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Original Contribution

Detection of radicals in single droplets of oil-in-water emulsions with the lipophilic fluorescent probe BODIPY$^{665/676}$ and confocal laser scanning microscopy

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ABSTRACT

Lipid oxidation is a widespread phenomenon in foods and other systems of biological origin. Detection methods for early stages of lipid oxidation are in demand to understand the progress of oxidation in space and time. The fluorescence spectrum of the nonpolar fluorescent probe BODIPY$^{665/676}$ changes upon reacting with peroxyl radicals originating from 2,2′-azobis(2,4-dimethyl)valeronitrile and *tert*-butoxyl radicals generated from di-*tert*-butylperoxide. The excitation wavelength of the main peak of BODIPY$^{665/676}$ was 675 nm in the fluorometer, and 670 nm under the microscope, and the optimum excitation wavelength for the secondary peak of BODIPY$^{665/676}$ was 580 nm. Advantages of using BODIPY$^{665/676}$ are fewer problems with autofluorescence and the possibility of combining several fluorescent probes that are excited and emitted at lower wavelengths. However, because of the spectrum of the probe, specific lasers and detectors are needed for optimal imaging under the microscope. Furthermore, BODIPY$^{665/676}$ is resistant to photobleaching at both excitation wavelengths, 670 and 580 nm. In diffusion studies, BODIPY$^{665/676}$ is highly lipophilic, remaining in the lipid phase and not diffusing into the aqueous phase or between lipid droplets.

Lipid oxidation is a radical chain reaction having damaging effects on lipids in biological systems, which can cause structural changes and loss of biological functions [1] and lead to oxidative stress [2]. One of the major concerns, however, is that unsaturated fatty acids are especially prone to radical attacks, and the susceptibility to oxidation increases with the degree of unsaturation [3]. Therefore, the detection of radicals is of interest in various research fields dealing with oxidative damage caused by reactive oxygen species or reactive nitrogen species. So far, the focus of lipid oxidation in cells has been addressed and limited to polar membrane lipids, and methodologies to study lipid oxidation in neutral lipid domains in cells, such as adipose tissue, have been less developed. One complication of the detection of lipid oxidation is that the lifetime of radicals is in the range of nano- to microseconds [4], but also the availability of lipid peroxidation probes sets special requirements on the methods and equipment. A widely employed radical detection method has been electron spin resonance spectroscopy used either directly or indirectly via spin trapping. However, this technique is not suitable for obtaining information about localization of events on a microscopic scale. Moreover, fingerprinting, in which the end products of lipid oxidation are detected, could be used as an alternative method for detecting DNA damage or lipid peroxidation in low-density lipoproteins. Light emission generated by reactive oxygen species could also be measured directly, but because light can be emitted by numerous reactions and from several components, it can cause additional difficulties [5].

The fluorescent probe BODIPY$^{665/676}$ is a potential commercially available probe that is suitable for oxidation studies in nonpolar lipids. This probe belongs to the BODIPY (4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene) C-11 class of fluorescent probes, which have fluorescent properties in the red region of the visible spectrum [6]. BODIPY$^{665/676}$ is sensitive to peroxyl radicals owing to its conjugated polyene system [7], similar to the amphiphilic fluorescent probe BODIPY$^{581/591}$ C-11 (Fig. 1). These two fluorophores have similar chemical structure, both containing the conjugated phenylbutadiene system and both evoking a change in the fluorescence spectra upon oxidation caused by radicals [8]. The differences between the two probes lie in their phase behavior, excitation wavelength, sensitivity,

Abbreviations: MCT, medium-chain triglycerides; CLSM, confocal laser scanning microscopy; DTBP, di-*tert*-butylperoxide; AMVN, 2,2′-azobis(2,4-dimethyl) valeronitrile; BODIPY$^{665/676}$, (*E,E*)-3,5-bis-(4-phenyl-1,3-butadienyl)-4, 4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene; ROI, region of interest.

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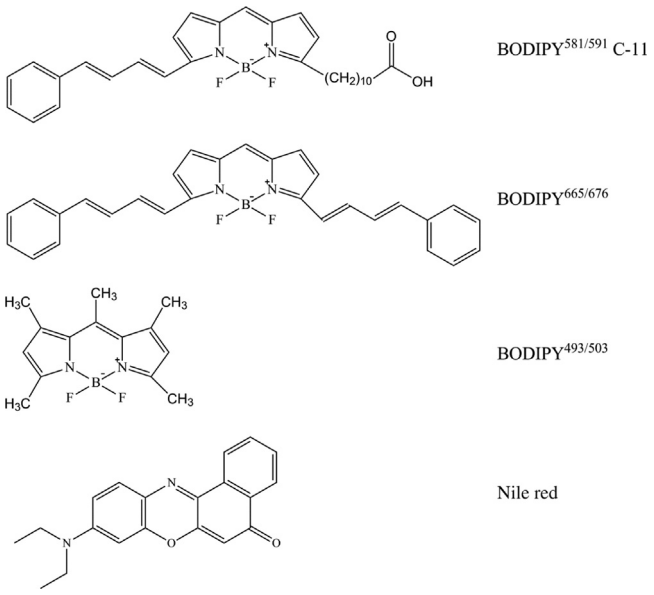

Fig. 1. Chemical structures of the four lipid-soluble fluorescent probes used in this work.

