J. Membrane Biol. 190, 75–82 (2002)
DOI: 10.1007/s00232-002-1025-5

The Journal of
Membrane
Biology

Fluorescent Probes DPH, TMA-DPH and C_{17}-HC Induce Erythrocyte Exovesiculation

C. Saldanha, N.C. Santos, J. Martins-Silva
Instituto de Bioquímica/Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa,
Av. Prof. Egas Moniz, P-1649-028 Lisboa, Portugal

Received: 15 May 2002/Revised: 9 July 2002

Abstract. An experimental approach has been developed to study human erythrocyte vesiculation, using the fluorescent probes diphenylhexatriene (DPH), trimethylamino-diphenylhexatriene (TMA-DPH) and heptadecyl-hydroxycoumarin (C_{17}-HC). Acetylcholinesterase (AChE) enzyme activity measurements confirmed the presence of exovesicles released from erythrocyte membranes labeled with DPH, TMA-DPH or C_{17}-HC. The fluorescence intensity and anisotropy values obtained showed that the amphiphilic probes TMA-DPH and C_{17}-HC are preferentially incorporated in the exovesicles (when compared with DPH). There is a significant decrease of the cholesterol content of the exovesicle suspensions with time, independently of the fluorescence probe used, reaching undetectable cholesterol levels for the samples incubated for 48 hr. The ratios between the concentration of cholesterol released in the exovesicles after 1 hr incubation with DPH, TMA-DPH or C_{17}-HC and the probe concentration used in the incubation were 84.7, 3.82 and 0.074, respectively. The size of the released vesicles was evaluated by dynamic light scattering spectroscopy. Some hypotheses are proposed that could explain the resemblance and differences between the results obtained for erythrocytes labeled with each probe, considering the present knowledge of membrane vesiculation mechanisms, lipid microdomains (rafts), erythrocyte membrane phospholipid asymmetry and AChE inhibition by TMA-DPH and C_{17}-HC. This work demonstrates that the fluorescent probes DPH, TMA-DPH and C_{17}-HC induce rapid erythrocyte exovesiculation; their use can lead to new methodologies for the study of this still poorly understood mechanism.

Key words: Erythrocyte membrane vesicle — Acetylcholinesterase — Membrane fluidity — Lipid microdomains — Cholesterol — Dynamic light scattering

Introduction

Erythrocyte membrane vesiculation occurs during erythrocyte differentiation, under red blood cell senescence and storage of blood samples (Geiduscheck & Singer, 1979; Waugh et al., 1992; Dumaswala et al., 1996). Also a wide variety of in vitro conditions such as ATP depletion, pH and temperature variations, or treatment with amphiphiles induces exovesicle release from erythrocytes (Bütikofer & Ott, 1985; Hägerstrand & Isomaa, 1989; Lelkes & Fodor, 1991). These microvesicles are enriched in acetylcholinesterase (AChE), which has been considered a marker of erythrocyte membrane integrity (Rumsby et al., 1977; Prall, Gambhir & Ampy, 1998). The influence of lipid membrane fluidity, assessed by 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence polarization, on AChE enzyme activity has been reported (Domenech, Domenech & Balegno, 1977; Saldanha, Quintão & Garcia, 1992).

The fluorescent molecules l-(4-(trimethylamino)-phenyl)-6-phenyl-l,3,5-hexatriene (TMA-DPH) and 4-heptadecyl-7-hydroxycoumarin (C_{17}-HC) are both amphiphilic compounds used as structural probes for the region close to lipid-water interface of phospholipid bilayers (Kuhry et al., 1983; Pal et al., 1985). TMA-DPH was also useful as a lipid marker for endocytosis and exocytosis (Kubina et al., 1987), and C_{17}-HC is an alkyl derivative of hydroxycoumarin, useful as a pH probe for the membrane surface (Pal et al., 1983).

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-(trimethylamino)-phenyl)-6-phenyl-1,3,5-hexatriene; 4-heptadecyl-7-hydroxycoumarin; AChE, acetylcholinesterase; DLS, dynamic light scattering; GPI, glycosylphosphatidylinositol; L_o, liquid ordered lipid phase.

Correspondence to: C. Saldanha; email: saldanha@medscape.com

After a fast incorporation in membranes, TMA-DPH remains in a partition equilibrium between the membranes (where it is fluorescent) and the aqueous buffer (where it is not) (Huang & Haugland, 1991). Previously, Kubina et al. (1987) showed that the fluorescence measurements of TMA-DPH could provide simultaneous information about platelet membrane fluidity and exocytosis.

It would be very useful if a similar approach could be applied to erythrocytes and to their vesiculation process, as described above. The aims of this work were: *i*) to develop an experimental approach to study erythrocyte vesiculation using the fluorescent probes DPH, TMA-DPH and C_{17}-HC; *ii*) to study the behavior and effect of these probes on the referred process; *iii*) to compare the membrane fluidity and AChE enzyme activity of the erythrocytes with the same parameters obtained for the isolated exovesicles; *iv*) to determine the effect of the incorporation of the fluorescence probes on the AChE enzyme activity; *v*) to determine the cholesterol content released on the exovesicles due to the incubation with the different membrane probes; and *vi*) to determine the size of the vesicles released during incubation with the different membrane probes.

Materials and Methods

Chemicals

The fluorescent probes 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-(4-(trimethylamino)-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) and 4-heptadecyl-7-hydroxycoumarin (C_{17}-HC) were purchased from Molecular Probes (Eugene, OR). The kit for the spectrophotometric determination of cholesterol concentrations was obtained from Roche Diagnostics (Mannheim, Germany). All other chemicals were from Sigma (St. Louis, MO).

