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Article

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Evidence for transfer of radicals between oil-in-water emulsion droplets as detected by the probe BODIPY$^{665/676}$.

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Abstract

BODIPY$^{665/676}$ is a lipophilic radical-sensitive fluorescent probe that can be used to study radical-driven lipid autoxidation. The sensitivity of BODIPY$^{665/676}$ was studied in the presence of radical initiators di-*tert*-butyl peroxide and 2,2'-azobis(2,4-dimethyl)valeronitrile (AMVN). In both cases the fluorescence of BODIPY$^{665/676}$ changed more in saturated MCT oil than in linseed or sunflower oils where the high degree of unsaturation is expected to give more pronounced radical derived lipid oxidation. It was suggested that BODIPY$^{665/676}$, as the only available oxidizable substance in the saturated oil, was directly attacked by radicals resulting in high rates of probe-oxidation, while in the unsaturated oils, radicals attacked either unsaturated fatty acids or BODIPY$^{665/676}$ resulting in lower rates of probe-oxidation. Confocal microscopy studies with BODIPY$^{665/676}$ as a radical-sensitive probe combined with oxygen consumption measurements of mixtures of oil-in-water emulsions showed that radicals could be transferred between oil droplets and thereby spread radical driven oxidation between neighboring droplets.

Keywords: BODIPY$^{665/676}$; radicals; confocal laser scanning microscopy; autoxidation; emulsion.

Introduction

There has for a long time been an interest in understanding the mechanisms of progression of radical-initiated lipid autoxidation in oil-in-water emulsions. The initial radicals are believed to be mainly generated by metal-catalyzed reactions in the aqueous phase of the emulsions, where radicals move towards the surface of the oil droplet, interact with the unsaturated fatty acids close to the interface, and initiate radical chain reactions, which eventually spread throughout the oil droplet.[1,2] However, despite the huge interest in preventing lipid autoxidation in food emulsions, practically no studies that examine the progression of autoxidation on microscopic scale have been carried out. For oil-in-water emulsions, incorporation of a radical-sensitive lipophilic fluorescent probe into the oil droplets would allow investigations of the presence and localization of radicals by microscopic techniques, thus providing more detailed understanding of the oxidation processes taking place inside and between the droplets. A number of fluorescent probes are available for studying oxidation-related phenomena in biological systems, but a significant part of these probes have either hydrophilic or amphiphilic properties that are suitable for studies of membranes or cells.[3,4,5] As a result, these probes are less applicable for studies of lipid oxidation taking place in microscopic domains of triglyceride phases, such as oil droplets in oil-in-water emulsions, since their amphiphilic behavior favors the probes to be located at the surfaces of the droplets rather than in the interiors. Moreover, the surface activity makes these probes more prone to diffusion between oil droplets complicating the studies of the exact localization of radical reactions on a microscopic scale. A requirement for such microscopic studies would be a probe with high lipophilicity and low water solubility in order to ensure its position within oil droplets and eliminating its diffusion between oil droplets.

The lipophilic fluorescent probe BODIPY$^{665/676}$ ((*E,E*)-3,5-bis-(4-phenyl-1,3-butadienyl)-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene) has been suggested as a peroxidation sensor. However, experiments have indicated that the probe is not monitoring lipid peroxidation directly, but is, instead, very sensitive to radical reactions in lipophilic systems.[6] Upon reacting with radicals, the fluorescence of BODIPY$^{665/676}$ changes from maximum emission intensity at ~685 nm to ~605 nm. Based on the experiments carried out with bulk saturated oil, and saturated oil emulsions, it was found that BODIPY$^{665/676}$ was a potentially useful probe for reporting lipid oxidation. Also, it was demonstrated that the lipophilic fluorescent probe BODIPY$^{665/676}$ is suitable for confocal microscopy detection of radicals in single droplets in oil-in-water emulsions made with saturated oil.[6]

In the present study, the use of BODIPY$^{665/676}$ for radical detection by fluorometric methods and confocal laser scanning microscopy (CLSM) in bulk unsaturated oil and unsaturated oil-in-water emulsions has been investigated. This has been carried out by using combinations of different emulsions, where neighboring oil droplets have different compositions, and thus allowing studies of transfer of radicals and oxidation between different oil droplets on a microscopic scale. The fluorescence-based studies have been complemented with electron spin resonance (ESR) spectroscopy for detection of radical-related reactions, and oxygen consumption for monitoring the overall extent of lipid oxidation in emulsions.

Materials and methods

Materials

Medium-chain triglyceride (MCT) oil was obtained from Cognis GmbH, Ludwigshafen, Germany and used as received. Linseed oil (LSO) and sunflower oil (SFO) were purchased from a local supermarket and purified from peroxyl radicals, trace metals, and tocopherols by alumina column chromatography according Yoshida et al. with few changes:[7] the oil was mixed with hexane 1:1, and extracted from hexane by rotational evaporator (Büchi Rotavapor R-144, Labortechnik AG, Flawil, Switzerland). The probe (*E,E*)-3,5-bis-(4-phenyl-1,3-butadienyl)-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY$^{665/676}$ (B-3932)) was purchased from Life Technologies Corporation, Oregon, USA. N-*tert*-butyl-α-phenylnitrone (PBN), methyl linoleate, methyl oleate, and Tween-20 were purchased from Sigma-Aldrich Inc., St Louis, Missouri, USA. Di-*tert*-butyl peroxide (DTBP) was purchased from Merck Schuchardt OHG, Hohenbrunn, Germany, and 2,2'-azobis(2,4-dimethyl)valeronitrile (AMVN) was purchased from Santa Cruz Biotechnology Inc., Dallas, USA.

