# Fluorometric Assay for Detection of Sterol Oxidation in Liposomal Membranes

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# Fluorometric Assay for Detection of Sterol Oxidation in Liposomal Membranes

**Parkson Lee-Gau Chong and Michelle Olsher**

**Summary**

The authors have developed a fluorescence assay to measure the rate and extent of sterol oxidation in lipid bilayers. Dehydroergosterol (DHE), a fluorescent cholesterol analog, is used as a probe and at the same time as a membrane component. The assay can also be performed on bilayers containing a mixture of sterols including DHE and nonfluorescent sterols, such as cholesterol and ergosterol. The fluorescence intensity of DHE decreases on oxidation, so the rate and extent of free radical- or enzyme-induced sterol oxidation can be measured as a function of temperature and membrane composition. For the studies, two-component (e.g., phosphatidylcholine (PC)/DHE) and multicomponent (e.g., DHE/PC/bovine-brain sphingomyelin) large unilamellar vesicles were used, and sterol oxidation was initiated either by the peroxy radical generator 2,2′-azobis (2-amidinopropane) dihydrochloride or by the enzyme cholesterol oxidase. The data gathered from this assay may be used to examine the effects of water- and lipid-soluble antioxidants on membrane sterol oxidation produced by free radicals. This assay can be used to test the potency of antioxidants and pro-oxidants, and can be used to determine whether unknown substances demonstrate antioxidant activity against sterol oxidation. The assay can also be used as a tool to examine the effect of sterol lateral organization on sterol oxidation (in the presence or absence of antioxidants). In agreement with the sterol regular distribution model, it is found that both free radical- and enzyme-induced sterol oxidation vary with membrane sterol content in a well defined alternating manner.

**Key Words:** Antioxidants; cholesterol; dehydroergosterol; fluorescence; free radicals; membrane; sterol oxidation.

## 1. Introduction

The authors have developed a novel fluorescence assay that allows them to measure the rate and extent of sterol oxidation in both the presence and absence of antioxidant. *In vivo* oxidation of membrane cholesterol has important biomedical implications; the oxysterol products produced have been implicated in a broad list of pathophysiological changes in the human body, and are believed to be a major factor associated with many disease states. Cholesterol can be oxidized in the body by enzymes and/or reactive oxygen species. A proportion of oxysterol production in the body allows for removal of excess cholesterol as a component of bile acids. However, when the oxidative load becomes too great as a result of overproduction of reactive oxygen species and/or depletion of antioxidant protective mechanisms, oxysterol production can induce deleterious changes. For example, the accumulation of oxidized low-density lipoprotein (LDL) in the subendothelial space is correlated with many pathological changes in vascular cells and thought to lead to the formation of the atherosclerotic plaques that are seen in coronary heart disease ***(1)***. Recent evidence points to the oxysterol contained within the oxidized-LDL particle as the cytotoxic agent ***(2)***. Oxidation products of cholesterol are highly toxic to arterial smooth muscle cells ***(3)*** and vascular cells ***(2)***

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as well as many other cell types ***(4)***. Oxysterol production is associated with the etiology of many diseases, such as Alzheimer's Disease, diabetes, and cancer ***(3,5,6)***. Elucidation of the mechanisms involved in the regulation of cholesterol oxidation would be useful in the development of therapeutic options for the prevention and treatment of many human diseases and conditions.

The conventional way to analyze the products of cholesterol oxidation requires: (1) the extraction of total lipids using organic solvents, (2) the enrichment, separation, and detection of oxycholesterols by thin-layer chromatography and high-performance liquid chromatography (HPLC), and (3) the confirmation of structural identity by mass and nuclear magnetic resonance spectrometry ***(7)***. Although this approach is necessary for understanding the chemistry underlying the oxidation reaction, it also perturbs membrane organization and is too cumbersome for kinetic studies. Ultraviolet (UV) light has been used to detect particular cholesterol oxidation products in lipid vesicles, but its use is rather limited. This is because oxycholesterols do not absorb UV light with a reasonably high extinction coefficient, except for cholest-4-en-3-one ***(8)*** and 7-ketocholesterol ***(9)***. The UV method is even less useful in intact cell membranes because of the presence of many other membrane constituents, which also absorb UV light.

The fluorescence assay described here is sensitive and can be used to measure the rate and extent of oxidation of sterol contained within a membrane without much membrane organization disruption. In this chapter, how to use a peroxy radical generator such as 2,2′-azobis [2-amidinopropane] dihydrochloride (AAPH) to induce sterol oxidation has been illustrated, but rates and extent of sterol oxidation induced by other pro-oxidants, such as 2,2′-azobis (2,4-dimethylvaleronitrile), a lipophilic peroxy radical generator, and by enzymes, such as cholesterol oxidase ***(10)*** can also be monitored.

It is also possible to get a relative measure of the degree of antioxidant protection afforded against sterol oxidation by looking at the length of the antioxidant-induced lag phase. The lag phase is a period of slow oxidation during which the antioxidant is scavenging some proportion of the free radicals being generated (*see* **Fig. 3**). Once the antioxidant has been depleted, the rate of sterol oxidation increases. By comparing the length of the lag phase along with the rate of sterol oxidation after the lag phase, it is possible to rank lipid- and water-soluble antioxidants according to their effects on sterol oxidation. When this assay is used to examine sterol oxidation by free radicals or by enzymes, either with or without the addition of antioxidant, only one sample preparation is required. The lipid system must include the fluorescent sterol, dehydroergosterol (DHE); however, other sterols (such as cholesterol or ergosterol) may be mixed with DHE (*see* **Note 1**).