Samples

Human venous blood samples were collected with anticoagulant (10 I.U. of heparin/ml of blood) from 91 healthy male donors, with their previous informed consent, following our protocol with the Portuguese Blood Institute. Freshly collected whole-blood samples were centrifuged for 10 min at $1000 \times g$ in a Sorvall TC6 centrifuge (Du Pont, Bad Nauheim, Germany). Erythrocytes were isolated by plasma and buffy-coat removal, resuspended in phosphate buffer pH 7.4, 155 mM, and divided into aliquots.

Fluorescence Spectroscopy

Erythrocyte suspension aliquots were incubated for 30 min, at room temperature, with the lipophilic fluorescent probes DPH, TMA-DPH or C_{17}-HC. The final total concentrations of DPH, TMA-DPH and C_{17}-HC were 0.22 μM in 0.037% haematocrit, 5.4 μM in 0.01% haematocrit, and 0.11 mM in 0.01% haematocrit, respectively. These values were optimized for the fluorescence measurements in erythrocytes according to the membrane/water partition coefficient and fluorescence quantum yield of each probe. As the fluorescence probes reach an equilibrium between the aqueous and lipid phases, and the unincorporated probes do not fluoresce (Huang & Haugland, 1991), there was no need for a washing procedure to be carried out. Membrane fluidity was assessed by fluorescence anisotropy measurements (for review *see*, e.g., Lentz, 1988). This parameter (r) is defined by the following equation (e.g., Lakowicz, 1999):

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are the fluorescence intensities and the subscripts indicate the vertical (V) or horizontal (H) orientations of the excitation and emission polarizers. $G = I_{HV}/I_{HH}$ is the instrumental factor. All the fluorescence intensity data points used for calculation were the average of two identical aliquots, corrected by blank subtraction. DPH fluorescence measurements were carried out with an excitation wavelength (λ_{exc}) of 352 nm and an emission wavelength (λ_{em}) of 430 nm, TMA-DPH fluorescence measurements with λ_{exc} = 340 nm and λ_{em} = 425 nm, and C_{17}-HC fluorescence measurements with λ_{exc} = 375 nm and λ_{em} = 450 nm. The results were not corrected for turbidity (Lentz et al., 1979) or energy transfer from DPH to hemoglobin (Plášek, Cermáková & Jarolím, 1988), as these experimental artifacts do not differ significantly among the aliquots. Total fluorescence intensity measurements were also carried out for all the samples. All the fluorescence spectroscopy measurements were carried out in a Hitachi F-3000 fluorescence spectrophotometer (Tokyo, Japan), with 5 nm bandwidths on both monochromators. After the erythrocyte membrane fluidity measurements, the aliquots were centrifuged for 10 min at $1000 \times g$ and the measurement procedure was repeated for the supernatants. These supernatant fluorescence measurements were carried out two more times, with identical aliquots centrifuged 24 and 48 hr after the initial incubation.

AChE Activity

AChE activities were measured using a Beckman 35 UV/Vis spectrophotometer (Fullerton, CA) at 37°C, by the colorimetric method proposed by Ellman et al. (1961). The measurements were carried out in the supernatants of aliquots centrifuged 1 hr after erythrocyte incubation at room temperature with the fluorescent probes DPH, TMA-DPH and C_{17}-HC. Enzyme activity data points were calculated with the average absorbance of two identical aliquots, corrected by blank subtraction. AChE activity measurements were also carried out with samples incubated with identical volumes of the organic solvents used for the incorporation of the fluorescent probes DPH, TMA-DPH and C_{17}-HC: acetone, N,N-dimethylformamide and tetrahydrofuran, respectively.

Cholesterol Concentration

Cholesterol concentrations were measured using a specific spectrophotometric/enzymatic kit, based on the technique of Ott, Binggeli & Brodbeck (1982) and adapted to these samples by us. The measurements were carried out in the supernatants of aliquots centrifuged 1, 24 and 48 hr after erythrocyte incubation, at room temperature, with the fluorescent probes DPH, TMA-DPH and C_{17}-HC.

Dynamic Light Scattering Spectroscopy

The dynamic light scattering (DLS) measurements were carried out in a BI-2030AT with a 128-channel digital autocorrelator (Brookhaven Instruments Corporation, Holtsville, NY), equipped with a

Spectra-Physics He-Ne Laser, model 127 (632.8 nm, 35 mW). DLS provides information on the dynamic properties of the scattering molecules or aggregates, on the μsec time scale, by performing an auto-correlation with the scattering intensity data (e.g., Santos & Castanho, 1996; Santos et al., 2002). This technique allows the determination of the diffusion coefficient (D) of the molecule or supramolecular aggregate under evaluation. The values of D can be used to calculate the hydrodynamic radius, R_h, using the Stokes-Einstein equation,

$$D \approx \frac{kT}{6\pi\eta R_h} \tag{2}$$

where k is the Boltzman constant, T the absolute temperature and η the solvent viscosity. Since we are dealing with polydisperse samples, the size distributions of the samples were obtained using the standard CONTIN method (Provencher, 1982a, 1982b), considered to be the most suitable for the study of systems with broad size distributions (its potentialities and drawbacks were tested and reviewed in Johnsen & Brown, 1992). The measurements were carried out with the supernatants of aliquots centrifuged 1, 24 and 48 hr after erythrocyte incubation, at room temperature, with the fluorescent probes DPH, TMA-DPH and C_{17}-HC. The light scattering data were collected at 25°C, using a scattering angle of 90°, a total count above 2×10^7 and a sample time of 50 μsec.