Radical-initiated fluorometric studies

UV-irradiation-induced radical reactions

Fluorometric measurements of UV-irradiation-induced radical reactions were performed with a 1-cm path length quartz cuvette using a spectrofluorometer (Perkin Elmer Instruments, LS55 Luminescence spectrometer, Seer Green, UK). UV-irradiation was conducted with Rayonet Mini-Photochemical Reactor (Model RMR-500, The Southern New England Ultraviolet Co., Hamden, Connecticut, USA) using four 300-nm UV-lamps as described earlier[6]. Briefly, a sample in a quartz cuvette was placed in the middle of the minireactor, and UV-light was exposed from four sides making the irradiation uniform throughout the whole sample. The irradiation procedure was carried out on a control (oil with 1 μM BODIPY$^{665/676}$), and a sample (oil with 1 μM BODIPY$^{665/676}$ and 5.7 μM DTBP) for specific periods of time. Subsequently, the fluorescence spectra at excitation wavelengths (λ_{ex}) 675 nm and λ_{ex} 580 nm

were measured. Samples included MCT oil, and linseed oil (LSO). In the case of LSO, handling of the oil was carried out under a nitrogen atmosphere. The extent of oxidation was quantified as a relative decrease of the fluorescence intensity measured at λ_{ex} 675 nm, and a relative increase of the fluorescence intensity at λ_{ex} 580 nm in the samples compared to the non-irradiated controls. The measurements were conducted in triplicate.

Fluorescence studies of radical reactions in oils

Fluorometric measurements of heat-induced radical reactions were performed with a 1-cm path length quartz cuvette using a luminescence spectrometer (Aminco-Bowman Series 2, Thermo Fisher Scientific, Waltham, USA). Heating in a thermo chamber at 37 ºC was carried out on the control (oil with 1 μM BODIPY$^{665/676}$), and on the sample (oil with 1 μM BODIPY$^{665/676}$ and 13 mM AMVN) for 3 hours. Fluorescence spectra were recorded at 1-minute intervals at λ_{ex} 580 nm. Samples included MCT oil, sunflower oil (SFO), mixtures of MCT oil and SFO in various ratios, MCT oil mixed with methyl linoleate (10 wt%), and MCT oil mixed with methyl oleate (10 wt%). The samples containing unsaturated fatty acids were handled under a nitrogen atmosphere. The extent of oxidation was quantified as percentage of increase of the fluorescence at λ_{ex} 580 nm in irradiated samples compared to the non-irradiated controls. The measurements were conducted in triplicate.

Electron spin resonance (ESR) spectroscopy studies

The rate of radical formation in oils containing radical initiator AMVN (6 mM) and spin trap PBN (20 mM) were determined by ESR. The oil phase was kept on ice while AMVN was dissolved, and subsequently PBN was added. The intensity of the generated spin adducts were recorded with an ESR spectrometer (Miniscope MS200, Magnettech, Berlin, Germany). The samples in the ESR measuring cavity were heated at 37 ºC by using a temperature controller

(Temperature Controller M01, Magnettech, Berlin, Germany). The oil samples included MCT oil, sunflower oil (SFO), and blends of MCT oil and SFO in various ratios. The samples containing unsaturated fatty acids were handled under a nitrogen atmosphere. The settings in all ESR measurements were as follows: sweep width 99.63 Gauss, sweep time 30 sec, modulation 1000 mG, and microwave attenuation 10 dB. The peak-to-peak amplitudes of the center doublet peaks of the ESR spectra were used to quantify the amount of spin adducts, and all results were expressed as an initial rate of the radical formation calculated from the slopes of the initial linear parts of plots of spin adduct ESR intensities as a function of heating time. However, in the case of MCT oil substituted with 10 wt% of methyl linoleate, MCT oil, MCT oil blended with SFO in the ratio of 7:3, and in the ratio of 9:1, the plots were fitted to exponential curves from which the initial rates of spin adduct formation were calculated.. The measurements were conducted at least in duplicate.

Oil-in-water Tween-20-stabilized emulsions

Oil-in-water emulsions stabilized by Tween-20 were made according to Berton et al.[8] Emulsions consisted of 30 wt% MCT oil or sunflower oil (SFO), 0.5 wt% of Tween-20, and 69.5 wt% sodium acetate-acetic acid buffer (pH = 4.65). Homogenization was done with Ultra Turrax T25 (IKA Works GmbH & Co. KG, Staufen, Germany) at 8000 rpm and with a dispersion element of 8 mm in diameter. The fluorescent probe BODIPY$^{665/676}$ and the radical initiator AMVN were added to the oil phases prior to homogenization. In the case of added AMVN, emulsion preparation and homogenization was done on ice. When SFO was used, the whole mixing process was additionally carried out under a nitrogen atmosphere.

Confocal scanning laser microscopy (CLSM) studies of radical reactions in oil-in-water emulsions

A series of emulsions were made as described above containing oil (MCT oil or SFO), radical initiator (with AMVN or without) and a fluorescent probe (with BODIPY$^{665/676}$ and without). Mixtures of two different emulsions were made such that each combination contained AMVN (6 mM) and BODIPY$^{665/676}$ (1 μM), although not necessarily present in the same lipid droplet of the original emulsions (Table 1). The controls were single emulsions and consisted only of oil, emulsifier and the aqueous phase. Radicals were generated by unimolecular decomposition of AMVN upon heating samples in a water bath at 37 °C during specific periods of time. The measurements were conducted with a confocal Leica TCS SP5-X MP microscope. The objective was an oil immersion 40x HCX PL APO CS, NA 1.30, pinhole 1 AU, and image resolution 1024 x 1024. The samples were scanned using a supercontinuum white light laser either at λ_{ex} 580 nm with signal detection using a hybrid detector at the emission band of 585 nm – 620 nm, or at λ_{ex} 670 nm with signal detection at the emission band of 675 nm – 785 nm. Droplets loaded with BODIPY$^{665/676}$ were selected to study the changes in the fluorescence emission intensities at λ_{ex} 580 nm and λ_{ex} 670 nm, and droplets without BODIPY$^{665/676}$ were selected to establish background intensities. Droplets with BODIPY$^{665/676}$ and droplets without BODIPY$^{665/676}$ were discernible at both excitation wavelengths, λ_{ex} 580 nm and λ_{ex} 670 nm, due to emitted fluorescence of BODIPY$^{665/676}$-loaded droplets and simultaneous use of transmitted light mode. For each combination of emulsions, five droplets loaded with BODIPY$^{665/676}$, and five droplets without BODIPY$^{665/676}$ were quantified. For that purpose, a region of interest (ROI) for about 4/5 of the diameter of the droplet, excluding the border regions, was drawn, average intensity of the ROI was acquired, and changes in the fluorescence intensities were expressed relative to the unexposed controls.

