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# Detergent-free isolation of native red blood cell membrane complexes

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ABSTRACT

Over the past few decades, studies on the red blood cell (RBC) membrane gave rise to increasingly sophisticated although divergent models of its structural organization, since investigations were often performed in denaturing conditions using detergents. To access soluble isolated RBC membrane complexes with the preservation of their interactions and conformations, we decided to apply the recent SMALP (Styrene Maleic Acid Lipid Particles) technology to RBC ghosts. Depending on the ionic strength of buffers in which ghost membranes were re-suspended, the isolated proteins within SMALPs could differ on Coomassie–stained gels, but with few changes when compared to ghost membrane SDS lysates. We subsequently produced SMALPs derived from ghosts from two different blood group phenotypes, RhD-positive and RhD-negative, both types of RBC expressing the RhCE proteins but only RhD-positive cells being able to express the RhD proteins. This allowed the isolation, by size exclusion chromatography (SEC), of soluble fractions containing the Rh complex, including the RhD protein or not, within SMALPs. The use a conformation-dependent anti-RhD antibody in immunoprecipitation studies performed on SEC fractions of SMALPs containing Rh proteins clearly demonstrated that the RhD protein, which was only present in SMALPs prepared from RhD-positive RBC ghosts, has preserved at least one important conformational RhD epitope. This approach opens new perspectives in the field of the erythroid membrane study, such as visualization of RBC membrane complexes in native conditions by cryo-electron microscopy (CryoEM) or immuno-tests with conformation-dependent antibodies against blood group antigens on separated and characterized SMALPs containing RBC membrane proteins.

## 1. Introduction

During these last five decades, the red blood cell (RBC) membrane has been extensively studied [1] and data on its composition have been useful as a model for studies of other plasma membranes [2]. Because of its specific biconcave shape allowing both deformability and stability, the erythrocyte can travel in the circulation and squeeze through the capillary network. This property is related to connections between transmembrane proteins and the membrane skeleton which have also been widely described [3]. Interest in the nature of this plasma membrane has been reinforced since many components of the RBC membrane carry blood group antigens that may be involved in blood type incompatibilities during transfusions or pregnancies [4].

To date, information on the erythrocyte membrane proteins has essentially been obtained from membranes ('ghosts') isolated by hypotonic hemolysis in solubilizing media of different ionic strengths followed by various detergent treatments. Major erythrocyte membrane proteins have thus been separated and characterized, clearly identified by their migration patterns in SDS-PAGE [5]. Less abundant membrane proteins can be detected by immunoblot analysis of the RBC membrane lysates. These studies, performed on normal and pathological RBCs, have yielded critical information on the organization of membrane complexes [6–9].

Based on these biochemical data as well as on determination of recent crystal structures and/or 3D structure modellings, several models of the RBC membrane have been proposed over the past 20 years [10–13]. However, use of detergents leads to loss of most protein/protein and protein/lipid interactions. Consequently, in RBCs,

*Abbreviations:* RBC, red blood cell; SMALP, Styrene Maleic Acid Lipid Particles; Rh, Rhesus; RhAG, Rh-associated glycoprotein; G3PD, glyceraldehyde 3-phosphate dehydrogenase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; MOPS, 3-(N-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; LIS, low ionic strength; VLIS, very low ionic strength

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though the major anchoring points of the membrane to the skeleton have been definitively established, contacts and relative positions of proteins within the complexes, essentially between the transmembrane proteins, remain unknown and may differ from one model to another [1]. For example, based on the crystal structure of non-erythroid homologue RhCG [14], a trimeric structure of Rh proteins is accepted in the recent representations of the RBC membrane, but no information is yet available regarding the precise subunit composition of the trimers in Rh proteins (RhAG, RhD and/or RhCE). Moreover, some models propose a location for the Rh trimer between two dimers of Band 3, the most abundant RBC membrane glycoprotein [10,11,13], while other models suggest a location next to Band 3 tetramers [1,12,15]. Detergent extracts from ghost membranes cannot help to decide between these hypotheses.