Results

Tables 1 and 2 present the fluorescence intensity and fluorescence anisotropy results, respectively, obtained for DPH, TMA-DPH and C_{17}-HC in erythrocyte suspensions and in their supernatants (centrifugation at 1, 24, and 48 hr after incubation). Two identical aliquots of each one of the N blood samples were used. The mean values ± standard deviations were calculated with the N averages of the two aliquots. For each blood sample, fluorescence intensities were normalized by dividing by the fluorescence intensity measured before centrifugation. Paired samples t-test shows that for supernatants with C_{17}-HC there is a significant ($p < 0.002$) increase of C_{17}-HC fluorescence anisotropy with time (Table 2). The variations of TMA-DPH and C_{17}-HC fluorescence anisotropies of the supernatants are represented in Figure 1.

AChE enzyme activities measured in the supernatants of aliquots centrifuged 1 hr after erythrocyte incubation with DPH, TMA-DPH or C_{17}-HC are shown in Figure 2. Enzyme activities are represented as mean value and standard deviation of 21 blood samples ($N = 21$). Paired samples t-test shows that there is a significant ($p < 0.002$) difference between the values obtained for samples with DPH and the values obtained for samples with TMA-DPH or C_{17}-HC. The supernatants of samples incubated with identical volumes of the organic solvents used for the incorporation of the fluorescent probes (acetone, N,N-dimethylformamide and tetrahydrofuran) showed no AChE activity.

Table 3 presents the cholesterol concentration results obtained for DPH, TMA-DPH and C_{17}-HC in erythrocyte suspensions and in their supernatants

Table 1. Fluorescence intensity results obtained with supernatants of centrifugations carried out 1 hr (t_1), 24 hr (t_{24}) and 48 hr (t_{48}) after incubation of erythrocyte suspensions with DPH, TMA-DPH or C_{17}-HC

Sample	Fluorescence intensity (a.u.)		
	DPH ($N = 39$)	TMA-DPH ($N = 73$)	C_{17}-HC ($N = 34$)
Supernatant t_1	≈0	0.89 ± 0.26	0.56 ± 0.25
Supernatant t_{24}	0.32 ± 0.16	0.81 ± 0.24	0.49 ± 0.25
Supernatant t_{48}	0.23 ± 0.17	0.82 ± 0.23	0.52 ± 0.35

Data are presented as mean value ± standard deviation of the averaged result (two aliquots) of each one of the N blood samples. For each erythrocyte suspension, fluorescence intensities were normalized by dividing by the fluorescence intensity measured before centrifugation.

Table 2. Fluorescence anisotropy results obtained for DPH. TMA-DPH and C_{17}-HC in erythrocyte suspensions and supernatants of centrifugations carried out 1 hr (t_1), 24 hr (t_{24}) and 48 hr (t_{48}) after incubation

Sample	Fluorescence anisotropy		
	DPH ($N = 54$)	TMA-DPH ($N = 34$)	C_{17}-HC ($N = 34$)
Erythrocyte suspension	0.261 ± 0.018	0.331 ± 0.027	0.296 ± 0.045
Supernatant t_1	*	0.338 ± 0.024	0.307 ± 0.080
Supernatant t_{24}	*	0.338 ± 0.023	0.339 ± 0.053
Supernatant t_{48}	*	0.339 ± 0.027	0.359 ± 0.050

* I_{VV}, I_{VH}, I_{HV}, and I_{HH} values too low for anisotropy calculation. Data are presented as mean value ± standard deviation of the averaged result (two aliquots) of each one of the N blood samples.

(centrifugation at 1, 24 and 48 hr after incubation). Statistical analysis shows that there is a significant ($p < 0.0005$) decrease of the cholesterol content of the exovesicle suspensions with time, independently of the fluorescence probe used, and that there are substantial differences between the values obtained for the three probes. As can be seen, the values obtained for t_{48} (independently of the fluorescent probe used to induce the vesiculation) were below the detection threshold of the determination method.

Due to the high relevance of the release of cholesterol to the supernatants, we defined and calculated a vesiculation induction yield, η_{vi}, as the ratio between the molar concentration of cholesterol released in the exovesicles and the total probe molar concentration used in the incubation:

$$\eta_{vi} = \frac{[\text{cholesterol}]_{\text{released}}}{[\text{probe}]_{\text{total}}} \tag{3}$$

The results obtained for this parameter (using each probe for t_1 and t_{24}) are presented in Table 4.