Oxygen consumption measurements of emulsions

A Clark-type oxygen electrode (Radiometer, Copenhagen, Denmark) was used to determine the oxygen concentration in emulsions. The data was recorded using a Unisense picoammeter (Unisense PA2000, Aarhus, Denmark) after a method described earlier.[2] Briefly, a sample was transferred into a 2.7 ml glass vessel with a ground glass stopper, a capillary-size hole through the center, and a magnetic stirring bar, and was then placed in a water bath at 37 °C. Oxygen concentrations were recorded every 30 sec. The rates of oxygen consumption were calculated as slopes of the linear part of plots of per cent of oxygen concentration as a function of time in minutes. The samples studied included (1) MCT emulsion, (2) SFO emulsion, (3) SFO emulsion containing BODIPY$^{665/676}$, and (4) blended oil emulsions with MCT oil and SFO blended in weight ratios 3:7 or 7:3. Various concentrations of AMVN (0 mM, 1.8 mM, 4.2 mM or 6 mM) were added to oils prior to mixing the emulsions. In addition, MCT and SFO emulsions mixed in a ratio 3:7 and 7:3 were studied, where only one of the two original emulsions contained AMVN.

Statistical analysis

All data shown are presented as the mean value and standard deviation of at least three measurements. Box and whisker diagrams (Figure 5) illustrate mean values, ranges of scores between 25 per cent and 75 per cent, and outliers.

Results

BODIPY$^{665/676}$ *as radical probe in bulk triglyceride oils*

The effect of radicals on the fluorescence of BODIPY$^{665/676}$ was tested in purified unsaturated linseed oil (LSO), where lipid oxidation after initial formation of radicals can be maintained through radical chain reactions. The probe has been demonstrated to be able to detect radicals in saturated medium-chain triglyceride (MCT) oil.[6] The bulk LSO samples contained di-*tert*-

189 butyl peroxide (DTBP), which upon irradiation with 300-nm UV-light generate alkoxyl
190 radicals that can initiate lipid autoxidation. The relatively low concentration of added DTBP
191 (5.7 μM) was chosen based on previous experiments to provide an adequate radical flux,
192 ensure propagation of radical-induced processes, and reduce the effect of terminating
193 bimolecular radical reactions.[6] The UV-irradiation was carried out in 3-minute intervals
194 during the initial 18 min of irradiation, and a final measurement was carried out after a total of
195 30 min irradiation. The fluorescence of BODIPY$^{665/676}$ was measured at λ_{ex} 675 nm and 580
196 nm (Figure 1). Control experiments with saturated MCT oil showed that the fluorescence
197 intensity at λ_{ex} 580 nm increased initially linearly with irradiation time, but after the first 12
198 min of irradiation, the increase in the fluorescence intensity began to level off. For MCT oil
199 the total λ_{ex} 580 nm fluorescence intensity increased around 3000 % relative to a non-
200 irradiated control sample, whereas the λ_{ex} 675 nm fluorescence intensity decreased around 50
201 % in comparison with the unexposed control (Figure 1B). Accordingly, the change in the
202 fluorescence intensity measured at λ_{ex} 675 nm was not as pronounced as in the case of λ_{ex} 580
203 nm. The experiments with bulk LSO gave a linear increase in the fluorescence intensity of
204 BODIPY$^{665/676}$ at λ_{ex} 580 nm with irradiation time (Figure 1A). However, the maximum
205 intensity reached only 900 % relative to the unexposed control, and the variations between the
206 repetitions were smaller with LSO than with MCT oil. Also, with LSO the fluorescence
207 measured at λ_{ex} 675 nm was constant in contrast to MCT oil where it decreased during the
208 irradiation (Figure 1B).
209
210 The fluorescence changes of BODIPY$^{665/676}$ in the two oils were not in agreement with the
211 expected levels of oxidation, because highly unsaturated LSO should be oxidatively unstable
212 and be able to sustain pronounced radical-chain lipid oxidation. The response of
213 BODIPY$^{665/676}$ towards lipid oxidation was therefore tested in an alternative set of

experiments based on the temperature-dependent lipid-soluble radical initiator AMVN in blends of oxidatively stable MCT oil and purified unsaturated sunflower oil (SFO). Oil samples containing BODIPY$^{665/676}$ were heated at 37 °C, and the rate of fluorescence change was measured as the initial change of the fluorescence intensity with heating time (Figure 2). The blends of oils with high levels of SFO gave low rates of fluorescence changes in comparison with the pure MCT oil, where the rates were substantially higher. In addition, blends of MCT oil with 10 wt% methyl oleate, and MCT oil with 10 wt% of methyl linoleate were included in the experiment, and these blends gave similar high rates of changes in fluorescence intensity as the pure MCT oil.

The two sets of experiments with different unsaturated oils and different modes of radical generation both indicated that BODIPY$^{665/676}$ was most sensitive towards radicals in the pure saturated MCT oil, while the response in the unsaturated LSO and SFO was low. To further understand the underlying mechanism of radical reactions and differences in radical sensitivity of the probe, electron spin resonance (ESR) spectroscopy was used to evaluate the level of radical reactions initiated by AMVN in the oils and their blends (Figure 3). The detection of radicals was based on the formation of long-lived spin adducts with the spin trap PBN, which traps radicals generated directly from AMVN as well as radicals formed during radical-chain lipid oxidation reactions. The ESR experiments demonstrated that rates of spin adduct formation in the oil blends decreased with increasing concentration of MCT oil up to 70 wt%. However, at MCT oil concentrations above 70 wt%, rates of spin adduct formation increased to levels similar to the pure SFO. The high rate of spin adduct formation in the SFO was expected, since the high degree of unsaturation should give a high level of oxidative chain reactions, thus sustaining a high steady-state concentration of radicals.[9,10] At the same time, the high rate of spin adduct formation in MCT oil was surprising. A blend of MCT oil

with 10 wt% methyl linoleate gave the same high rate of spin adduct formation as the MCT oil blend with 10 wt% SFO.

