J Membrane Biol (2009) 227:39–48
DOI 10.1007/s00232-008-9142-4

Resistance of Human Erythrocyte Membranes to Triton X-100 and $C_{12}E_8$

Cleyton Crepaldi Domingues · Annarita Ciana · Armando Buttafava · Cesare Balduini · Eneida de Paula · Giampaolo Minetti

Received: 12 September 2008 / Accepted: 11 November 2008 / Published online: 9 December 2008

Abstract Lipid rafts are microdomains enriched in cholesterol and sphingolipids that contain specific membrane proteins. The resistance of domains to extraction by non-ionic detergents at 4°C is the commonly used method to characterize these structures that are operationally defined as detergent-resistant membranes (DRMs). Because the selectivity of different detergents in defining membrane rafts has been questioned, we have compared DRMs from human erythrocytes prepared with two detergents: Triton X-100 and $C_{12}E_8$. The DRMs obtained presented a cholesterol/protein mass ratio three times higher than in the whole membrane. Flotillin-2 was revealed in trace amounts in DRMs obtained with $C_{12}E_8$, but it was almost completely confined within the DRM fraction with Triton X-100. Differently, stomatin was found distributed in DRM and non-DRM fractions for both detergents. We have also measured the order parameter (*S*) of nitroxide spin labels inserted into DRMs by means of electron paramagnetic resonance. The 5- and 16-stearic acid spin label revealed significantly higher *S* values for DRMs obtained with either Triton X-100 or $C_{12}E_8$ in comparison to intact cells, while the difference in the *S* values between Triton X-100 and $C_{12}E_8$ DRMs was not statistically significant. Our results suggest that although the acyl chain packing is similar in DRMs prepared with either Triton X-100 or $C_{12}E_8$ detergent, protein content is dissimilar, with flotillin-2 being selectively enriched in Triton X-100 DRMs.

Keywords Detergent-resistant membrane · Lipid raft · Membrane-skeleton · EPR · Nitroxide spin label · Flotillin

C. Crepaldi Domingues · E. de Paula
Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, SP, Brazil

A. Ciana · C. Balduini · G. Minetti (✉)
Dipartimento di Biochimica "A. Castellani", Università di Pavia, Via Bassi, 21, 27100 Pavia, Italy
e-mail: minetti@unipv.it

A. Buttafava
Dipartimento di Chimica Generale, Università di Pavia, Pavia, Italy

Introduction

Lipid rafts are transient membrane microdomains observed in vivo that are rich in cholesterol, sphingolipids, and specific membrane proteins (Simons and Ikonen 1997; Brown and London 1998; Simons and Vaz 2004; Pike 2004; Lichtenberg et al. 2005). These membrane rafts, which exist in a liquid-ordered phase (Lichtenberg et al. 2005; Delaunay et al. 2007), have been implicated in numerous cellular functions, such as signal transduction, endocytosis, parasite or virus infection, and vesicular and cholesterol trafficking (Samuel et al. 2001; Haldar et al. 2002; de Gassart et al. 2003; Pike 2004, 2006).

In the last few years, different techniques have been suggested to study lipid rafts, but the vast majority of reports describing the properties of these structures are based on methods that involve their extraction with non-ionic detergents at 4°C (Brown and Rose 1992; Schuck et al. 2003; Pike 2004). In this way, the operational definition for rafts as detergent-resistant membranes (DRMs) has been widely accepted. While, on the one hand, the detergent-soluble membrane fraction obtained by this treatment is enriched in phospholipids containing unsaturated acyl chains, on the other, DRMs are enriched in sphingolipids that, because of their saturated acyl chains,

strongly contribute to a more organized bilayer environment.

Although different studies with DRMs from erythrocyte cells have been reported (Samuel et al. 2001; Salzer and Prohaska 2001; Nagao et al. 2002; Salzer et al. 2002; Murphy et al. 2004, 2007; Ciana et al. 2005), the association of rafts with membrane-skeletal proteins, the cholesterol regulation of the raft's protein content, and the role of rafts in erythrocyte function are topics that remain unclear (Grzybek et al. 2006). An optimized method to prepare DRMs from ghost membranes has been described by our group (Ciana et al. 2005), and these results strongly suggest the association of DRMs to the erythrocyte membrane-skeleton. More recently, the contribution of rafts to Gsα-mediated signal transduction in erythrocyte membranes has been evidenced (Kamata et al. 2008).

Qualitative and quantitative differences in the properties of DRMs are observed by the use of different detergents (Schuck et al. 2003; Pike 2004). Nonionic detergents are very useful in membrane studies because of their capacity to solubilize the lipid bilayer without affecting important structural features, and Triton X-100 is a classical nonionic detergent used in the majority of DRM studies so far. In the present study, we have isolated, characterized, and compared DRMs obtained from human erythrocytes with two nonionic detergents, Triton X-100 and $C_{12}E_8$. The use of $C_{12}E_8$, in particular, is very common for the solubilization, crystallization, and reconstitution of membrane proteins (Rigaud et al. 1995; le Maire et al. 2000; Santos et al. 2002). It has been used for extracting erythrocytes' band 3 because of its capacity to preserve the in vivo oligomeric state of the protein (Casey and Reithmeier 1991). Furthermore, the lytic effect of the $C_{12}E_8$ occurs in the same range of that of Triton X-100 because their physicochemical properties, such as molecular weight, critical micellar concentration, and hydrophilic/lipophilic balance, are very similar (Preté et al. 2002). We show here that flotillin-2 was found only in the DRMs obtained with Triton X-100.

Moreover, to our knowledge, for the first time, electron paramagnetic resonance (EPR) was used to characterize purified DRMs (isolated in a sucrose gradient) obtained from erythrocytes. The results indicate that the acyl chain packing is similar in DRMs prepared with the two detergents.

Methods

Preparation of Erythrocyte Membranes

Blood was collected from normal human donors after informed consent was obtained. Blood was mixed with 0.1 volumes of 3.8% (*w/v*) sodium citrate. After centrifugation at 1000*g*, the plasma was removed and the packed cells were suspended in an equal volume of phosphate-buffered saline (5 mM Na-phosphate, 154.5 mM NaCl, 4.5 mM KCl, 300 mOsmol/kg H_2O, pH 7.4). The suspension was then filtered through α-cellulose/microcrystalline cellulose to isolate erythrocytes from platelets and leukocytes (Beutler et al. 1976). The purified erythrocyte suspension was washed three times in phosphate-buffered saline, and the packed cells were used to prepare DRMs. For the preparation of ghost membranes, the purified erythrocytes were mixed with 10 volumes of hypotonic phosphate buffer (5 mM Na-phosphate, 0.5 mM EDTA, 5 mM di-isopropyl fluorophosphate, pH 8.0) at 4°C, followed by centrifugation at 26,000*g*; the supernatant was discharged and the washing repeated four times with the same buffer, according to Dodge et al. (1963).

