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ORIGINAL PAPER

Solvatochromic Effect in the Optical Spectra of Calcofluor and its Relation to Fluorescent Staining of Yeast Cell Walls

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Abstract Fluorescence spectral properties of calcofluor (a popular stain used to visualize cell walls of bacteria, yeast and fungi) has been studied. The analysis of calcofluor fluorescence emission spectra measured in a wide range of solvents (including media containing chitin), and in yeast cell suspensions has revealed that the solvatochromic properties of calcofluor ensue essentially from the by solvent-solute hydrogen bonding, or from the hydrogen bonding to cell wall polysaccharides with an eventual contribution of calcofluor aggregation at the cell surface. Preliminary data suggest that calcofluor emission spectra can be employed as a practical marker of variations in the quality of yeast cell wall.

Keywords Calcofluor · Absorption · Fluorescence · Solvatochromism · Yeast cell wall · Hydrogen bonds

Introduction

Calcofluor has been used for nearly half a century as a vital fluorescent stain enabling the rapid detection of yeasts, fungi and parasitic organisms [1–5], often with the aim to ensure their quantitative determination, e.g., [6]. It is a member of the class of fluorescent whitening agents (also known as optical brightening agents or fluorescent whiteners) that is marketed under many different names[1], see also [7, 8] and references therein. For the most of its published biomedical applications, calcofluor has been purchased as Calcofluor White M2R (also known as, e.g., Fluorescent Brightener 28, see Scheme 1).

Some discrepancies in calcofluor identification exist, which stem partly from the fact that the commercial calcofluor products are available as either a disulfonic acid (CAS registry number 4404-43-7; 4,4′-bis[6-anilino-4-bis-(2-hydroxyethylamino)-1,3,5-triazin-2- ylamino]stilbene-2,2′-disulpfonic acid), or its disodium, dipotassium, and potassium sodium salts (their respective CAS registry numbers being 4193-55-9, 70942-01-7, 71230-67-6, respectively). Another identifiable difference between calcofluor molecules from various sources consists in the substituents attached to the triazine rings of this molecule. This case can be illustrated with a calcofluor structure presented in older literature [9], and two recent fluorescent brighteners marketed as Calcofluor White, i.e., Calcofluor White RC (CAS 16090-02-1), Calcofluor White MR (CAS 133-66-4).[2] It should be noted here that Calcofluor White LD (CAS 87-01-4) and Calcofluor White RW (CAS 96538-19-1) are simple derivatives of coumarine. Thus their properties cannot be compared with those of Calcofluor White M2R and related products.

In contrast, the members of the latter group share a number of key features. In particular, they contain sulfonate groups, which makes these molecules to be well soluble in alkaline aqueous media, owing to the electrostatic repulsion of calcofluor anions. In practice, however, the actual solubility of calcofluor may differ between the disulfonic acid forms and

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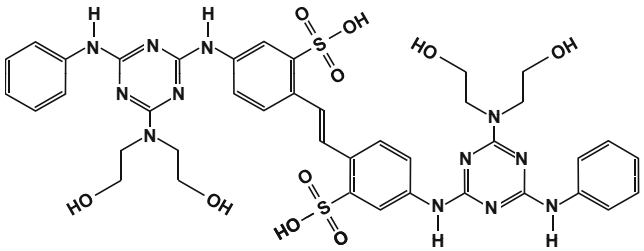
[1] http://www.zarc.com/english/other_sprays/hazardous_chemicals/2606-93-1.html.

[2] Search for Product "calcofluor" on www.lookchem.com.



Scheme 1 Chemical structure of calcofluor: Fluorescent Brightener 28, CAS 4404-43-7

their salts [10]. A further similarity between different calcofluor structures concerns the arrangement of hydrogen-bond donors and acceptors. This aspect seems to be extremely important because infrared spectra measured with model hexopyranose polymers have implied that the high affinity of calcofluor for polysaccharides is due to a hydrogen bonding [11]. Regarding the UV-Vis absorption spectra of calcofluor, it is shown in our paper that the above structural variations in the triazine ring substituents do not play a significant role. While the major properties of calcofluors are practicaly independent of the variability in these substituents, this variability may still be a culprit of some conflicting reports on recommended staining protocols, as noted, e.g., in [12].

Varying degrees of specificity in calcofluor binding to different polysaccharides were reported in existing literature. In particular, calcofluor has often been incorrectly declared as a cytochemical marker for chitin, see for example a recent contribution to the benchmarking in yeast fluorescence microscopy [13]. However, this common belief stems from an indirect evidence only [9], such as (i) a correlation between the localization of chitin in scar margins of *S. cerevisiae* cell walls and maximum fluorescence intensity emitted from these structures, (ii) the finding that fungal cells treated with 10% potassium hydroxide (to whose action their chitinous walls are somewhat resistant) can still be fluorescent upon staining with calcofluor, and (iii) the effect of calcofluor staining on the rate of chitin polymerization in growing *G. lactis* and *S. cerevisiae* cells if this dye s used at high concentrations (>25 μM), [14, 15] and references therein. On the other hand, a solid argument against the calcofluor specificity for chitin is that a bright calcofluor emission can be observed in the bud scars of *Schizosaccharomyces pombe* whose cell walls contain very little if any chitin [16, 17]. Moreover, a strong affinity of calcofluor to cellulose [18] and various β-glucans has also been reported, e.g., [19–21]. Calcofluor-flow-injection analysis was even applied for the quantitative determination of β-glucans, see [22] and references therein.

A key aspect of the analysis of the optical properties of calcofluor is the role of its stilbene moiety. Neither the absorption nor fluorescence spectra of closely related bis (triazinylamino)stilbene structures depend considerably on the nature of a particular substituent in the triazine ring [23]. Therefore, no significant difference in fluorescence properties is expected to exist between various chemical forms of calcofluor. Typical absorption and emission spectra of calcofluor in aqueous solutions can be found in [24]. These spectra are quite broad. Thus, the walls of yeast cells stained with calcofluor can fluoresce blue-white or apple-green, depending on the filter combination used [5, 25, 26].