Oxidation in emulsions

Confocal laser scanning microscopy (CLSM) together with BODIPY$^{665/676}$ was used to study the progression of oxidation in emulsion systems on the scale of single oil droplets. The fluorescent probe BODIPY$^{665/676}$ has been shown not to diffuse between oil droplets, having remained localized in the droplets where it has been initially dissolved during the preparation of the emulsion.[6] In order to study the transfer of radicals between oil droplets, a series of experiments were carried out where two Tween-20-stabilized emulsions made from different oils were mixed in a way so that the temperature-dependent radical initiator AMVN was present in some oil droplets, and the fluorescent probe BODIPY$^{665/676}$ was present in the same droplets or in the droplets without AMVN, or no AMVN was present at all (Table 1). When the respective combinations were investigated under a confocal microscope, the images showed that droplets loaded with BODIPY$^{665/676}$ were easily recognizable by their fluorescence at λ_{ex} 670 nm, while droplets without BODIPY$^{665/676}$ could be detected and located in the transmitted light mode. In the case when no radicals were released (samples not heated), no fluorescence was seen at λ_{ex} 580 nm. Therefore, no differentiation based on fluorescence between the two emulsions could be made, and the droplets were visible and could be defined only in transmitted light. Upon heating the AMVN-containing mixtures of emulsions, an increase in the fluorescence at λ_{ex} 580 nm could be noticed in the BODIPY$^{665/676}$-loaded oil droplets indicating the presence of radicals. During the heating of the samples and consequent constant generation of radicals for up to 60 min, it was seen that the fluorescence intensities were rising independent of the location of the radical initiator AMVN and the probe BODIPY$^{665/676}$ (Figure 4). Extended heating for more than 60 min

resulted in a drop in the fluorescence intensity of the mixtures of emulsions, where SFO droplets contained the probe, while in the combination where MCT oil droplets was loaded with BODIPY$^{665/676}$ a further increase was measured up to 120 min. Regardless of the oil medium of the droplets carrying the probe, the oil droplets without the probe showed no increase in fluorescence during the experiments (Figure 4).

The combination of SFO emulsion containing AMVN and BODIPY$^{665/676}$ mixed with MCT oil emulsion had a high level of fluorescence (~100 fluorescence units (FU)) already before heating. This resulted in a stable initial fluorescence, which did not change during the heating (data not shown). No change in the fluorescence intensity was seen in the two emulsion combinations, which did not contain AMVN, serving as internal controls. Similarly, MCT oil emulsion with BODIPY$^{665/676}$, and control experiments of emulsions without BODIPY$^{665/676}$ did not show any changes in the fluorescence intensities upon heating (data not shown). The presence of radicals also influenced the fluorescence intensities at λ_{ex} 670 nm. At this wavelength, a weaker contrast between the BODIPY$^{665/676}$-loaded droplets and their counterparts was seen. The fluorescence decreased in oil droplets observed at λ_{ex} 670 nm correlated with the increases of fluorescence observed at λ_{ex} 580 nm for all the studied emulsions (data not shown).

The effect of the location of the radical initiator AMVN in the mixtures of emulsions on lipid oxidation was also studied on a macroscopic scale by measuring rates of oxygen consumption (Figure 5). The rates of oxygen consumption in MCT oil emulsions both with and without added AMVN were very low (Figure 5A). Mixing MCT emulsion containing AMVN with SFO emulsion gave rates of oxygen consumption, which were even higher than the rates of oxygen consumption in a pure SFO emulsion without AMVN (Figures 5A and 5B). The level

of oxidation in these combinations increased with the amount of SFO from 30 wt% to 70 wt%, although the total concentration of AMVN decreased, from 4.2 mM to 1.8 mM, at the same time. These results demonstrate that oxidation was accelerated in the mixed emulsions.

SFO emulsions with different concentrations of AMVN gave rates of oxygen consumption very similar when 6 mM or 4.2 mM AMVN was used, whereas in the case of 1.8 mM AMVN the rate of oxygen consumption was significantly lower (Figure 5B). Moreover, the presence of $BODIPY^{665/676}$ with AMVN in the SFO emulsion slightly increased the oxidation rate indicating that $BODIPY^{665/676}$ did not inhibit the progression of lipid oxidation as it has been reported earlier.[11,12]

Experiments were also carried out where the total concentration AMVN in the mixed emulsions was kept constant at either 4.2 mM AMVN or 1.8 mM AMVN, but where the localization of AMVN within the oil droplets, either in MCT oil or SFO, was different (Figure 5C). The rates of oxygen consumption in the samples, which contained a combination of two SFO emulsions, were similar when AMVN was located with a higher local concentration in either 70 wt% or 30 wt% of the droplets as compared to being present in all droplets (Figure 5C). The mixture of SFO emulsion containing AMVN with MCT emulsion gave a lower rate of oxygen consumption in the case of a total concentration of 4.2 mM AMVN. However, the combinations, where MCT oil emulsion and SFO emulsion with added AMVN were mixed in a ratio 7:3 (corresponding to a total concentration of 1.8 mM AMVN), seemed to have a critical combination of components, and despite several repeated measurements, a rather large variation of the oxygen consumption rates was observed.

Oxygen consumption was also measured in emulsions made with blended MCT and SFO oils (Figure 6). Emulsions based on blended oils with 70 wt% MCT oil gave very low rates of oxygen consumption independent of the concentration of AMVN, whereas the emulsions

made with 30 wt% MCT oil gave high rates of oxygen consumption, and the emulsion with 4.2 mM AMVN gave the highest rate. These results correspond to the evaluation of radical formation in bulk oil blends by ESR, where higher level of radicals were observed in the 30 wt% MCT oil blend rather than in the 70 wt% MCT oil blend (Figure 3).

