EFFECT OF THE INCORPORATION INTO UNILAMINAR VESICLE ON THE PHOTODEGRADATION OF INDOLES SENSITIZED BY FLAVINS

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ABSTRACT

In this work we study the incorporation into dipalmitoylphosphatidylcholine (DPPC) small unilamellar vesicle (SUV), of three indols: Trp, indol-3-acetamide and N-acetyl tryptophan ethyl ester (NATE), and of three flavins: riboflavin (RF), lumiflavin (LF), and 2'3'4'5' riboflavin tetrapropionate (TPRF). The incorporation into the vesicle was determined by selective fluorescence quenching for the indols, and by equilibrium dialysis for the flavins. The more hydrophobic compound, the greater incorporation into the vesicle; therefore, TPRF and indol-3-acetamide and NATE are the flavins and indols, with the higher incorporation. Consequently, the higher efficiency for the indol photodegradation and the photoproducts generation was obtained when the hydrophobic compounds were irradiated with monochromatic light in the presence of the vesicle.

1. INTRODUCTION

Riboflavin (RF), commonly called vitamin B₂, is one of the eight water-soluble B vitamins. RF plays a crucial role in certain metabolic reactions as precursor of the redox cofactors FMN and FAD. Upon UVA light exposure, RF can also act as a photochemical sensitizer, promoting the photodegradation of other molecules (1-3).

In the last decades, numerous reports have proposed photodynamic therapy (PDT) as a feasible non-invasive approach for the treatment of several diseases including cancer and other pathologies (4-6). In aqueous solutions and in the presence of oxygen and visible light, RF induces substrate oxidation through the generation of singlet oxygen (type II mechanism) and/or radical species (type I mechanism) (7,8). Notably, under low oxygen pressure, RF mediated tryptophan (Trp) photooxidation occurs through a mixed mechanism, being type I mechanism more effective than type II (8,9). The higher efficiency of the electron transfer mechanism can be explained by the change of the redox potential of the triplet excited state of flavins (5) and the large quenching efficiency of Trp towards RF triplet (ca. $k_q \approx 1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) (7,8).

Several photoproducts of RF and Trp have been in fact isolated by our group, such as: (i) photoadducts between RF and Trp; (ii) aggregation photoproducts of RF; (iii) aggregation photoproducts of Trp and photodegradation products from both Trp and RF (9,10). The performance of RF-Trp photoproducts against to human and mice tumour cells in culture has also been studied by our group (11,12), where nuclear and cytoplasmic damage, similar to that described in apoptotic cells processes, have been observed. The damage is higher when the photoproducts are generated under anaerobic condition, which favours the formation of radical species and therefore, RF-Trp adducts formation (11).

Photochemical and pharmacokinetic properties for several flavins esters (FE) have also been studied by our group (6,13). Our findings indicate that flavin esters are more stable to visible light irradiation than RF. Furthermore, remarkable differences in their pharmacokinetic behaviour in C57/BL6 mice were observed upon esterification. RF, lumiflavin (LF) and the shorter-chain RF esters appear to be rapidly cleared from the serum. However, long-chain esters exhibit longer lifetime in serum (13).

Little is known about the photochemical behaviour of RF and FE in membrane like systems, although there has been described that the photophysical properties of RF are affected upon compartmentalization (14,15). In this work, we have studied the incorporation of flavinic and indolic derivatives in small unilamellar vesicle (SUV) of dipalmitoylphosphatidylcholine (DPPC), and the effect of their incorporation in the efficiency of the degradation of three indolic derivatives photosensitized by flavins.

2-MATERIALS AND METHODS

2.1 Chemicals

Riboflavin (RF), lumiflavin (LF), tryptophan (Trp), N-acetyl tryptophan ethyl ester (NATE), indole-3-acetamide, acrylamide, pyrene, and

1,2-dipalmitoyl-sn-glycero-3-phosphocoline (DPPC) were purchased from Sigma-Aldrich (USA), and used as received. Na₂HPO₄, NaH₂PO₄, KH₂PO₄, NaCl, KCl and ethanol were purchased from Merck (Germany).

2.2 Synthesis

The synthesis of 2'3'4'5' riboflavin tetrapropionate was performed as previously described (16,17). The compound was recrystallized from 95% methanol and characterized by IR and ¹H NMR spectroscopy (16).