Though fluorescence emission spectra can provide valuable insight into the nature of a fluorochrome microenvironment, calcofluor studies dealing with this issue are rather rare. Meadows used fluorescence spectroscopy to study the binding of calcofluor to cellulose during the process of cell wall regeneration in plant protoplasts [27]. Low-resolution fluorescence emission spectra of calcofluor in aqueous solutions and butanol have been presented by Albani and Plancke, and compared with those of calcofluor bound to human serum albumin, cellulose and α_1-acid glycoproteins [28–30]. Wood measured both the absorption and fluorescence emission spectra of calcofluor in pure water and alkaline carbonate buffer, and has published spectral effects caused by the binding of calcofluor to oat β-D-glucan [20]. The fluorescence emission spectrum of calcofluor bound to β-glucan has also been published by Kim and Inglett [22]. They used in their study β-glucan standards of several molecular weights dissolved in phosphate buffers of different ionic strengths, but have not specified the nature of specimen used to measure the calcofluor emission spectrum. It is therefore difficult to interpret a 4 nm shift of their spectrum relative to the data of Wood. Since a very limited set of solvents have been used in existing studies, it is difficult to interpret the solvatochromic behaviour of this fluorochrome. In view of current lack of spectral data on calcofluor fluorescence, this study is aimed at obtaining concise information on its spectral response to physico-chemical properties of fluorochrome microenvironment, including the specific case of calcofluor bound to yeast cell walls.

Experimental

Yeast strains and cell culturing

S. cerevisiae US 50–18C (*MATα, PDR1-3, ura3, his1*) and its mutant AD1-3 (Δ*yor1::hisG*, Δ*snq2::hisG*, Δ*pdr5::hisG*) have been used [31]. Yeast was pre-cultured in YEPG medium (1% yeast extract, 1% bactopeptone, 2% glucose) at 30°C for 24 h. A volume of 150 μl inoculum was added to 20 ml fresh YEPG medium and the main culture was grown for different periods of time to reach the different growth phases. The post-diauxic cells were harvested, and suspended directly without any washing in either distilled water or citrate-phosphate (CP) buffer of pH 7.8 to OD_{578}=0.05.

Calcofluor solutions

Calcofluor was from Sigma-Aldrich (Fluorescent Brightener 28, Product # F3543). A stock solution (10^{-3}M) was prepared with distilled water of pH tuned to 10.9 using NaOH, under which condition an electrostatic repulsion between ionized calcofluor molecules boosts their solubility. In neutral and acidic media this interaction is missing, and thus the calcofluor solubility is much less. All other solvents, except citrate-phosphate buffers, were of analytical grade. Chitin from crab shells, practical grade, was from Sigma-Aldrich; we used 1% (w/v) solution in citrate-phosphate buffer (pH 7.3).

Absorption and fluorescence spectroscopy

Absorption spectra were measured with Lambda-12 (Perkin Elmer) UV/Vis spectrophotometer using 1×1 cm quartz cuvettes, and dye concentration of 2×10^{-5} M. Fluorescence spectroscopy has been performed with Fluoromax 3 (Horiba Jobin-Yvon, USA) using disposable 1×1 cm UV-grade fluorimetric cuvettes (Kartell spa, Italy), and 1×10^{-6} M calcofluor solutions (apart from the spectra shown in Fig. 3). The bandwidths used for the excitation and the emission were 3 nm and 2 nm, respectively. Fluorescence emission spectra of calcofluor solution were measured with λ_{exc}=365 nm, which choice matches a typical excitation mode used in the fluorescence microscopy of calcofluor stained microorganisms. All emission spectra have been corrected for the instrument spectral sensitivity.

Relative fluorescence intensities from different calcofluor solutions have also been corrected for the inner filter effect, see e.g., [32], as follows: The optical densities of analyzed calcofluor solutions, OD_{365}, were determined for λ_{exc}=365 nm, and used to assess relative intensities, I_{exc}^{C}, of excitation light transmitted to the centre of sample cuvette. Then, the experimental fluorescence intensities were normalized with respect to corresponding I_{exc}^{C} values. Finally, the above normalized fluorescence intensities have been divided by the value of molar extinction coefficient, ε_{365}. In this way, the calcofluor fluorescence intensities measured under various conditions have been converted to a scale that reflects relative values of fluorescence quantum yields.

The emission spectra of fluorescence from calcofluor stained yeast cell

Calcofluor was added to yeast cell suspensions (OD_{578}= 0.05) to a final concentration of 1×10^{-5} M. The binding of calcofluor to yeast cell walls has been practically immediate. The cuvettes with stained cell suspensions were gently shaken by hand before measuring fluorescence emission spectra. To get pure calcofluor emission spectra from cells, fluorescence from supernatant was measured and subtracted from the overall emission. The cell suspensions have been centrifuged directly in the fluorimetric cuvettes in which the emission spectra of cell suspensions were measured. For this purpose, a standard centrifuge tube holder designed for the cylindrical tubes of 1.5 cm diameter has been used without any adaptation. After centrifugation, the cuvettes with samples were carefully transferred to the fluorimeter without removing the pellet, and the spectra from the cell supernatant measured.

Results and discussion

Absorption spectra of calcofluor aqueous solutions and their pH-dependence

The calcofluor absorption spectra measured in CP buffers of pH ε 6.4 exhibit a distinct absorption band located in a near-UV region. The spectral maximum of this band shifts slightly towards longer wavelengths on going from alkaline media to the moderately acidic ones (λ_{max}=331 nm at pH= 8.0, and 345 nm at pH=6.4). For CP buffers of pH≈ 7, the maximum of near-UV band is found close to 348 nm, which finding matches earlier data on calcofluor spectral properties [24]. Upon increasing the acidity of calcofluor solutions to pH<6, a new absorption band occurs that is located around 410 nm, whose growth is accompanied by the decrease of absorptivity of the near-UV band, Fig. 1.