Preparation of DRMs

DRMs were prepared either from intact erythrocytes or ghost membranes following a previously detailed protocol (Ciana et al. 2005). Briefly, the packed cells (approximately 1.25×10^9 cells) were incubated with TNE buffer (25 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4) containing Triton X-100 or $C_{12}E_8$, so that the final volume was 0.625 ml and the final detergent concentration 16 mM, and the detergent/lipid molar ratio approximately 12. After 30 min, the sample was mixed with an equal volume of 80% sucrose solution in TNE containing 0.3 M Na_2CO_3. The sample was then transferred to ultracentrifuge tubes, and 2.5 ml of a 30% sucrose solution in TNE was gently layered on top of the sample, followed by 1.25 ml of a 5% sucrose solution in TNE. All procedures were conducted at 4°C, and the samples were subjected to ultracentrifugation in a bench-top ultracentrifuge (Optima Max, equipped with a swinging-arm MLS50 rotor, Beckman Coulter, Milan, Italy) at 225,000g_{max} for 16 h at 4°C. The top 1 ml was collected as the first fraction. Proceeding down the gradient, fractions 2–6 were collected as 0.8-ml aliquots and saved for subsequent characterization. Fractions 2 and 3 contain the low-density material corresponding to DRMs. DRMs from ghost membranes were prepared by using the same protocol; samples of ghost membranes were treated with the detergent so that the final protein concentration was set to 1.0 mg/ml.

Cholesterol was quantified with a colorimetric assay kit (N. 10139050035, R-Biopharm Italia Srl, Milan, Italy), directly on a suitable dilution of ghost membranes and DRMs, and after lipid extraction of whole erythrocytes (Rose and Oklander 1965). Protein content was determined with bicinchoninic acid (Pierce Biotechnology, Rockford, IL) with bovine serum albumin used as a standard.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Testing

Samples were separated in 10% SDS-PAGE gels according to Laemmli (1970). The gels were either stained with Coomassie Brilliant Blue or electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 1 h in TNE containing 5% skim milk and 0.05% Tween-20, then incubated with the relevant primary antibody: mouse monoclonal anti-flotillin-2 (BD Italia, Milan, Italy), goat polyclonal anti-human stomatin and mouse monoclonal (BRIC 10) anti-human glycophorin C (GPC) (Santa Cruz Biotechnology, Heidelberg, Germany), mouse monoclonal anti-human band 3 (Sigma-Aldrich, Milan, Italy), and mouse polyclonal anti-protein 4.1 (Abnova Corporation, Taipei, Taiwan). The membranes were then washed and incubated with the appropriate peroxidase-conjugated secondary antibody; proteins were revealed with the Amersham ECL Western blotting kit (GE Healthcare, Milan, Italy). For the quantitative Western blot technique described in Fig. 5, densitometric quantification of the protein bands on digital images of the photographic films was performed by the software Scion Image (Scion Corporation, Frederick, MD).

EPR Experiments

The *n*-doxyl-stearic acid spin labels, SASL probes (Sigma Chemical Co., St. Louis, MO) with $n = 5$ or 16, were incorporated up to approximately 2 mol% of lipids into the DRM fractions by 30-min incubation at room temperature. The EPR spectra were recorded at 20–25°C and 9.4 GHz in a Bruker EMX (X band) spectrometer. For the SASL probes, whose long molecular axis is approximately parallel to the bilayer normal, the order parameter (S) was calculated according to Eq. 1:

$$S = \frac{2A// - 2A\perp}{2\left[A_{zz} - \left(A_{xx} + A_{yy}\right)/2\right]} \quad (1)$$

where $A//$ and $A\perp$ are the hyperfine splittings corresponding to spin labels whose long molecular axes are oriented approximately parallel or perpendicular, respectively, to the external magnetic field. From oriented SASL spectra (as in the membrane), the hyperfine splittings can be directly determined as half the separation between the outer and the inner extrema, respectively (Hubbel and McConnel 1971), as exemplified in Fig. 6. A_{xx}, A_{yy} (6.0 G each), and A_{zz} (32.0 G) are the values of the principal components of the hyperfine tensor (Griffith and Jost 1976), determined in a rightly oriented sample. The order parameter is related to the tilt angle of the acyl chains and to *trans-gauche* distribution of chain dihedrals, but the relationship is indirect. The larger the value of S, the smaller the amplitude of motion and the more ordered the lipid chains (Schreier et al. 1978).

Results

Characterization of Cholesterol and Protein Content of DRMs Obtained from Whole Erythrocytes with Triton X-100 and $C_{12}E_8$

To compare DRMs obtained with the two different detergents, the purified insoluble materials were prepared using the same detergent/lipid molar ratio (12:1) as described in Materials and Methods.

Figure 1 shows the DRM material (interface between 5% and 30% sucrose) obtained from ghost membranes (tubes 1 and 2) and intact cells (tubes 3 and 4) with each detergent. Visually, one can observe that a more diffuse band was formed when $C_{12}E_8$ was used, revealing density differences in comparison to that obtained with Triton X-100. In spite of this difference, the DRM bands from ghosts and intact erythrocytes were very similar when the same detergent was used. Furthermore, as opposed to what may be observed in Fig. 1, DRMs did not float to the 5–30% sucrose interface when sodium carbonate was not present after the treatment with the detergent (data not shown).

The cholesterol and protein contents of the isolated low-density material (Fig. 1) were determined, and the results

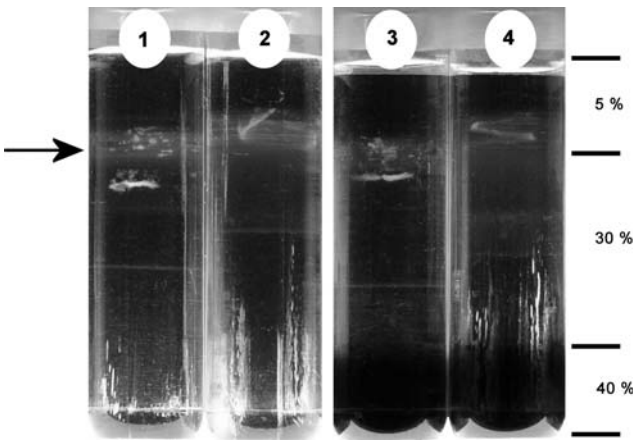

Fig. 1 Sucrose density gradients after centrifugation of detergent-treated ghost membranes (tubes 1 and 2) and whole erythrocytes (tubes 3 and 4). Tubes 1 and 3 contain samples treated with Triton X-100; tubes 2 and 4 contain samples treated with $C_{12}E_8$. The arrow indicates the position of the DRMs. The percentages to the right indicate the sucrose concentration of the various layers. The dark color present in the bottom (40% sucrose) of the tubes 3 and 4 is due to the presence of hemoglobin released after detergent treatment. The image is representative of at least five independent experiments with similar results

