ESR ASSOCIATED TO SPIN LABEL METHOD IN THE STUDY OF PHOTOSENSITIZATION IN LIPOSOMAL SOLUTION

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Introduction

Photosensitized reaction is primarily defined as a reaction in which a chemical species having absorbed light undergoes no practical change but some other species undergo a certain reaction without absorption of photons. A species which absorbs photons and induces sensitized phenomenom is called a sensitizer.

Sensitized photomodifications of biological membranes have been studied as long as the photosensitization phenomenom itself. Among many applications, photodynamic therapy (PDT) is an excellent exemple in which light in combination with sensitizer agents are exploited to treat disease (1).

PDT is based (i) on selective accumulation of a particular photosensitizer in abnormal or hyperproliferative cells such as those in cancerouse tissue and (ii) on tumoral cell destruction by activation of the drug with light. A special attention is devoted to sensitizers presenting a tumor cells preferential retention and absorbing above 600 nm. In this spectral region, endogenous tissular components are transparent to the incident radiation, minimizing the risk of photodamage at the level of cells or tissues not containing the photosensitizers. Moreover, under light irradiation, these dyes must produce reactive oxygen species (ROS) able to react with the surrounding biological components inducing lethal damages.

Indeed, evidence is accumulating that the generation of ROS is intimately associated with the PDT effect of many sensitizers involved in cancer therapy. A light-activated molecule can transfer energy from its triplet state by two processes, directly to molecular oxygen with generation of singlet oxygen (Type 2 reaction) or by interaction with solvent or substrate by electron or proton transfer with generation of radicals (Type 1 reaction). While singlet oxygen is believed to be the major mediator of photochemical cell damage for many types of photosensitizers, oxygen species like the superoxide anion and the hydroxyl radical can also induce deleterious effects including lipid peroxidation and membrane damage.

However, it is very complicated to draw conclusions on the exact mechanism which induces tumour necrosis since during PDT, the reaction pathway strongly depends on the oxygenation within the tumour, the singlet oxygen lifetime in a particular environment and the stability of generated radicals. Type 1 reaction processes are expected to be favoured in polar media while, in contrast, Type 2 mechanisms predominate in hydrophobic environments. So, the antitumoral activity of a sensitizer appears to be mainly governed by its solubilization inside or outside the membrane. Its primary localization in biomolecules strongly depends on its lipophilic or hydrophobic character.

Electron spin resonance (ESR) spectroscopy of nitroxide spin labels has been used successfully for diverse biomembrane studies, including studies of membrane structure and membrane fluidity. In addition, the kinetics of chemical reduction of spin labels can be used to determine their distribution inside the membrane.

Liposomes (2) are often used as well-defined and easily controlled model systems to study , in a more simple way, the influence of several parameters like membrane viscosity or surface charge density , on the distribution properties of hydrophobic sensitizer in normal and tumor tissue.

ESR associated to spin label method (3, 4, 5, 6) provides detailed informations on the sensitizer intraliposomal localization. The aim of this report is to described the basic principles of this method.

Liposomes

Liposomes or phospholipid vesicles are self-assembled colloidal particles that occur naturally and can be prepared artificially (dispersion in aqueous media above the gel-to-liquid crystalline phase transitions). At first, they were used to study biological membranes, several practical applications, most notably in drug delivery, emerged in the 1970s.
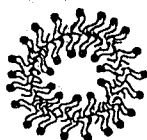
Unilamellar liposome

Liposomes can be simply defined as vesicles in which an aqueous phase is enterely enclosed by one or several membranes composed of phospholipid molecules. In these self-assembled structures, the polar head group of the molecules is in contact with the polar environment. They are conventionally classified into three groups by their morphology and size which may range from tens of nanometers to tens of microns in diameter : (i) small unilamellar vesicle (SUV) and (ii) large unilamellar vesicle (LUV) possess just one phospholipid bilayer while in (iii) multilamellar liposome(MLV), several concentric bilayers alternate with aqueous layers. Stable bilayer membrane vesicles can be made with a wide range of phospholipids, natural or artificial, saturated or unsaturated, charged or neutral .

ESR associated with spin label method

ESR spectroscopy is a technique for detection of unpaired electrons. Spin labels are usually molecules containing a nitroxyl radical which possesses an unpaired electron localized on the nitrogen and oxygen atoms.

ESR spectrometers measure an absorption of microwave energy by spin labels in the samples as function of magnetic field strength (figure 1). A measurable net absorption of energy by the sample can occur when the unpaired electrons associated with spin labels move from the lower permitted energy state to the higher state. This transition detectable as the ESR spectrum, depends on the quantity, orientation, motion and other magnetic properties of the environment of the nitroxyl radical. The ESR spectrum represents the first derivative of energy absorbed against the magnetic field.