Discussion

The lipophilic fluorescent probe BODIPY$^{665/676}$ has previously been shown to be suitable for oxidation studies in single droplets of oil-in water emulsions made with saturated MCT oil, since it does not diffuse between droplets, and its fluorescence changes in the presence of radicals.[6] In the present work, it was found that the fluorescence of BODIPY$^{665/676}$ changed more in the oxidatively stable saturated MCT oil than in the oxidation-sensitive unsaturated linseed oil (LSO) and sunflower oil (SFO). The fluorescence changes were low in oil blends with high levels of SFO, and only at high levels of MCT, the rate of fluorescence intensity change increased. These experiments were based on the radical initiator AMVN, which produces radicals at a constant rate. The amounts of radicals generated in the different oil blends can therefore be assumed to be similar. The experiments with BODIPY$^{665/676}$ in the various blends of MCT oil and SFO taken together with the concentration of radicals evaluated by ESR suggest that BODIPY$^{665/676}$ was most sensitive to the initial radicals generated from AMVN and less sensitive to bulky lipid autoxidation-derived radicals. It has been reported that probes belonging to the C11-class, including BODIPY$^{665/676}$ and BODIPY$^{581/591}$ C-11, are overestimating the oxidation of lipids due to their own high susceptibility to oxidation.[11,13] Moreover, Yoshida et al.[12] reported that the rates of reactions between unsaturated fatty acids and peroxyl radicals were half of the reaction rates between BODIPY$^{581/591}$ C-11 and peroxyl radicals, which suggest that BODIPY$^{665/676}$ will react with radicals even more readily since it is more sensitive towards peroxyl radicals than

BODIPY$^{581/591}$ C-11.[14] In the experiments based on the radical initiator DTBP, radicals in MCT oil interacted directly with the probe, mainly because the probe was the only available substance prone to oxidation. In LSO, on the other hand, probably due to the competition between BODIPY$^{665/676}$ and the unsaturated lipids, fewer radicals attacked the radical-sensitive fluorescent probe. The fluorometric experiments with fatty acid methyl esters in MCT oil gave higher rates of fluorescence intensity changes than in the LSO triglycerides indicating that the reaction between the lipid-derived radicals and BODIPY$^{665/676}$ was affected by steric effects, and thus mitigated the response to the oxidation reported by the probe.

The spin trap PBN also gave varying levels of detectable spin adducts in the different oil blends. The decrease of spin adduct formation when increasing the level of MCT oil up to 50 wt% in the oil blends could be due to the dilution of the unsaturated SFO, which is in agreement with the notion that the level of oxidation decreases with the decreasing level of unsaturation. In these oil blends, trapping of lipid oxidation-derived radicals is dominating.[15] Under the present experimental conditions at 37 °C, the PBN spin adducts with peroxyl radicals are very unstable, thus PBN formed detectable stable adducts probably with alkoxyl and alkyl radicals.[16,17,18,19] The high levels of spin adduct formation at the high concentrations of MCT in the oil blends were surprising, and suggest PBN was more efficiently reacting with the initial radicals generated from AMVN when the extent of competing reactions between the AMVN-derived radicals and unsaturated fatty acids were low due to low levels of unsaturation.

The experiments with oil blends illustrate that the fluorescence intensity changes of BODIPY$^{665/676}$ can be used for detecting reactive radicals in triglyceride systems, however, the structure of the radicals can significantly affect the level of intensity changes – bulky triglyceride-derived radicals apparently give smaller changes due to steric effects. The degree of unsaturation should therefore always be taken into consideration when comparing the

spectral changes of BODIPY$^{665/676}$ between different triglyceride blends. In addition, a low level of intensity changes in highly unsaturated oil cannot be directly interpreted as indicative of a low level of lipid oxidation.

In the confocal microscopy studies of two mixed Tween-20-stabilized emulsions with different compositions of the oil phases, the probe BODIPY$^{665/676}$ gave a clear indication of transfer of radicals between oil droplets. Having BODIPY$^{665/676}$ and the radical initiator AMVN localized within the same droplets lead, as expected, to changes of fluorescence spectra measured at λ_{ex} 580 nm and λ_{ex} 670 nm. However, more interestingly, when BODIPY$^{665/676}$ and AMVN were located in separate droplets, the fluorescence intensity of the probe also changed showing that radicals could be detected in droplets neighboring the AMVN-loaded droplets. The concentration of Tween-20 was higher than the critical micelle concentration, and the emulsifier micelles could potentially solubilize smaller compounds, including AMVN and AMVN-derived radicals, providing transporter mechanism for radical reactions. [20,21] The escape of AMVN-derived radicals from lipid containing particles has been observed in the case of LDL particles where it was found that they could initiate lipid oxidation in neighboring particles.[22,23] Krainev and Bigelow found that negligible amounts of AMVN-derived radicals were able to leave the lipophilic environment of rabbit skeletal sarcoplasmic reticulum membranes and egg phosphatidylcholine liposomes.[24]

The hypothesis that radicals are able to move between the oil droplets, either by transporter mechanisms or diffusion, was supported by determining the rates of oxygen consumption in the mixtures of emulsions. The oxygen consumption was expected to reflect the overall extent of lipid oxidation, and accordingly, the rates of oxygen consumption were very low in MCT emulsions, even in the presence of AMVN, whereas the rates were very high in the SFO

emulsions. Experiments with combinations of emulsions showed that AMVN located in MCT oil droplets led to increased oxidation when mixed with SFO droplets indicative of the transfer of radicals between droplets. A mixture of SFO emulsion containing 6 mM AMVN and MCT oil emulsion in the ratio of 7:3 decreased the rate of oxygen consumption compared to SFO emulsion with added 4.2 mM AMVN. Furthermore, a combination of SFO emulsion containing 6 mM AMVN and SFO emulsion in the ratio of 7:3 gave comparable rates of oxygen consumption with SFO emulsion with added 4.2 mM AMVN. Since the total concentration of AMVN in all these mixed systems was the same, and identical amounts of radicals were generated, the presence of MCT oil must have had an effect on the level of oxidation as has previously been observed with mixed mayonnaises.[2] This suggests the transfer of radicals from radical-loaded SFO droplets to saturated MCT oil droplets may inhibit the efficiency of radical transfer to other SFO droplets, and therefore lead to reduction of the overall rate of oxidation in the mixture of emulsions since MCT oil droplets are not expected to be able to sustain the transfer of radicals.

