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Fluorescence energy transfer in lipid vesicles. A time-resolved analysis using stretched exponentials

Guy Duportail [a,*], Fabienne Merola [b], Panagiotis Lianos [c]

[a] *Laboratoire de Biophysique, Centre de Recherches Pharmaceutiques, Université Louis Pasteur, B.P. 24, 67401 Illkirch Cedex, France*

[b] *Laboratoire pour l'Utilisation du Rayonnement Electromagnétique (LURE), CNRS-MEN-CEA, Bat. 209-D, Centre Universitaire Paris-Sud, 91405 Orsay, France*

[c] *Physics Section, School of Engineering, University of Patras, 26500 Patras, Greece*

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Abstract

Fluorescence energy transfer in lipid vesicles between *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-labelled phosphatidylethanolamine (acting as donor) and *N*-(lissamine-rhodamine B)-labelled phosphatidylethanolamine (acting as acceptor) was studied by steady state and time-resolved fluorescence quenching analysis. Both fluorescent phospholipids were incorporated as minor components in four different types of lipid vesicle: dipalmitoylphosphatidylglycerol vesicles in their L_β gel phase at 20 °C and in their L_α liquid crystalline phase at 50 °C, and egg yolk phosphatidylethanolamine vesicles at 40 °C in their L_α liquid crystalline phase at pH 9.5 and in their H_{II} inverted hexagonal phase at pH 5.0. The quenching of the donor fluorescence by energy transfer is diffusion controlled in all cases, except in the L_β gel phase. The dimensionality and type of constraints imposed on diffusion are different in each case, with the most efficient diffusion-controlled quenching in the hexagonal phase.

Keywords: Fluorescence energy transfer; Time-resolved fluorescence; Lipid vesicles; Diffusion processes; Lipid polymorphism

1. Introduction

Fluorescence resonance energy transfer has been extensively used in model membrane systems, such as lipid vesicles, and appears to be a powerful tool for obtaining information on membrane biophysics. As an example, resonance energy transfer between *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-labelled phosphatidylethanolamine (NBD-PE) (acting as donor) and *N*-(lissamine-rhodamine B)-labelled phosphatidylethanolamine (Rh-PE) (acting as acceptor) is commonly used. This pair of fluorescent phospholipids, as minor components in lipid bilayers, has been used to monitor the transfer of lipids between vesicles [1] and the lipid mixing during aggregation and fusion processes [2], as well as to determine the transbilayer distribution of lipids [3].

This work essentially deals with a time-resolved study of the fluorescence quenching of NBD-PE by energy transfer to neighbouring Rh-PE. We have registered the fluorescence decay profiles of NBD-PE, incorporated at 1 mol.% into different types of lipid vesicle, in the presence of varying amounts of Rh-PE, and have analysed the data using a model resulting from the theory of random walks in fractal domains. In this model, presented previously [4–7], the fluorescence decay profiles are fitted by stretched exponentials according to the following equation

$$I(t) = I_0 \exp(-k_0 t) \exp(-C_1 t^f + C_2 t^{2f}) \quad (1)$$

where k_0 is the decay rate of the donor in the absence of acceptors and C_1, C_2 and f are constants ($0 < f < 1$). A first-order decay rate is deduced through the following equation

$$K(t) = fC_1 t^{f-1} - 2fC_2 t^{2f-1} \quad (2)$$

It should be noted that an equation similar to Eq. (1) has also been used by Takami and Mataga [8] to analyse energy transfer data between charged porphyrins in surfactant vesicles.

The above model of stretched exponentials was chosen instead of other existing models (e.g. Refs. [9,10]) for the following two reasons: (1) energy transfer in any medium, and particularly in restricted media, is always

* Corresponding author.

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analysed by stretched exponential decay, where the non-integer exponent f is related to dimensionality. Thus $f = 0.5$ for a three-dimensional random distribution of donors and acceptors [11,12]; (2) diffusion-controlled reactions in restricted media should also be modelled by stretched exponentials [4–7,13,14]. As the present work deals with energy transfer between diffusing reactants, the choice of the above model is fully justified.

