# Detergent-resistant membranes in human erythrocytes and their connection to the membrane-skeleton

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In cell membranes, local inhomogeneity in the lateral distribution of lipids and proteins is thought to exist *in vivo* in the form of lipid 'rafts', microdomains enriched in cholesterol and sphingolipids, and in specific classes of proteins, that appear to play specialized roles for signal transduction, cell-cell recognition, parasite or virus infection, and vesicular trafficking. These structures are operationally defined as membranes resistant to solubilization by nonionic detergents at 4°C (detergent-resistant membranes, DRMs). This definition appears to be necessary and sufficient, although additional manoeuvres, not always described with sufficient detail, may be needed to ensure isolation of DRMs, like mechanical homogenization, and changes in the pH and/or ionic strength of the solubilization medium. We show here for the human erythrocyte that the different conditions adopted may lead to the isolation of qualitatively and quantitatively different DRM fractions, thus contributing to the complexity of the notion itself of lipid raft. A significant portion of erythrocyte DRMs enriched in reported lipid raft markers, such as flotillin-1, flotillin-2 and $GM_1$, is anchored to the spectrin membrane-skeleton via electrostatic interactions that can be disrupted by the simultaneous increase in pH and ionic strength of the solubilization medium.

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## 1. Introduction

Lipid microdomains enriched in cholesterol and sphingolipids are thought to exist *in vivo* in the membranes of living cells (Edidin 1997, 2001; Brown and London 1998; Pike 2004). These structures are sites of selective enrichment of specific classes of proteins, and are thus believed to play specialized roles for the transduction of signals (Sargiacomo *et al* 1993; Ilangumaran *et al* 1999; Simons and Toomre 2000; Drevot *et al* 2002), for cell-cell recognition processes, parasite or virus infection (Scheiffele *et al* 1997; Nguyen and Hildreth 2000; Rosenberger *et al* 2000; Samuel *et al* 2001; van der Goot and Harder 2001; Haldar *et al* 2002; Slimane *et al* 2003), vesicular trafficking (de Gassart *et al* 2003).

The human erythrocyte has served for decades as a model of election for the study of biological membranes. However only a few, relatively late studies have so far reported on the existence and properties of lipid rafts in this cell type (Civenni *et al* 1998; Samuel *et al* 2001; Salzer and Prohaska 2001; Nagao *et al* 2002; Salzer *et al* 2002; Murphy *et al* 2004).

Although direct visualization of rafts with various techniques has been proposed as possible (Schutz *et al* 2000; Suzuki *et al* 2000), a widely accepted operational definition of lipid rafts is that of membranes resistant to solubilization by nonionic detergents at 4°C (detergent-resistant membranes, DRMs). Whether or not DRMs are a true representation of the organization of lipid rafts in the plasma membranes *in vivo* is, however, a matter of

**Keywords.** Band 3; detergent-resistant membrane; erythrocyte; flotillin; lipid raft; membrane skeleton

Abbreviations used: DRMs, Detergent-resistant membranes; IOVs, inside-out-vesicles; PMSF, phenyl-methyl-sulphonyl-fluoride; SLP, stomatin-like-protein-2; TX-100, Triton X-100.

debate (Munro 2003), and the notion itself of lipid rafts as stable entities has been deeply criticised (Mayor and Rao 2004). Studies with artificial membranes have shown that, although microdomains may originate by spontaneous segregation of cholesterol and sphingolipids from the surrounding phospholipids, they are easily perturbed by temperature fluctuations and by the presence of even trace amounts of detergents. Moreover, the detergent itself may contribute to the generation of liquid–ordered phases (Heerklotz *et al* 2003). The present view suggest that, if rafts exist in living cells, they are dynamic structures of nanometer size, that bear little or no relationship with the DRMs that are commonly isolated from a given cell type to provide material for biochemical characterization. More recently, this critical approach has fostered research into the development of detergent-free methods for the isolation of lipid rafts (Macdonald and Pike 2005).

Despite the delicate nature of lipid rafts and of DRMs, many protocols have been implemented for the isolation of DRMs, based on what appeared to be the necessary and sufficient operational definition; i.e. the insolubility in nonionic detergents at 4°C. However, even this relatively cogent definition appears insufficient since, in many studies, additional manoeuvres are adopted to ensure isolation of DRM by density gradient fractionation. Thus, beside treatment at 4°C with the nonionic detergent, one or more of the following steps are included in the protocols: mechanical manipulation with homogenizers, sonication, passages through syringe needles of various gauges, and changes in the pH and/or ionic strength of the solubilization medium.

Such manoeuvres are, however, poorly characterized, and their important role rarely emphasized in the published protocols, although they probably represent an important step for the isolation of DRMs. Qualitative and quantitative differences in the properties of DRMs obtained from a given cell type, may be due not only to the use of different nonionic detergents (Schuck *et al* 2003; Shogomori and Brown 2003; Pike 2004), but also to different, less characterized conditions adopted for the isolation. For instance, DRMs from erythrocytes have been described as enriched in ganglioside $GM_1$, flotillin-1, flotillin-2, stomatin, GPI-linked proteins, and devoid of membrane-skeletal proteins like spectrin, actin, and of integral membrane proteins, band 3, glycophorins. We show here a more complex scenario, whereby different families of DRMs are obtained from erythrocyte membranes, depending on the conditions adopted for extraction. The type of DRMs with previously reported features, i.e. enriched in flotillins and $GM_1$, and depleted of band 3 and spectrin, could only be obtained if detergent-treated membranes were further processed in media of high pH and ionic strength. This suggests the involvement of electrostatic interactions between one or more still unidentified components of the DRMs and the membrane skeleton, that need to be disrupted to allow their isolation as material of low buoyant density in sucrose gradients.