As a further difficulty, blood group antigens carried by RBC membrane proteins are mainly recognized by conformation-dependent antibodies and thus cannot be extracted in their native form with conventional techniques that use detergents, nor be detected by these antibodies following extraction. This is the case for highly polymorphic Rh proteins which carry 54 antigens encoded by two highly homologous genes (*RHD* and *RHCE*) [16]. RhD-negative Caucasian individuals, do not express RhD protein, due to deletion of the *RHD* gene, but express only RhCE protein carrying the RhC/c and RhE/e antigens. RhD-positive Caucasian individuals express both RhD protein, carrying the RhD antigen, and RhCE proteins [17]. The RhD antigen can be considered as a collection of epitopes along the entire RhD protein [18]. All available antibodies against D epitopes recognize only native RhD protein, except for one monoclonal anti-D which is able to detect both native and denatured protein [19].

The application of an amphipathic copolymer, Styrene Maleic Acid (SMA) to plasma membrane has allowed the successful extraction, in the absence of detergents, of proteins embedded in nanodiscs of ~10 nm diameter [20], giving access to isolated membrane complexes, while preserving their conformation and native environment, for further biochemical and structural analysis [21].

In the present study, we solubilized the RBC membranes from ghosts treated by buffers of different ionic strengths, in order to produce SMALPs either bound to, or devoid of, skeletal proteins. We then characterized SMALPs produced from RBC ghosts of RhD-positive and RhD-negative blood group phenotypes respectively. Finally, we showed that, once presented in SMALPs, the RhD blood group antigen could be detected by an anti-RhD antibody which, like almost all anti-RhD immunoreagents currently used in immunohematology, is conformation-dependent.

## 2. Materials and methods

### 2.1. Reagents and antibodies

The ready-to-use Lipodisq® (Styrene:Maleic Anhydride Copolymer 3:1, pre-hydrolyzed) and the protein A-Sepharose CL-4B were purchased from Sigma-Aldrich (Saint Quentin, France).

Conformation-independent human monoclonal anti-RhD LOR15C9 which exceptionally recognizes a linear epitope of RhD [19], was a generous gift of Pr. A. Blancher from the CHU Purpan, Toulouse, France. Conformation-dependent human monoclonal anti-RhD F5 recognizing Epitopes 6 and 7 [22], and rabbit polyclonal antibody anti-RhD/RhCE MPC8 raised against a linear C-terminal epitope common to both RhD and RhCE proteins [23], were developed in our Institute (the National Institute for Blood Transfusion- INTS, Paris, France).

### 2.2. Red blood cells

In this study, RBCs are from the "Panel National de Référence du CNRGS" and are reagents used for red cell antibody identification, and as positive or negative controls for RBC phenotype analysis. This panel has received the CE marking according to the 98/79/EC European directive, and certification for the EN ISO 13485 standard in 2005. The present study was conducted according to institutional ethical guidelines of the National Institute for Blood Transfusion (INTS, Paris, France). All procedures were carried out in accordance with the Declaration of Helsinki. Written informed consent was given by the donors.

### 2.3. Ghost preparation and solubilization

Ghosts were obtained from RBCs according to the protocol in Steck [24]. Briefly, 200 µl of pelleted RBCs were washed three times in D-PBS (Life Technologies, Fisher Scientific, Illkirch, France) and submitted to hypotonic lysis by an addition of 40 volumes of low ionic strength buffer (LIS) composed of 5 mM sodium phosphate, 0.1 mM EDTA, pH 8. Membranes were pelleted by centrifugation 15 min, 27,000*g*, 4 °C (rotor JA25.50, Beckman Coulter). Supplementary washes in LIS buffer were carried out until the pellet became white.

A part of these white ghosts were further submitted to very low ionic strength buffer (VLIS) composed of 0.3 mM sodium phosphate, 0.1 mM EDTA, pH 8, in order to remove the spectrin-based skeleton [25]. In this condition, after a wash in VLIS buffer, they were resuspended in 10 volumes of VLIS buffer and incubated 30 min at 37 °C on a wheel. After centrifugation, the pelleted ghosts were kept for solubilization by SMA, and the supernatant assessed for spectrin removal.