The pH-dependent variations in calcofluor absorption spectra are clearly illustrated in titration plots of molar absorption coefficients measured at 340 nm and 410 nm,

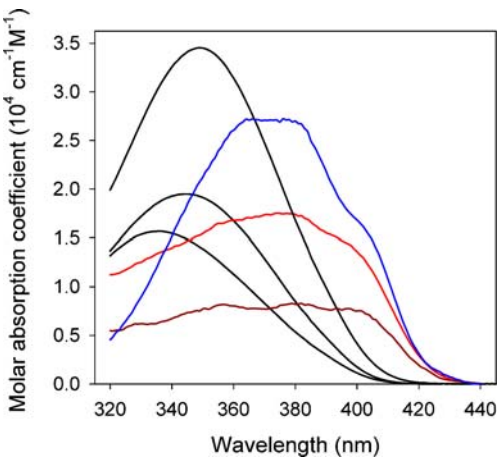


Fig. 1 Absorption spectra of calcofluor solutions in citrate-phosphate buffers. *Black lines* correspond to pH values of 7.80, 6.35, and 6.90 (the *lower*, *middle*, and *uper curve*, respectively); *red line*, pH 4.70; *dark red line*, pH 3.04. *Blue line* represents the absorption spectrum of calcofluor bound to chitin (1×10^{-5} M calcofluor and 1% w/v chitin solution in CP buffer, pH 6.90)

Fig. 2. On going from acidic to alkaline media, these plots reveal a series of consecutive proton dissociation steps, in which calcofluor molecule proceeds from its neutral form (with two sulfonate groups fully protonated) to the monoanionic, and finally to the dianionic one. An absorption band located around 410 nm, which is red-shifted relative to the monomeric one, is a well-known attribute of existence of collective energy levels of electrons in aggregated supramolecular assemblies, for more information on this phenomenon see, for example, [33].

Calcofluor ions present in alkaline solutions exhibit a strong mutual electrostatic repulsion. This interaction, which can counterbalance a hydrophobic attraction of the solute, is evidently absent in the case of neutral molecules existing in acidic media. Therefore, the possibility of calcofluor aggregation must be taken into account when dealing with the acidic calcofluor solutions. Note that a direct evidence of the formation of aggregates can be obtained if calcofluor stock solutions are injected to CP buffers. While a clear stream of liquid leaves a pipette tip during the injection of calcofluor solutions into alkaline CP buffers (pH>7), their injection into acidic solvents (pH<4) results in a hazy cloud, which is the effect of light scattering by microscopic calcofluor precipitates. The onset of ε_{410} curve in Fig. 2 indicates that the formation of supramolecular assemblies of calcofluor starts below pH≈6.

In contrast to this situation, calcofluor molecules in alkaline CP buffers exist as perfectly soluble divalent anions. Calcofluor absorption spectra with their first band located around 331 nm (as found in CP buffers of pH close to 8.0) seem to indicate such a monomeric state of the solute. At intermediate pH values, calcofluor molecules will reach series of pH-dependent equillibria between the respective concentrations of divalent and monovalent cations, or monovalent cations and neutral molecules. The condition under which calcofluor molecules may exist preferentially as the monovalent anions is very likely revealed by a marked maximum in the ε_{340} titration curve found around pH 7. Unfortunately, the onset of the light absorption by aggregates, have varied considerably with the concentration of calcofluor solution (data not shown). Therefore, we do not attempt here to assess pKa values for the protonation of calcofluor sulfonate groups.

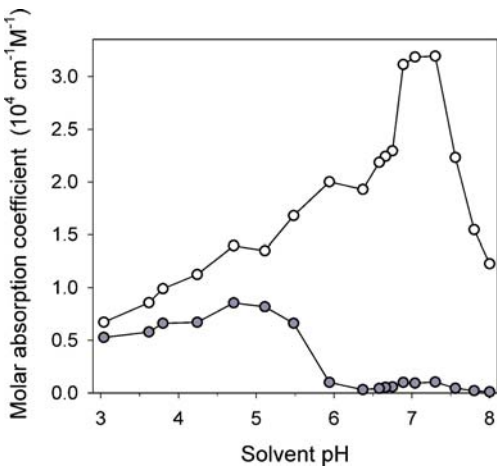


Fig. 2 The pH dependence of molar absorption coefficients of calcofluor solutions in citrate-phosphate buffers. The values of molar absorption coefficients measured at λ=340 nm and 410 nm are marked with *empty* and *full circles*, respectively

Fluorescence emission spectra of calcofluor aqueous solutions

Fluorescence emission spectra of calcofluor aqueous solution are shown in Fig. 3. Their pH-dependence is rather modest compared to the corresponding absorption spectra. The emission spectra of calcofluor solutions measured for pH ranging from alkaline to mildly acidic values (pH>6.9) are practically identical, apart from small differences within the long-wavelength tail of the spectrum.

The fluorescence emission spectra of acidic calcofluor solutions exhibit a clear shift towards longer wavelengths. Besides, the above spectral changes are associated with a marked increase in fluorescence intensity. The relative fluorescence quantum yields in fresh calcofluor solutions of pH 3.04 and pH 7.80 are 1.9 and 1.0, respectively. The red shift of fluorescence emission spectra observed in acidic media can again be plausibly interpreted as an attribute of the formation of calcofluor aggregates. To learn how large the spectral shifts due to calcofluor aggregation might be, we measured fluorescence emission spectra from calcofluor