The hydrodynamic diameter maxima of the size distributions (obtained by DLS) of the exovesicles

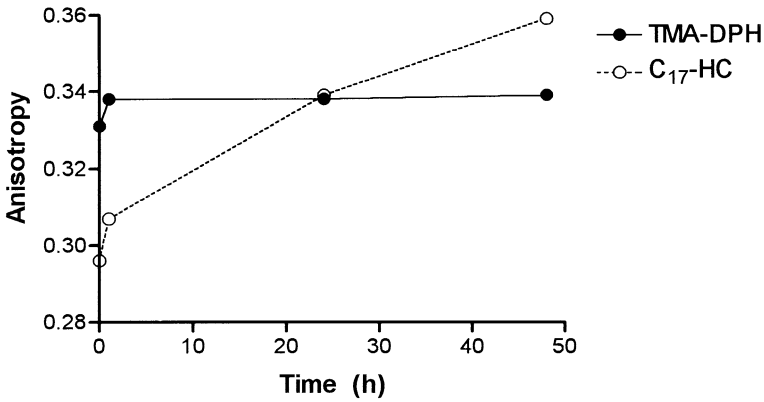

Fig. 1. Variation of TMA-DPH and C_{17}-HC fluorescence anisotropies for erythrocyte suspensions (zero time value) and its supernatants (centrifugation 1, 24, and 48 hr after incubation). Data are presented as mean values of the averaged result (two aliquots) of each one of the $N = 34$ blood samples. Paired samples t-test shows that for supernatants with C_{17}-HC there is a significant ($p < 0.002$) increase of C_{17}-HC fluorescence anisotropy with time.

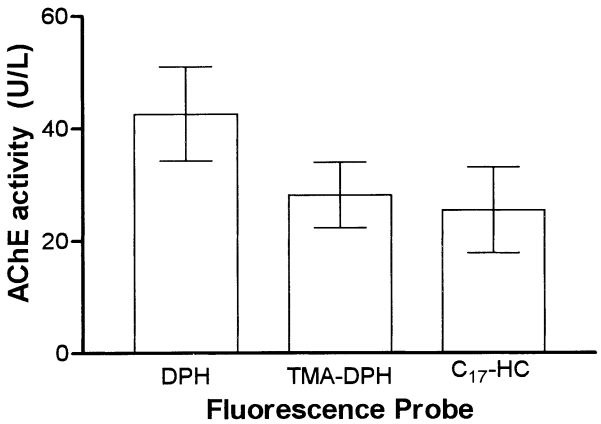

Fig. 2. AChE activities measured in the supernatants of aliquots centrifuged 1 hr after erythrocyte incubation with the lipophilic fluorescent probes DPH, TMA-DPH or C_{17}-HC. Enzyme activity data points were calculated with the average absorbance of two identical aliquots, corrected by blank subtraction, and are represented as mean value and standard deviation of 21 blood samples ($N = 21$). The supernatants of samples incubated with identical volumes of the organic solvents used for the incorporation of the fluorescent probes (acetone, N,N-dimethylformamide and tetrahydrofuran) showed no AChE activity.

Table 3. Cholesterol concentration results obtained for the supernatants of centrifugations carried out 1 hr (t_1), 24 hr (t_{24}) and 48 hr (t_{48}) after incubation of erythrocyte suspensions with DPH, TMA-DPH or C_{17}-HC

Sample	[Cholesterol] (μM)		
	DPH ($N = 18$)	TMA-DPH ($N = 18$)	C_{17}-HC ($N = 18$)
Supernatant t_1	18.6 ± 2.6	20.6 ± 2.5	8.2 ± 2.6
Supernatant t_{24}	18.1 ± 2.6	20.0 ± 2.6	7.4 ± 2.6
Supernatant t_{48}	≈0	≈0	≈0

Data are presented as mean value ± standard deviation of the results obtained for each one of the N blood samples.

Table 4. Vesiculation induction yields η_{vi}

Sample	η_{vi}		
	DPH ($N = 18$)	TMA-DPH ($N = 18$)	C_{17}-HC ($N = 18$)
Supernatant t_1	84.7	3.82	0.0741
Supernatant t_{24}	82.3	3.71	0.0675

Ratios η_{vi} between the concentration of cholesterol released in the exovesicles and the probe concentration used in the incubation obtained for the supernatants of centrifugations carried out 1 hr (t_1) and 24 hr (t_{24}) after incubation of erythrocyte suspensions with DPH, TMA-DPH or C_{17}-HC.

released at t_1 and t_{24}, after incubation with each of the three fluorescence probes, are presented in Table 5. Beside these size distribution peaks, all the samples presented higher-diameter structures that (in contrast to the indicated peaks) were also present in some of the blank suspensions.

Discussion

We have developed an experimental approach to study erythrocyte membrane vesiculation in the presence of the membrane probes DPH, TMA-DPH and C_{17}-HC. The same experiment provides fluorescence intensity and anisotropy values both in erythrocyte suspensions and in its supernatants (Table 2). AChE enzyme activities (Fig. 2), were measured in the supernatants obtained after centrifugation of erythrocyte suspensions labeled with DPH, TMA-DPH or C_{17}-HC. These results, associated with the fluorescence anisotropy and intensity data determined in the same supernatant aliquots, indicated the presence of erythrocyte exovesicles.

As AChE belongs to the glycosylphosphatidylinositol (GPI)-linked protein family (Lisanti, Rodriguez-Boulan & Saltiel, 1990; Hägerstrand & Isomaa, 1991), one of the membrane vesicles' major components, the presence of AChE enzyme activity in the supernatant is a good marker for the exovesiculation process. Unless exovesiculation occurs, AChE can only be removed from the cell membrane by some detergents or phospholipase treatment (Brodbeck,

Table 5. Hydrodynamic diameter maxima of the size distribution obtained by dynamic light scattering (DLS)

Sample	Diameter (nm)		
	DPH	TMA-DPH	C_{17}-HC
Supernatant t_1	83	57	58
Supernatant t_{24}	129	139	99

Maxima for the supernatants of centrifugations carried out 1 hr (t_1) and 24 hr (t_{24}) after incubation of erythrocyte suspensions with DPH, TMA-DPH or C_{17}-HC. The typical coefficient of variation for these parameters is 5%.