## 2. Materials and methods

### 2.1 *Blood processing*

Blood was collected from normal human donors after informal consent and mixed with 0·1 volumes of 3·8% (w/v) Na-citrate. After centrifugation at 1000 *g*, plasma was removed and packed cells resuspended in an equal volume of PBS (5 mM Na-phosphate, 154·5 mM NaCl, 4·5 mM KCl, pH 7·4, 300 mosmol/kg $H_2O$); this suspension was filtered through *α*-cellulose/microcrystalline-cellulose to purify erythrocytes from platelets and leukocytes as previously detailed (Beutler *et al* 1976). Purified ghost membranes were prepared by hypotonic haemolysis of erythrocytes with 10 volumes of 5P8 [5 mM Na-phosphate, 0·5 mM EDTA, 0·2 mM phenyl-methyl-sulphonyl-fluoride (PMSF), pH 8·0] at 4°C, followed by centrifugation at 26000 *g* and repeated washings with the same buffer, according to the method of Dodge *et al* (1963). Spectrin-free membranes were obtained as 'inside-out-vesicles' (IOVs) following the method of Bennett (1983). Briefly, 5 ml of ghost membrane suspension were treated with 250 ml of spectrin-extraction-buffer (0·5 mM EDTA, pH 8·5, 0·33 mM dithiotreitol, 0·15 mM PMSF) and incubated at 37°C for 1 h. IOVs were collected by centrifugation at 27000 *g* at 4°C for 40 min, and resuspended to 5 ml with 5P8 buffer. By this method, 80–90% of the initial ghost membrane is recovered as spectrin-free IOVs. For the purpose of this study, the IOVs were used for DRM extraction without further purification or characterization of vesicle sidedness.

### 2.2 *Preparation of DRMs*

DRMs were prepared from purified ghost membranes either by following a basic protocol as described by others (Samuel *et al* 2001; Murphy *et al* 2004) or by several modified versions of the basic method. Ghosts were always used within one week of preparation from freshly drawn blood, and stored at – 20°C until use. Additional experiments with ghosts that had never been frozen, gave identical results (not shown). The basic protocol was the following: an aliquot of ghost suspension was mixed with TNE buffer (25 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, pH 7·4) containing Triton X-100 (TX-100), from a 10% aqueous solution (Pierce Biotechnology, Rockford, IL, USA) so that the final protein concentration was in the range between 0·8 and 2·0 mg/ml (approximately 0·2

and 0·50 mg/ml cholesterol) and the final detergent concentration was 1%, and carefully homogenized by repeated pipetting. After 30 min the sample was mixed with an equal volume of 80% sucrose solution in TNE, to give a final sucrose concentration of 40%. The sample was then transferred (1·25 or 0·84 ml/tube) in ultracentrifuge tubes (5 ml total volume). A 30% sucrose solution in TNE was gently layered on top of the sample (2·5 or 2·9 ml/tube), followed by a solution of 5% sucrose in TNE (1·25 ml/tube). All procedures were carried out at 4°C. Samples were spun in the swinging-arm rotor (MLS50, Beckman Coulter, Milan, Italy) of a bench-top ultracentrifuge (Optima Max Beckman Coulter, Milan, Italy) at 46000 rpm corresponding to 225000 *g* max, for 4 h at 4°C. At the end, 6 to 10 fractions were collected by suction from the top of each gradient and aliquots from each fraction were saved for subsequent characterization.

2.2a *Variations of the basic protocol.* For some experiments, the 80% sucrose solution used to dilute the detergent-treated sample before gradient separation, was obtained by dissolving the sucrose in one of the following solutions: 0·6 M NaCl to raise the ionic strength to 0·3 after mixing with the sample in TNE; 0·050 M, 0·053 M, 0·056 M or 0·070 M NaOH to obtain a pH increase to 10·5, 11·2, 12·0 or 12·5, respectively (the latter values were experimentally determined to take into account the presence of TNE in the sample and the available water of the highly concentrated sucrose solution); 0·05 M NaOH plus 0·6 M NaCl to obtain both pH = 10·5 and I = 0·3; 0·2 M $Na_2CO_3$ (Nagao *et al* 2002), that gives a pH of approximately 10·5 and a final ionic strength of 0·3 (after mixing with the sample in TNE).

In some experiments a detergent-free procedure was followed: ghost suspensions were treated with 4 volumes of 0·2 M $Na_2CO_3$, and either mixed by pipetting or homogenised in a Dounce homogenizer. After 20 min at 4°C the sample was mixed with an equal volume of an 80% solution of sucrose in 0·2 M $Na_2CO_3$, before fractionation in the sucrose density gradient. Under these conditions, the final ionic strength was I = 0·6 and pH = 11·7.

### 2.3 *Analytical methods*

Cholesterol was measured using a colorimetric assay kit (N. 10139050035, R-Biopharm Italia srl, Milan, Italy). Protein content was determined with bicinchoninic acid (Pierce Biotechnology, Rockford, IL, USA), using bovine serum albumin as a standard. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) and electro-transferred to nitrocellulose membranes for Western blotting according to previously detailed protocols (Towbin *et al* 1979). Samples from sucrose density gradient fractions and ghost membranes were treated with 0·5 volumes of SDS-PAGE sample buffer [50 mM Tris/HCl, 5% SDS (w/v), 5 mM EDTA, 200 mM dithiotreitol, 35% sucrose (w/v), 0·01% bromophenol blue, pH 6·8] and incubated at 60°C for 15 min.

Ganglioside $GM_1$ was determined in the fractions isolated from sucrose gradients, by dot-blotting on nitrocellulose membranes and binding of horseradish-peroxidase-conjugated cholera toxin subunit B (Calbiochem-Merck Biosciences, Darmstadt, Germany) followed by chemiluminescence detection (ECL kit, Amersham-Biosciences, Milan, Italy).

Western blotting of the relevant proteins was carried out using the following primary antibodies at the specified dilution: mouse monoclonal, clone 29, anti flotillin-2 [1 : 8000] (Becton Dickinson Italia, Milan, Italy); mouse monoclonal, clone BIII-139, anti human band 3 [1 : 40000] (Sigma-Aldrich, Milan, Italy); rabbit polyclonal anti N-terminal domain of human $p56/53^{lyn}$ [1 : 200] (Santa Cruz Biotechnology, Heidelberg, Germany). Binding of primary antibody was assayed after incubation with a proper secondary antibody conjugated with horseradish peroxidase, followed by chemiluminescence detection (Becton Dickinson Italia, Milan, Italy). All other reagents used were of analytical grade.

## 3. Results

### 3.1 *Basic protocol for the isolation of DRMs from human erythrocyte membranes*

Several attempts to isolate DRMs from human erythrocyte ghosts following the basic protocol described in § 2 gave the results summarized in figure 1. The sucrose gradients displayed, at the end of the separation, a faint and diffuse band at the interface between 5% and 30% sucrose, where DRMs are expected to migrate (arrow a); and additional variable bands in the lower part of the 30% sucrose region (arrows b); or in the loading zone (arrow c).