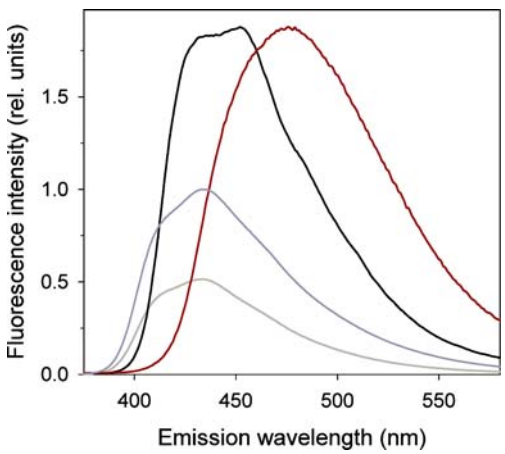


Fig. 3 Fluorescence emission spectra of calcofluor solutions in citrate-phosphate buffers (dye concentration 2×10^{-5} M, λ_{exc}= 365 nm). *Gray*, *dark gray*, and *black line* corresponds to pH 6.90, 7.80, and 3.04, respectively. *Dark red line* is the emission spectrum of fluorescence from calcofluor microcrystals (the raw powder of Fluorescent Brightener 28 from Sigma) suspended in hexane. The relative intensity of the latter spectrum is not in scale with the spectra from aqueous solutions

microcrystals (as found in Fluorescent Brightener 28 powder from Sigma) suspended distilled water. These spectra are structureless, and extremely shifted towards longer wavelengths, λmax≈480 nm, compared to the fluorescence from calcofluor monomers in alkaline solutions.

In acidic media that promote calcofluor aggregation, the actual fluorescence emission spectra may depend considerably on the history of a calcofluor solution. If acidic calcofluor solutions are left to equilibrate for about 5–15 min, the long wavelength part of the emission spectrum will increase (data not shown). Upon shaking vigorously such an equilibrated solution, which may partly re-dissolve large supramolecular aggregates, the shape of its fluorescence emission spectrum becomes again similar to that one typical of fresh solutions.

Solvatochromic behaviour of calcofluor fluorescence

If bound to polysaccharides, calcofluor exhibits fluorescence whose emission spectra are significantly different from those found in simple aqueous solutions. This well-known fact has usually been interpreted in terms of the effects of polarity- and viscosity-dependent orientational relaxation of Franck-Condon states, e.g., [20, 28–30]. To verify whether these plain physical factors alone may actually control the solvatochromic behaviour of calcofluor fluorescence, we measured both the absorption and emission spectra of this fluorochrome in a series of solvents, including polar aprotic solvents acetone and dimethyl sulfoxide, polar protic solvents water and ethylene glycol, and amphiprotic alcohols (methanol, ethanol, n-propanol, isopropanol, n-butanol). In Fig. 4, the fluorescence emission spectra from calcofluor solutions in ethanol and methanol are shown as examples, together with the corresponding emission spectra of fluorescence from calcofluor in both the neutral aqueous solutions and chitin-containing solutions.

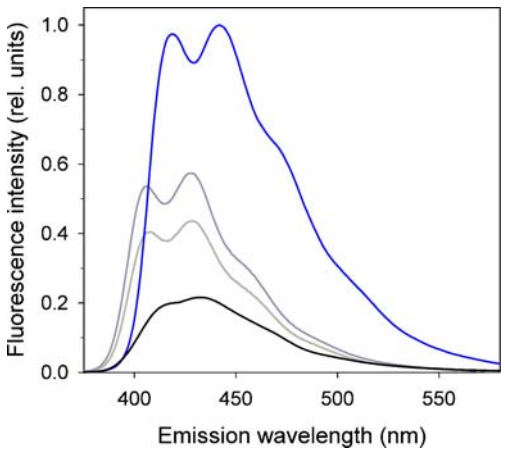


Fig. 4 Fluorescence emission spectra of calcofluor solutions in CP buffer of pH 7.30 (*black line*), methanol (*gray line*), ethanol (*dark gray line*), and 1.1% w/v aqueous solution of chitin (*blue line*). The insert shows the spectra in ethanol, CP buffer and chitin solution as normalized to unity at maximum. The concentration of calcofluor solutions has been 2×10^{-5} M, λ_{exc}=365 nm

In all the non-aqueous slightly polar solvents we used, the fluorescence emission spectra of calcofluor exhibit a pronounced vibronic structure compared to the calcofluor aqueous solutions. Such a difference is usually indicative of less solvent relaxation around the fluorochrome excited state than in the aqueous media [34]. The solvent-relaxation controlled solvatochromic effects are adequately described by Lippert-Mataga model, which predicts a linear dependence between the Stokes shift (Δν) and so-called orientational polarizability

$$\Delta f = \frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1} \quad (1)$$

where ε and n are the dielectric constant and refractive index of a solvent [32, 35, 36].

For our set of solvents, the dielectric parameters of which are summarized in Table 1, no significant linear relationship has been found between the Stokes shift of calcofluor fluorescence (assessed as a wavenumber difference between the absorption maximum and the first vibronic peak of the emission spectrum) and the orientational polarizability:

$$\Delta v = 6312 - 7297\,\Delta f \quad (2)$$

with n=9, R (the correlation coefficient)=0.247, and SE (the standard error)=607. Furthermore, two correlation tests have been performed (Pearson Product Moment and Spearman Rank Order), which also indicated that there is no significant correlation between Δf and Δv values (P>0.050).

The absence of a correlation between Δν and Δf values suggests that the orientational polarization is not a major factor controlling the magnitude of solvent-dependent Stokes shift of calcofluor fluorescence. This is not a surprising result, because the Lippert-Mataga theory treats only long range dipolar interactions. Hence it is not appropriate to systems with specific short range solute-solvent interactions, including hydrogen bonding that definitely take place in the alcoholic solutions of calcofluor. Furthermore, it may be expected from the above theory that the effect of orientational polarization should be low for nonpolar molecules with high symmetry, such as anthracene [36]. The actual solvatochromic behaviour of calcofluor fluorescence is consistent with this expectation.