The liposomes can be as simple as two-component vesicles, for example, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)/DHE, or as complex as multicomponent vesicles, LDL particles, or cells with incorporated DHE. These samples can be used to test the potency of antioxidants and pro-oxidants, and can be used to determine whether unknown substances demonstrate antioxidant activity. In addition to the aforementioned possibilities, the assay can be used as a tool to probe the relationship between membrane sterol lateral organization and sterol oxidation. The fluorescence assay is most suited for this application. For this specialized use of the assay, a more rigorous sample preparation is required.

The relationship between membrane lateral organization and sterol oxidation can be described by the sterol superlattice theory. This theory (***11,12***; reviewed in **refs. *13*** and ***14***) states that at certain mathematically predicted mole fractions of sterol, the sterol molecules

are maximally organized into a type of regular distribution called a sterol superlattice. It is hypothesized that differences in sterol lateral organization can lead to large variations in membrane packing between components at a critical mole fraction compared with those at a noncritical mole fraction. Many membrane-associated properties, such as the activity of some surface-acting enzymes and the partitioning of nystatin into the bilayer, show multiple biphasic changes as a function of sterol mole percent ***(10,15,16)***. The fluorescence assay can be used to examine the rate of sterol oxidation, which is another property showing multiple biphasic changes as a function of sterol mole fraction ***(10,17)***. The rate of sterol oxidation should be proportional to sterol availability, and sterol is expected to be more accessible to the aqueous phase (containing water-soluble pro-oxidants or enzymes) at critical mole fractions (reviewed in **ref. *13***). The authors observed an increase in the rate of sterol oxidation at critical predicted mole fractions, and a decrease in the oxidation rate as mole percent deviated from critical ***(10,17)***. When the assay was performed in the presence of antioxidants, a decrease in the length of the lag phase at or near predicted critical mole fraction as compared with the length of the lag phase at noncritical mole fractions was observed (Olsher and Chong, unpublished results). Thus, this application of the assay not only provides supporting evidence for the sterol superlattice theory, but also reveals a new type of regulation for sterol oxidation, that is, sterol oxidation varies with the extent of sterol superlattice in the membrane, and changes biphasically with sterol content at critical mole fractions.

The most important procedure involved in this application of the assay is sample preparation. A typical sample set contains between nine and fifteen independently prepared samples, each differing from the next by a very small increment (~0.3 mol%) in mole percent of sterol. The absolute amount of sterol remains constant from sample to sample; the mole fraction of sterol is varied by changing the amounts of the nonsterol lipid components in small increments from sample to sample. Thus, the end result is a series of samples in which the mole percent of sterol changes by small steps over an entire range of less than 3–4 mol%. Because such small differences in sterol mole fraction are used, all aspects of sample preparation must be carried out with extreme precision. Determination of stock solution concentrations, analysis of phosphorus concentration after extrusion and pipetting of lipids (or liposomes) must all be done accurately. The thermal history of each sample must be the same. Careful sample preparation insures that the differences observed between samples can only be attributed to differences in sterol mole percent (hence, differences in the lateral organization of sterol in the bilayer).

The assay protocol involves time trace monitoring of DHE fluorescence intensity after addition of a free radical generator (e.g., AAPH) or an oxidizing enzyme at desired temperature (37°C, in the study) (*see* **ref. *17***). The initial rate of sterol oxidation is then calculated from the decay of DHE fluorescence intensity. The assay may be performed in the presence or absence of antioxidants.

## 2. Materials

### *2.1. DHE Concentration Determination*

1. DHE (Sigma, St. Louis, MO) is purified by HPLC before being dissolved in $CHCl_3$ to make a stock solution (*see* **Note 2**). Stock solution is sensitive to light, heat, and oxygen, and must be sealed with Teflon tape and parafilm and stored in darkness at −20°C.
2. 1,4-Dioxane (Burdick & Jackson, Morris Township, NJ).
3. Small glass culture tube with phenolic screw cap that has a chloroform-resistant Teflon interface (Pyrex 9826) (Corning, distributed by Fisher Scientific, Suwanee, GA).

4. Spectrophotometer and two-matched quartz cuvettes (also used in phospholipid and cholesterol determinations).
5. Microman pipettes (Gilson, Middleton, WI).

### 2.2. Phospholipid Concentration Determination

1. Phospholipids: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and DMPC (Avanti Polar Lipids, Alabaster, AL) are prepared by dissolving lipids in $CHCl_3$ to produce a stock solution of approx 1 m*M*. Phospholipid stock solution is sealed with Teflon tape and parafilm and stored at –20°C.
2. Phosphorus standard solution (0.65 m*M*) (Sigma P3, 869).
3. 13 × 100 mm Disposable culture tubes.
4. 50% $H_2SO_4$, 30%$H_2O_2$, 5% ammonium molybdate, sodium bisulfite.
5. Fiske-Subbarow reagent (FSR) (Sigma).
6. Heating plate and heating blocks (20 wells).