Gentinetta & Ott, 1981; Richier, Arpagaus & Toutant, 1992). The presence of AChE activity in the supernatants after incubation of erythrocyte suspensions with DPH (at variance with the control samples) indicated that a vesiculation process had been occurring. However, the DPH fluorescence intensities in these samples were too low for fluorescence anisotropy evaluation, indicating that the exovesiculation process does not favor DPH partition to the released vesicles. On the contrary, the supernatants of erythrocyte suspensions incubated with TMA-DPH or C_{17}-HC showed high normalized fluorescence intensities. These results demonstrate that the amphiphilic probes TMA-DPH or C_{17}-HC, when compared with DPH, have higher partition coefficients (as previously defined, Santos, Prieto & Castanho, 1998) between the exovesicles and the remaining erythrocyte membrane, i.e., these probes incorporate more extensively in the exovesicles. Due to their polar groups (Fig. 3), after incorporation in a lipid bilayer, TMA-DPH or C_{17}-HC will be located in a shallow position, near the lipid-water interface. At variance, DPH molecules are incorporated in the highly disordered region near the center of the bilayer (Cranney et al., 1983). It could be assumed that the deeper location of DPH may be related with its different behavior. However, despite the erythrocyte vesiculation process being far from being completely understood (e.g., Civenni et al., 1998), the most probable scenario is that the depth of the probe has no relevance for the exovesiculation process. Kubina et al. (1987) explained a similar behavior, in platelet exovesicles induced by thrombin, as being a consequence of the permeant behavior of DPH and its incorporation in the granules inside the cell.

It is generally accepted that erythrocyte vesiculation involves the detachment of the membrane from the cytoskeleton, leading to the release of exovesicles enriched in endogenous GPI-linked proteins, such as AChE, in a higher proportion than transmembrane proteins (e.g., de Jong et al., 1996). Recently, radioisotope-labeling results (Civenni et al., 1998) proved to be a serious setback to the idea that the high mobility of GPI-linked proteins allows them to move to the membrane spicules that lead to the release of a exovesicle. Alternatively, the enrichment in AChE

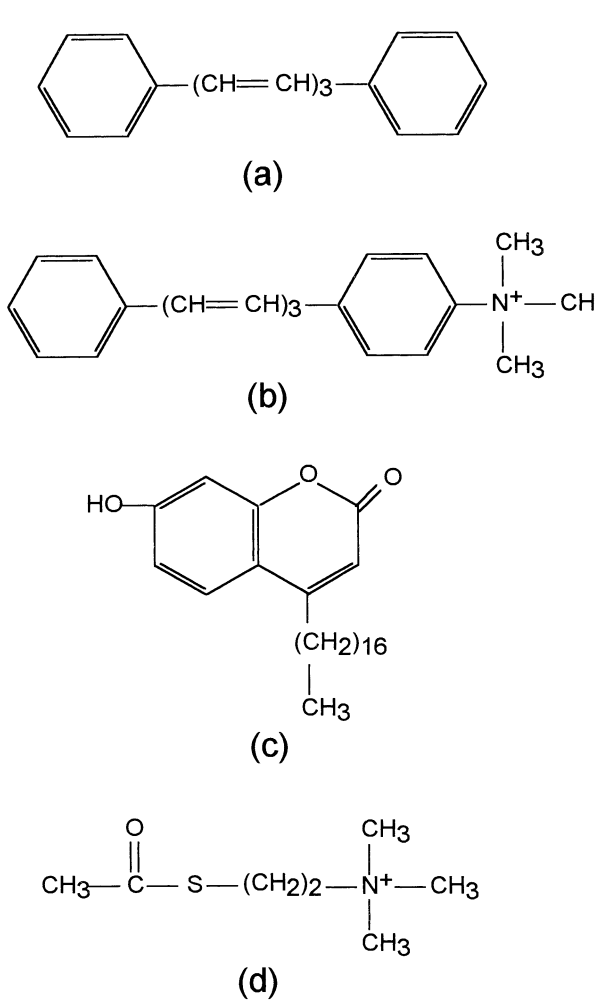

Fig. 3. Molecular structures of: (*a*) 1,6-diphenyl-1,3,5-hexatriene (DPH); (*b*) 1-(4-(trimethylamino)-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH); (*c*) 4-heptadecyl-7-hydroxycoumarin (C_{17}-HC); (*d*) acetylcholine.

can be related to the pre-existence of erythrocyte membrane domains (or lipid rafts) in the liquid ordered (L_o) state, with a higher surface concentration of GPI-linked proteins (for review *see*, e.g., Edidin, 1997; Rietveld & Simons, 1998; London & Brown, 2000). Depending on the size of these microdomains (a question that is yet to be clarified, e.g., London & Brown, 2000), erythrocyte exovesicles may be released from large lipid domains or include a higher proportion of small lipid rafts than that existing on the native erythrocyte membrane. Considering the already accepted involvement of lipid rafts on endovesiculation processes (e.g., Rietveld & Simons, 1998), their involvement in the formation of exovesicles deserves some attention. According to this hypothesis, our results could be explained by a higher partition coefficient of TMA-DPH and C_{17}-HC to those domains.

