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Excitation wavelength-specific changes in lignocellulosic autofluorescence

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Abstract Lignocellulose (paper sheets of thermomechanical pulp) was irradiated with specific wavelengths in an epifluorescence microscope equipped with filter cubes with excitation and emission filters commonly used for excitation of fluorophores with blue, green, and red emissions. The irradiation was shown to affect the relative autofluorescence intensity over time. Shorter wavelength excitation (λ_{ex} = 340–380 nm) induced photoyellowing that increased the autofluorescence. Filter cubes allowing irradiation at longer wavelengths (λ_{ex} = 450–490 nm and λ_{ex} = 515–560, respectively) caused photobleaching and a decrease in lignocellulosic autofluorescence. The increase and decrease in autofluorescence was reversible; prephotoyellowed samples could be photobleached and prephotobleached samples regained autofluorescence by shorter wavelength irradiation. Thus, the specificity of the excitation wavelength and the time-dependent autofluorescence might affect long-term imaging experiments of lignocellulose-based samples.

Key words Autofluorescence · Lignocellulose · Photobleaching · Photoyellowing · Thermomechanical pulp

Introduction

The autofluorescence of wood depends on the structural and chemical properties of the natural organic fluorophores in lignocellulosic fibers, and on the excitation energy (intensity and wavelength).[1–3] Fluorescence microscopy is a common tool for analyzing distribution of immunolabeled epitopes in plant cell material.[4,5] In order to specifically excite and detect emissions from commonly used fluorescent-protein probes, different filter cubes with excitation and emission wavelength filters are employed depending on the fluorescent probe used. Due to the organic nature of the fluorescent-protein probes used for immunolocalization, they suffer from fading of fluorescence. However, newly developed fluorescent probes based on semiconductor nanocrystals are very photostable under conditions normally used in fluorescence-based immunolocalization using fluorescence microscopy.[6–8] Prolonged irradiation of lignocellulose is known to induce photobleaching or photoyellowing,[9–11] depending on the wavelength, and the lignocellulose autofluorescence is also known to be affected by chemical and irradiation treatments.[1–3] Thus, long term epifluorescent imaging of a lignocellulosic material would also affect the autofluorescence. Therefore, the use of photostable probes or new analytical methods involving prolonged irradiations of lignocellulose might consequently result in alterations of background autofluorescnce (and unlabeled reference samples) of possible importance for (semi)quantitative analyses. Therefore, in this study, the effects on lignocellulosic autofluorescence were analyzed when irradiated over time using a conventional epifluorescence microscope equipped with commercial filter cubes typically used for excitation of common fluorophores with blue, green, and red emissions, respectively.

Experimental

As a source of a homogenous lignocellulosic fiber material of uniform thickness and density, commercial unbleached newsprint of thermomechanical pulp was used ("Standard News," SCA, Sweden). Cut samples of the paper were placed in between a glass slide and a coverslip and analyzed dry or hydrated using a Leica fluorescence microscope. A commercial set of three excitation/emission filter cubes were used (Leica): excitation (λ_{ex}) = 340–380 nm and emission (λ_{em}) ≥ 425 nm (filter cube A, blue emission); λ_{ex} = 515–560 nm and λ_{em} ≥ 590 nm (filter cube N2.1, red emission); λ_{ex} = 450–490 nm and λ_{em} ≥ 515 nm (filter cube I3, green emission). The samples were subjected to continuous irradiation in the microscope using the respective filter

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cubes, and photomicrographs were recorded (exposure time 98.1 ms or 41.6 ms) at time intervals using a CCD camera. The autofluorescence was analyzed as mean gray value using ImageJ software. All experiments were performed in triplicate and presented as mean values ± standard deviation.