### 2.3. Sample Preparation

1. DHE/$CHCl_3$ stock solution and phosphatidylcholine/$CHCl_3$ stock solution.
2. Storage tubes: Pyrex round bottom culture glass tubes that can be closed with a phenolic screw cap having a Teflon fluorocarbon-resin-faced rubber liner that is resistant to chloroform and many other organic solvents (*see* **Note 3**).
3. 50 m*M* Tris-HCl buffer, pH 7.0, 0.02% $NaN_3$, 0.1 m*M* ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, MO) (*see* **Note 4**), filtered through a syringe filter (0.2 µm).
4. Nitrogen (compressed gas) and Reacti-Vap III, Reacti-Therm III manifold (Rockford, IL).
5. Flexi-dry freeze-dryer (FTS Systems, Stone Ridge, NY).
6. Extruder (Lipex Biomembranes, Vancouver, Canada) and Nucleopore polycarbonate membranes (Whatman Int., Kent, UK).
7. Zetasizer HS-100 spectrometer (Malvern, Worce, UK).

### 2.4. Assay Protocol

1. Phosphatidylcholine/DHE large unilamellar vesicles (LUVs) are pipetted into reaction cuvette and mixed with calculated volume of Tris-HCl buffer.
2. AAPH (Aldrich, Milwaukee, WI) stock is prepared by mixing with Millipore (Billerica, MA) water. AAPH is stored at 4°C at all times; only remove from this temperature when it needs to be added to the reaction cuvette (*see* **Note 5**).
3. For assays including antioxidants, ascorbic acid is dissolved in (Millipore) water. Ascorbyl palmitate is dissolved in 100% ethanol.
4. Fluorometer (SLM 8000C) (SLM, Urbana, IL).

## 3. Methods

### 3.1. DHE Concentration Determination

1. Before pipetting DHE stock solution for concentration determination, DHE must be brought to room temperature and mixed gently to assure even distribution of molecules.
2. Pipette 2 mL of 1,4-dioxane (Burdick & Jackson) into small glass culture tube with chloroform-resistant screw cap.
3. Pipette 20 µL DHE stock solution into tube containing dioxane; mix well (overnight with rotation; cover tube with foil).
4. Wash out two matched quartz cuvettes (1-cm pathlength) and add DHE/dioxane mixture from screw-top tube.

5. Take absorbance measurements at 326 nm of DHE/dioxane mixture; collect data every 10 s; block light from spectrophotometer in between readings to prevent photodamage.
6. Collect for 50 s. Wait a few minutes and try again. Compare readings.
7. Use mean absorbance value $A_{326}$ to calculate the concentration of DHE from: [DHE] = ($A_{326}$/ε) where the extinction coefficient ε is equal to 10,600 $M^{-1}cm^{-1}$ in dioxane at 326 nm ***(18)***; multiply by dilution correction factor (total volume divided by volume of DHE added to dioxane).

### *3.2. Phospholipid Concentration Determination*

1. Phospholipid concentration in stock solution is determined by the method of Bartlett ***(19)***, with some modifications.
2. Pipette phosphorus standard solution (0.65 m*M*) into 13 × 100 mm disposable culture tubes. Two tubes are left empty to serve as blanks; the remaining six tubes contain 13, 26, 39, 52, 65, and 78 nmols of phosphate, and will be used to produce a standard curve.
3. Pipette phospholipid stock solution for phosphate concentration determination. Calculate approximately how much sample would be required so that the unknown concentrations will fall within the standard curve values.
4. Add 200 µL of 50% $H_2SO_4$ to each tube and vortex gently.
5. Place tubes in preheated aluminum block (200°C) for 5 min.
6. Add 200 µL (*see* **Note 6**) of 30% $H_2O_2$ to each tube and char in aluminum heating block for 30 min.
7. Remove tubes from block and allow cooling.
8. Add 2.3 mL of $H_2O$ (*see* **Note 7**) to each tube; then add 115 µL of 5% ammonium molybdate to each tube and vortex gently. If liquid becomes yellow after addition of ammonium molybdate, add a few crystals of sodium bisulfite and vortex until liquid becomes clear.
9. Prepare FSR according to manufacturer's instructions; add 115 µL of FSR to each tube and vortex gently.
10. Place tubes in a water bath at 100°C for 20 min or until samples turn blue.
11. Allow tubes to cool. Read absorbance at 660 nm (start with least concentrated samples).

### *3.3. Sample Preparation*

When the assay is used for screening antioxidants or pro-oxidants, the sample preparation is simplified, because only one sample is required. However, the studies have shown that properties such as antioxidant protection and pro-oxidant potency may vary at different mole fractions of sterol because of differences in sterol lateral organization. This should be kept in mind when designing the experiment, so that the mole fraction of sterol remains constant for each sample being compared.

When this assay is used to examine the relationship between sterol oxidation and sterol lateral organization, then strict attention to all aspects of sample preparation is required. In each set, sterol mole fraction is increased by small increments (~0.3 mol%) for each sample. The absolute number of sterol molecules per sample does not vary; rather, sterol mole percent is changed by varying the amount of the other lipid components. *See* **Table 1** for a typical sample preparation design.