Assay of cholesterol and total proteins in six fractions collected from the gradient showed that the light fraction had a cholesterol/protein ratio of approximately 0·5. This corresponds to 2·5 times the cholesterol/protein ratio of whole-ghost membranes (approximately 0·2). An increase in the cholesterol/protein ratio is one of the reported properties of detergent-resistant membranes. However, the recovery of DRMs was very low in our hands. In fact, less than 5% of the total cholesterol of the original sample was found in the light fraction, compared to the 10% or above (see also § 4) expected on the basis of previously published work (Samuel *et al* 2001; Murphy *et al* 2004). Most importantly, analysis of $GM_1$, flotillin-2 and

the tyrosine kinase p56/53$^{lyn}$ – which are, reportedly, selective markers of lipid rafts – revealed that the isolated material was not only quantitatively but also qualitatively different from the expected. Flotillin-2 and p56/53$^{lyn}$ remained almost entirely confined in the dense fractions of the gradient, while $GM_1$ was only present in minor amounts in the putative DRM fraction (figure 1C, arrow). The basic protocol was initially varied to take into account possible factors that could have determined this major discrepancy as listed here: (i) samples treated with 1% TX-100 in TNE were homogenized with a glass Dounce homogeniser before gradient separation; (ii) the total protein concentration of TX-100-treated ghosts was varied between 0·14 mg/ml and 2·4 mg/ml; (iii) the concentration of TX-100 was varied between 0·5% and 1·5% at a constant protein concentration of 1·0 mg/ml during detergent-extraction; (iv) the freshly-drawn blood from which ghost membranes were prepared was either leukodepleted or not; or (v) it was stored for one week at 4°C before processing without leukodepletion. As none of the modifications listed above resulted in the recovery of DRMs in the expected quantity and quality, at the interface between 5% and 30% sucrose, the detailed results are not shown here, being virtually superimposable to those shown in figure 1.

### 3.2 *Further variations to the basic protocol for the isolation of DRMs*

As we suspected that the difficulties in isolating DRMs by the sole treatment with a nonionic detergent were due to failure to detach them from larger, high-density com-

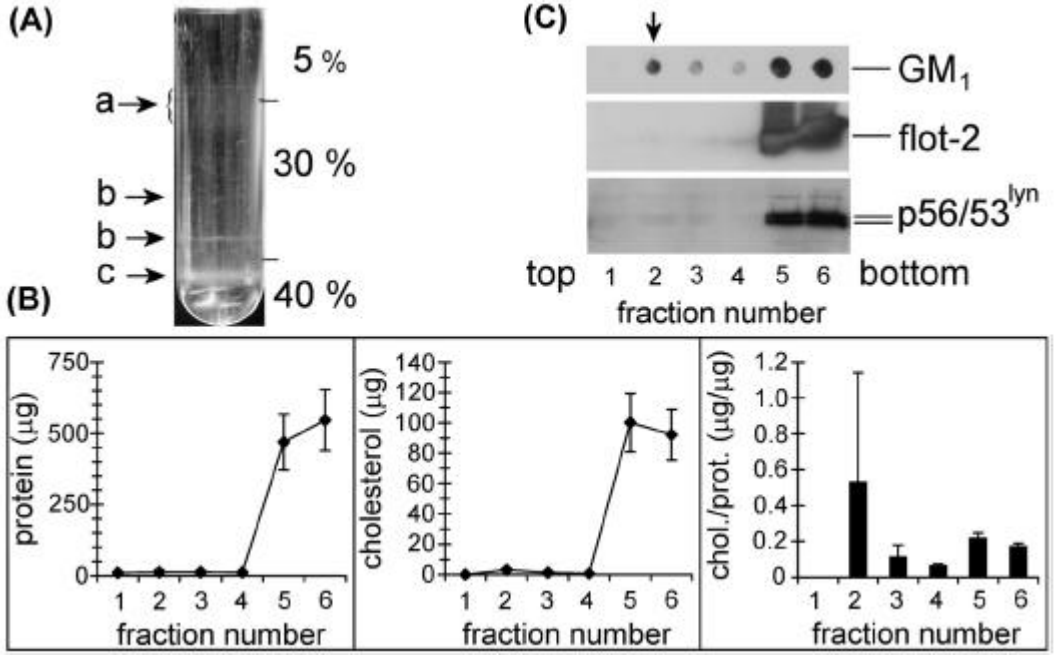


**Figure 1.** Basic protocol for the isolation of DRMs from erythrocyte ghosts. Erythrocyte ghosts were treated with 1% TX-100 in TNE. After incubation for 20 min at 4°C, the sample was diluted with an equal volume of 80% sucrose solution in TNE, and 0·84 ml of this mixture were loaded in the tube and subjected to density fractionation, as described in § 2. (**A**) Aspect of the sucrose density gradients after centrifugation. A faint band of refractive material was detected at the interface between 5% and 30% sucrose (arrow a). Depending on the experiment, various bands of detergent-insoluble material were also visible in the lower part of the tubes, above (arrows b) or within the sample-loading zone (arrow c). (**B**) Quantification of protein content, cholesterol content and cholesterol/protein ratio of six fractions collected from the top of the gradient in four independent experiments. Protein and cholesterol contents are expressed as total micrograms per fraction from one tube. Fraction volumes were 1·0 ml for fraction 1 and 0·8 ml for fractions 2–6. The percent of cholesterol recovered in fraction 2 (arrow a) was 1·6 ± 0·4 (mean ± standard deviation; $n = 4$) with respect total sample cholesterol. The high variability in the cholesterol/protein ratio in fraction 2 (error bar) is due to the low amounts of material collected in this fraction. Error bars represent the standard deviation. (**C**) $GM_1$, flotillin-2 and p56/53$^{lyn}$ immunodetection in the gradient fractions. Flotillin-2 is absent in the low-density DRM fraction (5%–30% sucrose), while p56/53$^{lyn}$ and $GM_1$ are present in trace amounts. Results shown in (**A**) and (**C**) are representative of four independent experiments.

plexes in the membrane, attempts were made to weaken these interactions, hypothetically electrostatic in nature, by varying the pH and the ionic strength of the medium during detergent extraction.