Several types of phospholipid multilamellar vesicles (MLVs) were studied: dipalmitoylphosphatidylglycerol (DPPG) vesicles either in the lamellar gel phase (L_β) at 20 °C or the lamellar liquid crystalline phase (L_α) at 50 °C, and egg yolk phosphatidylethanolamine (EYPE) vesicles at 40 °C either in the L_α phase at pH 9.5 or the inverted hexagonal phase (H_{II}) at pH 5.0. The major purpose of this study is to obtain an insight on the influence of polymorphism on diffusion processes in model membranes.

2. Materials and experimental details

2.1. Chemicals

Dipalmitoylphosphatidylglycerol, ammonium salt (DPPG) (99% purity grade) and egg yolk phosphatidylethanolamine (EYPE) (98% purity grade) were purchased from Sigma, St. Louis, MO and were used without further purification. *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE) and *N*-(lissamine-rhodamine B sulphonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Rh-PE) were purchased from Molecular Probes, Eugene, OR.

2.2. Preparation of liposomes

MLVs containing 1 mol.% NBD-PE and varying proportions of Rh-PE (0.2, 0.4, 0.8 and 1.2 mol.%) were prepared by mixing phospholipid and probes in a chloroform–methanol solution (9 : 1), followed by solvent rotary evaporation. The vacuum was maintained for 30 min after evaporation to remove any residual solvent. The lipid film was then dispersed in buffer by vigorous vortex shaking for 2 min, at 20 °C and 50 °C for EYPE and DPPG vesicles respectively, thus directly giving MLVs. These vesicles, at a concentration of 2×10^{-4} M in lipids, were used for fluorescence decay measurements about 2 h after preparation. The buffers used were 2 mM N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)/2 mM L-histidine/100 mM NaCl (pH 7.4) for DPPG vesicles and 5 mM borate/150 mM NaCl/0.1 mM ethylenediaminetetraacetic acid (EDTA) (pH 9.5) for EYPE vesicles. In the latter case, a concentrated (1 mM) citric acid solution was used to lower the pH from 9.5 to 5.0 (7 μl was needed for an initial 3 ml volume) as described previously [7].

2.3. Fluorescence measurements

Fluorescence spectra were recorded using an SLM-48000 spectrofluorometer with an excitation wavelength of 455 nm. Fluorescence decay profiles were recorded with the photon-counting technique using synchrotron radiation provided by the Super-ACO of LURE, Orsay, France, as described previously [4,6]. The excitation wavelength was again 455 nm, while the emission was monitored at 525 nm.

3. Results

3.1. Steady state fluorescence quenching

The fluorescence of NBD-PE is very efficiently quenched by energy transfer when this probe is co-solubilized with Rh-PE in lipid vesicles. At 1 mol.% NBD-PE, 1 mol.% Rh-PE is sufficient for almost complete quenching. The fluorescence spectra, obtained by excitation in the absorption band of NBD-PE (455 nm), for the four types of vesicles studied, are shown in Figs. 1–4 representing: (1) DPPG vesicles in the L_β gel phase at 20 °C; (2) DPPG vesicles in the L_α liquid crystalline phase at 50 °C; (3) EYPE vesicles in the L_α phase at 40 °C and pH 9.5; (4) EYPE aggregates in the H_{II} inverted hexagonal phase at 40 °C and pH 5.0. The concentration of the donor was kept at 1 mol.%. The concentration of the acceptor was varied from 0.2 to 1.2 mol.%. In all cases, as expected, the fluorescence of the donor decreased with increasing concentration of the acceptor. The most efficient transfer was observed in the case of inverted hexagonal EYPE