1. Before pipetting, DHE/$CHCl_3$ and phospholipid/$CHCl_3$ stock solutions must be brought to room temperature to assure even distribution of molecules.
2. Pipette calculated amounts of phospholipid and DHE stock solutions; stock solution tubes should remain in ice while pipetting to slow evaporation. All solutions (stock, vesicles, and so on) containing DHE must be protected from light (use opaque tubes or wrap tubes in foil).
3. Dry lipids under nitrogen gas stream until no visible solvent remains; then dry lipids overnight under vacuum.

**Table 1**
**Typical Sample Preparation Scheme**

| Tube no. | Sterol (mol%) | DHE (nmol) | POPC (nmol) |
|---|---|---|---|
| 1 | 18.4 | 125 | 554 |
| 2 | 18.8 | 125 | 540 |
| 3 | 19.1 | 125 | 529 |
| 4 | 19.4 | 125 | 519 |
| 5 | 19.7 | 125 | 510 |
| 6 | 20.0 | 125 | 500 |
| 7 | 20.3 | 125 | 491 |
| 8 | 20.6 | 125 | 482 |
| 9 | 20.9 | 125 | 473 |
| 10 | 21.2 | 125 | 465 |
| 11 | 21.5 | 125 | 456 |

A typical set of DHE/POPC mixtures shows increases in sterol mole percent by increments of 0.3 mol%. The absolute number of moles of sterol (DHE, in this case) remains constant from sample to sample, whereas the number of moles of the second component (POPC) varies from sample to sample. In this sample set, 20.0 mol% sterol is the only predicted critical sterol mole fraction for maximal superlattice formation ***(23)***.

4. The next day, reconstitute lipids with 5 mL of filtered Tris-HCl buffer (pH 7.0) at the desired temperature (*see* **Note 8**). After reconstitution, flush samples with argon, cover tubes with parafilm and vortex for 2 min at the chosen temperature to form multilamellar vesicles (MLVs).
5. MLVs are then subjected to three cooling/heating cycles (for POPC/sterol or DMPC/sterol LUVs, 4°C for 30 min, and then 37°C for 30 min were used).
6. Let MLVs sit at room temperature for at least 4 d to allow them to come to thermal equilibrium or semiequilibrium for membrane lateral organization. Store vesicles under argon to avoid auto-oxidation of sterol before sterol oxidation measurements.
7. Prepare LUVs from MLVs by extrusion. Extrude at desired temperature 10 times through two stacked Nucleopore polycarbonate filters under nitrogen gas pressure to form homogeneous LUVs of desired size (*see* **Note 9**).
8. Store LUVs under nitrogen or argon gas at room temperature before activity measurements to avoid auto-oxidation of sterols.
9. Incubate at room temperature for 7 d. Measure the phospholipid concentration of each LUV sample by the method of Bartlett (*see* **ref. *19***) to determine the new lipid concentration after loss because of extrusion (*see* **Note 10**). Also, measure vesicle size by photon correlation spectroscopy (Malvern Zetasizer HS-1000 spectrometer) (*see* **Note 11**). The previous study ***(10)*** shows that the sterol mole percents in vesicles before and after extrusion differ by less than 0.2 mol%. Thus, for convenience, the sterol mole percents in MLVs were used to assess the relationship between sterol content and sterol oxidation in LUVs.

### *3.4. Assay Protocol*

1. Turn on circulating water bath connected to fluorometer until it reaches the desired temperature (*see* **Note 12**). For the experiments on DHE/POPC and DHE/DMPC, the authors used a 15 min incubation period to allow samples to reach 37°C.
2. Turn on fluorometer (SLM 8000C) and set excitation wavelength to 325 nm with a bandpass of 0.5 nm. Observe emission through a monochromator set at 396 nm with a bandpass of 8 nm.
3. Add calculated amounts of phospholipid/DHE LUVs and Tris-HCl buffer to a fluorescence cuvette (total sample volume = 1.6 mL; final cuvette concentration = ~8 μ*M* sterol and ~30 μ*M* total lipid).
4. If antioxidant is to be used in the assay, it is added at this time.