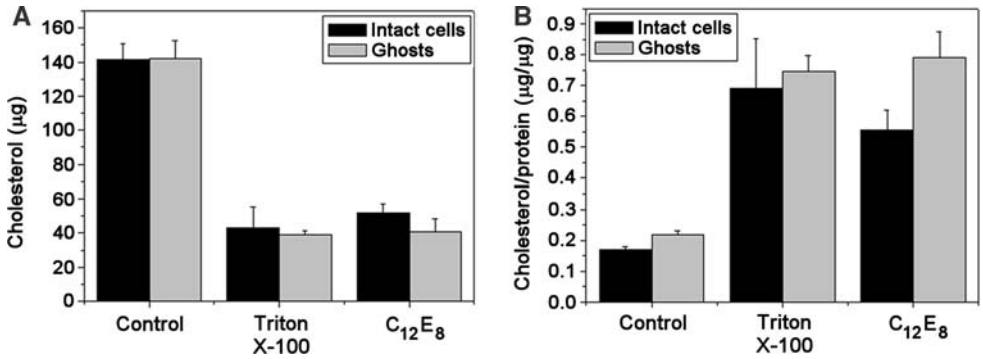

Fig. 2 Quantification of cholesterol content (**a**) and cholesterol/protein ratio (**b**) of DRM fractions 2 and 3, obtained with Triton X-100 and $C_{12}E_8$. The cholesterol in (**a**) was quantified in 1.25×10^9 intact erythrocytes or in a volume of ghost membranes corresponding to 0.625 mg protein (control), and in the DRMs obtained after centrifugation of the given amounts of starting material. Error bars represent the standard deviation ($n = 3$). The amount of membrane proteins present in intact erythrocytes in B (control) was estimated according to the lipid:membrane–protein mass ratio (1:1.2) and the total membrane lipid concentration, according to Dodge et al. (1963) and Malheiros et al. (2000)

revealed that for both detergents, approximately 30% of total cholesterol of the original samples (ghosts or intact cells) was recovered in the DRM fractions 2 and 3 (Fig. 2a). No differences were detected when DRMs were prepared with Triton X-100 or $C_{12}E_8$.

A remarkable feature of the DRMs is their increased cholesterol-to-protein mass ratio with respect to the parent cell membrane. When DRMs were obtained from ghost membranes, there was no significant difference in the cholesterol-to-protein mass ratio of the DRM fractions between the detergents used (Fig. 2b). Although the cholesterol-to-protein mass ratio is smaller in $C_{12}E_8$ DRMs prepared with intact cells, we observed a 3-fold increase in this ratio for all DRM fractions obtained with that detergent, in comparison to intact cells or ghost membranes (controls, Fig. 2b).

Figure 3 shows the distribution of different proteins in Triton X-100 and $C_{12}E_8$ DRMs from whole erythrocytes; no differences were observed when ghosts or intact cells were treated with the same detergent (data not shown). The distribution of the raft markers flotillin-2 and stomatin was very different: whereas stomatin was recovered in both soluble and insoluble (Triton X-100 and $C_{12}E_8$ DRMs) fractions, flotillin-2 was highly enriched in Triton X-100 DRMs and poorly detected in $C_{12}E_8$ DRMs. Band 3 and GPC show up as a barely visible signal in Western blots of DRMs obtained with Triton X-100, and are not detected when $C_{12}E_8$ is used (Fig. 3). A more quantitative estimation of the band 3 and GPC content in DRMs obtained with Triton X-100 was carried out here by quantitative Western blot testing using carefully prepared purified ghost membranes as a standard for building calibration curves. As shown in Fig. 4, band 3 content in DRMs is approximately 2% and GPC content 3%, with respect to the content of each protein in the intact cell membrane. The same

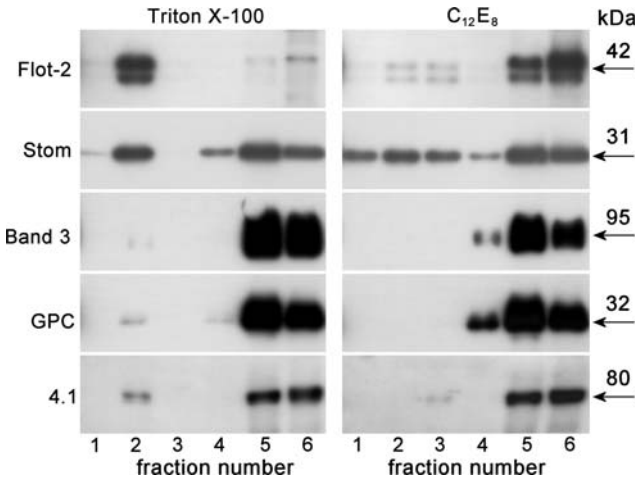

Fig. 3 Distribution of flotillin-2 (flot-2), stomatin (stom), band 3, GPC, and protein 4.1 (4.1) in different sucrose density fractions numbered from top to bottom. Fractions 2 and 3 correspond to DRMs obtained from whole erythrocytes with Triton X-100 (left) and $C_{12}E_8$ (right). Results are representative of five independent experiments

quantification for DRMs obtained with $C_{12}E_8$ was not conducted because of the much lower band 3 and GPC content in these samples (see Fig. 3).

Interestingly, we found that membrane-skeletal proteins, such as spectrin, actin and protein 4.1, were present in both Triton X-100 and $C_{12}E_8$ DRMs (Figs. 3 and 5). The presence of these proteins in erythrocyte DRMs is also a matter of debate in the literature. It was previously observed by others (Murphy et al. 2004) that membrane-skeletal proteins tend to associate to the DRMs when a relatively low detergent-to-protein ratio is used during the solubilization of erythrocyte ghosts with Triton X-100. However, in our hands, membrane-skeletal proteins are detected in variable amounts depending on the blood sample, even when DRMs are extracted by a relatively high detergent-to-protein ratio,