As can be seen in Table 2, the values of fluorescence anisotropy obtained with TMA-DPH were

higher than those determined with DPH (both molecules have similar chromophores and excited state lifetimes), which characterizes a membrane region with a higher degree of order (e.g., Donner & Stoltz, 1985). Attending to their structures (Fig. 3), these results are in agreement with the shallower location of TMA-DPH chromophore, due to its charged trimethylammonium group fixed to the phospholipid polar head and the nonpolar DPH moiety inserted in the hydrophobic domain of phosphoglycolipid (Prendergast, Haugland & Callahan, 1981).

The supernatants of aliquots incubated with TMA-DPH have higher normalized fluorescence intensity values than those obtained for aliquots incubated with C_{17}-HC (Table 1). These results indicate either a lower C_{17}-HC partition coefficient for the released vesicles or different microvesicle populations. C_{17}-HC, anchored by its hydrocarbon chain in the hydrophobic phospholipid region, due to its fluorescent coumarin moiety, is also used to study the organization of the phospholipid polar region near the lipid-water interface (Pal et al., 1985). There is evidence that C_{17}-HC has almost no effect on the acyl chains of the phospholipids in the cell membrane, with its chromophore inserted in the head-group region of the bilayer, at variance with the chromophore of TMA-DPH residing in the hydrocarbon region of the bilayer interface (Pal et al., 1985). A lower C_{17}-HC quantum yield when inserted in the vesicle membrane, due to a higher exposure to the bulk aqueous phase (Pal et al., 1985), can also explain the lower fluorescence intensity values obtained in the supernatant aliquots. This higher exposure can result from the increased curvature of the vesicle membrane.

As presented in Table 2 and Fig. 1, there is a significant increase in C_{17}-HC fluorescence anisotropy with time, without a concomitant increase in TMA-DPH anisotropy. It is to be noted that although C_{17}-HC ionization is strongly affected by small changes in the pH of the solution (Pal et al., 1983), its fluorescence anisotropy is not significantly affected by the degree of ionization of the probe (Pal et al., 1985). Thus, the pH decrease to be expected with time (due to lactate production by the erythrocytes) cannot be the cause of this effect on C_{17}-HC anisotropy. Taking together, the increase in C_{17}-HC fluorescence anisotropy and the constant values obtained for TMA-DPH, suggest that with longer periods before supernatant separation, the membranes of the exovesicles obtained present a higher degree of order at the lipid-water interface (at the level of the polar headgroups), without any significant change at the level of the hydrocarbon chains (even near the interface). The results can also be explained by the existence of two vesicle populations or different lipid domains on the vesicles, with the prevalence of the most ordered ones at longer vesiculation times, as long as C_{17}-HC is present in both populations and TMA-DPH in only one of them. This hypothesis is in agreement with the finding that in some types of vesicles the phospholipid asymmetry between inner and outer monolayers, characteristic of erythrocyte membranes, is preserved (e.g., de Jong, Beleznay & Ott, 1986), whereas in other types of erythrocyte vesicle there is a scrambling of the phospholipids (e.g., Dressler et al., 1984). A slower lateral diffusion and incorporation of C_{17}-HC in L_o lipid domains or in the annular phospholipids surrounding the membrane proteins (with a concomitant increase on fluorescence anisotropy) can also explain the results presented in Fig. 1.

The hypothesis of the existence of different vesicle populations when longer vesiculation times are used (as mentioned above), is reinforced by the dynamic light scattering data. As can be seen in Table 5, the diameters calculated for t_{24} samples are clearly higher than those obtained for t_1, independently of the probe used to induce the vesiculation. However, there are also significant differences between the diameters calculated for each probe.

As depicted in Fig. 2, the presence of AChE enzyme activity in the supernatants of erythrocyte suspensions incubated with DPH, TMA-DPH or C_{17}-HC (and not in the supernatants of control samples incubated with identical volumes of the organic solvents used for the incorporation of the fluorescent probes), indicates that all these probes trigger a vesiculation process, despite of their low (DPH) or high (TMA-DPH and C_{17}-HC) partition to the exovesicle. From these and other results it can be reasoned that erythrocyte vesiculation is nonspecifically triggered by most of the alterations on the native erythrocyte membrane composition, such as the incorporation of exogenous molecules or the impairment of the native phospholipid asymmetry between inner and outer membrane monolayers associated with the ageing process.

The increased values of AChE enzyme activity of DPH incubation supernatants (Fig. 2), when compared with those with the TMA-DPH and C_{17}-HC, can either result from: (*i*) a higher degree of vesiculation in samples incubated with DPH, (*ii*) a higher enrichment in AChE of the vesicles released due to the incubation with DPH, or (*iii*) the inhibition of AChE enzyme activity by the two amphiphilic probes (TMA-DPH may act as an AChE inhibitor due to its structural resemblance with acetylcholine, as it can be depicted in Fig. 3). Other authors had previously demonstrated the inhibition of AChE activity by another coumarin derivative (Crone, 1974) and by phenyltrimethylammonium groups attached to Sepharose 4B (Rosenberry, Chang & Chen, 1972).

As can be seen on Tables 3 and 4, the amount of cholesterol released in the exovesiculation process (considering either the cholesterol concentration or the η_{vi}) is considerably different depending on the probe used to induce the release. These differences cannot be

solely explained by the different membrane/water partition coefficients of the probes (*data not shown*). Independently of the probe used, there is also a decrease in the cholesterol level of the supernatants with time, with no concomitant variation of the fluorescence intensities. Further experiments are being carried out to explain this behavior and to relate it with the preexistence of cholesterol-rich membrane microdomains.