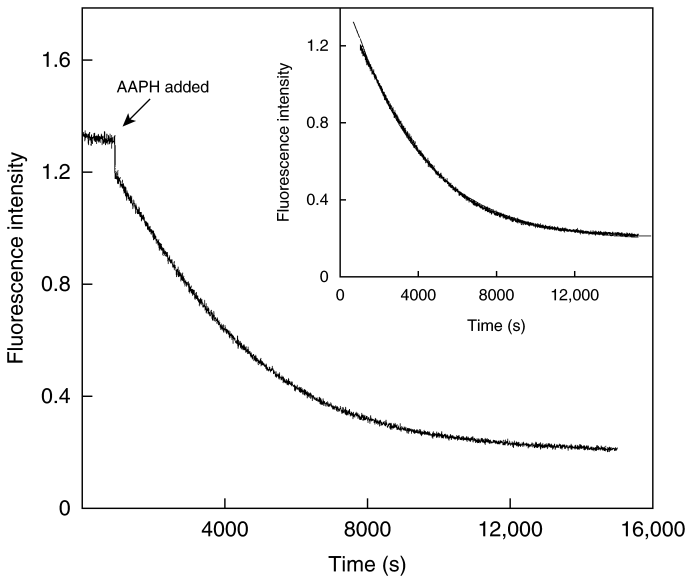


Fig. 1. Profile of a typical assay showing DHE fluorescence intensity decay. DHE (21.2 mol%)/POPC LUVs were incubated at 37°C; 30 μL of 300 m*M* AAPH was added at 1000 s (*see* **Note 15**) and rate of intensity decay was monitored at 395 nm. The sharp drop seen on addition of AAPH is because of energy transfer (*see* **Note 16**) and the asymptotic value at end of assay is referred to as "$F_\infty$" (*see* **Note 17**). **(Inset)** Data of the AAPH-induced DHE fluorescence decay in 21.2 mol% DHE/POPC LUVs measured at 37°C are fitted well by first-order kinetics using the equation $[DHE]/[DHE]_0 = F/F_0 = (F_{obs} - F_\infty)/F_0 = \exp(-kt + b)$. Here, b is a constant, and $[DHE]_0$ and $F_0$ denote the DHE concentration and fluorescence intensity, respectively, at reaction time zero (upon addition of AAPH), and a correlation coefficient $R = 0.999$ was obtained. k is the apparent rate constant of the reaction, and $F_\infty$ is the DHE fluorescence intensity at a long time. For the data fitting, all the fluorescence intensities are normalized against the intensity at reaction time zero (reproduced from ***[17]*** with permission).

5. Place cuvette into sample compartment of the fluorometer and incubate under gentle magnetic stirring for chosen incubation period to allow sample to reach desired temperature.
6. After incubation, begin time trace monitoring. Monitor background fluorescence for at least 60 s. If background fluorescence is stable (i.e., no photobleaching is apparent, *see* **Note 13**), pause the data acquisition and add free radical generator or oxidizing enzyme by pipetting into center of reaction cuvette (*see* **Note 14**).

### *3.5. Data Analysis*

#### *3.5.1. Apparent Rate Constant*

The authors have used data from this fluorescence assay to calculate three different parameters: initial rate of sterol oxidation, length of lag phase in the presence of antioxidant, and the apparent reaction rate constant (k). To calculate k, the reaction must run until DHE fluorescence intensity reaches an asymptotic value **(Fig. 1)**.

In the absence of antioxidant, the steady decay of DHE fluorescence intensity induced by AAPH is best described by first order kinetics. A curve-fitting program (Kaleidagraph; v. 3.52; Synergy Software, Reading, PA) allows calculation of the apparent reaction rate constant (k) using equation ***(1)***: $F = F_\infty + e^{(-kt + b)}$ **(Fig. 1**, **inset)**. To calculate k in the presence of antioxidants, fit all data after lag phase has ended.

### 3.5.2. Initial Rate

Most of the assays performed to examine the rate of sterol oxidation focus on the parameter of initial rate rather than the apparent rate constant. As this kind of experiment is very tedious, and it is crucial to keep the sample thermal history identical for all the samples in the same sample set, the approach requiring a short operational time is preferred. An initial rate calculation requires substantially less time per assay than does solving for k. The initial rate is also particularly more useful for the purpose of examining the relationship between sterol oxidation and sterol lateral organization. When free radical oxidation occurs, the oxidized product is likely to cause some membrane perturbation and/or change in original membrane lateral organization because of the fact that the oxidized sterol product has a larger mean molecular cross-sectional area than native sterol ***(20)***, and has a weaker interaction with the acyl chains of the neighboring phospholipids ***(21)***. Initial rate of sterol oxidation will give us the most accurate snapshot of initial sterol organization in the bilayer (*see* **Note 18**).

Initial rate of sterol oxidation is calculated from the slope of DHE fluorescence intensity decrease *vs* time. By examining a typical plot (*see* **Fig. 1**), it is apparent that the first 15 min of sterol oxidation can be roughly fit to a straight line. However, a snapshot of the initial rate of oxidation at the very beginning of free radical production, before any change in lateral organization has occurred is needed. Each set of samples spanning a critical mole fraction must be analyzed separately in order to get an accurate picture for that particular set of LUVs. There are variations in the time used to define the initial rate calculation per sample set. One reason for these slight variations is because of the high sensitivity of fluorescence signals.

Fluorescence intensity is a relative value, depending on the amount of fluorophore in the cuvette, sample mixing, temperature fluctuations, and quenching because of impurities in the sample. For this reason, each complete set is analyzed separately, but all sets are normalized so that they can be compared with each other. Each sample set is analyzed by examining the first 5–10 min of fluorescence decay in a sequential manner. The first 3 min are plotted, and then new plots are created for the first 4, 5, 6, and so on minutes. The trend of the slopes of each sequential reaction profile shows the point at which the reaction rate first begins to slow. When the slope begins to become smaller in a systematic manner (meaning deviation from linearity), then the data points at shorter time should be used. By looking at the reaction profiles in this fashion, details are visible that would be overlooked if just the larger profile is examined. It is possible to visualize the beginning of deviation from linearity, which usually occurs between 4 and 7 min, depending on the sample set. Within a sample set, the deviation from linearity can be determined and, thus, the number of minutes that will be used to calculate the initial rate can be defined. An acceptable linear fit for that particular time span is usually a correlation coefficient ($R$) value of at least 0.8. However, the validity of a particular $R$ value is based on a power analysis, which takes into account the sample size and calculates the lowest acceptable correlation coefficient that will still give a 95% confidence interval ($p < 0.05$). The correlation coefficient can be somewhat lower than normally would be accepted because its value is based not only on the spread of points, but on the number of points in the plot. If an initial rate is chosen for the first 5 min, the $R$ values may be systematically lower than those for a set where the initial rate is defined as the first 6 min. The initial rate is defined as the smallest time frame that gives a linear plot and a reasonable correlation coefficient and power analysis.