2.3 Spectral measurements

Absorption and emission spectra were recorded at 25°C. The absorption measurements were carried out with a Hewlett-Packard 8453 diode array spectrophotometer. Fluorescence spectra were recorded on a Perkin-Elmer LS-55 spectrofluorimeter. The emission intensity was proportional to the fluorophore concentration. ($\lambda_{\text{ex}} = 450 \text{ nm}$ and $\lambda_{\text{em}} = 510 \text{ nm}$ for flavins; $\lambda_{\text{ex}} = 280 \text{ nm}$ and $\lambda_{\text{em}} = 360 \text{ nm}$ for indolic compounds; $\lambda_{\text{ex}} = 340 \text{ nm}$ and $\lambda_{\text{em}} = 400 \text{ nm}$ for Pyrene). In all cases, the absorption at the excitation wavelength was kept < 0.1.

Fluorescence lifetimes were measured in an *Edinburgh Instrument OB-900* time-correlated single-photon-counting fluorimeter. H₂ gas was used in the flash lamp. Analysis of the fluorescence decays were carried out by a least-squares iterative convolution method based on the Marquardt algorithm by means of the analysis routine provided by *Edinburgh Instrument* (Edinburgh, UK). The quality of the fit was assessed by χ^2 values together with the distribution of the residuals.

2.4 Photodegradation studies

The photodegradation studies were carried out for 60 min at 37°C in a thermostatically controlled cell with nitrogen bubbling. Light from a 150 W slide projector lamp, equipped with an interference filter, was employed for irradiation at 450 nm. The energy flow rate ($2.8 \text{ J m}^{-2} \text{ s}^{-1}$) was measured at the level of the irradiated solution with an YSI-Kettering 65A radiometer (Yellow Springs Instruments Co., OH, USA). Concentration for the indolic derivatives was kept below 0.1 mM and 35 mM for the flavins. All measurements were carried out in phosphate buffer saline (PBS) pH 7.3, prepared with bidistilled water. Changes in the absorption spectra between 300-500 nm were monitored over time and the photoconversion determined as the decrease in the fluorescence intensity of each fluorophore (see 2.2 *Spectral measurements*) after 15, 30, 45, and 60 min. of irradiation. Photoproduct generation was monitored by the increase at the absorbance at 320 nm.

2.5 DPPC vesicle (SUV).

Preparation of SUV and incorporation of flavin and indolic compounds, were performed following the injection protocol described by Kremer et al. (18). Briefly, 5 mg of DPPC were solubilized in 700 μL of pure ethanol or in 700 μL of an ethanolic solution of flavin and/or indolic derivatives. Note that a 1:3 ratio was employed when both compounds were incorporated. Solutions were slowly injected (20 $\mu\text{L}/\text{sec}$) into 10 mL of PBS, pH 7.3, at 55°C.

2.6 Incorporation Studies

The incorporation studies for indoles and flavins were carried out by two different methods. The selective quenching of the fluorescence of the compound in the aqueous phase was used as a tool for measuring the incorporation of indolic compounds. Acrylamide (up to 0.04M) was used as quencher of the indolic derivatives. According to the model proposed by Quina and Toscano (19), the fluorophore fraction present in the aqueous phase (*f_w*) can be obtained from Eq. 1, with a plot of $\frac{I_0}{I_0 - I_q}$ vs $\frac{1}{[Q]}$ (see figure 2B)

$$\frac{I_0}{I_0 - I_q} = \frac{1}{f_w} + \frac{1}{f_w K_{sv}} \frac{1}{[Q]} \quad (\text{Eq 1})$$

where *I₀* and *I_q* are the fluorescence emission in the absence and in the presence of the quencher, respectively.

In the case of flavins, the equilibrium dialysis method was used (20,21). Briefly, the dialysis bags were filled with dispersions of vesicle with the

flavin, then they were immersed in a fixed volume (50 mL) of PBS at 5°C and the system was gently shaken for 24 h. After completion of dialysis the fluorescence of the liquid inside the membrane bag was measured at 520 nm, and the concentration of the incorporated flavin was determined by interpolation in a standard calibration curve for the given flavin.