Ghosts, subjected or not to spectrin removal, were washed by centrifugation in 50 mM sodium phosphate, 300 mM NaCl buffer pH 8. One volume of a 5% SMA solution (in 50 mM sodium phosphate buffer, pH 8) was added to 40 µl of ghost pellet exhibiting a wet weight of 20–40 mg, so that the final concentration of NaCl was 150 mM, and solubilization was performed in the SMA buffer (50 mM sodium phosphate, 150 mM NaCl, pH 8), as described in [26]. Under gentle agitation with a magnet, the white mixture became clear within a few minutes. Incubation was performed for 1 h at room temperature, then overnight at 4 °C. SMALPs were then centrifuged 1 h at 26 psi corresponding to approximately 100,000*g* in a Beckman Airfuge apparatus to remove aggregates. Supernatant was harvested and stored at 4 °C.

### 2.4. Gel filtration (size exclusion chromatography, SDS-PAGE, Coomassie staining)

SMALP supernatant was injected into a Superdex 200 increase 3.2/300 column, using an Akta Purifier (GE Healthcare Life Sciences, Velizy-Villacoublay, France), at a 0.08 ml/min flow rate. The column was previously equilibrated with one column volume of SMA buffer. 100 µl fractions were collected and stored at 4 °C. Absorbance was monitored at 280 nm.

Samples from elution fractions, SMALPs and ghosts were denatured with loading buffer containing SDS and β-mercaptoethanol and loaded onto a NuPAGE 4–12% acrylamide gradient gel (Life Technologies, Fisher Scientific, Illkirch, France). Migration was performed in MOPS buffer (Life Technologies, Fisher Scientific, Illkirch, France) at 130 V. The gels were stained with Instant Blue (Expedeon, VWR, Fontenay-sous-Bois, France) and destained with deionized water.

### 2.5. Western blot analysis

Proteins were transferred onto nitrocellulose membrane using Bio-Rad turbo transfer kit and apparatus (Biorad, Marnes la Coquette, France). Membrane were saturated with PBS- or TBS-milk 5% then probed with antibodies LOR15C9 (undiluted) or MPC8 (1/4000) overnight at 4 °C. Washes were carried out in PBS- or TBS-Tween 0.1%. HRP-conjugated secondary antibodies (Abliance, Compiègne, France) were incubated for 30 min at room temperature. After final washes, ECL Prime reagent (GE Healthcare Life Sciences, Velizy-Villacoublay, France) was added and signals were detected by a Chemidoc MP

apparatus (Biorad, Marnes la Coquette, France).

### 2.6. Immunoprecipitation

Immunoprecipitation was performed from RhD-positive gel filtration fractions. To decrease background, fractions were first submitted to a preclearing step. Protein A-Sepharose resin was equilibrated in the SMA buffer then SMALPs were added and incubated for 1 h at room temperature on a wheel. After centrifugation for 3 min at 15,000*g*, the supernatant was collected. Meanwhile, 800 µl of monoclonal antibody F5 or SMA buffer were incubated with 60 µl of settled resin for 4 h at room temperature on a wheel. Unbound antibodies were removed by washing steps with the SMA buffer. Precleared SMALPs were added on F5-resin or free resin and the mixtures were incubated overnight at 1000 rpm and 4 °C in a thermomixer (Eppendorf, Montesson, France). The following centrifugation steps were performed for 3 min at 15,000*g*. Flow-through was first harvested, then the resin was washed twice with SMA buffer and twice with 50 mM sodium phosphate, 300 mM NaCl, pH 8. Bound proteins were eluted by incubation for 10 min at room temperature with 1 × Laemmli buffer.

## 3. Results and discussion

This paper describes a solubilization of the RBC membrane, performed, for the first time, in detergent-free conditions. This was accomplished using the capacity of Styrene-Maleic Acid (SMA) amphipathic copolymer to incorporate into membranes and spontaneously form nanoscale Styrene Maleic Acid Lipid Particles (SMALPs) in which the conformation of the embedded proteins is preserved [20]. RBC membranes (named ghosts) are easy to prepare and it is possible to obtain quite pure membranes devoid of cytosolic proteins, in particular of hemoglobin, and thus composed only of integral membrane and skeletal proteins surrounded by the lipid bilayer. Addition of SMA to ghosts resulted in a rapid and almost complete solubilization of the membranes. Here, we chose to test solubilization of ghosts prepared from small amounts of RBCs, so that experiments described in the present study from small-scale solubilized material could afterwards easily be extended to samples of various RBC phenotypes.