It is therefore necessary to analyze the likely role of solvent-solute hydrogen bonding in the calcofluor solvatochromism. In this study, we have focused on calcofluor absorption because the spectral range in which solvent-dependent absorption maxima are found is more than three fold larger than its fluorescence counterpart. Moreover, the

Table 1 Solvent parameters and solvatochromic effect in calcofluor absorption spectra: ε, dielectric constant; n, index of refraction, $\Delta f = \frac{\varepsilon-1}{2\varepsilon+1} - \frac{n^2-1}{2n^2+1}$, orientational polarizability; π*,α, β, Kamlet-Taft parameters[a]; λ_{max}, absorption maximum (nm)

Solvent	ε	n	Δf	π*	α	β	λ_{max}
Acetone	20.7	1.36	0.284	0.71	0.08	0.50	334
Dimethyl sulfoxide	47.2	1.48	0.263	1.00	0.00	0.76	360
Methanol	33.0	1.33	0.308	0.60	0.93	0.62	354
Ethanol	24.3	1.36	0.289	0.54	0.83	0.77	352
n-propanol	20.0	1.38	0.275	0.52	0.78	0.825[b]	345
2-propanol	18.3	1.38	0.272	0.48	0.76	0.95	341
n-butanol	17.0	1.40	0.262	0.47	0.79	0.88	342
Ethylene glycol	37.0	1.39	0.288	0.92	0.90	0.52	358
Water	80.0	1.33	0.321	1.09	1.17	0.18	336[c]

[a] data from [37]

[b] an interpolation between β values for ethanol and n-butanol, based on the analogy with known β_2^H values for ethanol, n-propanol and n-butanol as published in [47]

[c] measured in CP buffer of pH 7.8

absorption spectra reveal Franck-Condon states, free of orientational polarization effects.

To date, there have been put forward a few quantitative scales for hydrogen-bond acidity/basicity, amongst which the Kamlet-Taft approach seems to be the most successful [37]. Based on the averaging of multiple normalized solvent effects on a variety of properties of many diverse types of indicators, three scales of solvatochromic parameters, π*, α, and β, have been constructed, which are to serve towards correlation of solvent effects on many reaction rates, equillibria, and spectroscopic properties through a schematic equation of the form

$$XYZ = XYZ_0 + s\pi^* + a\alpha + b\beta \tag{3}$$

The π* parameter measures the ability of the solvent to stabilize the charge and/or the dipole of a solute by virtue of dielectric effects [38, 39]. The α-scale represents the ability of solvent to donate a proton into a solvent-to-solute hydrogen bond, i.e., it measures a hydrogen-bond donor (HBD) acidity [40]. The β scale of HBA (hydrogen-bond acceptor) basicity quantifies the ability of solvents to accept a proton (donate an electron pair) in a solute-to-solvent hydrogen bond [41]. The values of solvatochromic parameters we have used in the evaluation of calcofluor solvatochromism are presented in Table 1, together with the apparent wavelengths of calcofluor absorption maxima observed in individual solvents.

In Fig. 5, these solvatochromic parameters are plotted against the actual wavenumbers of absorption maxima, ν_{max}. A clear distinction can be seen between two aprotic solvents and the group including ethylene glycol and amphiprotic alcohols, while the data for aqueous solutions are quite singular. For ethylene glycol and alcohols, the actual ν_{max} values exhibit various degree of linear relationship to individual Kamlet-Taft parameters, Eqs. 4a–4c.

$$\nu_{max} = 30264.8 - 2713.7\pi^* \tag{4a}$$
$$n = 6, R = 0.811, SE = 418.2$$

$$\nu_{max} = 35149.0 - 7740.9\alpha \tag{4b}$$
$$n = 6, R = 0.898, SE = 314.4$$

$$\nu_{max} = 26136.3 + 3335.4\beta \tag{4c}$$
$$n = 6, R = 0.959, SE = 201.6$$

The above set of linear regressions indicates that the solvatochromism of calcofluor absorption in ethylene glycol and alcoholic solutions is satisfactorily measured by the solvent β-scale basicity. Particulary, Eq. 4b suggests that the calcofluor absorption spectra will shift towards longer wavelengths if the hydrogen bonding of calcofluor to its surroundings is dominated by the proton-donating ability of this surroundings. As linear regressions to more complex combinations of solvatochromic parameters are concerned, they have provided negligible if any improvement compared to a simple fit to he β-scale:

$$\nu_{max} = 3148.3(8.51 - 0.19\alpha + \beta) \tag{5a}$$
$$n = 6, R = 0.960, SE = 206.8$$

$$\nu_{max} = 3148.3(5.91 + 0.13\pi^* + 0.17\alpha + \beta) \tag{5b}$$
$$n = 6, R = 0.961, SE = 249.0$$

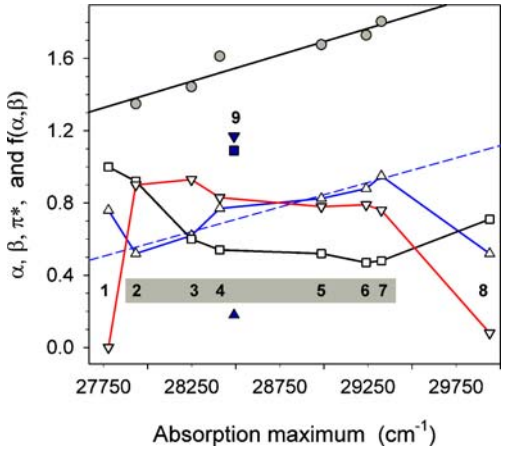


Fig. 5 The correlation between Kamlet-Taft parameters and calcofluor absorption maxima in the series of solvents (data from Table 1). Solvents used: *1*) dimethyl sulfoxide, *2*) ethylene glycol, *3*) methanol, *4*) ethanol, *5*) n-propanol, *6*) n-butanol, *7*) 2-propanol, *8*) acetone, *9*) water. Kamlet-Tafte parameters: π^*—squares/black line, α—triangles down/red line, β—triangles up/blue line; the singular data for aqueous solutions are shown in *dark blue*. *Dash blue line*—linear regression of the β vs ν_{max} dependence for solvents numbered from (2) to (7) within the gray box. *Gray circles*—f(α, β) data that match Eq. 5a (calculated according to the equation $f(\alpha, \beta)=1.3-0.19\alpha+\beta$, which differs from exact linear regression result $\nu_{max}/3148.3=8.51-0.19\alpha+\beta$ by a suitable constant only)