Figure 2A (tube 2) shows that increase of the ionic strength of the detergent-treated sample before density gradient separation to a value of I = 0·3 (a double ionic strength compared to TNE), did not alter the sucrose gradient profile with respect to the control (tube 1). Increasing only the pH of the solubilization medium from 7·4 to 12·5 resulted in a progressive migration of detergent-insoluble material to less dense regions of the gradients (tubes 3–5). Only at pH 12·5 was the interface 5%–30% sucrose (arrow) populated with relatively abundant DRM material (tube 5). Most importantly, while I = 0·3 or pH = 10·5 were ineffective for the isolation of appreciable amounts of DRMs if tested separately, the combination of the two conditions (tube 6) brought about apparently the same result obtained with pH 12·5. Simultaneous increases in ionic strength and pH to values similar to those described above may be obtained by mixing the TX-100-treated sample with 80% sucrose in 0·2 M $Na_2CO_3$ (tube 7). Sodium carbonate has been used, in combination with nonionic detergents or alone, in a number of studies of lipid rafts (Song *et al* 1996; Nagao *et al* 2002), although we could not trace an explicit mention of its rationale. Analysis of proteins and cholesterol in 6 fractions of the gradient (tube 7) revealed that approximately 30% of total cholesterol was recovered in fraction 2 (figure 2B), which had a cholesterol to protein ratio 2·5 times higher than that of intact ghosts. $GM_1$ was enriched in the DRM fraction of carbonate-treated samples, thus confirming the selectivity of the procedure (figure 2C). The distribution of flotillin-2 in ten fractions collected

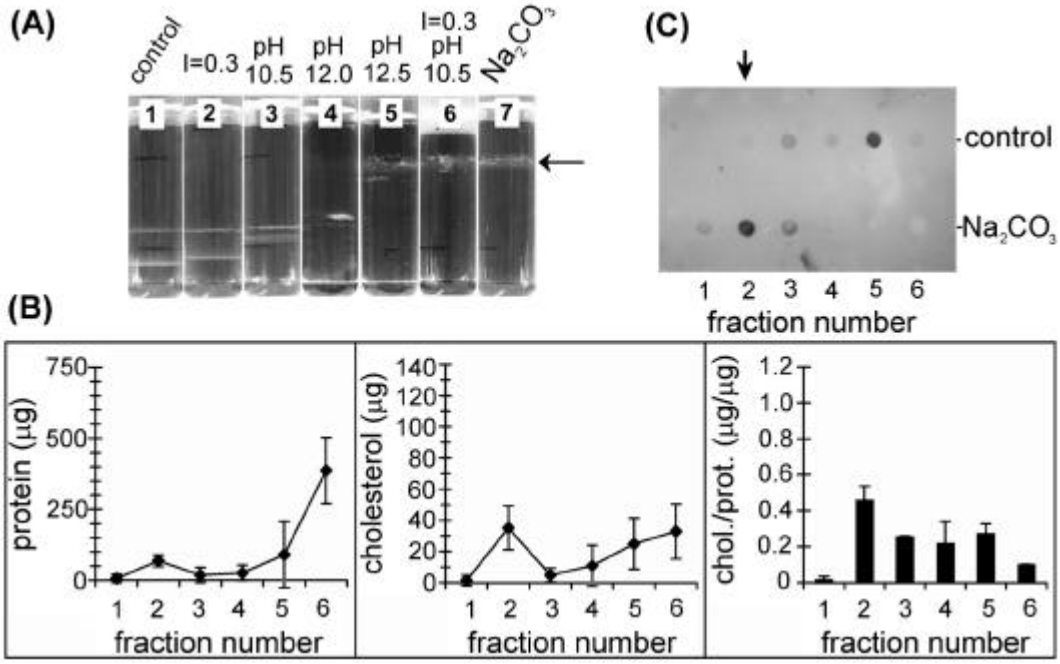


**Figure 2.** Variations of the basic protocol for the isolation of DRM from erythrocyte ghosts. Erythrocyte ghosts were treated with 1% TX-100 in TNE at a protein concentration of approximately 0·8 mg/ml (0·2 mg/ml cholesterol). After incubation for 20 min at 4°C, the sample was diluted with an equal volume of 80% sucrose solution in TNE (control) or in the various solutions described under § 2 to adjust the ionic strength and/or pH of the sample as required, before gradient fractionation. In each tube, 1·25 ml of this mixture were loaded. (**A**) Aspect of the sucrose gradients after centrifugation. Tubes 5–7, corresponding to the conditions pH = 12·5, I = 0·3/pH = 10·5, and $Na_2CO_3$, respectively, contain a visible band of low-density DRMs (arrow). (**B**) Quantification of protein content, cholesterol content and cholesterol/protein ratio of six fractions collected from the top of the gradient corresponding to the $Na_2CO_3$-treated sample, in three independent experiments. Protein and cholesterol contents are expressed as total micrograms per fraction from one tube. Fraction volumes were 1·0 ml for fraction 1 and 0·8 ml for fractions 2–6. The per cent of cholesterol recovered in the DRM fraction 2 was 28 ± 6 (mean ± standard deviation; $n = 3$) with respect to total sample cholesterol. Error bars represent the standard deviation. (**C**) $GM_1$ content in gradient fractions, showing selective enrichment of the ganglioside in DRMs obtained by treatment of TX-100-extracted ghosts with $Na_2CO_3$ before gradient fractionation. Results shown in (**A**) and (**C**) are representative of three independent experiments.

from the gradients of figure 2 is shown in figure 3. Again, DRMs obtained by simple treatment of ghosts with the nonionic detergent at 4°C (control) were devoid of flotillin-2, which remained concentrated in the dense region of the gradient. When carbonate was added, DRMs were both abundant and selectively enriched in flotillin-2, which, in fact, was almost entirely recovered with the DRMs (figure 3, $Na_2CO_3$). Fractions 2–4 also displayed higher amounts of $p56/53^{lyn}$ and flotillin-1 than in control samples (not shown). Band 3, the main integral protein of the erythrocyte membrane, was, in turn, absent in DRMs, or present in trace amounts, as previously observed (Samuel *et al* 2001; Salzer and Prohaska 2001; Murphy *et al* 2004).

### 3.3 *Isolation of DRMs from spectrin-free erythrocyte membranes*

We reasoned that, if ghost membranes were freed of the membrane skeleton before treatment with the nonionic detergent, the additional increase in pH and ionic strength would no longer be necessary for the isolation of DRMs. Removal of spectrin can be effected by treating ghost membranes with buffers of very low ionic strength. This method, which results in endovesiculation of the membrane, was originally exploited for the preparation of IOVs, after the early work of Steck and Kant (1974). Figure 4 shows the result of the treatment of spectrin-free membranes with TX-100 alone, compared with samples of intact ghosts treated either with TX-100 alone (control) or with TX-100 and $Na_2CO_3$. Spectrin-free membranes yielded an appreciable band of light-density DRMs even in the absence of carbonate treatment (figure 4A, arrow). These DRMs were enriched in flotillin-2 (figure 4B) in a selective manner, being band 3 largely depleted, although less than in DRMs obtained from ghosts by the combined treatment with TX-100 and carbonate (see § 4).