### 3.1. Production of SMALPs bound to, or depleted of, skeletal proteins from RBC ghosts incubated with buffers of different ionic strengths

SMALPs were first produced from RBC ghosts prepared with the commonly used low ionic strength (LIS) buffer [24]. A rapid analysis of the solubilized material was performed by SDS-PAGE and Coomassie-staining. A comparison with the well-known pattern of SDS lysates of ghosts [5] revealed the presence, in the LIS-SMALPs, of most of the major membrane proteins as well as skeletal proteins, which likely remain in interaction with transmembrane proteins during the solubilization (Fig. 1A). This first result suggests that most RBC membrane proteins can be efficiently extracted by SMA.

Treatment of ghosts with buffers displaying different ionic strengths is known to modify the interactions between the integral proteins and skeleton and/or enzymes [27]. We used this property to remove most of the spectrin-based skeleton by very low ionic strength (VLIS) buffer, as described in [25], in order to more specifically separate integral proteins from the RBC membrane. The spectrin-depleted ghosts were then submitted to solubilization by SMA. For VLIS-SMALPs, the pattern obtained on a Coomassie-stained gel was similar to that of SDS lysates of spectrin-depleted ghosts (Fig. 1B), with a substantial decrease of skeletal proteins that are known to belong to the junctional complex [28].

It was interesting to find that glyceraldehyde-3-phosphate dehydrogenase (G3PD), an enzyme linked to Band 3 [29] was partially retained in these VLIS-SMALPs. In these conditions, the interaction between G3PD and Band 3 seems to be stabilized in very low ionic strength buffer, a stabilization that appears to be strong enough to be subsequently maintained in the higher ionic strength conditions necessary to produce SMALPs (Supplementary Fig. 1).

In contrast, in SMALPs from LIS-ghosts, proteins of the spectrin-based skeleton were retained, but not G3PD, which was totally absent. This loss from the membrane is assumed to be a consequence of the incubation of ghosts in high ionic strength SMA buffer, leading to a modification of the ionic interaction with its binding partner Band 3. Physiologically, G3PD is inactive when bound to its partner [30] but can be displaced and activated by deoxyhemoglobin (Hb) [31] or by tyrosine phosphorylations within the binding sites of Band 3 [30].

### 3.2. Separation by size exclusion chromatography of SMALPs prepared from LIS- and VLIS-ghosts

In order to better characterize the SMA solubilized material, we performed size exclusion chromatography. When preliminary experiments were carried out with larger volumes of ghosts, and after injection of the corresponding SMA-solubilized material into a gel filtration column (Supplementary Fig. 2), the resulting profiles were similar to those previously described for other SMALP preparations [32–34].

In the present study, the use of a smaller column did not modify the elution profiles but led to a separation with an expected lower resolution. LIS- and VLIS-SMALPs were injected into a small column of size exclusion chromatography. The removal of spectrin from ghosts did not change the overall elution profile, since elution profiles at 280 nm were similar and, in particular, peaks and shoulders can be observed for the same elution volumes (Fig. 2A and B). A calibration of the elution fractions using standards revealed very high molecular weight of SMALPs containing RBC complexes (from about 500 kDa to more than 670 kDa, Supplementary Fig. 3).

The different fractions were analyzed by SDS-PAGE and Coomassie staining. No protein was detected in fraction A1 of both gel filtrations (Fig. 2C and D), this fraction corresponding to the first peak of the chromatography and probably to lipid aggregates incompletely removed by the last centrifugation step before injection. All fractions from gel filtration of VLIS-SMALPs were, as expected, depleted in spectrin (Fig. 2C) and, interestingly, fraction A3 was enriched in SMALPs containing Band 3 with less skeletal proteins. After gel filtration of LIS-SMALPs, the largest SMALPs (fractions A2 and A3) were, as expected, those that were physically bound to spectrin. Actin, protein 4.1, 4.2 and p55 were found in the same fractions (mainly A5 and A6), while stomatin was more widely distributed, along with Band 3 (A2 to A5), as were spectrins (Fig. 2D). The distribution observed suggested that the proteins were either in SMALPs of similar size or, alternatively, co-isolated in a given SMALP.