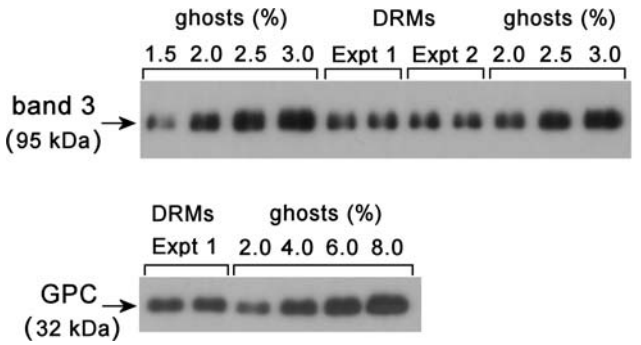

Fig. 4 Quantification by Western blotting of band 3 and GPC in DRMs obtained from whole erythrocytes with Triton X-100. Purified ghost membranes were prepared from an aliquot of erythrocytes with particular attention to avoid loss of cells during the preparation, so that the final suspension of white ghosts contained the same amount of cells. DRMs were prepared from whole erythrocytes. In the SDS-PAGE gels, appropriate amounts of ghost membranes were loaded as calibration curves suitably chosen to comprise the intensity signal of the unknown DRM samples, as determined in preliminary tests. The calibration curves are labeled in percent, meaning the percentage of protein with respect to that present in the whole cell membrane. After transfer to PVDF membranes, the protein of interest was revealed by immunodetection with chemiluminescence. The integrated densities of the corresponding bands in the photographic films were measured and the calibration curves were used to extrapolate the amount of protein in the unknown samples. In the images shown, for band 3, two calibration curves and two different DRM samples (two replicate lanes for each experiment) are shown from independent experiments loaded in the same gel; for GPC, one calibration curve and two lanes loaded with DRMs from the same experiment are shown. The image is representative of four independent experiments for band 3, and three independent experiments for GPC. The mean ± SD of the values obtained for the protein content in DRMs, expressed as the percentage of the content in the original cell membrane, were band 3, 1.86 ± 0.07%; and GPC, 2.92 ± 0.37%

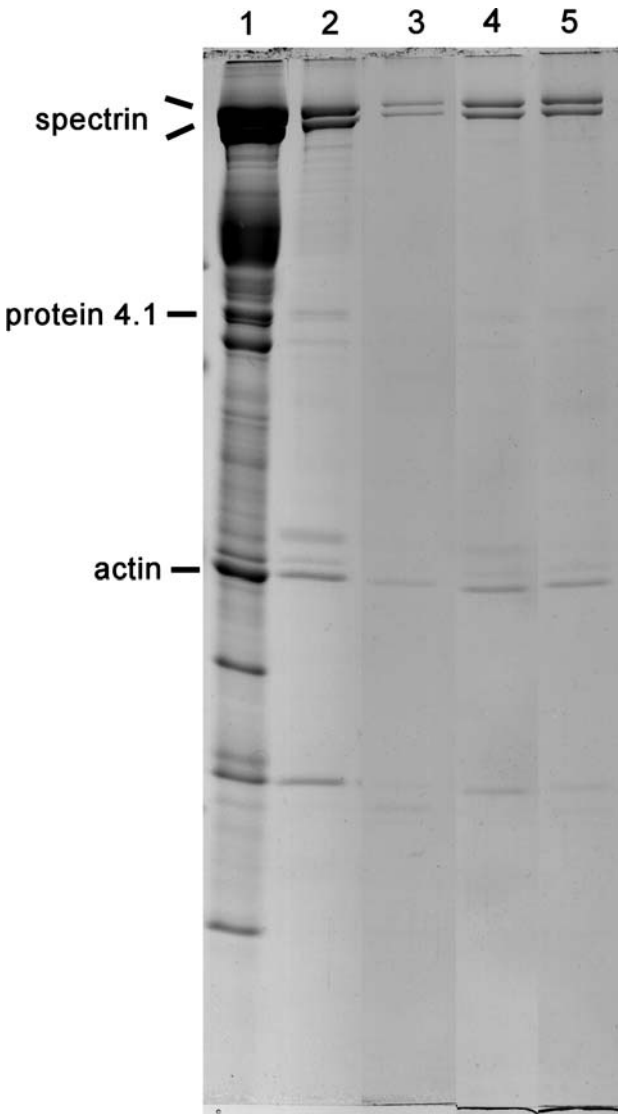

Fig. 5 SDS-PAGE of proteins present in the DRM fractions 2 and 3 prepared from whole erythrocytes with Triton X-100 (lanes 2 and 3) and $C_{12}E_8$ (lanes 4 and 5). Lane 1 corresponds to an amount of ghost sample proportional to the total amount loaded for the DRM fractions 2 and 3. Results are representative of three independent experiments

at which Murphy et al. (2004) did not observe membrane-skeletal proteins in DRMs (Ciana et al. 2005).

EPR Spectroscopy of DRMs Obtained from Whole Erythrocytes with Triton X-100 and $C_{12}E_8$

To investigate the acyl chain packing in the DRMs, we have monitored two different regions of the bilayer by means of the spin probes 5- and 16-SASL.

Figure 6 shows the EPR spectra of 5-SASL in erythrocyte membranes, as well as in Triton X-100 and $C_{12}E_8$ DRMs disclosing the measure of the outer and inner extrema ($2A//$ and $2A\perp$). 5-SASL sensed a highly ordered region of the membrane, with S control values around 0.76. There is no significant difference in S values between whole erythrocytes and purified ghost membranes, nor between the DRMs obtained from them, when Triton X-100 was used (Table 1). The S values in DRMs were significantly higher (>0.82) than those from intact membranes ($P < 0.001$), showing an increase in the orientation of the acyl chains (increased order) consistent with the liquid-ordered state of the DRMs. Furthermore, no significant difference ($P > 0.05$; $n = 5$) was observed between S values of DRMs obtained from intact cells extracted by Triton X-100 and by $C_{12}E_8$ (Table 1).

As Table 1 shows, 16-SASL monitored a less organized region of the membrane so that S values lower than those obtained with 5-SASL were found for control samples and their respective DRMs obtained with either Triton X-100 or $C_{12}E_8$. The small order parameters reflect the increased trans-gauche isomerization of the acyl chain at deeper acyl chain positions, as expected from the profile of acyl chain

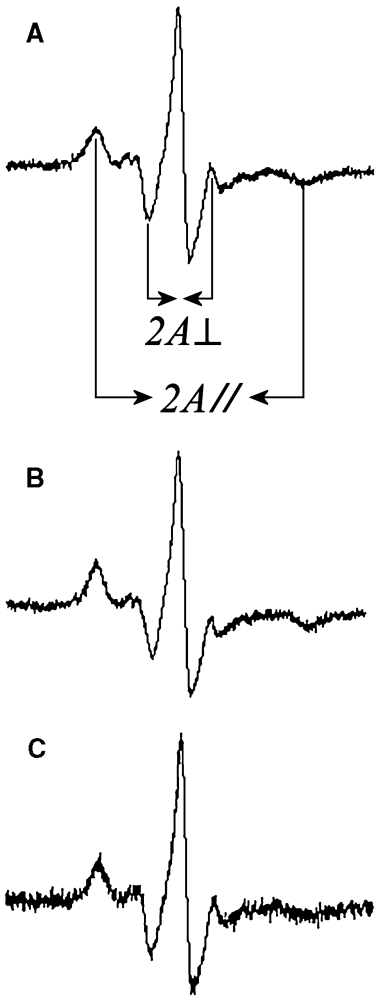

Fig. 6 EPR spectra of 5-SASL in whole erythrocytes (**a**), Triton X-100 DRMs (**b**), and $C_{12}E_8$ DRMs (**c**). Results are representative of five independent experiments