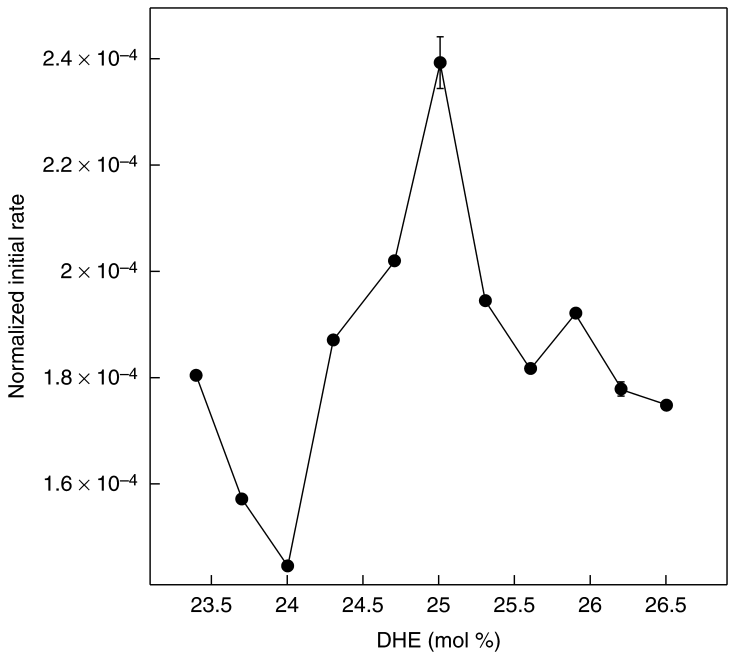


Fig. 2. Normalized initial rate of sterol oxidation in DHE/POPC LUVs (diameter ~200-nm). Assays were performed at 37°C; 30 μL of 300 m*M* AAPH was used to initiate oxidation. The vertical bars are the standard deviations of the measurements from three independently prepared samples. These data show that sterol oxidation rate undergoes a biphasic change at the critical sterol mole fraction 25.0 mol%, which is the only critical sterol mole fraction for maximal superlattice formation in this mole percent range examined ***(23)***. Similar biphasic changes in AAPH-induced sterol oxidation were observed at other critical sterol mole fractions in DHE/POPC LUVs and in other membrane systems such as DHE/bovine-brain sphingomyelin/POPC LUVs ***(17)***. Cholesterol oxidase-induced sterol oxidation exhibited similar multiple biphasic changes with membrane sterol content ***(10)***.

### *3.5.3. Applications for This Assay*

Any of the assay applications described can be extrapolated to more complex systems, such as multicomponent vesicles, lipoprotein particles, and cells. DHE can be incorporated into cells by using either the DHE-methyl-β-cyclodextrin conjugate, or by allowing spontaneous transfer of cholesterol and DHE between membranes through the aqueous phase ***(22)***.

This fluorescence assay is an excellent tool for relating the rate of sterol oxidation to membrane sterol lateral organization, particularly because DHE is used not just as a fluorescent probe to monitor oxidation rate, but also serves as an actual membrane component. Thus, a view of a lipid bilayer that is undisturbed by extraneous probe molecules can be observed.

To look at sterol lateral organization, the initial rates of oxidation from samples can be compared within a sample set. Because all samples contain the same absolute amount of sterol, differences in initial rate of sterol oxidation must be a result of changes in the lateral organization of sterol molecules in the membrane at different sterol mole fractions. The plot above (*see* **Fig. 2**) shows a biphasic change in initial rate of sterol oxidation, with a maximum oxidation rate at one of the critical mole percents (25.0 mol%), as predicted by the sterol