### 3.4 *DRM connection to the membrane-skeleton*

We suspected that the membrane skeletal proteins, mostly spectrin, acted as ballast to prevent the migration of

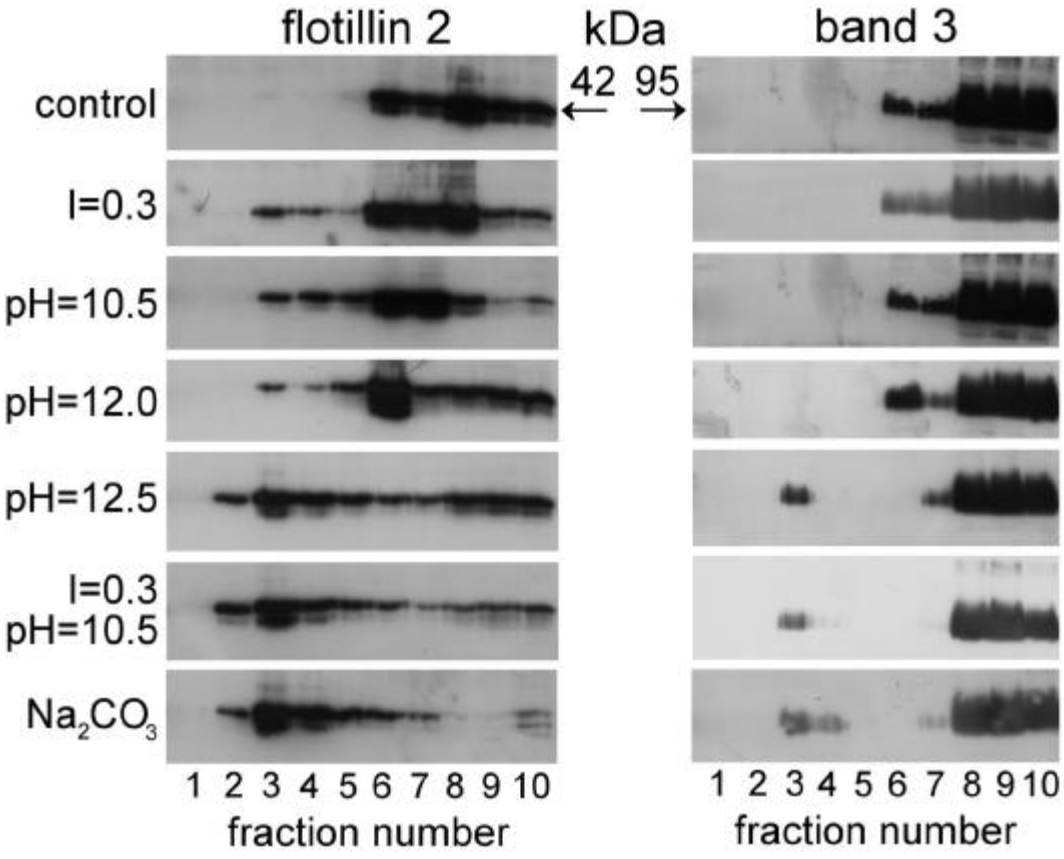


**Figure 3.** Immunodetection of flotillin-2 and band 3 in DRMs isolated from erythrocyte ghosts by various treatments. Flotillin-2 (left) and band 3 (right) Western blotting in ten fractions of 0·5 ml each, obtained from the sucrose gradients described in figure 2 and numbered from top to bottom. Flotillin-2 is enriched in DRMs (fractions 2–4) when the extraction conditions include, besides TX-100, higher pH (pH 12·5), or high pH and ionic strength (I = 0·3/pH = 10·5, or $Na_2CO_3$).

DRMs in the low-density region of the gradient. It could be argued, however, that failure to isolate DRMs under these conditions was rather due to the spectrin skeleton preventing the assembly of lipid microdomains and raft-specific proteins into DRMs of proper density. To address this issue, we exploited the fact that when ghost membranes were treated with TX-100 in buffers of either high pH (10·5–12) or high ionic strength alone, a band of refractive material was often clearly visible in the gradients at the interface between the sample loading zone (40% sucrose) and the overlying 30% sucrose (see figure 2, tubes 2–4). Under our hypothesis, these bands should contain DRMs similar, both quantitatively and qualitatively, to those obtained with TX-100 plus carbonate, except for a higher amount of spectrin. On the other hand, if spectrin prevented the assembly of membrane microdomains, these bands should either represent a minor amount of DRMs with the expected properties, or a different species of detergent-resistant membranes, of mixed composition and no enrichment in specific markers.

Figure 5 shows results compatible with our hypothesis. In fact, the material isolated with TX-100 at pH 11·2 (tube 2), corresponding to the band at the 40%–30% sucrose interface, contained 30% of total sample cholesterol, was enriched in flotillin-2 and devoid of band 3, thus being indistinguishable from a sample of DRMs isolated with TX-100 plus carbonate (tube 1), except for the higher amount of spectrin. This indicates that spectrin does not prevent the assembly of DRMs, but rather increases their buoyant density.

Preliminary experiments were dedicated to the isolation of lipid domains with a detergent-free procedure. Treatment of erythrocyte membranes with carbonate alone, at a higher concentration than that used in combination with TX-100, resulted only in a band of refractive material at the interface 30%–40% sucrose (figure 5, tube 3) which contained most of the membrane cholesterol and band 3 protein, but was not enriched in flotillin-2. Interestingly, spectrin was strongly depleted, suggesting that the effect of carbonate was indeed the selective displacement of the membrane-skeleton from the membrane, without, however, inducing a selective enrichment in flotillin-2. When membranes treated with carbonate alone were also subjected to homogenization (with a Dounce homogeniser), a band of material, amounting to 2% of total sample cholesterol, was isolated from the 5%–30% sucrose interface (figure 5, tube 4). However, this band was devoid of flotillin-2, thus resembling the material isolated with 1% TX-100 alone (see figure 1).