Results and discussion

Exposed to irradiation at or below 400 nm, lignocellulose undergoes photoyellowing,[9,10] which is a discoloration phenomenon that is disadvantageous for the usage of high-yield pulp. However, brightening of high-yield pulp is known to occur when it is exposed to wavelengths between 400 and 550 nm.[11] This is in accordance with the results shown in Fig. 1; 20 min irradiation at λ_{ex} = 340–380 nm clearly resulted in an increase in blue emission intensity ($\lambda_{em} \geq 425$ nm) using filter cube A, whereas prolonged irradiation using longer excitation wavelengths resulted in a considerable decrease in autofluorescence (using red and green emission filter cubes). The obtained autofluorescence using the filter cube for green emission (λ_{ex} = 450–490 nm and $\lambda_{em} \geq 515$ nm, filter cube I3) clearly indicated a similar effect to filter cube N2.1 for red emission, although the longer excitation wavelengths (λ_{ex} = 515–560) for red emission had a more evident effect. Therefore, to facilitate comparisons, the extremes (the shorter and longer wavelength filter cubes for blue and red emissions) are presented in Figs. 2–4, which show the changing autofluorescence over time. In Fig. 2, the total average mean emission intensities were measured over time, and it was notable how the exposure using the red emission filter cube (N2.1) instantly caused photobleaching of the lignocellulosic paper sheet, with a resulting decrease in autofluorescence. The shorter excitation wavelength (λ_{ex} = 340–380 nm) using the blue emission filter cube also rapidly affected the sample, with increased emission intensity. The increased autofluorescence can be explained as photoyellowing, because the irradiated spot on the paper sample was clearly discolored after prolonged exposure. Yellowing of lignocellulose is the result of photooxidation forming new chromophores.[9–11] When prebleached samples (using filter cube N2.1; λ_{ex} = 515–560 nm) were subsequently irradiated using λ_{ex} = 340–380 nm (blue emission filter cube) the lignocellulose regained its autofluorescence (Fig. 3). Preyellowed samples initially showed strong autofluorescence when analyzed with red emission filter cubes (therefore photomicrographs were taken using shorter exposure times), but the autofluorescence quickly faded during prolonged irradiation at λ_{ex} = 515–560 nm (Fig. 3). Thus, the lignocellulosic autofluorescence was showed to be, to some extent, reversible. The results from Figs. 1–3 showed that prolonged exposure to the light beam in an epifluorescence microscope affected the autofluorescence of lignocellulosic samples (i.e., any higher plant fiber material) very differently depending on the filter cubes used.[12–14] In addition, the nature and state of the sample will affect the resulting autofluorescence. In Fig. 4, hydrated thermomechanical pulp samples were subjected to the same irradiations as in Fig. 2. The relative fading of the red emission using filter cube N2.1 autofluorescence was smaller when the samples were wetted; plausibly the higher water absorption coefficient at longer wavelengths combined with the light-scattering effect of the water reduced the light intensity,[15] which resulted in reduced excitation and subsequently reduced photobleaching.

Autofluorescence and long-term imaging

In conventional immunolocalization, using widefield fluorescence microscopy or confocal microscopy, fluorescent-

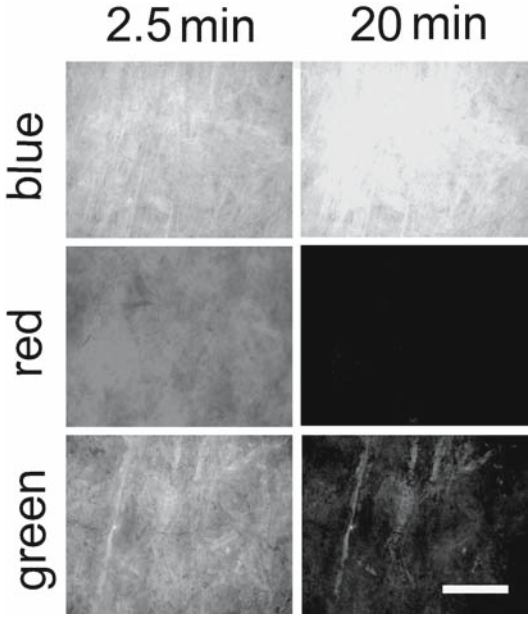

Fig. 1. Lignocellulosic paper sheets irradiated using a fluorescence microscope, with different filter cubes (indicated by emitted color). *Bar* 100 μm