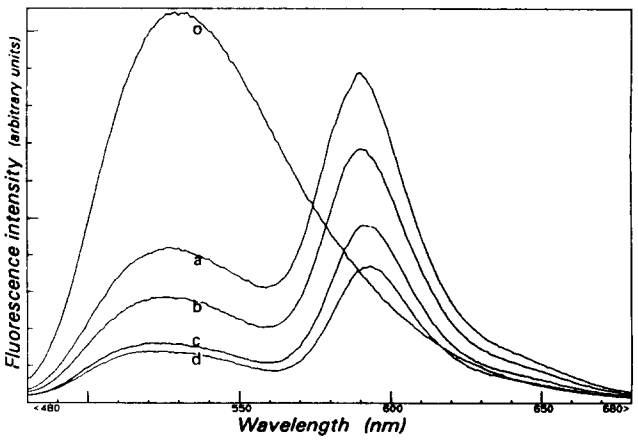

Fig. 1. Fluorescence spectra of DPPG vesicles at 20 °C (case 1), labelled with 1 mol.% NBD-PE and varying concentrations of Rh-PE: (o) 0 mol.%; (a) 0.2 mol.%; (b) 0.4 mol.%; (c) 0.8 mol.%; (d) 1.2 mol.%. Excitation wavelength, 455 nm.

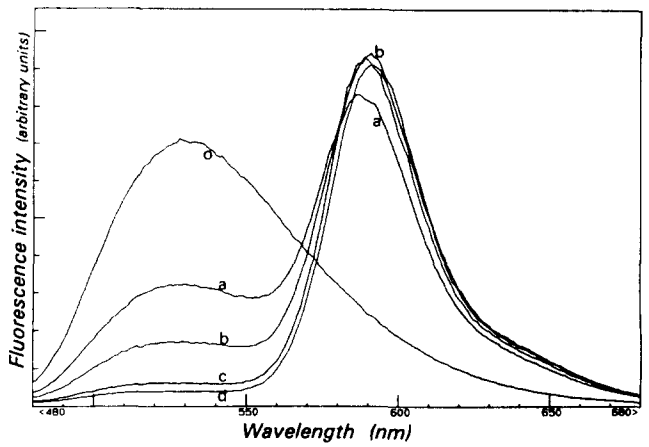

Fig. 2. Fluorescence spectra of DPPG vesicles at 50 °C (case 2). Labelling concentrations and lettering as for Fig. 1.

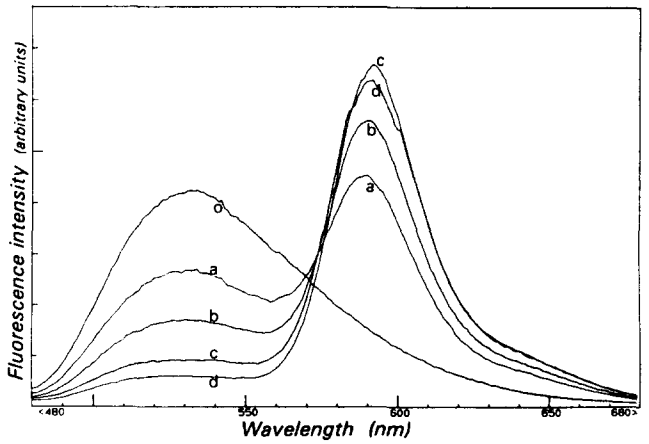

Fig. 3. Fluorescence spectra of EYPE vesicles at 40 °C and pH 9.5 (case 3). Labelling concentrations and lettering as for Fig. 1.

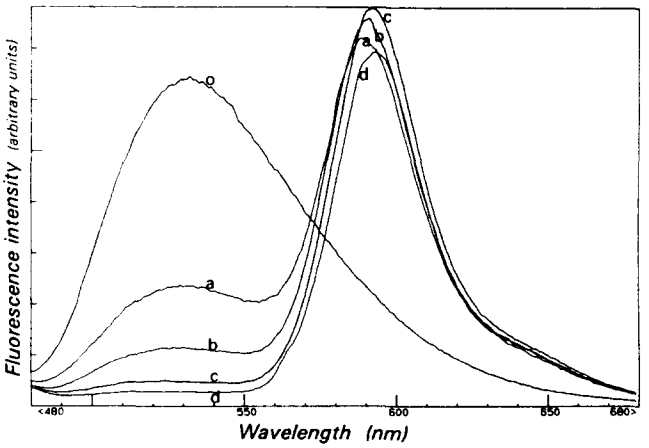

Fig. 4. Fluorescence spectra of EYPE aggregates at 40 °C and pH 5.0 (case 4). Labelling concentrations and lettering as for Fig. 1.

aggregates and liquid crystalline DPPG vesicles. Energy transfer was substantially less efficient in the other two cases. An increase in Rh-PE fluorescence, almost proportional to its concentration, was observed for EYPE vesicles in their fluid lamellar phase (L_α), whereas a decrease was observed in DPPG vesicles in their gel phase. This decrease is probably due to self-quenching of Rh-PE. The other two cases are obviously a mixture of these two extreme behaviours.