In literature, a report is available on the solvatochromic behaviour of a close calcofluor analog—disodium 4,4′-bis [4-methoxy-6- phenoxy-1,3,5-triazin-2-ylamino]stilbene-2,2′-disulphonate (further called BTSD), which differs moderately from calcofluor in the substituents attached to the triazines of the bis(triazinylamino)stilbene moiety [42]. Since the set of alcoholic solvents used in the BTSD study has been practically identical to our current choice (except for 2-propanol), it is possible to compare the absorption solvatochromic shifts in these two related dyes. The correlation between wavenumbers of BTSD and calcofluor solvent-dependent absorption maxima, denoted below as ν_{BTSD} and ν_{CF}, respectively, is extremely strong:

$$\nu_{BTSD} = 1892.7 + 0.948\,\nu_{CF}, \quad (6)$$

$$n = 5, R = 0.995, SE = 57.0$$

Interestingly, for ethylene glycol and alcoholic solvents the difference between the respective BTSD and calcofluor absorption maxima is nearly solvent independent, and equal to 413±57 cm^{-1}. Therefore, the solvatochromic behaviour of calcofluor appears to be a general feature of bis (triazinylamino)stilbene structures, to which family both calcofluor and BTSB belongs.

The simplest interpretation of the above findings is that the solvatochromism of calcofluor solutions is considerably affected by the hydrogen bonding ability of a solvent. Considering the strong dependence of ν_{max} on the basicity of alcoholic solvents, it seems that the secondary amines of calcofluor play a major role in this interaction as weak proton donors. Unfortunately, it is not possible to understand in depth the hydrogen bonding pattern around calcofluor molecules in solutions without analysing the cooperativity between four different amine sites, and/or proton-acceptor behaviour of the triazine part of calcofluor molecule. Such an analysis is far behind the scope of current study since the hydrogen bonding between calcofluor and its cell wall surroundings is likely to be controlled by a fixed spatial distribution of proton donors and acceptors on polysaccharides. Nevertheless, the study on solvatochromic behaviour of calcofluor alcoholic solutions has revealed that the orientational polarization alone cannot account for the spectral effect in calcofluor fluorescence whenever hydrogen bonds between calcofluor and its surroundings can be formed. Obviously, the discrepancy between simplified models based on the phenomenon of orientational polarization only and the actual properties of calcofluor fluorescence can be even higher if calcofluor is allowed to aggregate.

As the aqueous solutions of calcofluor are concerned, the situation might evidently become more complicated because the electronic states of calcofluor are influenced by the presence of ionized sulfonate groups. This fact may obviously account for the singular position of water data in the solvatochromic diagrams of Fig. 5.

Fluorescence emission spectra from calcofluor bound to chitin

The emission spectrum of fluorescence from calcofluor bound to chitin is nearly identical with the spectra of calcofluor bound to β-d-glucan [20]. At the same time, this spectrum is very similar to those from the calcofluor alcoholic solutions, Fig. 4, but shifted about 13 nm towards longer wavelengths. In spite of this similarity in emission spectra, the resultant value of Stokes shift (about 3,600 cm^{-1}) is much less than it has been found in calcofluor alcoholic solutions because the absorption spectrum of chitin-bound calcofluor is also shifted considerably towards longer wavelengths, Fig. 1. In view of both the weak correlation between decreasing ν_{CF} of calcofluor absorption maxima and proton-donating ability of solvents, α, and the evidence for the binding of calcofluor to proton-donating hydroxy groups of polysaccharides [11], we can assumed that the extreme long-wavelength position of the chitin-bound calcofluor absorption spectrum results largely from such a hydrogen bonding.

Fluorescence emission spectra from calcofluor-stained yeast cells

The measurement of calcofluor emission spectra from yeast cells has been repeated twice, in different days and with

different cell preparations. The major trends concerning the difference between calcofluor emission spectra from various yeast cell suspensions have been significantly reproducible.

A remarkable feature of the fluorescence from calcofluor stained cell suspensions is a considerable dependence of its emission spectra on the ionic strength of cell medium, Fig. 6. If suspended in distilled water, calcofluor-stained yeast cells emit fluorescence with structureless emission bands whose maxima are close to those of the fluorescence from calcofluor supramolecular aggregates, cf., Fig. 4. In the particular case of US50-18C strain, the apparent calcofluor emission band is practically identical with the fluorescence from calcofluor microcrystals, Fig. 6. With cells suspended in CP buffer (pH 7.9), the emission spectra of calcofluor fluorescence are found in the wavelength range that is characteristic of calcofluor bound to polysaccharides. Besides this, the emission from the cells of AD1-3 mutant strain exhibits also a vibronic structure similar to that one found in the fluorescence of chitin-bound calcofluor.

The dependence of calcofluor fluorescence properties on the ionic strength of cell medium indicates clearly that the calcofluor binding to yeast cell walls is controlled to a large extent by the surface charge of yeast cells. Note that in CP buffers the yeast cells are negatively charged, with less negative surface charge produced in acidic cell media below pH5. Moreover, the negative surface charge of yeast cells is inversely related to ionic strength of the cell medium, due to a screening by counter-ions present in the medium [43, 44].

