents the first systematic lifetime study of the *autofluorescence* for individual grains. It is clear from the results that such measurements can, at least, provide data useful for the discrimination between grass and tree pollen. It may also prove possible to apply the approach to distinguish between catkin and other flowering mechanisms of pollen delivery. Therefore the study presented here clearly indicates that fluorescence lifetime measurements should represent a further useful technical target for the real-time PBAP detection instrumentation that is currently being developed. Furthermore the fact that distinctive fluorescence spectra for pollen can be measured using excitation wavelengths, $\lambda > 400$ nm, may provide the basis for future instrumentation, employing suitable blue/purple, visible region diode lasers, to be developed that can likely reduce or eliminate many potential interferences from chemical species such as certain Polycyclic Aromatic Hydrocarbons (PAHs).

Acknowledgements

We would like to thank the SFI NAP programme for financial support and also the Irish EPA for financial support under its Doctoral Scholarship Scheme (to DJOC).

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