### 3.3. Characterization of SMALPs from RhD-positive and RhD-negative ghosts by size exclusion chromatography and western blot analysis

As had been previously described for SMA-isolated protein assemblies, such as the ACIII-cyt *aa3* supercomplex from *Flavobacterium johnsoniae* membranes [35] or the RC-LH1-PufX, LH2 and cyt$bc_1$ complexes from *Rhodobacter sphaeroides* chromatophores [36], complexes from the RBC membrane were expected to be isolated in their native conformation. To test the preservation of protein structures within SMA-solubilized materials, conformation-dependent antibodies directed against a member of the complex are one efficient tool. For the RBC membrane, the Rh multimolecular complex represents a model of choice, because it is composed of proteins carrying the Rh blood group antigens, including the major antigen RhD which is essentially recognized by conformation-dependent antibodies.

For this study, we obtained SMALPs from RhD-negative and RhD-positive RBCs: ghosts were prepared from small volumes (200 µl) of RhD-positive and RhD-negative RBC pellets in LIS conditions, SMALPs were then produced from the two types of ghosts, and proteins were analyzed by size exclusion chromatography. The two elution profiles

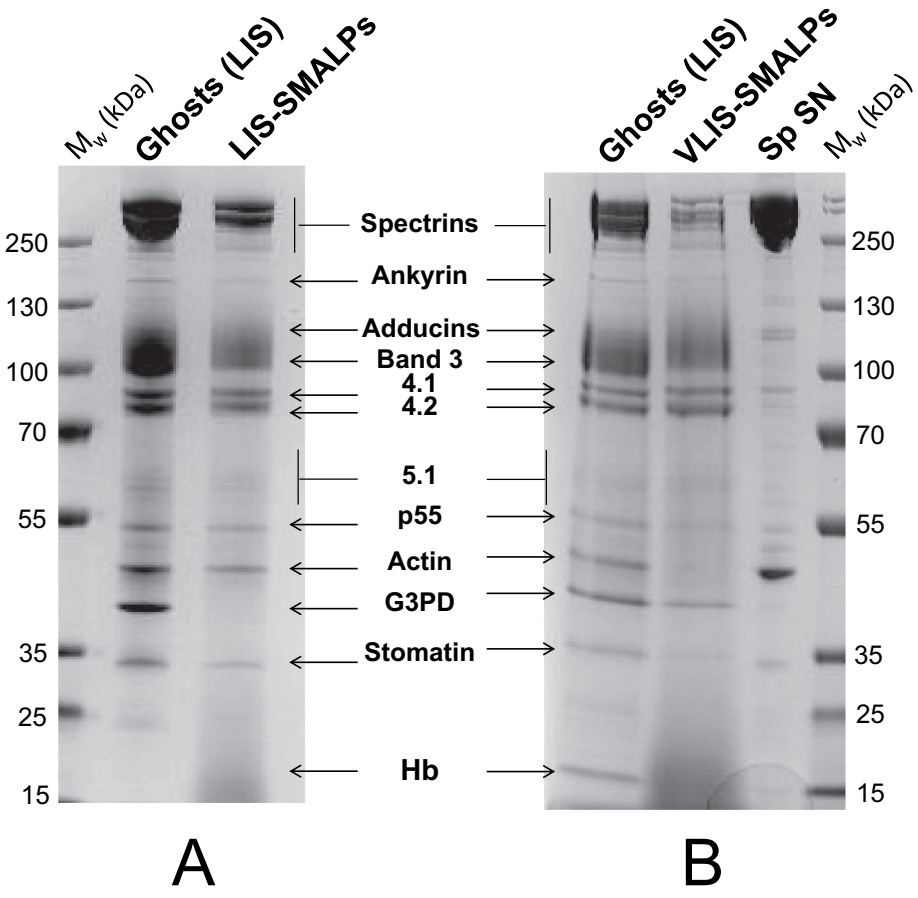


**Fig. 1.** SDS-PAGE and Coomassie staining analysis of major RBC ghost proteins and SMALPs obtained after incubation of ghosts with either low ionic strength (LIS) buffer, giving rise to LIS-SMALPs (A), or very low ionic strength (VLIS) buffer (B), producing VLIS-SMALPs. Erythroid transmembrane and skeletal proteins are identified from their well-known migration patterns. The efficiency of spectrin removal was assessed by testing in Coomassie stained SDS-PAGE the supernatant (Sp SN) after centrifugation of VLIS-Ghosts, revealing large amount of spectrins and actin but also the presence of other proteins that belong to the junctional complex, such as adducins, protein 4.1, p55 and stomatin.

were similar (the first, presumably lipidic, peak -see above-, was absent from this Rh-negative sample, but not others) (Fig. 3A and B).