In conclusion, with our work we have developed an experimental approach to study erythrocyte vesiculation, using the fluorescent probes DPH, TMA-DPH and C_{17}-HC. Acetylcholinesterase enzyme activities of the supernatants were measured, confirming the presence of exovesicles released from erythrocyte membrane labeled with the fluorescent probes DPH, TMA-DPH and C_{17}-HC, indicating that these probes can be used to induce erythrocyte vesiculation. The fluorescence intensity and anisotropy values obtained in the exovesicles showed that, when compared with DPH, the amphiphilic fluorescence probes TMA-DPH and C_{17}-HC have higher partition coefficients between the vesicle and erythrocyte membranes. However, the fluorescence, dynamic light scattering and cholesterol concentration data also indicated that there are significant differences between the behavior of TMA-DPH and C_{17}-HC in terms of incorporation on the erythrocyte vesicle membrane and in the dimensions of the released vesicles.

The authors thank Prof. Miguel A. R. B. Castanho (University of Lisbon and Technical University of Lisbon) and Dr. Luís M. S. Loura (University of Évora and Technical University of Lisbon) for reading the typescript and for valuable discussions, and to Mrs. Teresa Santos for her excellent technical assistance. The authors also acknowledge the use of the light scattering spectroscopy facilities of the Centro de Química-Física Molecular (Instituto Superior Técnico, Technical University of Lisbon). This work was supported by the Fundação para a Ciência e Tecnologia (FCT) of the Portuguese Ministry of Science and Technology (MCT) and by the Calouste Gulbenkian Foundation (Portugal).

References

Brodbeck, U., Gentinetta, R., Ott, P. 1981. Purification by affinity chromatography of red blood cell membrane acetylcholinesterase. *In:* Membrane proteins. A laboratory manual. A. Azzi, U. Brodbeck, P. Zhaler, editors, pp. 85–96. Springer-Verlag, Berlin

Bütikofer, P., Ott, P. 1985. The influence of cellular ATP levels on dimyristoylphosphatidylcholine-induced release of vesicles from human erythrocytes. *Biochim. Biophys. Acta* **821:**91–96

Civenni, G., Test, S.T., Brodbeck, U., Bütikofer, P. 1998. In vitro incorporation of GPI-anchored proteins into human erythrocytes and their fate in the membrane. *Blood* **91:**1784–1792

Cranney, M., Cundall, R.B., Jones, G.R., Richards, J.T., Thomas, I.W. 1983. Fluorescence lifetime and quenching studies on some interesting diphenylhexatriene membrane probes. *Biochim. Biophys. Acta* **735:**418–425

Crone, H.D. 1974. Can allosteric effectors of acetylcholinesterase control the rate of ageing of the phosphorylated enzyme? *Biochem. Pharmacol.* **23:**460–463

de Jong, K., Beleznay, Z., Ott, P. 1996. Phospholipid asymmetry in red blood cells and spectrin-free vesicles during prolonged storage. *Biochim. Biophys. Acta* **1281:**101–110

Domenech, C., Domenech, E.E.M., Balegno, H.F. 1977. Pesticide action and membrane fluidity. Allosteric behavior of rat erythrocyte membrane-bound acetylcholinesterase in the presence of organophosphorous compounds. *FEBS Lett.* **74:**243–246

Donner, M., Stoltz, J.F. 1985. Comparative study on fluorescent probes distributed in human erythrocytes and platelets. *Biorheology* **22:**385–397

Dressler, V., Haest, C.W., Plasa, G., Deuticke, B., Erusalimsky, J.D. 1984. Stabilizing factors of phospholipid asymmetry in the erythrocyte membrane. *Biochim. Biophys. Acta* **775:**189–196

Dumaswala, U.J., Dumaswala, R.U., Levin, D.S., Greenwalt, T.J. 1996. Improved red blood cell preservation correlates with decreased loss of bands 3, 4.1, acetylcholinesterase, and lipids in microvesicles. *Blood* **87:**1612–1616

Edidin, M. 1997. Lipid microdomains in cell surface membranes. *Curr. Opin. Struct. Biol.* **7:**528–532

Ellman, G.L., Courtney, K.D., Andres Jr., V., Featherstone, R.M. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7:**88–95

Geiduscheck, J.B., Singer, S.J. 1979. Molecular changes in the membranes of mouse erythroid cells accompanying differentiation. *Cell* **16:**149–163

Hägerstrand, H., Isomaa, B. 1989. Vesiculation induced by amphiphiles in erythrocytes. *Biochim. Biophys. Acta* **982:**179–186

Hägerstrand, H., Isomaa, B. 1991. Amphiphile-induced antihaemolysis is not causally related to shape changes and vesiculation. *Chem. Biol. Interact.* **79:**335–347

Huang, Z., Haugland, R.P. 1991. Partition coefficients of fluorescence probes with phospholipid membranes. *Biochem. Biophys. Res. Commun.* **181:**166–171

Johnsen, R., Brown, W. 1992. An overview of current methods of analyzing QLS data. *In:* Laser light scattering in biochemistry. S.E. Harding, D.B. Sattelle, V.A. Bloomfield, editors, pp. 77–91. The Royal Society of Chemistry, Cambridge

Kubina, M., Lanza, F., Cazenave, J.-P., Laustriat, G., Kuhry, J.-G. 1987. Parallel investigation of exocytosis kinetics and membrane fluidity changes in human platelets with the fluorescent probe, trimethylammonio-diphenyl hexatriene. *Biochim. Biophys. Acta* **901:**138–146