Table 1 Order parameters (S) obtained from electron paramagnetic resonance spectra of the spin labels 5- and 16-SASL in ghost membranes, intact erythrocytes, and their respective detergent-resistant membranes (DRMs) prepared with Triton X-100 or $C_{12}E_8$[a]

Sample	$S_{control}$	S_{DRM} Triton X-100	S_{DRM} $C_{12}E_8$
5-SASL			
Ghosts	0.759 ± 0.016	0.823 ± 0.014**	ND
Intact cells	0.762 ± 0.009	0.836 ± 0.005**	0.827 ± 0.017**
16-SASL			
Ghosts	0.277 ± 0.009	0.321 ± 0.004*	ND
Intact cells	0.268 ± 0.001	0.319 ± 0.001**	0.302 ± 0.002*

* $P < 0.01$, ** $P < 0.001$ (paired Student's *t*-test, $n = 3$–5)

[a] Data are expressed as mean ± SD. Statistical differences are between DRMs versus control samples (ghosts or intact cells). ND, not determined

order (Seelig 1977). As for 5-SASL, an increase in the orientation of the acyl chains sensed by 16-SASL was observed in the DRMs obtained with both detergents, as reflected by higher *S* values measured in DRMs with respect to the parent cell membrane (Table 1).

Discussion

Studies with DRMs from human erythrocytes have been reported, showing that variations in the protocols and/or some maneuvers result in DRMs with different properties. For other cell types, the use of different detergents has been suggested to produce differences in the properties of DRMs (Schuck et al. 2003; Pike 2004). To isolate DRMs from human erythrocytes, Triton X-100 has been the only detergent tested so far. In the present study, we compare DRMs obtained with Triton X-100 and $C_{12}E_8$, which are strongly lytic nonionic detergents (Preté et al. 2002).

The use of sodium carbonate in addition to the nonionic detergent for the isolation of DRMs from erythrocytes has only been described by Nagao et al. (2002) and by our group (Ciana et al. 2005), and it has been shown to aid in isolating DRMs containing only integral membrane proteins (Salzer and Prohaska 2001) as an alternative to otherwise effective methods not requiring carbonate in addition to the detergent (Samuel et al. 2001; Murphy et al. 2004; Wilkinson et al. 2007; Kamata et al. 2008). The isolation of DRMs from rabbit erythrocytes has also been described to require carbonate (Motoyama et al. 2006). The inclusion of EDTA in the solubilization buffers has been suggested as a possible cause for the failure in isolating DRMs without carbonate (Wilkinson et al. 2007). However, some of the published methods that do not involve carbonate also include EDTA in their buffers (Samuel et al. 2001; Murphy et al. 2004; Kamata et al. 2008). Moreover, in our hands, the simple omission of EDTA does not permit to isolate DRMs in the low-density region of the gradient without carbonate (not shown). The question as to why some methods do allow isolating DRMs without the use of carbonate is thus still open. Our results strongly suggest that both for intact erythrocytes and ghost membranes, sodium carbonate helps to disrupt possible electrostatic interactions between DRMs and the membrane-skeleton.

Approximately 30% of total cholesterol of the original samples (ghosts or intact cells) was recovered in the isolated DRMs (Fig. 2a), a finding that agrees well with previous reports (Murphy et al. 2004; Ciana et al. 2005). Moreover, a remarkable increase in the cholesterol-to-protein mass ratio (Fig. 2b) was observed in DRMs obtained from both detergent treatments. These results show that DRMs are cholesterol enriched relative to intact

membranes, thus exhibiting characteristics consistent with the properties of membrane rafts.

DRMs from erythrocytes have been reported as enriched in proteins such as flotillin-1, flotillin-2, stomatin, Gsα, a number of glycosyl-phosphatidyl-inositol (GPI)-linked proteins and the ganglioside GM1 (Salzer and Prohaska 2001; Ciana et al. 2005; Wilkinson et al. 2007; Kamata et al. 2008). Although the cholesterol results presented here revealed no differences between Triton X-100 and $C_{12}E_8$ DRMs, we wondered whether integral and/or membrane-skeletal proteins would be differentially solubilized by each detergent.

One of the remarkable results of the application of the solubilization at 4°C with nonionic detergents of the erythrocyte membrane is the almost complete exclusion of band 3 protein from the DRMs (Ciana et al. 2005). Although the presence of band 3 as a significant component of DRM fractions has only been reported by Murphy et al. (2004), our evidence is in good agreement with that of other groups (Salzer and Prohaska 2001; Wilkinson et al. 2007), and we observed the same results with $C_{12}E_8$, which better preserves the oligomeric state of band 3 (Casey and Reithmeier 1991) (Figs. 3 and 4). This evidence is striking in view of the fact that band 3 is the most abundant integral protein of the erythrocyte membrane, and a significant portion of the membrane itself is detergent resistant (when considering that DRMs contain approximately 30% of the whole membrane cholesterol), yet DRMs remain virtually band 3–free. GPC is also strongly depleted in DRMs. We concluded provisionally from our previous work (Ciana et al. 2005) that band 3 is only present in trace amounts in DRMs obtained from ghosts with Triton X-100, and that it is therefore unlikely that band 3 could significantly contribute to the anchoring of DRMs to the membrane-skeleton. Although it could be reconsidered, after the results of the quantification presented here, the possibility that a low but significant percent of band 3 and GPC in DRMs could be responsible for their carbonate-sensitive linkage to the membrane-skeleton, the fact that DRMs obtained with $C_{12}E_8$ lack band 3 and GPC and yet can be obtained in similar amounts (expressed as cholesterol content) as with Triton X-100 only when carbonate is used, seems to rule out this possibility.

Three models for the structure of rafts have been proposed by Pike (2004) to account for the observed experimental results. In model I, a lipid raft has a layered structure with a central core (enriched in cholesterol and sphingolipids) surrounded by layers of decreasing lipid order. The detergents act on this type of raft by solubilizing the layers, eventually leaving an insoluble portion that is more or less enriched in cholesterol/sphingolipids depending on the detergent. In model II, rafts have a homogeneous composition and detergents act by selectively extracting some components (lipids and proteins) and leaving others as insoluble material. In model III, rafts are heterogeneous, i.e. different populations of rafts, with distinct lipid and protein composition, coexist in the membrane. The detergents, according to their strength and selectivity, solubilize some populations while leaving others as insoluble membranes.