## 4. Discussion

According to recent views of how lipid rafts are organized in the membrane of living cells, DRMs may result from the coalescence of lipid nano-domains that exist as dynamic entities in the lipid bilayer and assemble into larger complexes while selectively including – depending on any given biochemical procedure adopted – some proteins and excluding others. While trying to implement what seemed to be a relatively standard procedure for the isolation of DRMs from erythrocyte membranes, we realised that the complexity of the variables involved prevented the reproduction of previously reported results. Our difficulties in isolating DRMs conform to previously

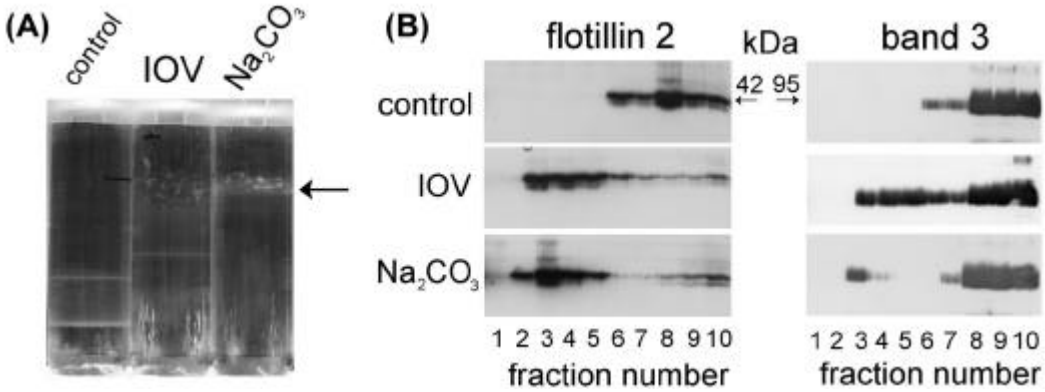


**Figure 4.** Isolation of DRMs from spectrin-free erythrocyte membranes. (**A**) DRMs (arrow) can be isolated from spectrin-free membranes (IOVs) with 1% TX-100 at 4°C in TNE without further adjustments of pH and/or ionic strength. Samples 'control' and '$Na_2CO_3$' were obtained as described in the legend to figure 2. (**B**) Immunodetection of flotillin-2 (left) and band 3 (right) in fractions from density-fractionated spectrin-free membranes treated with TX-100 alone, as compared to ghost membranes treated with TX-100 alone (control) or with the detergent and then with $Na_2CO_3$. Flotillin-2 is enriched, compared to band 3, in DRMs isolated from spectrin-free membranes under the conditions described above.