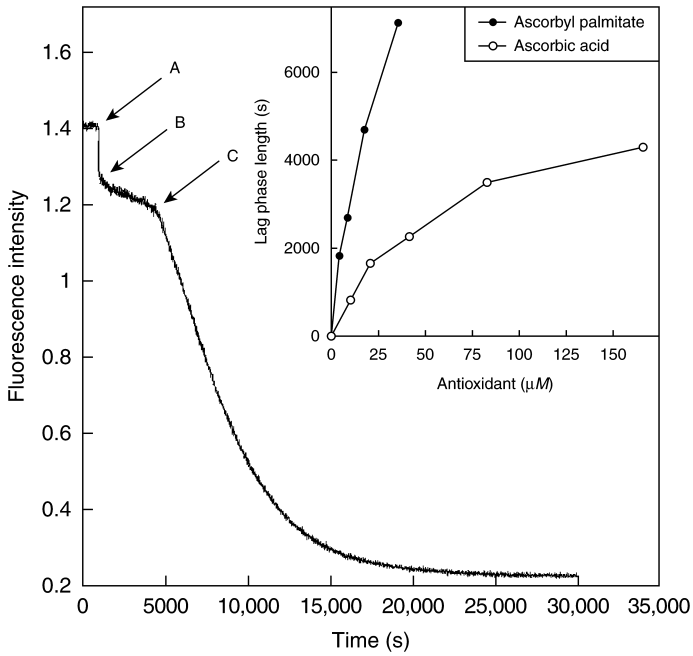


Fig. 3. Profile of a typical assay showing DHE fluorescence intensity decay after incubation with an antioxidant. DHE (19.1 mol%)/POPC LUVs were incubated with 22.5 μ*M* ascorbyl palmitate for 15 min at 37°C before beginning of assay. Point A: addition of 30 μL of 300 m*M* AAPH; points A to B: energy transfer; points B to C: lag phase (slow oxidation step); after point C: beginning of late phase (fast oxidation step). **(Inset)** Plot showing comparison between length of lag phase produced by a water-soluble antioxidant (ascorbic acid) and a lipid-soluble antioxidant (ascorbyl palmitate) (Olsher and Chong, unpublished results). It is clear from the inset that, at the same given apparent dose, ascorbyl palmitate is much more potent in protecting against sterol oxidation than its water-soluble counterpart, ascorbic acid.

superlattice theory. Also the rate of sterol oxidation can be examined in the presence of an antioxidant. When the assay includes an antioxidant, the typical profile contains an additional lag phase (*see* **Fig. 3**).

Both lag phase and initial rate data immediately after lag can be fitted to separate linear lines; the cross point of these fitted lines minus the time before addition of AAPH equals the lag phase length. Generally, the end of lag phase is clearly visible and easily defined even without using the intersection of two fitted lines. By using the same DHE concentration for each sample, the length of the lag phase can be used to describe the relative protection from sterol oxidation given by different antioxidants (*see* **Fig. 3**, **inset**), or by a specific dose of one antioxidant. This procedure can be useful in ranking the potency of different antioxidants, or in examining the effect of water- and lipid-soluble antioxidants on sterol lateral organization. In addition, by assaying bilayers in the presence of a lipid-soluble antioxidant, such as ascorbyl palmitate, one can explore issues relating to membrane perturbation. If we choose to relate lateral organization to the rate of sterol oxidation in the presence of an antioxidant, we would follow the protocol described previously for examining sterol lateral organization, except that we incubate the sample with an antioxidant before adding pro-oxidant to the sample cuvette.

## 4. Notes

1. DHE is structurally similar to and behaves in the same fashion as cholesterol in terms of membrane incorporation, spontaneous transfer, and membrane lateral organization ***(22–25)***. For this reason, multicomponent vesicles can be made with cholesterol or ergosterol as the main sterol component, provided that a small amount (e.g., 1 mol%) of DHE is added as a probe.
2. Results produced by using unpurified DHE are often quite different from those produced using HPLC-purified DHE incorporated into LUVs. This may be because of the presence of oxidized DHE in the unpurified stock. To purify DHE, a Waters Symmetry $C_{18}$ column is used (5 µm; 3.9 × 150 mm) (Waters Corp., Milford, MA); mobile phase: methanol: acetonitrile 2:1, v/v; flow rate 1.5 mL/min; detection of absorbance at 326 nm. It is possible that the presence of oxidized DHE introduces error by affecting membrane packing in a nonsystematic way, and hence interferes with the measurement of the rate of sterol oxidation and the length of the lag phase (if antioxidants are used).
3. All glassware used for lipid storage and vesicle preparation should first be washed with polar and nonpolar solvents. For the vesicle preparation, the authors use one wash with chloroform:methanol (2:1 v/v), followed by one wash with ethanol, and a final washing with deionized water. Glassware must be dried completely. Gilson Microman pipettes are used to transfer organic solvents or lipid samples dissolved in organic solvents.
4. EDTA is used to chelate metal ions in solution. It was found that metal chelation is required for the measurement of the antioxidant capacity of ascorbyl palmitate. In the presence of free metal ions, ascorbyl palmitate either does not produce a lag phase at all or produces a greatly attenuated lag phase, and therefore does not provide typical antioxidant protection. Water-soluble vitamin C (ascorbic acid) demonstrates antioxidant protection both in the absence and presence of free metal ions, but EDTA was added to all buffer preparations.
5. Oxidizing agents, such as water- or lipid-soluble free radical generators or enzymes, can be used in this assay to oxidize sterol. AAPH is a water-soluble peroxy radical generator that undergoes thermal decomposition to yield $N_2$ plus two carbon radicals. Most of the carbon radicals then react rapidly with oxygen molecules to yield peroxy radicals ***(26)***. The rate of AAPH decomposition is determined primarily by temperature and pH. At 37°C in neutral water, the half-life of AAPH decomposition is about 175 h ***(27)***. Based on these calculations, the rate of peroxy radical formation is essentially constant for the duration of the assay, even when a significant lag phase is observed. However, if the sample set is large and must be run over the course of four or more days, there is some loss of potency of radical production. The potency is tested by comparing initial rates of sterol oxidation using the same sample (POPC/DHE LUVs; 19.1 mol% DHE; 30 µL of 300 m*M* AAPH per assay; 8 nmols DHE per assay) on four consecutive days, and calculating the percent decrease per day. Between daily assays, AAPH is stored at 4°C. After 4 d of storage, the initial rate of sterol oxidation is about 20% less than the original measurement. All samples comprising a single sample set should be assayed within 3 d.
6. The original procedure calls for a smaller amount of $H_2O_2$; however, it was found that the samples would sometimes turn brown after 30 min in the heating block if there was an insufficient amount of $H_2O_2$. To avoid this, add extra $H_2O_2$; 100–200 µL is sufficient to prevent this color change. If the samples are not clear on removal from heating block, the final absorbance readings will be affected.
7. The original assay calls for 2 mL of $H_2O$, 100 µL of ammonium molybdate and 100 µL of FSR reagent. The volume may be adjusted to allow for differences in the final volume required for a particular spectrophotometer, as long as the proportions remain consistent.
8. The temperature required for vortexing step (*see* **Subheading 3.3.**, **step 4**), heating/cooling step (*see* **Subheading 3.3.**, **step 5**) and the temperature at which the assay is performed (*see* **Subheading 3.4.**, **step 1**) all depend on the membrane system used. The temperature chosen is usually about 10°C more than the main transition temperature of the matrix lipid. For the studies, 37°C was used.