3-RESULTS AND DISCUSSION

3.1 Incorporation Study in DPPC vesicle

3.1.1 Indolic derivatives

The incorporation of the different indolic derivatives (see Figure 1 for structures) into DPPC SUV was determined by following the quenching of the indole fluorescence in the aqueous phase. Acrylamide was chosen as selective quencher because no quenching on pyrene fluorescence (compound known to be completely incorporated to lipid bilayers) (22) was observed. This indicates that acrylamide does not migrate into the lipid bilayer, and thus, it is suitable as quencher for indolic fluorescence in the outer aqueous phase (23).

Figure 1: Chemical structure for the Flavins (left) and Indole derivatives (right) employed in this work.

Downwards curved Stern-Volmer plots (see Figure 2A) indicates the presence of a fluorophore population not affected by acrylamide, thus the data treatment was carried out by the method described by Quina and Toscano (19).

Figure 2: Stern Volmer plots for the quenching of Trp by acrylamide: in PBS pH 7,3 (red) and in DPPC SUV (purple). Error bars have been included for comparison purposes.

Table 1 summarizes the incorporation percentage for the different derivatives, K_{sv} values and the absorption and emission maxima. In general, the incorporation of indolic derivatives does not exceed 50 % and the best incorporation was observed for the more hydrophobic compound: indol-3-acetamide. The K_{sv} values in SUV are similar to those in homogeneous media (see Table 1).

Table 1: K_{sv} values in PBS and SUV and Incorporation Percent of Indol and Flavin compounds into SUV

Compound	Octanol/ water Distribution ratio	λ_{max} emission	$K_{sv}^{M^{-1}}$ PBS	$K_{sv}^{M^{-1}}$ SUV	(%)Incor- poration into SUV
RF	0.13	515	-	-	54
LF	1.33	515	-	-	65
TPRF	21.8	513	-	-	76
Trp	0.43	357	69	70	32
NATE	1.23	350	65	48	49
INDOL-3- ACETAMIDE	2.3	350	149	139	50

On the basis of the octanol/water distribution and the blue shift of the fluorescence (respect to Trp emission in PBS) of indolic derivatives shown in Table 1, we postulate that, probably, a low amount of the Trp molecules are localized into the intravesicular aqueous phase, while most of the hydrophobic indolic compounds (NATE; indol-3-acetamide) are localized in the lipid bilayer near the aqueous inter-phase.

The quenching rate constant (k_q) is of the order of those for diffusion-controlled processes in water ($\approx 7 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$), due to the fact that the quenching occurs always in the aqueous phase. The fluorescence lifetimes measured in SUV are shown in Table 2. The fluorescence decays of Trp and NATE are characterized by a bi-exponential curve (Table 2). This behaviour

can be attributed to the presence of different conformers or rotamers, originated from two or more different configurations of the alanyl side chain in reference to the indolic nucleus (24). The bi-exponential behaviour of indolic derivatives is dependent on the α -carbon substituent. The acrylamide quenching efficiency of indole fluorescence decreases when the substituents on the 3-position change in the following order: $-\text{CO}_2\text{H} \geq -\text{CO}_2\text{C}_2\text{H}_5 \geq -\text{CONH}_2$, $-\text{CO}_2$, and this agrees with a decrease in the fluorescence lifetime of the corresponding indolic compound (25). On the other hand, indol-3-acetamide fluorescence decay was mono-exponential. This single decay may be the result of unresolved lifetimes from rapid rotational motions of different conformers (26).

Table 2-Singlet excited state lifetime of Indol and Flavin derivatives in different media.

Compound	Solvent	τ (ns)	a_i (%)
Trp	PBS Glycerol DPPC	3.13	*
		0.51	*
		5.44	*
		3.33	89
		0.31	11
NATE	PBS DPPC	1.84	*
		0.5	*
		2.61	78
		0.67	22
Indol-3-Acetamida	PBS Hexano Glycerol/PBS DPPC	5.48	100
		2.80	100
		4.76	89
		1.3	11
		5.60	100
RF	Buffer MeOH DPPC	3.9	100
		5.6	100
		4.30	100
LF	PBS CHCl_3 DPPC	4.23	100
		6.96	100
		4.67	100
TPRF	PBS CHCl_3 Hexane Glicerol/PBS DPPC	5.0	100
		6.5	100
		7.2	100
		5.4	100
		6.1	51
		3.7	49