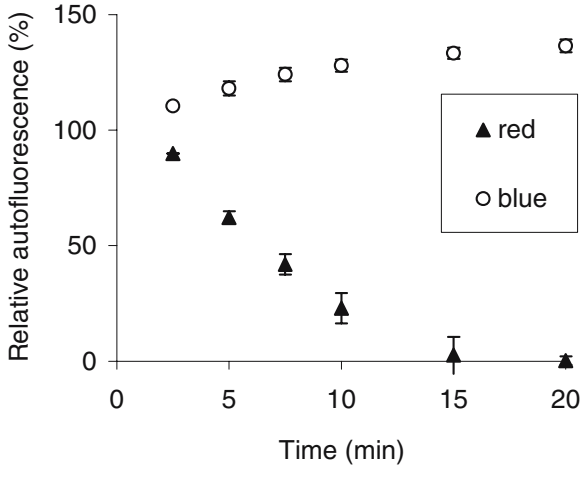

Fig. 2. Changes of relative autofluorescence over time. Normalized emission spectra intensities, time 0 min = 100% autofluorescence (blue and red refers to the emitted colors, exposure time = 98.1 ms)

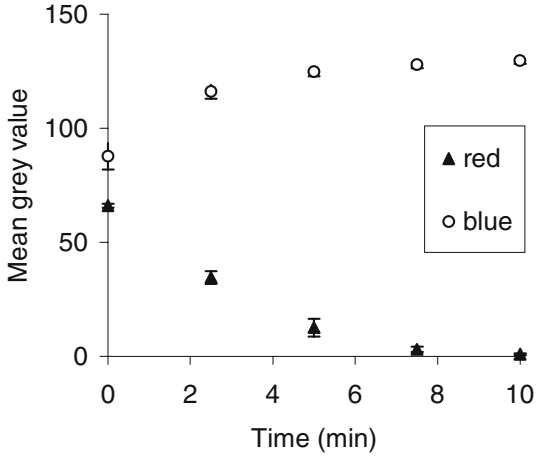

Fig. 3. Changes of mean gray value (autofluorescence) over time. The sample analyzed using the filter cube for red-light emission was first irradiated for 20 min at λ_{ex} = 340–380 nm, and the blue emission analysis was performed on λ_{ex} = 515–560 nm preirradiated samples (red-light emission filter cubes, 20 min, exposure time = 41.6 ms)

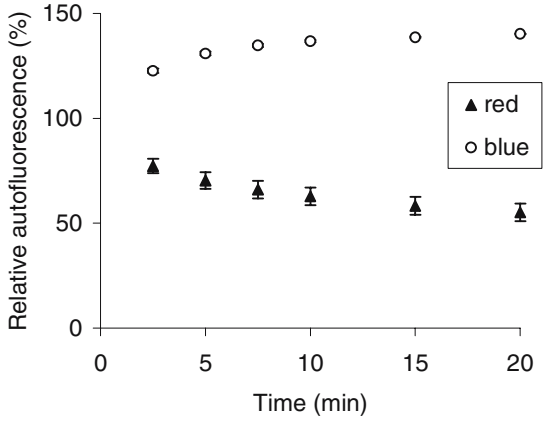

Fig. 4. Relative autofluorescence of hydrated thermomechanical pulp over time. Normalized emission spectra intensities, time 0 min = 100% autofluorescence (exposure time = 98.1 ms)

protein based probes are sensitive to photobleaching. When antifading reagents, which quench the excited state of the fluorophores and thereby retard the photooxidative degradation of the fluorescent probe,[16] are used, there is still a noticeable fading of the fluorescence. However, the emerging labeling technology based on semiconductor nanocrystals (so called quantum dots) allows for photostable labeling. To date, the use of semiconductor nanocrystals as inorganic fluorophores for labeling of proteins and biomolecules has been limited by their restricted biocompatibility, but new methods are being developed and studies have recently been conducted on various organic tissues.[7,8] Semiconductor nanocrystals have some advantages over organic fluorophores; excitation by ultraviolet (UV) or any visible wavelength give sharp and tunable emission spectra, they are highly resistant to chemical and biological degradation, and they can retain a high emission intensity over time. Thus, semiconductor nanocrystals can be used for long-term imaging of labeled structures in samples without suffering from photodegradation. However, if the sample matrix is made of lignocellulose, prolonged imaging would cause photobleaching or photoyellowing depending on the excitation wavelength used, which could significantly change the sample autofluorescence. However, the autofluorescence could also be used for live in situ monitoring of photobleaching and photoyellowing effects.

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