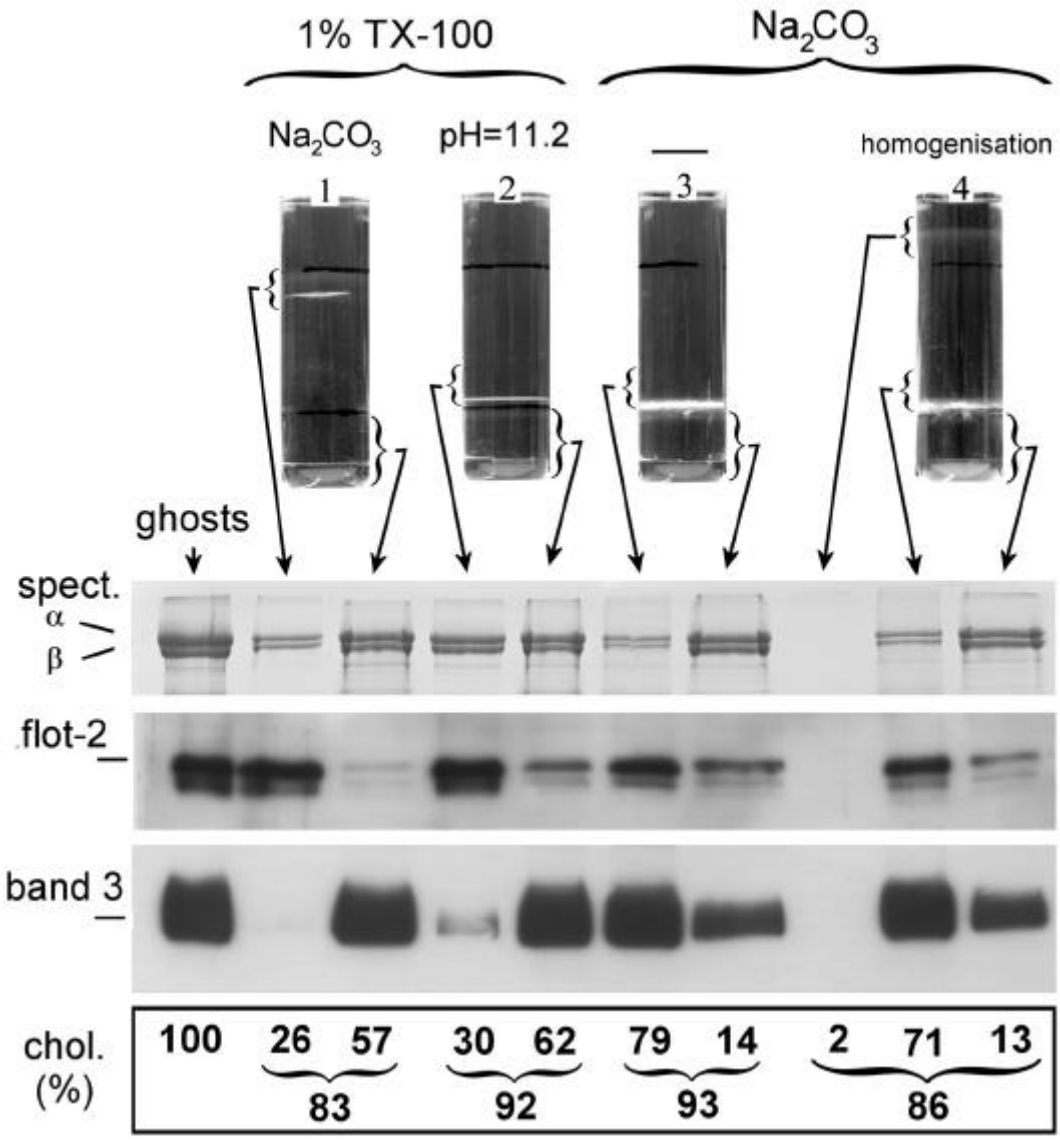


**Figure 5.** Density properties of DRMs in relation to their spectrin content. Erythrocyte ghosts were treated with TX-100 in TNE (final protein concentration = 0·8 mg/ml; detergent concentration = 1%) for 20 min at 4°C then mixed with an equal volume of 80% sucrose in 0·2 M $Na_2CO_3$ (tube 1), resulting in a final pH = 10·5 and I = 0·3 after mixing, or 0·053 M NaOH (tube 2) to give a final pH = 11·2 after mixing, and separated in sucrose density gradients as described under § 2. Alternatively, as a detergent-free method, erythrocyte ghosts were treated with 0·2 M $Na_2CO_3$ (final protein concentration = 0·8 mg/ml; final $Na_2CO_3$ concentration = 0·16 M, corresponding to I = 0·48, pH = 11·4) and incubated at 4°C for 20 min without (tube 3) or with (tube 4) homogenization with 20 strokes in a 1 ml glass Dounce homogeniser. Samples were then mixed with an equal volume of 80% sucrose in 0·2 M $Na_2CO_3$ resulting in a final I = 0·6 and pH = 11·7, and subjected to fractionation in sucrose gradient. Various fractions were collected from the gradients as indicated by the arrows, and subjected to SDS-PAGE for visualization of spectrin (spect. *a*,*b*) by Coomassie-blue staining, or for Western blotting to detect flotillin-2 and band 3, as indicated. The first lane in the gels (ghosts) contains an amount of ghost membranes proportional to the total amount loaded in the gradients. Samples from the gradient bands were loaded in amounts proportional to the collected volume for each band. Cholesterol (bottom panel, chol. %) was quantified only in the fractions indicated by the arrow and expressed as percent of the total cholesterol of a ghost sample corresponding to the amount subjected to each treatment. Results shown are representative of two similar experiments.

characterized rafts in erythrocytes (enriched in $GM_1$ and flotillins) by following, as a basic protocol, the operational definition of rafts, i.e. their insolubility in nonionic detergents (in our case, TX-100) at 4°C, suggested that additional factors were involved. Several modifications of the basic method of isolation were tested, by decreasing sample protein concentration during TX-100 treatment, or by varying the concentration of detergent between 0·5 and 1·5%, as suggested by some (Murphy *et al* 2004), or by mechanical homogenization of the sample with a Dounce homogenizer. None of these modifications was effective: not only the cholesterol recovered as DRM amounted to less than 5% of total cholesterol of the original ghost sample, but also, most importantly, some of the characteristic markers of rafts, like $GM_1$, flotillin-2, $p56/53^{lyn}$, were absent or present only in trace amounts in the isolated material. Recent work from another group seems to confirm that simple extraction of human erythrocyte ghosts with cold TX-100 and homogenization of the sample by passage through a narrow gauge needle, results in the recovery of less that 5% total lipids as DRMs (Koumanov *et al* 2005). Although this fraction was not characterized for the presence of raft markers like $GM_1$ or specific protein components, freeze-fracture microscopy revealed the absence of intramembrane particles, suggesting that this material might be similar to the DRM fraction obtained in low-yield in our study by following the basic protocol, which was largely devoid of proteins (flotillins, $p56/53^{lyn}$, and band 3). The low amount of DRMs recovered by us is in contrast with the much higher yield obtained by Samuel *et al* (2001) under similar conditions: in fact, although they state that "~ 10% of the total cellular cholesterol is in the DRM fraction" a figure of 15% can be recalculated from the results presented in figure 1C of the cited paper. By a similar recalculation on another set of data from the same group (Murphy *et al* 2004) a value of up to 40% for the cholesterol content in the DRM fraction, with respect to total cell cholesterol can be obtained (figures 1 and 2 in Murphy *et al* 2004). This discrepancy is difficult to explain, but it may be related to the relatively scarce information available, in the published protocols, regarding some peculiar step(s) for homogenizing the detergent-treated samples before density gradient fractionation. These manoeuvres are described, usually for cell types other than the erythrocyte, with variable details, ranging from none to relatively complex and drastic mechanical manipulations. Among the protocols in which sufficient explanation is provided, for example, isolation of DRMs from neuronal cortex cells, required homogenization by Dounce homogeniser in detergent-containing medium, followed by several cycles of sonication, before gradient fractionation (Parkin *et al* 1999). Similarly, neuroblastoma cells needed mechanical homogenization to release glycosphingolipid-enriched domains (Prinetti *et al* 1999). Porcine lung tissue was homogenized in a blender, the microsomal fraction treated with TX-100 and repeatedly passed through a narrow-gauge syringe needle, stirred for 2 h at 4°C, then again passed through a syringe needle, and finally in a Dounce homogenizer (Parkin *et al* 1996). Surprisingly, the need of similar treatments for DRM extraction from erythrocytes has not been reported. A number of studies simply refer to previously published protocols (Fricke *et al* 2003; Black *et al* 2004). More commonly, no additional steps besides solubilization with the nonionic detergent are described (Murphy *et al* 2004). In a single study, addition of sodium carbonate to detergent-treated whole erythrocytes appeared to be necessary for DRM isolation (Nagao *et al* 2002). Our own results seem to confirm the need for a similar treatment of erythrocyte ghosts, suggesting that the role of carbonate is to disrupt, by inducing a simultaneous increase in the pH and ionic strength of the solubilization medium, electrostatic interactions between the DRMs and the membrane skeleton. Although under these conditions the information on the pre-existing interactions between the lipid domains and the membrane-skeleton is lost, data presented here seem to indicate how to recover some of this information. The evidence that TX-100 treatment of spectrin-free membranes was sufficient *per se* to isolate higher amounts of DRMs enriched in flotillin-2 and $GM_1$, suggested that they might be bound to the membrane-skeleton in whole erythrocyte ghosts. In these DRMs, higher amounts of band 3 were also detected (see figure 4B), probably due to some non-specific migration, in the DRM region of the gradient, of band 3-containing membrane fragments or protein aggregates, which could have originated during preparation of IOVs. However, it must be noted that the procedure was still highly selective, since all flotillin-2 was present in the DRM fraction. Completely unspecific mixing of membrane fractions would have resulted in equal distribution of flotillin-2 between the DRM region and the sample-loading zone.