3.1.2 Flavin derivatives

A suitable flavin quencher is not currently available. Thus, the Quina and Toscano treatment could not be used. Equilibrium dialysis was chosen to determine the flavin incorporation into DPPC SUV. In this approach we assume that the flavin concentration measured outside of the dialysis bag corresponds to the free, not incorporated flavin, and that measured in the inside dialysis bag liquid corresponds to both flavin populations, that incorporated into SUV and free flavin. The later corresponds to that measured outside. As seen in Table 1, the more hydrophobic the flavin, the greater the incorporation into the vesicle. Furthermore, a 20 nm blue shift in the absorption (not shown), and 2 nm shift in the fluorescence spectra were observed for TPRF, suggesting that this compound is located in the lipid interphase (27). This is concordant with the resolved fine structure observed at the 450 nm absorption band of this hydrophobic flavin (27).

The RF and LF fluorescence lifetimes present a single exponential decay in both SUV and homogeneous medium. The fluorescence lifetimes of both flavins increase in SUV with respect to that in homogeneous medium (see Table 2). However, this increase is lower than that observed in organic solvents (27). These results are in agreement with the low incorporation into SUV. TPRF shows a single exponential decay in buffer and organic solvents

(Table 2). However, a bi-exponential fluorescence decay was observed in SUV, indicating that the compound is localized in two different environments. The lifetime values are similar to those observed in organic solvents and buffer, respectively, and the population of molecules in each of the different environments is ca. 50%, which is in agreement with the quantified percentage of incorporation (see Table 1). According with all these results the fraction of TPRF incorporated at the vesicle is probably localized in the lipid bilayer.

3.2 Photodegradation Studies.

The values of the pseudo-first-order rate constants for photodegradation of indolic derivatives, sensitized by flavins, are summarized in Table 3. All the experiments were carried out under N_2 saturated solutions, in order to promote type I mechanism, thus favouring the direct interaction between the flavins and the indole derivatives. The results indicate that when the photosensitizer is RF, the photodegradation process in SUV is slower than that in homogeneous media. This result agrees with the low incorporation of RF and the higher incorporation of the more hydrophobic indoles into vesicle. Hence, SUV is an entity that separates both compounds, thus avoiding the reaction. Moreover, when LF is the photosensitizer a higher efficiency was observed with the more hydrophobic indole derivatives (see Table 3).

Table 3: Pseudo first order rate constant (k) for the photodegradation of indolic derivatives sensitized by flavins in homogeneous medium (PBS pH 7.3) and in DPPC SUV. All measurements were performed under N_2 saturated solutions.

Indole	Flavin	$k \cdot 10^2 \text{ min}^{-1}$ PBS	$k \cdot 10^2 \text{ min}^{-1}$ SUV
	RF	0.80	0.70
Trp	LF	1.20	0.40
	TPRF	1.10	2.00
	RF	1.80	1.00
NATE	LF	0.80	1.20
	TPRF	1.36	5.60
	RF	2.00	0.80
Indol-3 acetamide	LF	0.55	0.60
	TPRF	1.60	4.20

When TPRF was used as photosensitizer, we observed that the photodegradation in SUV was greater than that in homogeneous medium for all the indole derivatives. Nevertheless, TPRF was the most photostable flavin derivative when compared with LF and RF in the same microenvironment (13,17). TPRF was localized in the lipid interphase; moreover, the photoprocess is very efficient because the microenvironment increases the triplet lifetime of the flavin (28).

Figure 3 shows the absorption spectra for indol-3-acetamide irradiated in the presence of TPRF in buffer and SUV. An increase in the absorption in the range at 300-400 nm, can be observed, indicating the generation of indol-flavin photoproducts (8).

The results of this study indicate that the behaviour of the indoles and flavins in DPPC SUV is determined by the locations of the compounds in the different microenvironment of the SUV, as is schematized in Figure 4.

According to all these results, we propose that the union of TPRF with the more hydrophobic indole derivatives (NATE and indol-3 acetamide) are the most appropriate systems for the generation of indol-flavin photoproducts, in view of future studies related with their cytotoxic properties.

Figure 3: Photodegradation of indol-3-acetamide in the presence of TPRF after irradiation at 450 nm: A) in PBS pH 7,3 B) in DPPC SUV.

Figure 4: Schematic representation of the location of representative indoles and flavins into SUV.

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