The data presented here on the differential distribution of flotillin, stomatin, band 3, and GPC in DRMs obtained with Triton X-100 and $C_{12}E_8$, can be discussed within the tripartite modelization described above. Thus, if layered domains (model I) or homogeneous domains (model II) existed in the erythrocyte membrane, then $C_{12}E_8$ would seem more powerful than Triton X-100 in solubilizing those layers where the above-cited proteins should be preferentially located (or to selectively extract lipids and proteins in the model of homogeneous domains), leaving a detergent-resistant portion that is depleted of those proteins. If heterogeneous microdomains existed in the membrane (model III), represented by flotillin-containing and flotillin-free domains, then Triton X-100 would seem less selective than $C_{12}E_8$ in solubilizing them, as it leaves, as detergent-resistant material, populations of DRMs that contain the mentioned protein, which is instead solubilized by $C_{12}E_8$. On the other hand, within model III, if $C_{12}E_8$ permitted the solubilization of only a subset of heterogeneous rafts (those which contain flotillin), then the amount of DRMs obtained should be less with $C_{12}E_8$ than with Triton X-100, which is not the case here, as approximately 30% of the cell membrane cholesterol is isolated as DRMs, with both detergents. Therefore, from this evidence it is not possible to propend for one of the models proposed to represent the lipid microdomains in the membrane, except, possibly, for model III. Further studies are needed to better understand the nature of these entities—for example, through immunological separation of DRMs obtained with a single detergent using antibodies against different protein species.

Recently, Wilkinson et al. (2007) have suggested that stomatin and actin are associated in rafts from erythrocytes, having a regulatory function in Ca^{2+}-induced vesiculation. In this way, the presence of actin in the DRM fractions prepared here (Fig. 5) is quite reasonable. The residual, variable content of membrane-skeletal proteins in our samples is difficult to explain, mainly in view of the fact that the supposed electrostatic interactions between them and the DRMs should be disrupted by the inclusion of carbonate in the solubilization medium. Moreover, high donor-to-donor variability has been observed in this respect during our study. What is invariant, however, in our experience, is the requirement of carbonate for the reproducible isolation of DRMs, which has led us to propose that DRMs in the erythrocyte interact electrostatically with the

membrane-skeleton (Ciana et al. 2005). One of the variables that has been suggested to modulate the interaction of DRMs with submembranous structures is the presence of the divalent cations Ca^{2+} and Mg^{2+} (Macdonald and Pike 2005; Parkin et al. 1996). However, a systematic investigation of the impact of the intracellular ionic composition on the interaction between the lipid bilayer and the underlying cytoskeleton, has not been conducted, so far. Thus, it would be possible that a subtle mechanism, regulating the supramolecular structure of the membrane-skeleton, on the one hand, and its interaction with domains of the lipid bilayer on the other, involved intracellular divalent cations and be at the basis of the observed variability in the amount of DRM-associated membrane-skeletal proteins. The importance of intracellular cations would be even more relevant for this cell type, where oscillations in the concentration of free magnesium ions occur at each oxygenation/deoxygenation cycle of hemoglobin. This aspect is now under study in our laboratory.

In order to further characterize the DRMs obtained from erythrocytes, we have used EPR spectroscopy with spin-labeled lipid molecules, an approach widely used to probe the order and dynamics of lipid bilayers and biological membranes (Cassera et al. 2002; Sulkowski et al. 2006; Rivas and Gennaro 2003; Rodi et al. 2006).

We have shown before in model membranes that S values measured at 22°C from the 5-SASL spectra in egg phosphatidylcholine (PC) liposomes ($S = 0.66$) (de Paula and Schreier 1995) increased ($S = 0.78$) in the presence of cholesterol (4:3 mol% egg PC:cholesterol) (Cereda et al. 2004), what is expected from the cholesterol effect in the liquid crystalline phase (McMullen et al. 1993). The order parameter of intact erythrocyte membranes ghosts, measured with 5-SASL ($S = 0.76$, Table 1) is in good agreement with previous reports in the literature (Rivas and Gennaro 2003). Thus, although the lipid packing in erythrocyte membranes could be comparable to that of egg PC:cholesterol liposomes, the high cholesterol content of the DRMs (as shown in Fig. 2) and the saturated acyl chains of the sphingolipids justify the higher molecular orientation of the detergent resistant fragments (Table 1), in the liquid ordered phase. Our EPR results with spin labels at different membrane depths revealed significantly higher S values in DRMs purified in a sucrose gradient with either Triton X-100 or $C_{12}E_8$, in comparison to intact cells. Other works have also reported increased S and A_{max} (2A//) values in EPR spectra from 5- and 16-SASL incorporated in insoluble pellets prepared from erythrocytes treated with cold Triton X-100 (Rivas and Gennaro 2003; Rodi et al. 2006). However, those authors did not use a sucrose gradient to isolate DRMs, so that their insoluble pellets could be contaminated by other possible insoluble (nonraft) components, which would present high density.

Curiously, EPR spectra did not reveal any difference in the DRMs obtained from intact erythrocyte membranes with Triton X-100 and $C_{12}E_8$ treatments. The similar S values found for Triton X-100 and $C_{12}E_8$ DRMs, both for 5- and 16-SASL, clearly show that the acyl chain packing is similar in these DRMs, as expected from their comparable cholesterol content (Fig. 2).

In summary, our results show that flotillin-2, a marker of lipid rafts, is selectivity enriched in Triton X-100 DRMs from erythrocytes, but it is solubilized by treatment with $C_{12}E_8$. On the other hand, the acyl chain packing of the lipids in these domains is not different, after treatment with Triton X-100 or $C_{12}E_8$. Thus, the absence of flotillin-2 from DRMs does not affect the order of the structures involved in those domains.

Atomic force microscopy and fluorescence correlation spectroscopy studies with model membranes have clearly shown the spontaneous formation of laterally segregated domains within lipid bilayers as well as the selective protein incorporation into such domains (Saslowsky et al. 2002; Bacia et al. 2004; Salamon et al. 2007). The association of certain membrane proteins with DRMs may depend on the concentration and the type of detergent (Ilangumaran et al. 1999; Matkó and Szöllősi 2005). Yet, from the point of view of membrane-protein extraction, the specific solubilization induced by detergents has been widely explored, $C_{12}E_8$ and Triton X-100 being powerful solubilizers for Na,K-ATPase and other eukaryote and prokaryote membrane proteins (Santos et al. 2002; Berger et al. 2005).