Kuhry, J.-G., Fontenean, P., Puportaril, G., Maechling, C., Laustriat, G. 1983. TMA-DPH: a suitable fluorescence polarization probe for specific plasma membrane fluidity studies in intact living cells. *Cell Biophys.* **5:**129–140

Lakowicz, J.R. 1999. Principles of fluorescence spectroscopy, 2nd edn. Kluwer Academic/Plenum Publishers, New York

Lelkes, G., Fodor, I. 1991. Formation of large, membrane skeleton-free erythrocyte vesicles as a function of the intracellular pH and temperature. *Biochim. Biophys. Acta* **1065:**135–144

Lentz, B.R. 1988. Membrane "fluidity" from fluorescence anisotropy measurements. *In:* Spectroscopic membrane probes, vol. I. M. Loew editor, pp. 13–41 CRC Press, Boca Raton, Florida

Lentz, B.R., Moore, B.M., Barrow, D.A. l979. Light-scattering effects in the measurement of membrane microviscosity with diphenylhexatriene. *Biophys. J.* **25:**489–494

Lisanti, M.P., Rodriguez-Boulan, E., Saltiel, A.R. 1990. Emerging functional roles for the glycosyl-phosphatidylinositol membrane protein anchor. *J. Membrane Biol.* **117:**1–10

London, E., Brown, D.A. 2000. Insolubility of lipids in Triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts). *Biochim. Biophys. Acta* **1508:**182–195

Ott, P., Binggeli, Y., Brodbeck U. 1982. A rapid and sensitive assay for determination of cholesterol in membrane lipids extracts. *Biochim. Biophys. Acta* **685:**211–213

Pal, R., Petri Jr., W.A., Barenholz, Y., Wagner, R.R. 1983. Lipid and protein contributions to the membrane surface potential of vesicular stomatitis virus probed by a fluorescent pH indicator, 4-heptadecyl-7-hydroxycoumarin. *Biochim. Biophys. Acta* **729:** 185–192

Pal, R., Petri Jr., W.A., Ben-Yashar, V., Wagner, R.R., Barenholz, Y. 1985. Characterization of the fluorophore 4-heptadecyl-7-hydroxycoumarin: a probe for the head-group region of lipid bilayers and biological membranes. *Biochemistry* **24:**573–581

Plášek, J., Cermáková, D., Jarolím, P. 1988. Fluidity of intact erythrocyte membranes. Correction for fluorescence energy transfer from diphenylhexatriene to hemoglobin. *Biochim. Biophys. Acta* **941:**119–122

Prall, Y.G., Gambhir, K.K., Ampy, F.R. 1998. Acetylcholinesterase: an enzymatic marker of human red blood cell aging. *Life Sci.* **63:**177–184

Prendergast, F.G., Haugland, R.P., Callahan, P.J. 1981. l-4-(trimethylamino)phenyl-6-phenylhexa-1,3,5-triene: synthesis, fluorescence properties and use as a fluorescence probe of lipid bilayers. *Biochemistry* **20:**7333–7338

Provencher, S.W. 1982a. A constrained regularization method for inverting data represented by linear algebraic or integral equations. *Comput. Phys. Commun.* **27:**213–227

Provencher, S.W. 1982b. CONTIN: a general purpose constrained regularization program for inverting noisy algebraic and integral equations. *Comput. Phys. Commun.* **27:**229–242

Richier, P., Arpagaus, M., Toutant, J.P. 1992. Glycolipid-anchored acetylcholinesterase from rabbit lymphocytes and erythrocytes differ in their sensitivity to phosphatidylinositol-specific phospholipase C. *Biochim. Biophys. Acta* **1112:**83–88

Rietveld, A., Simons, K. 1998. The differential miscibility of lipids as the basis for the formation of functional membrane rafts. *Biochim. Biophys. Acta* **1376:**467–479

Rosenberry, T.L., Chang, H.W., Chen, Y.T. 1972. Purification of acetylcholinesterase by affinity chromatography and determination of active site stoichiometry. *J. Biol. Chem.* **247:**1555–1565

Rumsby, M.G., Trotter, J., Allan, D., Michell, R.H. 1977. Recovery of membrane micro-vesicles from human erythrocytes stored for transfusion: a mechanism for the erythrocyte discocyte-to-spherocyte shape transformation. *Biochem. Soc. Trans.* **5:**126–128

Saldanha, C., Quintão, T., Garcia, C. 1992. Evaluation of bloodesterase in Alzheimer's disease. *Acta Med. Port.* **5:**591–593

Santos, N.C., Castanho M.A.R.B. 1996. Teaching light scattering spectroscopy: the dimension and shape of tobacco mosaic virus. *Biophys. J.* **71:**1641–1650

Santos, N.C., Prieto, M., Castanho, M.A.R.B. 1998. Interaction of the major epitope region of HIV protein gp41 with membrane model systems. A fluorescence spectroscopy study. *Biochemistry* **37:**8674–8682

Santos, N.C., Silva, A.C., Castanho, M.A.R.B., Martins-Silva, J., Saldanha, C. 2002. Evaluation of lipopolysaccharide aggregation by light scattering spectroscopy. *Chem. Biochem.* in press

Waugh, RE., Narla, M., Jackson, C.W., Mueller, T.J., Suzuki, T., Dale, G.L. 1992. Rheologic properties of senescent erythrocytes: loss of surface area and volume with red blood cell age. *Blood* **79:**1351–1358