Stearic acids labeled with a nitroxyl spin probe at different positions along a carbon chain are useful probes to study membrane properties . As shown in figure 2, in the bilayers, the spin labels (in that case, n-doxyl stearic acids , n-DSA) are oriented with their long axes parallel to the phospholipid chain. By variyng the position of the nitroxyl radical along the chain of a lipid spin label, it is possible to examine the lipidic structure of liposomes at various depths.
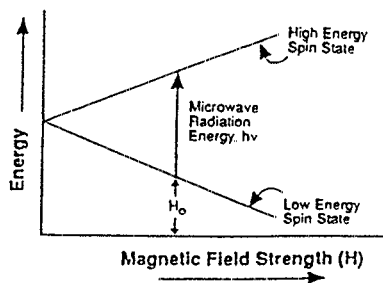
Figure 1. Energy of electron spin states as a function of magnetic field strength.

The location of n-DSA in liposome is well known. It is established that 5-DSA and 7-DSA explore the polar part of liposome while 12-DSA and 16-DSA explore the hydrophobic core. The mean distance between the fatty acid carboxyl carbon and the carbon atom to which the nitroxyl radical is bound has been calculated assuming that the molecule is linear and rigidly extended. It is about 8, 10.5, 17 and 21.5 Å for 5-, 7-, 12- and 16-DSA, respectively.
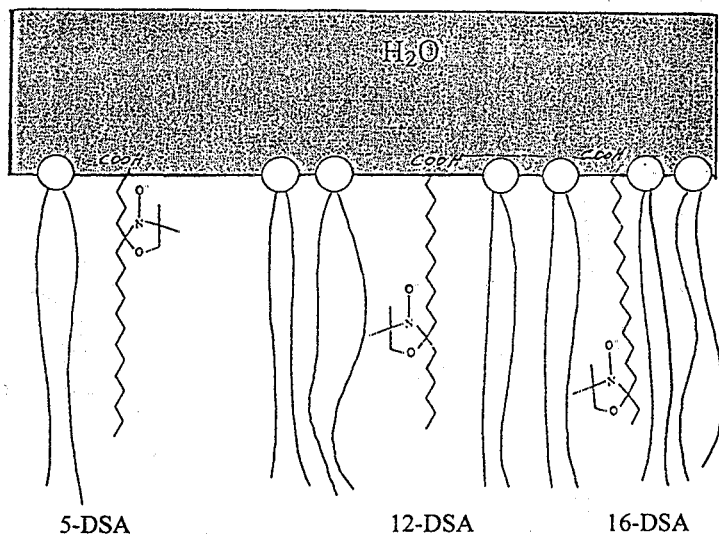

5-DSA   12-DSA   16-DSA

Figure 2 : the location of n-DSA in liposome membrane

In methanol, the ESR spectrum of the stearic acid spin probes (5-DSA, 7-DSA, 12-DSA and 16-DSA) consist of three symmetric absorption lines nearly indistinguishable from each other (figure 3)
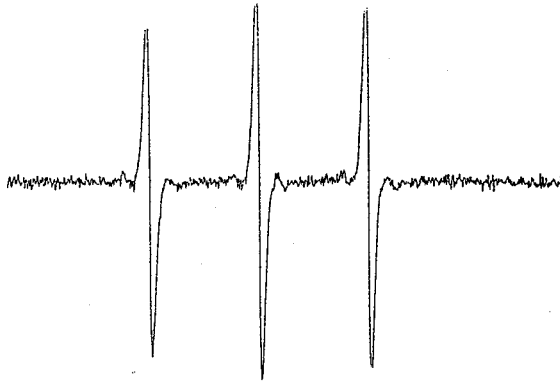
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Figure 3 : ESR spectra of the stearic acids spin probes (5-DSA) dissolved in methanol

The ESR spectrum of n-DSA is sensitive to the rotational mobility of the spin label. Drastic changes can occur when the paramagnetic entity is immobilized, compared with the spectrum obtained in which the paramagnetic entity is tumbling rapidly in solution. Then, depending on the location of the nitroxyl radical in the bilayer, the spin label spectrum reflects dynamics and order at different membrane depths.