In conclusion, BODIPY$^{665/676}$ is a useful lipophilic fluorescent probe for detecting and studying radical reactions in bulk oils and oil-in-water emulsions. However, the structure of radicals can significantly affect the level of fluorescence intensity changes, and a low level of BODIPY$^{665/676}$ intensity change cannot be directly interpreted as indicative of a low level of lipid oxidation. Studies of mixed emulsions with confocal laser scanning microscopy by using BODIPY$^{665/676}$ as a fluorescent probe supplemented with oxygen consumption studies demonstrated that radicals can be transferred between oil droplets, and lipid autoxidation can spread between neighboring oil droplets.

The possibility of radical transfer between oil droplets will have to be taken into account when discussing the mechanisms of lipid autoxidation in oil-in-water emulsions. Radical driven lipid autoxidation are not taking place as isolated events in individual droplets, but can spread between emulsion droplets. Although, it is expected that radicals mainly are generated by metal-catalyzed mechanisms in the aqueous phase, then the present results suggest that radicals generated in the interior of the oil droplets can contribute to the initiation of autoxidation in neighboring droplets. Such transfer of radicals between droplets could be the main mechanism of initiating and spreading lipid autoxidation at higher temperatures, where generation of radicals by cleavage of pre-formed lipid hydroperoxides inside oil droplets may begin to play a significant role. The design of emulsion protection schemes based on surface-active antioxidants would accordingly have to consider that radicals may be able to both enter and exit droplets.

Abbreviations

2,2'-azobis(2,4-dimethyl)valeronitrile (AMVN); (*E,E*)-3,5-bis-(4-phenyl-1,3-butadienyl)-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY$^{665/676}$); confocal laser scanning microscopy (CLSM); di-*tert*-butyl peroxide (DTBP); electron spin resonance (ESR); fluorescence units (FU); linseed oil (LSO); medium-chain triglyceride (MCT); N-*tert*-butyl-α-phenylnitrone (PBN); sunflower oil (SFO); excitation wavelength (λ_{ex}).

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438

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511 **Figure 1**. Fluorescence intensity changes of BODIPY$^{665/676}$ in MCT oil (□ and ■) and
512 linseed oil (LSO) (○ and ●). The oils contained DTBP (5.7 μM) and BODIPY$^{665/676}$ (1 μM),
513 and were irradiated with 300-nm UV-lamps. The fluorescence intensity was measured (A) at
514 λ_{ex} 580 nm (closed symbols), and (B) at λ_{ex} 670 nm (open symbols). The fluorescence
515 changes are reported relative to the non-irradiated control.

516 **Figure 2.** Rate of fluorescence change of BODIPY$^{665/676}$ in the blends of MCT oil and
517 sunflower oil (SFO) measured at λ_{ex} 580 nm. The samples of oil blends contained AMVN (13
518 mM) and were subjected to heating at 37 °C for 3 hours. The rate of fluorescence intensity
519 change is presented as a function of wt% of MCT oil in the oil blend. (□) Various blends of
520 MCT oil and SFO, (●) a blend of 90 wt% MCT oil and 10 wt% methyl linoleate, (▲) a blend
521 of 90 wt% MCT and 10 wt% methyl oleate.

522 **Figure 3.** Rate of spin adduct formation in blends of MCT oil and sunflower oil (SFO). The
523 samples of oil blends contained the spin trap PBN (20 mM), radical initiator AMVN (6 mM),
524 and were subjected to heating at 37 °C. The rate of spin adduct formation is calculated as an
525 increase of the spin adduct signal detected by ESR as a function of heating time, and reported
526 as a function of wt% of MCT oil in the oil blend. (□) Blends of MCT oil and SFO in various
527 concentrations. (▲) A blend of 90 wt% MCT oil and 10 wt% methyl linoleate.

528 **Figure 4.** Changes in the fluorescence intensity at λ_{ex} 580 nm of single oil droplets in
529 mixtures of emulsions observed with CLSM. The following systems were studied: (○ and ●)
530 MCT oil emulsion containing AMVN (6 mM) and BODIPY$^{665/676}$ (1 μM) mixed with SFO
531 emulsion; (□ and ■) MCT oil emulsion containing BODIPY$^{665/676}$ (1 μM) mixed with SFO
532 emulsion containing AMVN (6 mM); and (△ and ▲) SFO emulsion containing
533 BODIPY$^{665/676}$ (1 μM) mixed with MCT emulsion containing AMVN (6 mM). The
534 combinations were heated at 37 °C. Open symbols depict the fluorescence intensities of

droplets in the combinations of emulsions containing BODIPY$^{665/676}$, and closed symbols show the fluorescence intensities of droplets where BODIPY$^{665/676}$ was absent. The fluorescence intensities are reported relative to unexposed control samples.

Figure 5. Rate of oxygen consumption in Tween-20-stabilized emulsions and mixtures of emulsions measured at 37 °C, and presented as % of oxygen consumption per minute. (A) Experiments with MCT oil emulsion containing AMVN (6 mM), and MCT oil emulsion mixed with SFO emulsion. MCT emulsion without AMVN was included as a control. (B) Experiments with SFO emulsions with different concentrations of AMVN, and SFO emulsion with AMVN (1.8. mM) and BODIPY$^{665/676}$ (1 μM). (C) Experiments with SFO emulsions containing AMVN, and combinations of emulsions with MCT oil emulsion and SFO emulsions. An asterisk indicates the original emulsion that contained 6 mM AMVN.