3.2. Time-resolved fluorescence data

The analysis of the fluorescence decay profiles of NBD-PE with the help of Eqs. (1) and (2) gave the data presented in Table 1. A model of stretched exponentials should, theoretically, contain an infinite number of terms with non-integer powers of time [7]. However, two or, in some cases, even only one term may be sufficient to fit an experimental decay profile [4–7,15]. Thus the term containing C_1 is a first-order approximation and that containing C_2 is a second-order approximation. The importance of the second-order approximation term is seen by the value of the ratio C_2/C_1, shown in column 2 of Table 1. A second-order approximation is strictly necessary in case (1), i.e. for DPPG vesicles in their gel phase, and negligible in case (2), i.e. for DPPG vesicles in their fluid phase. EYPE vesicles give intermediate values. The values of the non-integer time exponent f were obtained with an accuracy of ± 0.05. As shown in column 3 of Table 1, f is not affected by the Rh-PE concentration, but substantially varies in the four different cases. For EYPE vesicles, it is larger in the hexagonal than in the lamellar phase. For DPPG vesicles, it is larger in the gel than in the liquid crystalline phase. The last three columns of Table 1 show three characteristic values of the rate constant, which is, of course, time

Table 1
Data obtained by analysis of the fluorescence decay profiles using Eqs. (1) and (2) (a_i pre-exponential factors)

Rh-PE concentration (mol.%)	C_2/C_1	f	K_1 (10^7 s^{-1})	K_L (10^7 s^{-1})	K_{AV} (10^7 s^{-1})
Case 1: DPPG vesicles at 20 °C; $\tau_1 = 6.0$ ns ($a_1 = 0.42$), $\tau_2 = 12.6$ ns ($a_2 = 0.58$)					
0.2	112	0.37	142	0	2.7
0.8	107	0.38	130	0	2.5
Case 2: DPPG vesicles at 50 °C; $\tau_1 = 1.7$ ns ($a_1 = 0.06$), $\tau_2 = 5.6$ ns ($a_2 = 0.94$)					
0.2	0	0.20	149	1.3	5
0.8	0	0.22	340	2.6	12
Case 3: EYPE vesicles at pH 9.5; $\tau_1 = 1.8$ ns ($a_1 = 0.09$), $\tau_2 = 4.7$ ns ($a_2 = 0.91$)					
0.2	78	0.27	166	1.6	6.2
0.4	75	0.30	231	2.6	9.5
Case 4: EYPE aggregates at pH 5.0; $\tau_1 = 2.1$ ns ($a_1 = 0.10$), $\tau_2 = 6.1$ ns ($a_2 = 0.90$)					
0.2	50	0.40	158	4.3	12
0.4	50	0.39	268	8.7	20

dependent, as shown in Eq. (2). K_1 is the value corresponding to the first and K_L to the last recorded time channels. K_{AV} is the average over all recorded time channels, 300 in our case (with a linearity of 0.078 ns per channel). K_{AV} values are always found between K_1 and K_L since $K(t)$ decreases smoothly with time. K_1 represents the probability of energy transfer immediately after excitation of NBD-PE. K_L represents the limited value obtained at long times. K_1 is the same, within experimental error, in all four cases when the concentration of Rh-PE is 0.2 mol.%. At higher acceptor concentrations, K_1 increases in all cases except for gel phase DPPG vesicles. K_L varies considerably from one case to another. It is zero in the case of gel phase DPPG vesicles and at a maximum in the case of hexagonal EYPE aggregates. Intermediate values are obtained in the other two cases. It should be noted that the average values are much smaller than K_1 and lie closer to K_L. This means that the energy transfer process is very efficient at short times and decreases rapidly thereafter. This situation is accompanied by relatively low f values, as is the case here. Finally, the values of τ_0 ($=1/k_0$), necessary to exploit Eq. (1), were obtained in the absence of Rh-PE and are also shown in Table 1 as τ_2. In fact, the decay profiles in the absence of acceptors were always found to be biexponential. The shorter component (minor) is also shown in Table 1 as τ_1.