The surface charge of yeast cells has been attributed to mannosyl phosphate residues of a outer layer of cell wall

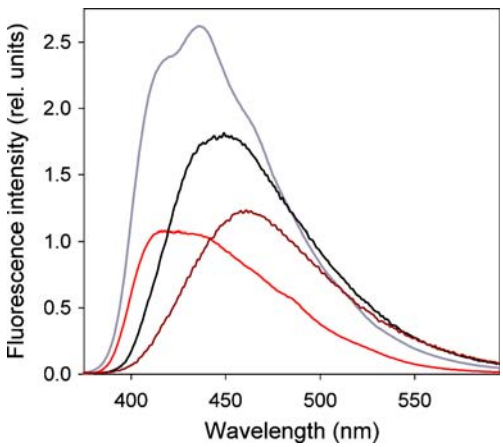


Fig. 6 Fluorescence emission spectra in calcofluor-stained cell suspensions. US50-18C strain: red line and dark red line—cell supension in CP buffer of pH 7.8 and distilled water, respectively, MDR pump deficient mutant strain AD1-3: *gray line* and *black line*—cell supension in CP buffer of pH 7.8 and distilled water, respectively. The cells were diluted into suspensions to a final $OD_{578}=0.05$, and stained with 1×10^{-5}M calcofluor

mannoproteins [45]. In addition to holding electric charge, this protein-containing layer seems to affect strongly the hydrophobicity of cells surface, see [46] and references therein. No doubt, both the electric charge and hydrophobicity of cell surface are factors that can jointly control the binding of calcofluor to yeast cell walls. Evidently, the rise of negative surface charge will result in the formation of an electrostatic barrier at the yeast cell surface. Because of this barrier, calcofluor anions attracted to the hydrophobic cell surface are hindered from passing further into an underlaid inner layer of cell wall, in which polysaccharides are localized. In these circumstances, we must take into account that a significant part of calcofluor molecules accumulated at the hydrophobic cell surface cannot bind to cell-wall polysaccharides. Instead, they will tend to aggregate if their local concentration is high enough. The microcrystal-like character of calcofluor emission spectra from US50-18C cells suspended in distilled water supports strongly this hypothesis. In contrast, the similarity between respective fluorescence emission spectra from calcofluor bound yeast cells in CP buffer and to pure polysaccharides indicates that if the surface charge yeast cells is screened by cations from the medium, calcofluor can easily reach the inner layer of polysaccharides. Then, it can bind to chitin and glucan via hydrogen bonds; very likely with a significant contribution of van der Waals forces mentioned by Woods, see [21] and references therein.

In conclusion, a variable extent of calcofluor hydrogen-bonding and calcofluor aggregation (both cell-specific and medium-dependent) should be invoked as an explanation of the character of fluorescence from calcofluor-stained yeast cells rather than an oversimplified view based on a solvent-solute orientational relaxation model. A significant difference between the calcofluor emission spectra from different yeast strains, as illustrated by the two examples shown in Fig. 6, is the subject of ongoing study.

Conclusion

The analysis of calcofluor fluorescence emission spectra measured in a wide range of solvents has revealed that the solvatochromic properties of calcofluor are controlled largely by solvent-solute hydrogen bonding. The fluorescence emission spectra from plain calcofluor solutions (including media containing chitin) have also been compared with those from calcofluor-stained yeast cell suspensions. This confrontation suggests that in the latter case the fluorescence properties of calcofluor ensue essentially from its hydrogen bonding to cell wall polysaccharides, with an eventual contribution of its aggregation in the negatively charged mannoprotein outer layer. Furthermore, the results of preliminary spectrofluorimetric studies of calcofluor-

stained yeast cells indicate that the emission spectra of calcofluor fluorescence could be employed as a practical marker of variations in the quality of yeast cell wall.