Since the procedure for preparing IOVs may cause a reorganization of the integral proteins and the lipid bilayer, possibly leading to partial loss of membrane architecture, a different approach was also followed to test the hypothesis of an electrostatic interaction between the DRMs and the membrane skeleton. Moreover, an answer was needed to the question whether spectrin, rather than increasing the density of fully formed DRMs, could simply prevent their assembly. We observed that, by increasing only the pH of the detergent-treated ghost membranes, it was possible to isolate additional bands of DRM-material of higher density, migrating in the gradients at the boundary between 40% and 30% sucrose. These bands had a composition very similar to that of low-density DRMs obtained by the combined action of

TX-100 and carbonate on whole ghost membranes (absence of band 3, enrichment in flotillin-2, a content of 30% of total sample cholesterol), except for a higher amount of spectrin. The interpretation of the results was two-fold: on the one hand that spectrin interacted with these DRMs and contributed to increase their density, and, on the other, it did not inhibit the formation of a 'normal' amount of DRMs.

As for the nature of the interactions between spectrin and the DRMs, that we have shown to be sensitive to pH and ionic strength, they may occur between one or more integral proteins and the spectrin skeleton. The two classical 'vertical' interactions between the lipid bilayer and the membrane skeleton, linking band 3 to spectrin through ankyrin, on the one hand, and glycophorin C to the 'junctional complex' (actin, protein 4·1, p55, spectrin) on the other, appear not to be good candidates since both band 3, as confirmed here and glycophorin C, as originally seen by others (Salzer and Prohaska 2001), are virtually absent in this species of DRMs. A more careful quantification of the residual band 3 and glycophorin in these fractions is under way. If it will be excluded that trace amounts of these proteins could tie the DRMs to the membrane skeleton, an alternative explanation is needed. A hint may be in the evidence that stomatin-like-protein-2 (SLP-2), an integral membrane protein present in the erythrocyte in approximately 4000 copies/cell, may contribute to such interaction, alone or in association with the more abundant stomatin (100000 copies/cell, enriched in red cell DRMs), since SLP-2 (unlike stomatin) appears to associate tightly with the membrane skeleton (Wang and Morrow 2000). Another candidate for the anchoring of lipid microdomains could involve $GM_1$, since it was known well before the notion of lipid-raft gained popularity that a fraction of membrane lipids selectively enriched in $GM_1$ resists solubilization by nonionic detergents at 4°C (Kellie *et al* 1983). However, since $GM_1$ does not span the membrane, the support of membrane proteins that could act as an anchor to the cell cytoskeleton was invoked (Lingwood *et al* 1980; Watanabe *et al* 1980). In the lipid-raft era, on the other hand, only relatively few studies, to our knowledge, report on the existence of lipid-rafts that are tethered to the cytoskeleton by specific protein-protein interactions (Oliferenko *et al* 1999; Foger *et al* 2001), while it may be worth reconsidering in a new perspective earlier reports on the co-isolation, from lymphoid cells, of actin, GPI-linked proteins, and specific lipids as detergent-insoluble fractions (Mescher *et al* 1981).

Alternatively, or in addition to protein-protein interactions, it may be speculated that the lipid moiety itself could contribute to the association of these domains with the membrane skeleton, according to the hypothesis previously suggested and supported by some evidence, of an affinity of spectrin for phospholipids (Haest 1982; O'Toole *et al* 2000). Interestingly, recent data seem to indicate that phosphatidylserine and phosphatidylinositol, which could establish such interaction, are present in both DRMs and in the parent cell membrane in similar proportions (Koumanov *et al* 2005).

In summary we have provided evidence that treatment of erythrocyte membranes with TX-100 produced at least two subfractions of detergent-resistant domains, one of which interacts with components of the membrane-skeleton. This association must be electrostatic in nature, being altered by varying pH and ionic strength. Moreover, such different classes of DRMs must also differ qualitatively, since extraction at physiological pH and ionic strength only produced a minor amount of low-density material, largely devoid of specific 'raft' markers. On the other hand, at higher pH and ionic strength the DRMs were selectively enriched in specific markers.

To better understand the relative role of nonionic detergents, of carbonate and mechanical manipulation, in some experiments a detergent-free method, based on treatment with carbonate alone, was adopted for the isolation of lipid rafts from ghost membranes. It was shown that a minor band of 'raft' material, qualitatively and quantitatively similar to the low density DRMs obtained by TX-100 alone, was isolated only after homogenization of the carbonate-treated samples. A major band of high-density extraction-resistant membranes was also obtained, whose protein composition was similar to that of untreated membranes, except for the lack of membrane skeletal components (figure 5). This would indicate that, both in detergent-based, and in detergent-free methods, carbonate plays the role of disrupting the interactions of raft domains with the cytoskeletal complex, while the specific composition of the domains is dictated by the detergent in the former methods, or the mechanical homogenization in the latter. The fact that we could not obtain a fraction enriched in raft markers by simple dispersion of the sample with a Dounce homogenizer, may simply indicate that more drastic methods for homogenization are needed, for instance sonication, which, however, is characterized by high variability (Macdonald and Pike 2005). Evidence in the literature indicates that, in other cell types, specific raft domains may be obtained with carbonate and mechanical manipulation (Song *at al* 1996), or even by mechanical homogenization alone (Smart *et al* 1995; Macdonald and Pike 2005). Work is in progress to determine whether the same could be obtained with erythrocytes.

Detergent-based methods for isolation of lipid rafts have been criticized mainly on the basis of studies on artificial membranes of known lipid composition, where the detergent itself has been shown to induce selective aggregations of lipids and formation of liquid-ordered phases, or

to affect the composition and dynamic equilibrium of pre-existing, spontaneously forming microdomains (Heerklotz *et al* 2003; Mayor and Rao 2004). These model systems are probably far from describing the situation in a real cell membrane, both because of the much higher number of different lipid classes present *in vivo*, and because the role of membrane proteins has not been taken into account. However, they provided results that serve as a caveat against a too superficial representation of lipid rafts as stable entities in the membrane, and of DRMs as a valid material for biochemical characterization. On the other hand, the mechanism by which DRMs with similar peculiar properties are obtained from different cell types, and by different procedures, is still largely unknown, and deserves further investigation. The present work shows that the interaction between the cytoskeleton and the lipid bilayer represents another variable amongst the many already at play in this complex scenario. It would also be interesting to know if a particularly strong association of DRMs to the membrane skeleton is a peculiar property of the erythrocyte, that might be relevant for the stability of the membrane and the specialized role played by this cell type, that faces intense mechanical stress in traversing the microvasculature during its circulatory life.

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