Figure 6. Rate of oxygen consumption in Tween-20-stabilized emulsions made with blended oils and with various concentrations of added AMVN measured at 37 °C, and presented as % of oxygen consumption per minute. Filled columns: blends of 30 wt% MCT oil and 70 wt% SFO. Empty columns: blends of 70 wt% MCT and 30 wt% SFO.

Table 1. Combinations of emulsions (1:1) investigated by CLSM studies.

	Emulsion II			
Emulsion I	SFO + AMVN + BODIPY$^{665/676}$	SFO + AMVN	SFO + BODIPY$^{665/676}$	SFO
MCT oil + AMVN + BODIPY$^{665/676}$				X
MCT oil + AMVN			X	
MCT oil + BODIPY$^{665/676}$		X		X
MCT oil	X		X	

*MCT (medium-chain triglyceride); SFO (sunflower oil); BODIPY$^{665/676}$ ((*E,E*)-3,5-bis-(4-phenyl-1,3-butadienyl)-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene); AMVN (2,2'-azobis(2,4-dimethyl)valeronitrile)

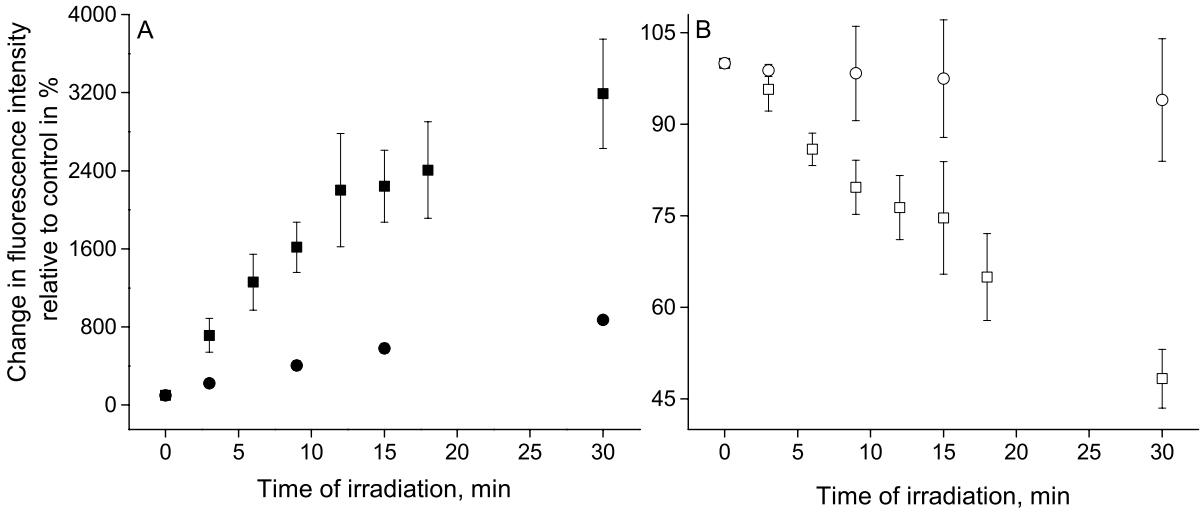
A
B
Change in fluorescence intensity relative to control in %
Time of irradiation, min
Time of irradiation, min
4000
3200
2400
1600
800
0
105
90
75
60
45
0
5
10
15
20
25
30

Figure 1

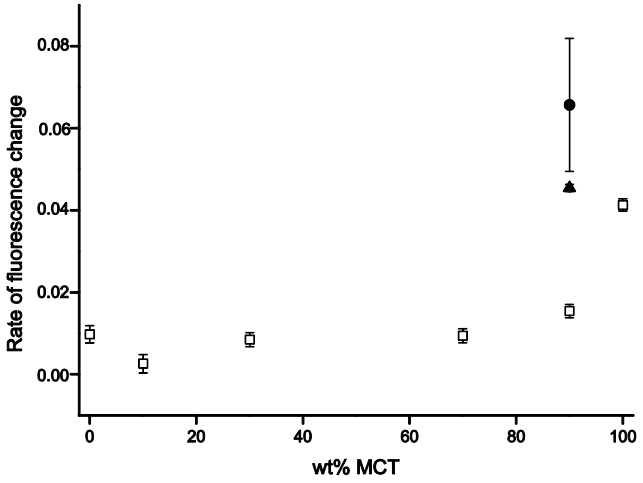
Rate of fluorescence change
0.08
0.06
0.04
0.02
0.00
0
20
40
60
80
100
wt% MCT

Figure 2

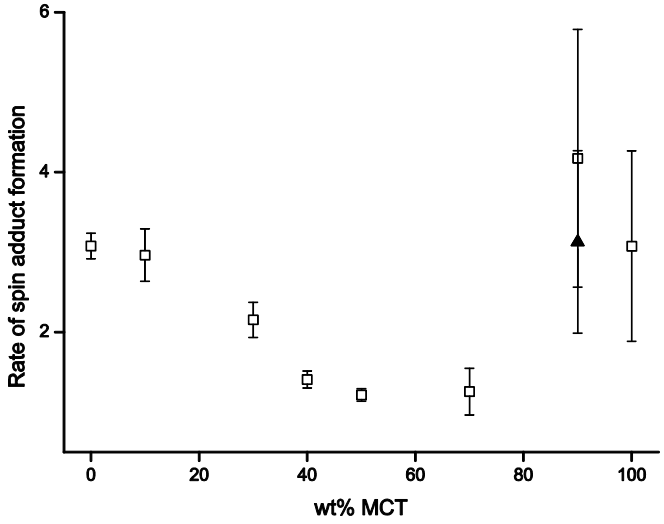

Figure 3

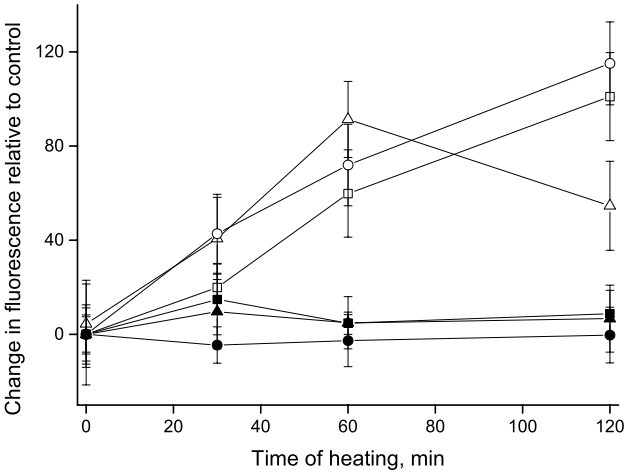
Change in fluorescence relative to control
120
80
40
0
0
20
40
60
80
100
120
Time of heating, min