4. Discussion

We first discuss the unquenched NBD-PE decay times, as measured in the absence of Rh-PE. NBD-labelled lipids are versatile fluorescent probes for structural studies of lipid vesicles [16–20]. NBD-PE has also been employed to detect the bilayer-to-hexagonal phase transition [21–24]. The fluorescence intensity of the probe was found to increase during this transition. As shown in Table 1, we have found a concomitant increase in the average decay time for NBD-PE incorporated in EYPE vesicles undergoing this phase transition. In the case of DPPG vesicles, the difference in the decay times between the two phases is more pronounced: the average decay time in the L_β phase is more than twice as high as that in the L_α phase. The lower value obtained at 50 °C can be attributed to the non-radiative de-excitation routes favoured by the higher bilayer fluidity and higher temperature. In our conditions of excitation ($\lambda_{exc}=455$ nm), unquenched NBD-PE does not give a single exponential decay. In all cases studied, the decay profile is composed of a major (τ_2, Table 1) and a minor (τ_1, Table 1) (with a much shorter lifetime) component, as described previously [17,25,26]. The reasons for biexponential decay are not clear, although a red-edge excitation effect has been evoked recently [26], giving good arguments for the existence of a shorter lifetime. As the contribution of this shorter lifetime is much less than that of the longer lifetime, only the latter (τ_2 in Table 1) was used in the analysis with Eq. (1).

We can distinguish three different possibilities of energy transfer in vesicles: (1) transfer between immobile species; (2) diffusion-controlled quenching by energy transfer between an excited donor and an acceptor which has come close enough by diffusion; (3) energy migration among the Rh-PE molecules once they have been excited by transfer from NBD-PE. Of course, these three possibilities can also occur as a mixture of two or more. Inspection of Figs. 1–4 reveals that, in each of the four cases, a different energy transfer process is expected. We believe that in the L_β gel phase of DPPG vesicles (cf. Fig. 1) we have a typical case of rapid energy transfer from NBD-PE to Rh-PE, followed by energy migration among Rh-PE molecules. The migrating energy is lost when a trap (i.e. a favourable non-radiative de-excitation condition) is encountered. Thus the rhodamine fluorescence intensity decreases when the Rh-PE percentage increases (i.e. the fluorescence is self-quenched). This is also reflected in the K values in Table 1 for case 1. $K(t)$ remains unaffected by the Rh-PE concentration, within experimental error. This is a case of quasi-static quenching which should not affect the NBD-PE radiative decay rate. Indeed, $K_L=0$ in case 1. This means that, once transfer from excited NBD-PE to close-lying Rh-PE is accomplished, the rest of the excited donors decay freely by emitting light with a decay time of 12.6 ns. The migration rate and the average number of steps before a trap is encountered can be estimated by analysing the Rh-PE fluorescence. This subject is currently under study.

A typical decay scheme by energy transfer between immobile species is given by the following equation [12,27]

$$I(t)=I_0 \exp(-k_0 t) \exp(-Ct^{D/6}) \quad (3)$$

where C is a constant and D is the dimensionality of the distribution of the reactive species. For a homogeneous distribution, the exponent $D/6$ takes the value of 1/6, 1/3 or 1/2 for one, two or three dimensions. We note that the decay is again expressed with a stretched exponential by using only one single time power. In case 1, it was impossible to fit our decay profiles using such an equation. A second power of time was necessary, as shown by the ratio C_2/C_1 which is highest in case 1. The need for the second term stems from the fact that transfer from NBD-PE to Rh-PE is accomplished between mobile species. The labelled phospholipids may not be allowed to diffuse in gel phase DPPG but they are not immobile. This means that the reaction distance varies during the lifetime of

the NBD excited state. The reaction rate then follows suit. A correction term to Eq. (3) is subsequently necessary [11] and thus Eq. (1) becomes valid. The value of f measured in the case of gel phase DPPG vesicles approaches 1/3, i.e. the two-dimensional case, which is justified by the two-dimensional structure of the lipid bilayer and the two-dimensional lateral displacement.