The types of proteins enriched in DRMs include acylated proteins, GPI-anchored proteins and certain membrane receptors and channels. However, many transmembrane proteins with hydrophobic alpha-helices, including specific tyrosine phosphatases, are largely excluded from membrane microdomains or DRMs (Moffet et al. 2000; Ridyard and Robbins 2003; Wong and Schlichter 2004; McIntosh 2007). Furthermore, the diversity observed among lipid rafts may be also explained by compartmentalization of raft-associated proteins by the underlying cytoskeleton (Munro 2003).

Flotillin is a well-known lipid-raft marker that is colocalized at the caveolae associated proteins, including GPI-linked receptors (Bickel et al. 1997; Glebov et al. 2006). It is myristoylated and multiply palmitoylated, and these lipid modifications are responsible for its membrane insertion because it does not have any intramembrane domain (Neumann-Giesen et al. 2004). Moreover, disruption or stabilization of the actin cytoskeleton modulate the lateral mobility of flotillin microdomains, as shown by fluorescence recovery after photobleaching, by Langhorst et al. (2007), showing flotillin intrinsic relation to the cytoskeleton. On the basis of cytoskeleton rupture, which also

depends on the solubilization of other proteins, it is not surprising that flotillin content in $C_{12}E_8$ and Triton X-100 DRMs is different because these detergents could have different selectivities in solubilizing other membrane proteins that are involved in the cytoskeleton.

Although the organization of DRM components in the bilayer is unclear, our results support the connection of DRMs to the membrane-skeleton, as we have suggested previously (Ciana et al. 2005). In this work, we also provide results that support the use of different detergents to improve the knowledge about the structures of DRMs from erythrocytes and their possible involvement in rafts in the plasma membrane in vivo.

Acknowledgments This work was supported by a research grant from FAPESP (Proc. 03/02394-4), Brazil, and by PRIN funds of the "Ministero dell'Università e della Ricerca," Italy. C.C.D. acknowledges a fellowship from CAPES (Proc. 3597/06-7) and CNPq (Proc. 141618/2005-1), Brazil.

References

Bacia K, Scherfeld D, Kahya N, Schwille P (2004) Fluorescence correlation spectroscopy relates rafts in model and native membranes. Biophys J 87:1034–1043

Berger BW, Garcia RY, Lenhoff AM, Kaler EW, Robinson CR (2005) Relating surfactant properties to activity and solubilization of the human adenosine A3 receptor. Biophys J 89: 452–464

Beutler E, West C, Blume KG (1976) The removal of leukocytes and platelets from whole blood. J Lab Clin Med 88:328–333

Bickel PE, Scherer PE, Schnitzer JE, Oh P, Lisanti MP, Lodish HF (1997) Flotillin and epidermal surface antigen define a new family of caveolae-associated integral membrane proteins. J Biol Chem 272:13793–13802

Brown DA, London E (1998) Functions of lipid rafts in biological membranes. Annu Rev Cell Dev Biol 14:111–136

Brown DA, Rose JK (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell 68:533–544

Casey JR, Reithmeier RAF (1991) Analysis of the oligomeric state of band 3, the anion transport protein of the human erythrocyte membrane, by size exclusion high performance liquid chromatography. J Biol Chem 266:15726–15737

Cassera MB, Silber AM, Gennaro AM (2002) Differential effects of cholesterol on acyl chain order in erythrocyte membranes as a function of depth from the surface. An electron paramagnetic resonance (EPR) spin label study. Biophys Chem 99:117–127

Cereda CM, de Araujo DR, Brunetto GB, de Paula E (2004) Liposomal prilocaine: preparation, characterization, and in vivo evaluation. J Pharm Sci 7:235–240

Ciana A, Balduini C, Minetti G (2005) Detergent-resistant membranes in human erythrocytes and their connection to the membrane-skeleton. J Biosci 30:317–328

de Gassart A, Géminard C, Février B, Raposo G, Vidal M (2003) Lipid raft-associated protein sorting in exosomes. Blood 102: 4336–4344

Delaunay JL, Breton M, Godine JW, Trugnan G, Maurice M (2007) Differential detergent resistance of the apical and basolateral NPPases: relationship with polarized targeting. J Cell Sci 120: 1009–1016

de Paula E, Schreier S (1995) Use of a novel method for determination of partition coefficients to compare the effect of local anesthetics on membrane structure. Biochim Bhiophys Acta 1240:25–33

Dodge JT, Mitchell C, Hanahan DJ (1963) The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. Arch Biochem Biophys 100:119–130

Glebov OO, Bright NA, Nichols BJ (2006) Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. Nat Cell Biol 8:46–54

Griffith OH, Jost OP (1976) Spin labels in biological membranes. In: Berliner LJ (ed) Spin labeling: theory and applications. Academic Press, New York, pp 454–523

Grzybek M, Chorzalska A, Bok E, Hryniewicz-Jankowska A, Czogalla A, Diakowski W, Sikorski AF (2006) Spectrin-phospholipid interactions. Existence of multiple kinds of binding sites? Chem Phys Lipids 141:133–141

Haldar K, Mohandas N, Samuel BU, Harrison T, Hiller NL, Akompong T, Cheresh P (2002) Protein and lipid trafficking induced in erythrocytes infected by malaria parasites. Cell Microbiol 4:383–395

Hubbel WL, McConnel HM (1971) Molecular motion in spin-labeled phospholipids and membranes. J Am Chem Soc 93:314–326

Ilangumaran S, Arni S, van Echten-Decker G, Borisch B, Hoessli DC (1999) Microdomain-dependent regulation of Lck and Fyn protein-tyrosine kinases in T lymphocyte plasma membranes. Mol Biol Cell 10:891–905

Kamata K, Manno S, Ozaki M, Takakuwa Y (2008) Functional evidence for presence of lipid rafts in erythrocyte membranes: Gsα in rafts is essential for signal transduction. Am J Hematol 83:371–375

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685

Langhorst MF, Solis GP, Hannbeck S, Plattner H, Stuermer CA (2007) Linking membrane microdomains to the cytoskeleton: regulation of the lateral mobility of reggie-1/flotillin-2 by interaction with actin. FEBS Lett 581:4697–4703

le Maire M, Champeil P, Moller JV (2000) Interaction of membrane proteins and lipids with solubilizing detergents. Biochim Biophys Acta 1508:86–111

Lichtenberg D, Goni FM, Heerklotz H (2005) Detergent-resistant membranes should not be identified with membrane rafts. Trends Biochem Sci 30:430–436

Macdonald JL, Pike LJ (2005) A simplified method for the preparation of detergent-free lipid rafts. J Lipid Res 46:1061–1067

Malheiros SVP, Meirelles NC, de Paula E (2000) Pathways involved in trifluoperazine-, dibucaine- and praziquantel-induced hemolysis. Biophys Chem 83:89–100