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References

1. Darken MA (1962) Absorption and transport of fluorescent brightener by microorganisms. Appl Microbiol 10:387–393
2. Wachsmuth ED (1988) A comparison of the highly selective fluorescence staining of fungi in tissue sections with Uvitex 2B and calcofluor white M2R. Histochem J 20:215–221
3. Perry JL, Miller GR (1989) Quality control slide for potassium hydroxide and cellufluor fungal preparations. J Clin Microbiol 27:1411–1412
4. Straton N, Hryniewicki J, Aarnaes SL, Tan G, de la Maza LM, Peterson EM (1991) Comparison of monoclonal antibody and calcofluor white stains for the detection of pneumocystis carinii from respiratory specimens. J Clin Microbiol 29:645–647
5. Harrington BJ, Hageage GJ (2003) Calcofluor white: a review of its uses and applications in clinical mycology and parasitology. Lab Med 34:361–367
6. Coleman T, Madassery JV, Kobayashi GS, Nahm MH, Little JR (1989) New fluorescence assay for the quantitation of fungi. J Clin Microbiol 27:2003–2007
7. Horobin RW, Kiernan JA (2002) CONN'S biological stains. A handbook of dyes, stains and fluorochromes for use in biology and medicine, 10th edn. BIOS Scientific, Oxford, pp 317–318
8. Miller V, Sasala K, Hogan M (2004) Report to Project Contract No. IOM-2794-04-00. The Center for Research Information, Brookville
9. Streiblova E (1984) in Nurse P, Streiblova E (eds) The microbial cell cycle. CRC, Boca Raton, pp 127–141
10. Anonym (2005) Stilbene fluorescent whitening agents category. report submitted to the US Environmental Protection Agency, ETAD Fluorescent Whitening Agent Task Force
11. Maeda H, Ishida N (1967) Specificity of binding of hexopyranosyl polysaccharides with fluorescent brightener. J Biochem 62: 276–278
12. Harrington BJ, Hageage GJ (1991) Calcofluor white: tips for improving its use. Clin Microbiol Newsl 13:3–5
13. Baggett JJ, Shaw JD, Sciambi CJ, Watson HA, Wendland B (2003) Fluorescent labeling of yeast. Curr Protoc Cell Biol Suppl. 20, Unit 4.13
14. Roncero C, Duran A (1985) Effect of calcofluor white and congo red on fungal cell wall morphogenesis: in vivo activation of chitin polymerization. J Bacteriol 163:1180–1185
15. Hickey PC, Swift SR, Roca MG, Read ND (2004) Live-cell imaging of filamentous fungi using vital fluorescent dyes and confocal microscopy. Methods Microbiol 34:63–87
16. Sietsma JH, Wessels JGH (1990) The occurrence of glucosaminoglycan in the wall of Schizosaccharomyces pombe. J Gen Microbiol 136:2261–2265
17. Bacon JSD (1981) Nature and disposition of polysaccharides within the cell envelope. In: Arnold WN (ed) Yeast cell envelopes: Biochemistry, biophysics and ultrastructure (chapter 5). CRC, Boca Raton
18. Harrington BJ, Raper KB (1968) Use of a fluorescent brightener to demonstrate cellulose in the cellular slime molds. Appl Microbiol 16:106–113
19. Fincher GB (1975) Morphology and chemical composition of barley endosperm cell-walls. J Inst Brew 83:116–122
20. Wood PJ (1982) Factors affecting precipitation and spectral changes associated with complex formation between dyes and β-d-glucans. Carbohyd Res 102:283–293
21. Wood PJ, Fulcher RG (1983) Dye interactions. a basis for specific detection and histochemistry of polysaccharides. J Histochem Cytochem 31:823–826
22. Kim S, Inglett GE (2006) Molecular weight and ionic strength dependence of fluorescence intensity of the Calcofluor/β-glucan complex in flow-injection analysis. J Food Comp Anal 19:466–472
23. Grabchev I, Philipova T (2000) Photophysical and photochemical properties of some triazinestilbene fluorescent brighteners. Dyes Pigm 44:175–180
24. Hoch HC, Galvani CD, Szarowski DH, Turner JN (2005) Two new fluorescent dyes applicable for visualization of fungal cell walls. Mycologia 97:580–588
25. Anonym (2001) Fungi-Fluor Kit. Technical Data Sheet 316. Polysciences, Inc, Warrington
26. Baron EJ, Finegold SM (1990) Baily & Scott's diagnostic microbiology, 8th edn. C.V. Mosby Company, St. Louis
27. Meadows MG (1984) A batch assay using calcofluor fluorescence to characterize cell wall regeneration in plant protoplasts. Anal Biochem 141:38–42
28. Albani JR, Plancke YD (1998) Interaction between calcofluor white and carbohydrates of α_1- acid glycoprotein. Carbohyd Res 314:169–175
29. Albani JR, Sillen A, Coddeville B, Plancke YD, Engelborghs Y (1999) Dynamics of carbohydrate residues of α_1-acid glycoprotein (orosomucoid) followed by red-edge excitation spectra and emission anisotropy studies of Calcofluor White. Carbohyd Res 322:87–94
30. Albani JR, Sillen A, Plancke YD, Coddeville B, Engelborghs Y (2000) Interaction between carbohydrate residues of α_1-acid glycoprotein (orosomucoid) and saturating concentrations of Calcofluor White. A fluorescence study. Carbohyd Res 327:333–340
31. Decottignies A, Grant AM, Nichols JW, de Wet H, McInstosh DB, Goffeau A (1998) ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p. J Biol Chem 273: 12612–12622
32. Lakowicz JR (2006) Principles of fluorescence spectroscopy, 3rd edn. Springer, New York
33. Wu JJ, Li N, Li KA, Liu F (2008) J-Aggregates of diprotonated tetrakis(4- sulfonatophenyl)porphyrin induced by ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate. J Phys Chem B 112: 8134–8138
34. Jaffe HH, Orchin J (1962) Theory and applications of UV spectroscopy. Wiley, New York
35. Lippert E (1955) Dipolmoment und Elektronenstruktur von Angeregten Molekulen. Z Naturforsch A 10:541–545 Naturforsch. Teit A, 10, 541, 1955
36. Mataga N, Kaifu Y, Koizumi M (1956) Solvent effects upon fluorescence spectra and the dipolemoments of excited molecules. Bull Chem Soc Japan 29:465–470
37. Kamlet MJ, Abboud J-LM, Abraham MH, Taft RW (1983) Linear solvation energy relationships. 23. A comprehensive collection of the solvatochromic parameters, π*, α and β and some methods for simplifying the generalized solvatochromic equation. J Org Chem 48:2877–2887

38. Kamlet MJ, Abboud JL, Taft RW (1977) The solvatochromic comparison method. 6. The π* scale of solvent polarities. J Am Chem Soc 99:6027–6038
39. Abboud J-LM, Kamlet MJ, Taft RW (1977) Regarding a generalized scale of solvent polarities. J Am Chem Soc 99:8325–8327
40. Taft RW, Kamlet MJ (1976) The solvatochromic comparison method. 2. The α-scale of solvent hydrogen-bond donor (HBD) acidities. J Am Chem Soc 98:2886–2894
41. Kamlet MJ, Taft RW (1976) The solvatochromic comparison method. I. The β-scale of solvent hydrogen-bond acceptor (HBA) basicities. J Am Chem Soc 98:377–383
42. Smit KJ, Ghiggino KP (1987) Fluorescence and photoisomerization of two stilbene-based dyes. Dyes Pigm 8:83–97
43. Vergnault H, Mercier-Bonin M, Willemot RM (2004) Physicochemical parameters involved in the interaction of Saccharomyces cerevisiae cells with ion-exchange adsorbents in expanded bed chromatography. Biotechnol Prog 20:1534–1542
44. Guillemot G, Lorthois S, Schmitz P, Mercier-Bonin M (2007) Evaluating the adhesion force between *Saccharomyces cerevisiae* yeast cells and polystyrene from shear-flow induced detachments. Chem Eng Res Design 85:800–807
45. Jigami Y, Odani T (1999) Mannosylphosphate transfer to yeast mannan. Biochim Biophys Acta 1426:335–345
46. Klis FM, Boorsma A, De Groot PWJ (2006) Cell wall construction in Saccharomyces cerevisiae. Yeast 23:185–202
47. Abraham MH, Grellier PL, Prior DV, Morris JJ, Taylor PJ (1990) Hydrogen bonding. Part 10. A scale of solute hydrogen-bond basicity using log K values for complexation in tetrachloromethane. J Chem Soc Perkin Trans 2:521–529