Since Coomassie staining of SDS PAGE of ghost lysates does not reveal most of the integral membrane proteins of the RBC, excepting Band 3 and stomatin, SMALPs and gel filtration fractions were tested by Western blot using specific antibodies raised against linear epitopes of membrane proteins, in particular those of RhD and RhCE.

Conformation-independent monoclonal anti-RhD LOR15C9 [19] revealed a band at a molecular weight corresponding to the RhD protein (~32 kDa) only in RhD-positive ghosts. In addition, only the SMALPs prepared from RhD-positive ghosts gave a positive signal in immunoblot corresponding to RhD protein, although with a slight upward shift that can be explained by a slower gel migration of proteins due to the presence of SMA (Fig. 3C and D). In lanes loaded with the SMALPs, free SMA could be detected by an unspecific interaction with the LOR15C9 antibody, giving a large positive signal at the bottom of the gel.

Most interestingly, RhD proteins, embedded in SMALPs and consequently produced in detergent-free conditions, could be solubilized and detected from RhD-positive samples only, as shown by the signal at 32 kDa obtained essentially in gel filtration fractions A4 and to a lesser extent in A3 and A5 (Fig. 3C).

When, after stripping, the same blot was incubated with antibody MPC8 recognizing the C-terminal segment common to both RhD and RhCE proteins [23], a signal at 32 kDa was seen, mainly in gel filtration fraction A4, from *both* RhD-positive and RhD-negative SMALPs (Fig. 3D). In RhD-positive elution fraction A4, this resulted from the presence of RhD and potentially RhCE, while, in RhD-negative elution fraction A4, it was due only to RhCE. These results highlighted the presence of specific Rh proteins in SMALPs produced from both RhD-positive and RhD-negative ghosts, a significant result, considering the propensity of these 12 transmembrane proteins to aggregate when isolated in the absence of detergents.

### *3.4. Immunoprecipitation of RhD-positive SMALPs using a conformation-dependent human anti-D monoclonal antibody*

Most anti-RhD antibodies are conformation-dependent, and immunoblots cannot show whether conformation-dependent epitopes are preserved in RhD proteins embedded in SMALPs. Consequently, we used a conformation-dependent monoclonal anti-RhD antibody, F5 [22] for immunoprecipitation studies of SMALPs from enriched gel filtration fraction A4 of RhD-positive or negative ghosts. For immunoblot revelation, conformation-independent anti-RhD LOR15C9 was used (Fig. 4A and B).

A positive signal was detected exclusively in the immunoprecipitation eluate from RhD-positive, but not RhD-negative, SMALPs incubated with monoclonal anti-RhD F5, and not from RhD-positive SMALPs incubated with buffer control (Fig. 4A and B). This shows that SMALPs containing RhD protein could be isolated with a conformation-dependent antibody and clearly demonstrates that the RhD protein present in the SMALPs has preserved at least one important conformational RhD epitope.

To further analyze the Rh complex immunoprecipitated by anti-RhD F5, the samples were also reacted with MPC8 antibody, raised against the C-terminal common to RhD and RhCE [23]. We have seen that this antibody reacted with ghosts and SMALPs from both Rh-positive and Rh-negative samples (Fig. 3D). After immunoprecipitation however (Fig. 4C), MPC8 could only react with the RhD-positive SMALPs, since the MPC8-reactive RhCE protein from RhD-negative SMALPs could not be immunoprecipitated using an anti-RhD antibody. While data presented here are in agreement with current knowledge of the Rh