Matkó J, Szöllősi J (2005) Regulatory aspects of membrane microdomain (raft) dynamics in live cells. In: Mattson MP (ed) Membrane microdomain signaling. Lipid rafts in biology and medicine. Humana Press, Totowa, NJ, pp 15–46

McIntosh TJ (2007) Overview of membrane rafts. In: McIntosh TJ (ed) Methods in molecular biology: lipid rafts. Humana Press, Totowa, NJ, pp 1–7

McMullen TPW, Lewis RNAH, McElhaney RN (1993) Differential scanning calorimetric study of the effect of cholesterol on the thermotropic phase behavior of a homologous series of linear saturated phosphatidylcholines. Biochemistry 32:516–522

Moffet S, Brown DA, Linder ME (2000) Lipid-dependent targeting of G proteins into rafts. J Biol Chem 275:2191–2198

Motoyama K, Arima H, Toyodome H, Irie T, Hirayama F, Uekama K (2006) Effect of 2,6-di-*O*-methyl-alpha-cyclodextrin on hemolysis and morphological change in rabbit's red blood cells. Eur J Pharm Sci 29:111–119

Munro S (2003) Lipid rafts: elusive or illusive? Cell 115:377–388
Murphy SC, Samuel BU, Harrison T, Speicher KD, Speicher DW, Reid ME, Prohaska R, Low PS, Tanner MJ, Mohandas N, Haldar K (2004) Erythrocyte detergent-resistant membrane proteins: their characterization and selective uptake during malaria infection. Blood 103:1920–1928
Murphy SC, Fernandez-Pol S, Chung PH, Murthy SNP, Milne SB, Salomao M, Brown HA, Lomasney JW, Mohandas N, Haldar K (2007) Cytoplasmic remodeling of erythrocyte raft lipids infection by the human malaria parasite *Plasmodium falciparum*. Blood 110:2132–2139
Nagao E, Seydel KB, Dvorak JA (2002) Detergent-resistant erythrocyte membrane rafts are modified by a *Plasmodium falciparum* infection. Exp Parasitol 102:57–59
Neumann-Giesen C, Falkenbach B, Beicht P, Claasen S, Lüers G, Stuermer CA, Herzog V, Tikkanen R (2004) Membrane and raft association of reggie-1/flotillin-2: role of myristoylation, palmitoylation and oligomerization and induction of filopodia by overexpression. Biochem J 378:509–518
Parkin ET, Turner AJ, Hooper NM (1996) Isolation and characterization of two distinct low-density, Triton-insoluble, complexes from porcine lung membranes. Biochem J 319:887–896
Pike LJ (2004) Lipid rafts: heterogeneity on the high seas. Biochem J 378:281–292
Pike LJ (2006) Rafts defined: a report on the keystone symposium on lipid rafts and cell function. J Lipid Res 47:1597–1598
Preté PSC, Gomes K, Malheiros SVP, Meirelles NC, de Paula E (2002) Solubilization of human erythrocyte membranes by non-ionic surfactants of the polyoxyethylene alkyl ethers series. Biophys Chem 97:45–54
Ridyard MS, Robbins SM (2003) Fibroblast growth factor-2-induced signaling through lipid raft-associated fibroblast growth factor receptor substrate 2 (FRS2). J Biol Chem 278:13803–13809
Rigaud J-L, Pitard B, Levy D (1995) Reconstituition of membrane proteins into liposomes: application to energy-transducing membrane proteins. Biochim Biophys Acta 1231:223–246
Rivas MG, Gennaro AM (2003) Detergent resistant domains in erythrocyte membranes survive after cell cholesterol depletion: an EPR spin label study. Chem Phys Lipids 122:165–169
Rodi PM, Cabeza MS, Gennaro AM (2006) Detergent solubilization of bovine erythrocytes. Comparison between the insoluble material and the intact membrane. Biophys Chem 122:114–122
Rose HG, Oklander M (1965) Improved procedure for the extraction of lipids from human erythrocytes. J Lipid Res 6:428–431
Salamon Z, Devanathan S, Tollin G (2007) Plasmon-waveguide resonance spectroscopy studies of lateral segregation in solid-supported proteolipid bilayers. In: McIntosh TJ (ed) Methods in molecular biology: lipid rafts. Human Press, Totowa, NJ, pp 159–178
Salzer U, Prohaska R (2001) Stomatin, flotillin-1, and flotillin-2 are major integral proteins of erythrocyte lipid rafts. Blood 97:1141–1143
Salzer U, Hinterdorfer P, Hunger U, Borken C, Prohaska R (2002) Ca^{++}-dependent vesicle release from erythrocytes involves stomatin-specific lipid rafts, synexin (annexin VII), and sorcin. Blood 99:2569–2577
Samuel BU, Mohandas N, Harrison T, McManus H, Rosse W, Reid M, Haldar K (2001) The role of cholesterol and glycosylphosphatidylinositol-anchored proteins of erythrocyte rafts in regulating raft protein content and malarial infection. J Biol Chem 276:29319–29329
Santos HL, Lamas RP, Ciancaglini P (2002) Solubilization of Na,K-ATPase from rabbit kidney outer medulla using only $C_{12}E_8$. Braz J Med Biol Res 35:277–288
Saslowsky DE, Lawrence J, Ren X, Brown DA, Henderson RM, Edwards JM (2002) Placental alkaline phosphatase is efficiently targeted to rafts in support lipid bilayer. J Biol Chem 277:26966–26970
Schreier S, Polnaszek CF, Smith ICP (1978) Spin labels in membranes. Biochim Biophys Acta 515:375–436
Schuck S, Honsho M, Ekroos K, Shevchenko A, Simons K (2003) Resistance of cell membranes to different detergents. Proc Natl Acad Sci USA 100:5795–5800
Seelig J (1977) Deuterium magnetic resonance: theory and application to lipid membranes. Q Rev Biophys 10:353–418
Simons K, Ikonen E (1997) Functional rafts in cell membranes. Nature 387:569–572
Simons K, Vaz WL (2004) Model systems, lipid rafts, and cell membranes. Annu Rev Biophys Biomol Struc 33:269–295
Sulkowski WW, Pentak D, Nowak K, Sulkowska A (2006) The influence of temperature and pH on the structure of liposomes formed from DMPC. J Mol Struct 792–793:257–264
Wilkinson DK, Turner EJ, Parkin ET, Garner AE, Harrison PJ, Crawford M, Stewart GW, Hooper NM (2007) Membrane raft actin deficiency and altered Ca^{2+}-induced vesiculation in stomatin-deficient overhydrated hereditary stomatocytosis. Biochim Biophys Acta 1778:125–132
Wong W, Schlichter LC (2004) Differential recruitment of Kv1.4 and Kv4.2 to lipid rafts by PSD-95. J Biol Chem 279:444–452