Figure 4

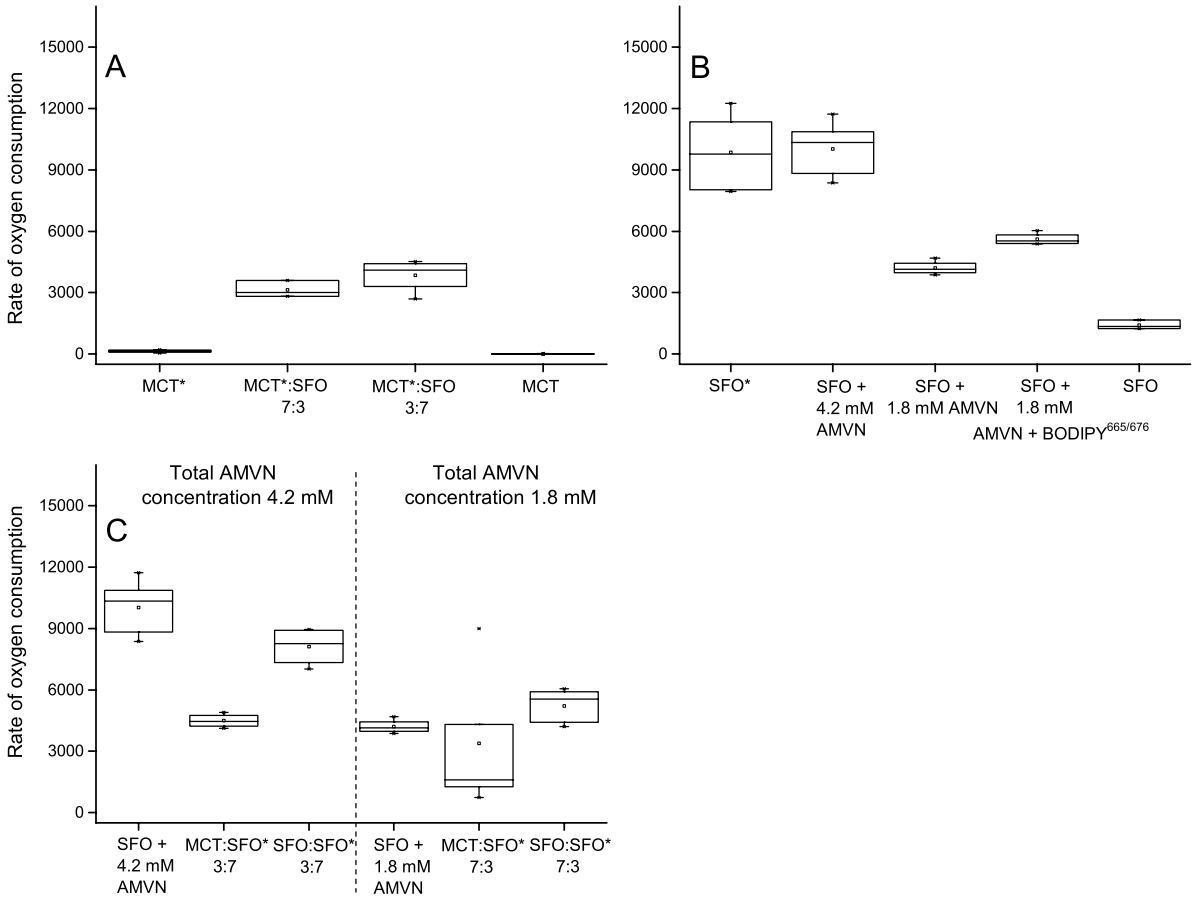
A
Rate of oxygen consumption
15000
12000
9000
6000
3000
0
MCT*
MCT*:SFO 7:3
MCT*:SFO 3:7
MCT
B
SFO*
SFO + 4.2 mM AMVN
SFO + 1.8 mM AMVN
SFO + 1.8 mM AMVN + BODIPY665/676
SFO
C
Total AMVN concentration 4.2 mM
Total AMVN concentration 1.8 mM
SFO + 4.2 mM AMVN
MCT:SFO* 3:7
SFO:SFO* 3:7
SFO + 1.8 mM AMVN
MCT:SFO* 7:3
SFO:SFO* 7:3

Figure 5

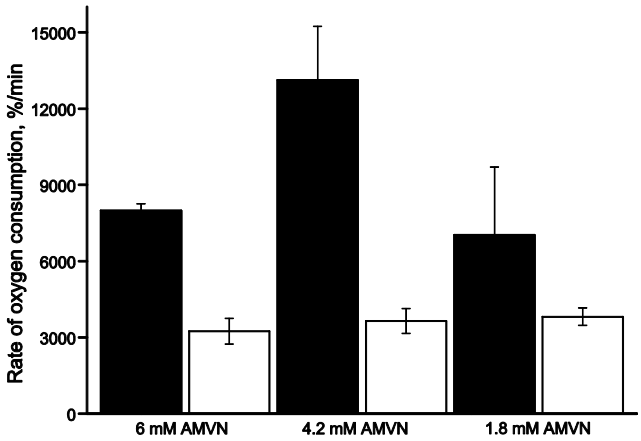
Rate of oxygen consumption, %/min
15000
12000
9000
6000
3000
0
6 mM AMVN
4.2 mM AMVN
1.8 mM AMVN

Figure 6

Graphic for table of contents