The incorporation of n-DSA into dimyristoyl-L-α-phosphatidylcholine (DMPC) liposomes results in a change in the ESR spectrum of the label which is characteristic of strongly or weakly immobilized species (figure 4). Indeed, the lipidic polar zone of the liposome explored by 5- and 7-DSA is rigid and relatively highly ordered whereas the hydrophobic core explored by 12- and 16- DSA is fluid.
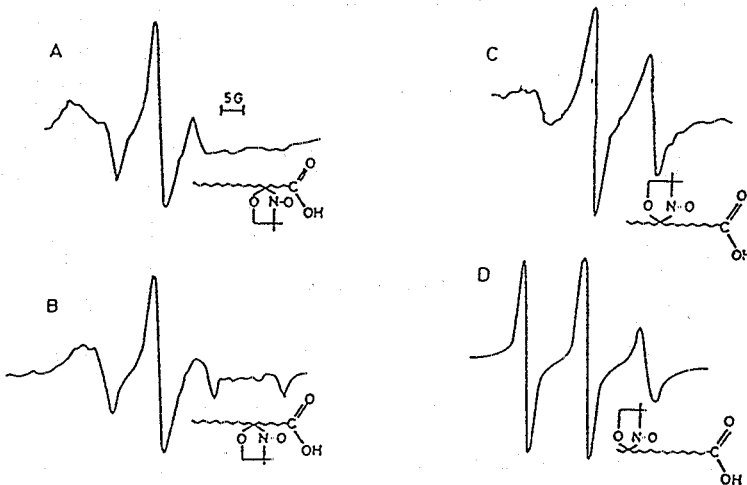

Figure 4 : ESR spectrum of 5-DSA (A), 7-DSA (B), 12-DSA (C) and 16-DSA (D) in DMPC liposome at 31°C.

From the ESR spectrum, the relative head group mobility in slow motion can be estimated with the order parameter S and in fast motion by calculation of empirical time correlation τ_c.

Correlation time parameter $\tau_c = (6.5 \times 10^{-10})\, W_0\, ((h_0/h_{-1})^{1/2} - 1)$

Order parameter
$$S = \frac{2\, T'_{//} \ - \ 2\, T'_{\perp}}{2\, T_{//} \ - \ 2\, T_{\perp}}$$

where W_0 is the with of the central line at half-height and h_0 and h_{-1} are the amplitudes of the center- and high-field lines, respectively ; $2 T_{//} \ - \ 2\, T_{\perp} = 52G$, $T'_{//}$ and $T'_{\perp}$ are the anisotropic hyperfine splitting constants measured from the ESR spectrum

Interaction between spin-label and sensitizer - Localisation

Some sensitizers are able to interact with spin label leading to a loss of ESR signal intensity. Under anaerobic conditions, in presence of photoexcited sensitizer able to act as electron donor/acceptor, electron-transfer reactions lead to nitroxyl radical reduction. The decrease of the signal amplitude can be followed as a function of the illumination time (figure 5).
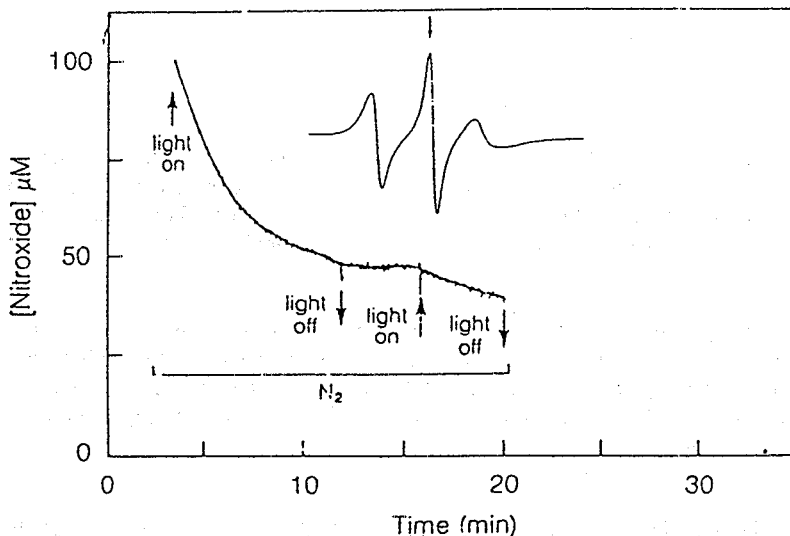

Figure 5 : exemple of nitroxyl radical reduction by photoexcited sensitizer (n-DSA in DMPC liposome in presence of Merocyanine 540 excited with visible light). The magnetic field is positioned at the maxima of the center line of the n-DSA spectrum and the field sweep turned off. The amplitude of the center line is then followed with time during irradiation under anaerobic conditions.

In homogeneous solvent like N_2-saturated methanol, the different n-DSA are reduced at similar rates in the presence of photoexcited sensitizer. Then, when the nitroxyl radicals are incorporated inside lipidic structure like DMPC liposomes, the kinetics of their paramagnetic signal decay under irradiation in the presence of the sensitizer give information about sensitizer location inside the structure as we are going to show in the exemple developped in the next paragraph.