9. If the LUVs are used to study sterol lateral organization, then the Nucleopore filter used for extrusion should have pores that are more than 60 nm ***(28)***. When vesicles are too small, the radius of curvature is large enough to disturb the lateral organization of the lipid molecules. For other studies, LUVs of any size can be used.
10. Lipid loss because of extrusion process was calculated to be about 40% ***(10)***.
11. Size was determined before and immediately after extrusion, as well as after the postextrusion 7-d incubation period. Vesicle size was also measured before and after some fluorescence assays, both in the absence and presence of antioxidants. It was found that there was no significant change in vesicle size because of addition of AAPH or any antioxidant used in the assay.
12. The recommended operation temperature range for this assay is 15–47°C. Below 15°C, it is necessary to flush the sample compartment of the fluorometer with nitrogen to get rid of moisture condensation on optical components. If the assay is performed at more than 47°C, an additional factor must be considered. At higher temperatures, sterol oxidation occurs at a faster rate, and the kinetic trace of the AAPH-induced DHE oxidation usually reaches an asymptotic value within 1 h. At this point, both initial rate of oxidation and apparent rate constant (k) can be calculated as described (*see* **Subheading 3.5.**). However, continuation of the time trace monitoring past this point (at temperatures >47°C) would result in the appearance of a sudden steep rise in fluorescence intensity over time (Yoon and Chong, unpublished results). This is probably owing to the formation of either an additional compound or an excited state complex. In either case, the initial rate of AAPH-induced sterol oxidation and the k value calculated from the DHE fluorescence intensity decay (once the profile has reached an asymptotic value) are meaningful, and can be interpreted in the same way as data retrieved at lower temperatures. Even at higher temperatures (>47°C), HPLC analysis clearly shows that, on addition of AAPH, the initial decay of DHE fluorescence intensity is because of the oxidation of DHE (Yoon and Chong, unpublished results).
13. Photobleaching is the irreversible destruction of an excited fluorophore on exposure to light. To measure the contribution of decreased fluorescence intensity by photobleaching, fluorescence intensity was measured for more than 5 h under the experimental conditions (37°C) in the absence of AAPH. The contribution to intensity loss by photobleaching was found to be approximately 0.5%. This is a contribution small enough to be ignored.
14. Thirty microliters of 300 m*M* AAPH was used for each reaction. AAPH solution was freshly prepared for each sample set and used within 3 d. Prepare AAPH by measuring appropriate amount and mixing with (Millipore) water. Store at 4°C in between assays; remove from refrigerator only when it is time to add AAPH to reaction cuvette and replace when finished. The final ratio in the reaction cuvette of DHE:AAPH is approx 1:1.
15. For purposes of illustration, AAPH is added here after 1000 s. In a typical assay, background intensity can be monitored for a much shorter time period to verify that there is no appreciable photobleaching or other artifacts that would result in DHE oxidation in the absence of peroxy-free radical.
16. On addition of AAPH, there is an immediate sharp drop in DHE fluorescence intensity, followed by a steady decrease in fluorescence intensity over time (*see* **Fig. 1**). The sharp drop in fluorescence intensity that occurs on addition of free radical generator is owing to resonance energy transfer, which is the transfer of excited state energy from an excited donor (DHE) to an acceptor (AAPH). As AAPH is nonfluorescent, the DHE fluorescence is thus reduced. Energy transfer does not affect the calculation of initial rate of sterol oxidation or the determination of lag phase length because energy transfer occurs much faster than sterol oxidation. The steady decrease in intensity over time results from AAPH-induced sterol oxidation.
17. The fluorescence intensity measured at a long time after addition of AAPH is designated as $F_\infty$, which is typically 10% of the initial intensity. The existence of a nonzero $F_\infty$ value is probably a result of either unreacted DHE in an inaccessible area or production of a weakly fluorescent product.
18. When comparative studies are conducted, the initial rates from different samples must first be normalized to unity.

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