and microscopic imaging. The conjugated diene system between the BODIPY core and the phenyl ring is the target for radical attacks [9–11], being most sensitive to hydroxyl, alkoxyl, and peroxyl radicals according to the study of BODIPY$^{581/591}$ C-11 [8]. Nevertheless, it has been stated that BODIPY$^{665/676}$ is more sensitive toward peroxyl radicals than BODIPY$^{581/591}$ C-11 [7]. This might be due to the two potential active sites of BODIPY$^{665/676}$ for radical attacks. Because it was reported that BODIPY$^{581/591}$ C-11 is more sensitive to radicals than to polyunsaturated fatty acids [9], it could be hypothesized that BODIPY$^{665/676}$ could be even more sensitive to radicals than to polyunsaturated fatty acids. As far as microscopic imaging is concerned, BODIPY$^{581/591}$ C-11 localizes owing to its polarity in bilayers or micelles, whereas BODIPY$^{665/676}$ can be used in nonpolar systems, such as in the lipid phase of Tween 20- or protein-stabilized emulsions. Employment of a fluorescent probe such as BODIPY$^{665/676}$ could aid in developing emerging novel therapeutic strategies to prevent adiposity-linked metabolic dysfunctions [12].

The objective of this study was to investigate (i) how suitable BODIPY$^{665/676}$ is as a reporter probe for radicals and (ii) how the reaction with radicals can be reported quantitatively. Lipid oxidation was induced in medium-chain triglyceride (MCT) oil by controlled generation of radicals from the heat-sensitive radical initiator 2,2′-azobis(2,4-dimethyl)valeronitrile (AMVN) and the UV-light-sensitive radical initiator di-*tert*-butylperoxide ((DTBP)). MCT oil was chosen as the lipid oxidation medium because it is an oxidatively stable nonpolar liquid triglyceride without unsaturated fatty acids [13]. Consequently, the reactions between probe and radicals could be studied directly without the interference of unsaturated lipids and possible side reactions due to autoxidation of unsaturated fatty acids. The changes in the fluorescence spectra of the probe have been characterized by spectrofluorometry and confocal laser scanning microscopy (CLSM).

Materials and methods

Materials

MCT oil was obtained from Cognis GmbH (Ludwigshafen, Germany). The lipophilic probe BODIPY$^{665/676}$ (B-3932) and the nonpolar fluorescent probe 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene (BODIPY$^{493/503}$ (D-3922)) were purchased from Life Technologies (Eugene, OR, USA). Nile red, fluorescent beads (InSpeck Red(580/605) microscope image intensity calibration kit, 6 μm), and Tween 20 were purchased from Sigma–Aldrich (St, Louis, MO, USA). DTBP was purchased from Merck Schuchardt OHG (Hohenbrunn, Germany), and AMVN was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The preparation of all samples containing AMVN was done on ice.

Spectrofluorometry

Fluorescence properties of BODIPY$^{665/676}$ were determined using a PerkinElmer spectrofluorometer (PerkinElmer Instruments, LS55, luminescence spectrometer, UK). Because the probe is sensitive to radicals, and evokes a change in the fluorescence, the excitation and emission spectra were recorded from 200 to 800 nm before and after oxidizing the probe. The spectra revealed that the probe has two excitation wavelengths: the main excitation at 675 nm, having maximum emission intensity at 685 nm, and a secondary excitation at 580 nm having maximum emission intensity at 595 nm.

UV-induced radical reactions

UV irradiation was conducted with a Rayonet mini-photochemical reactor (Model RMR-500, The Southern New England Ultraviolet Co., Hamden, CT, USA). A quartz cuvette containing a sample was placed in the middle of the minireactor, and the cuvette was exposed to light from all four sides making the irradiation uniform throughout the whole sample. A control sample (MCT oil + 1 μM BODIPY$^{665/676}$) and DTBP-containing samples (MCT oil + 1 μM BODIPY$^{665/676}$ + various concentrations of DTBP added to the oil phase, varying from 5.7 to 91.2 μM) were irradiated for specific intervals of time. The decrease in the fluorescence at excitation wavelength (λ_{ex}) 675 nm and increase in the fluorescence at λ_{ex} 580 nm were measured in a 1-cm path-length quartz cuvette using a PerkinElmer spectrofluorometer (PerkinElmer Instruments, LS55, luminescence spectrometer) and calculated relative to a nonirradiated control sample. The excitation and emission spectra of the resulting measurements were smoothed using a five-point moving average.

Heat-induced radical reactions

Heating of the control sample (MCT oil + 1 μM BODIPY$^{665/676}$) and AMVN-containing samples (MCT oil + 1 μM BODIPY$^{665/676}$ + various concentrations of AMVN added to the oil phase at concentrations of 6, 13, and 22 mM) was carried out in a water bath at 37 °C. The decrease in the fluorescence at λ_{ex} 675 nm and increase at λ_{ex} 580 nm were measured in a 1-cm path-length quartz cuvette using the spectrofluorometer and calculated relative to a nonheated control sample. The excitation and emission spectra of the resulting measurements were smoothed using a five-point moving average.

Fluorescence microscopy

The lipid oxidation and photobleaching experiments were carried out by CLSM with a Leica TCS SP5-X MP-equipped microscope. The concentration of the probe BODIPY$^{665/676}$ added to the oil phase before mixing emulsion was 1 μM. The objective was 40× HCX PL APO CS, NA 1.30 oil immersion. For excitation a supercontinuum white-light laser and for detection a hybrid detector were employed. Image resolution was 1024 × 1024, and the pinhole was set to 1 AU.

The probe diffusion experiments were carried out by CLSM on a Leica TCS SP5 II microscope.