Application : localisation of Bacteriochlorin *a* (BCA) inside DMPC liposome
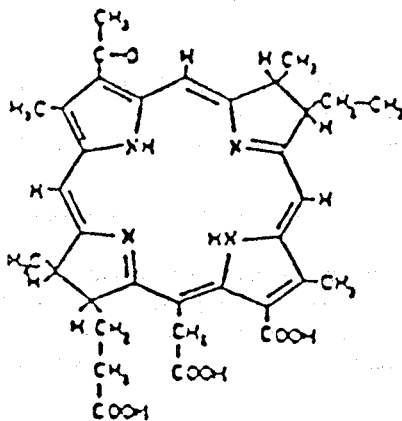
Bacteriochlorin *a*

Hydrophobic dyes like BCA are known to accumulate at different sites of the liposomal double membrane. The kinetics of nitroxide spin labels reduction can be used to assess the dye localisation in the biomembrane accurately. Indeed, photoexcited BCA is able to destroy the paramagnetism of spin-labelled doxyl stearic acid (n-DSA).

In homogeneous solvent like N_2-saturated methanol, the series of n-DSA ($2 \times 10^{-5}M$) studied are reduced at similar rates in the presence of photoexcited BCA ($2 \times 10^{-5}M$). The reduction rate is linearly proportional to the BCA concentration and, in the absence of the dye or under O_2 , no variation of the n-DSA signal amplitude is induced by irradiation of the sample (data not shown).

On the other hand, the kinetics of signal amplitude reduction in BCA- treated DMPC liposomes are affected by the position of the doxyl moiety along the stearic acid chain. As shown in figure 6 , the amount of 5-DSA whose doxyl moiety is close to the membrane surface decreases as a function of time whereas the 12-DSA and 16-DSA whose doxyl moieties are deep in the membrane hydrocarbon region do not show any significant reduction. Figure 6 also exhibits the reduction of the water soluble carbamoyl (CTPO) which kinetic is between those of 5-DSA and 12-DSA. In any case the shape of the label ESR spectra is unchanged. In the absence of BCA or light or under O_2, no variation of the spin label concentration is observed in the liposomes suspension.
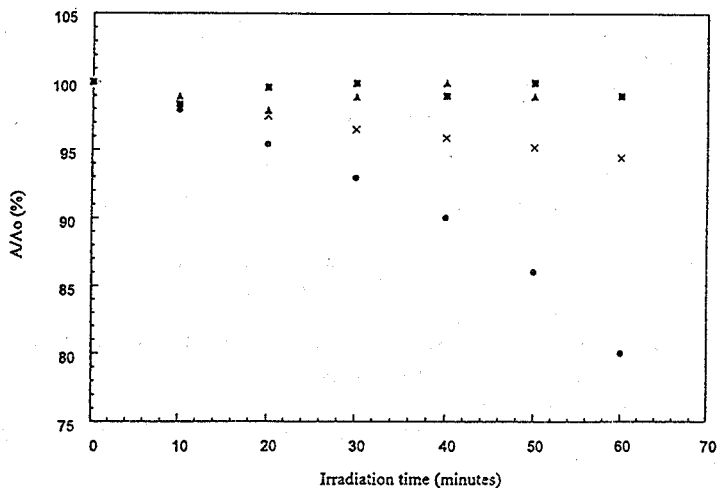
Figure 6 : The reduction of nitroxide spin labels, intercalated into BCA treated DMPC liposomes (n-DSA) or occupying the external aqueous phase (CTPO), is measured by ESR upon irradiation with visible and UV light under anaerobic conditions. (A/A$_0$ denotes the signal amplitude of nitroxide relative to the amplitude at time zero ; ■ 16-DSA, ◆ 12-DSA, X CTPO, ● 5-DSA)

       As shown in figure 6, the DSA reduction rates are affected by the position of the doxyl moiety along the fatty acid chain. The more efficient quenching of 5-DSA together with the absence of any significant reduction for 12-DSA and 16-DSA are in favour of a localisation of the dye near the polar head interface. Indeed, it is well established that the mean distance between the fatty acid carboxyl carbon and the carbon atom to which the doxyl group is bound is approximatively 8 Å . 5-DSA explores the polar part of the liposome and its rate of reduction is controlled by the accessibility of the doxyl moieties to the reducing agent, in that case the excited BCA. Thus, the high rate of reduction obtained for 5-DSA suggests that BCA is located near the membrane surface of DMPC liposomes. Moreover, the observed reduction of the water soluble carbamoyl clearly indicates that at least a portion of BCA molecules resides close to the external membrane surface.

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