In case 2, i.e. DPPG vesicles in their L_α liquid crystalline phase, the much higher fluidity allows easy lateral diffusion of lipids. We believe that, in this case, direct transfer to a distance is a much slower process than diffusion. Therefore the reaction is mainly diffusion controlled. The zero C_2 values observed in case 2 can be mistaken as indicating that Eq. (3) applies here. $C_2=0$, however, simply indicates a diffusion-controlled reaction in which the number of available sites for acceptor solubilization is very large compared with the number of acceptors, which is typical of vesicles in the fluid phase [5–7]. The quenching rate is high at short times, but still occurs at long times. This is again a characteristic of diffusion-controlled quenching. Thus $K_L \neq 0$ in case 2. $K(t)$ increases with the acceptor concentration. f is very small in case 2. If a diffusion-controlled reaction is modelled by the mutual random walk of the reacting species, then f is the spectral dimension of the random walk [7]. The low f values simply indicate that the random walk is very restricted. This, in turn, means either that the displacement is very compact (localized), which is unlikely to be the case here, or that the dimensionality of the walk is very low. Our finding therefore suggests that lateral diffusion in case 2 may even be one-dimensional (motion along a line). The type of interaction ascribed to case 2 should create one excited Rh-PE for each quenched NBD-PE. However, the evolution of the Rh-PE fluorescence intensity with the acceptor concentration (see Fig. 2) reveals a rather irregular variation at high concentration. Apparently, significant self-quenching of Rh-PE fluorescence occurs in this case.

Case 3, i.e. EYPE vesicles in their L_α liquid crystalline phase, appears to be a typical diffusion-controlled interaction without (or with only negligible) rhodamine self-quenching. Thus steady state fluorescence data show that approximately one excited acceptor is formed for each quenched donor. Contrary to case 2, C_2 presents a large value in lamellar EYPE vesicles. This and the fact that the average reaction rate is smaller in case 3 than in case 2 may indicate that lateral diffusion is somehow more restricted in EYPE vesicles at 40 °C and pH 9.5 than for DPPG vesicles at 50 °C, although these types of vesicle are both in the L_α phase. The nature of this restriction is not a question of dimensionality, as can be inferred from the fact that f is larger in case 3 than in case 2. It may stem from the existence of hydrogen bonds between the ammonium groups of ethanolamine and the unesterified phosphate oxygens of the adjacent phospholipids, including host probes. This restriction takes place at the polar head level and not at the lipid core level, since fluorescence anisotropy values measured with diphenylhexatriene are found to be similar in both cases ($r=0.066$ for EYPE vesicles at 40 °C; $r=0.062$ for DPPG vesicles at 50 °C), indicating an identical lipid core fluidity.

Finally, case 4, i.e. EYPE aggregates in the inverted hexagonal phase, gives fluorescence spectra similar to those obtained in case 2. We believe that here again we have a diffusion-controlled reaction accompanied by limited Rh-PE self-quenching. However, this case is clearly separated from the other three cases by the higher values of f and $K(t)$. It appears that the dimensionality of the reaction domain is higher in the hexagonal phase EYPE aggregates and the diffusion-controlled reaction is also more efficient, particularly at long times.

5. Conclusions

We have found that fluorescence energy transfer processes in lipid vesicles between two head-labelled phospholipids, namely excited NBD-PE acting as donor and Rh-PE acting as acceptor, can be successfully analysed using a model resulting from the theory of random walks in fractal domains, a model previously used for collisional fluorescence quenching [4,6] and pyrene monomer fluorescence quenching by excimerization [4–7]. The fluorescence energy transfer process was studied in four different lipid vesicles and was found to be diffusion controlled in all cases, except for DPPG vesicles in the L_β gel phase. The lateral diffusion of the labelled phospholipids is more efficient in EYPE aggregates in the inverted H_{II} hexagonal phase. However, characteristic features concerning the nature of diffusion (e.g. dimensionality, type of imposed restrictions) are observed for each case.

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