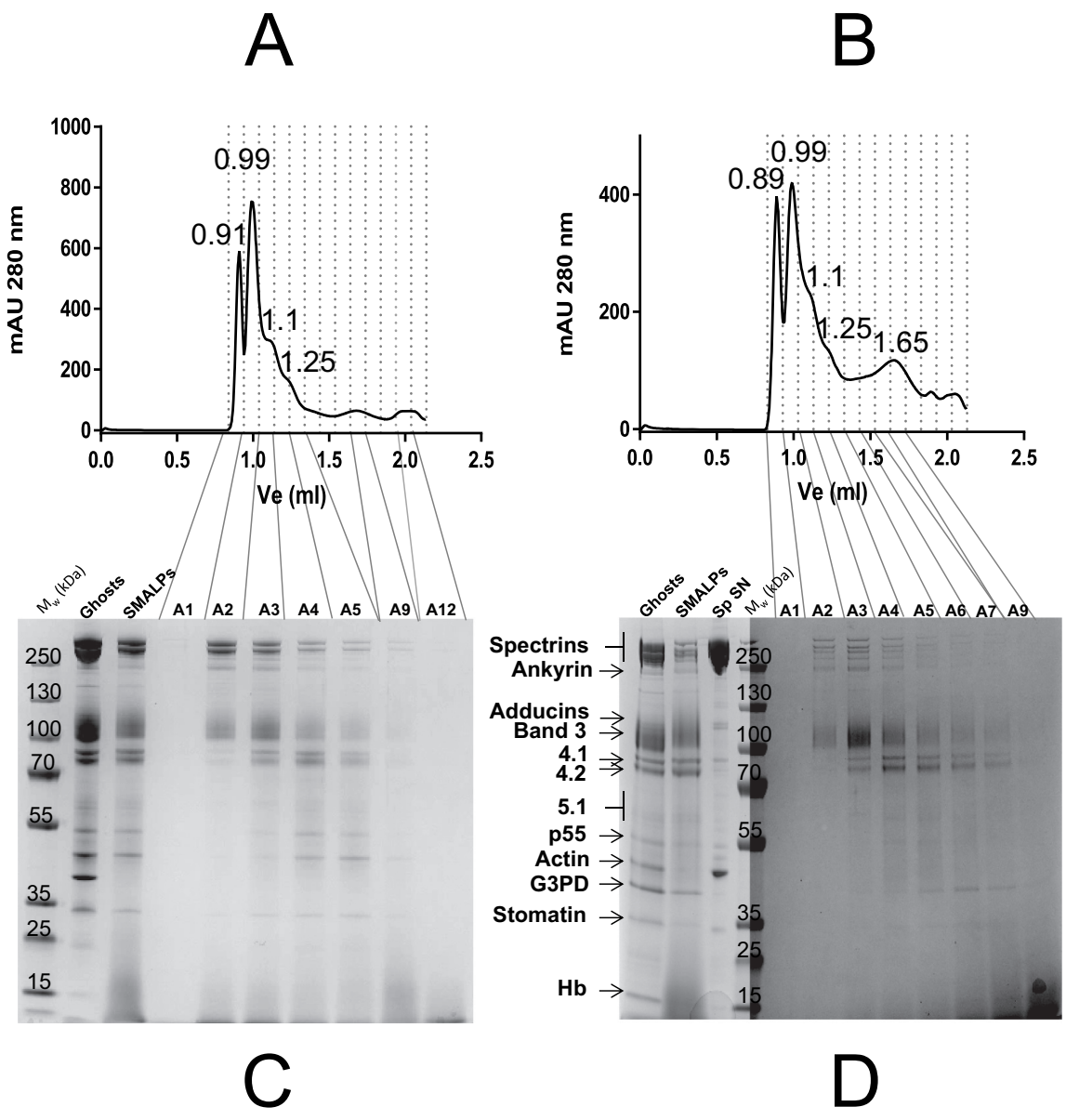


**Fig. 2.** Separation by size exclusion chromatography of SMALPs prepared from LIS- and VLIS-ghosts. The chromatograms correspond to absorbance at 280 nm to detect protein content (A and B) and the elution volume of peaks and shoulders are mentioned. Fractions are analyzed by SDS-PAGE and Coomassie staining (C and D). Intensities were homogeneously increased by Image Lab Software (Bio-Rad) for fractions in C in order to compensate low loading material on gel, compared to controls obtained by auto scale (left).

complex, further binding assays, using SMALPs containing Rh proteins, will be performed with conformation-dependent antibodies recognizing either different epitopes of RhD or antigens carried by the RhCE protein.

### *3.5. Conclusion and perspectives using SMA for the study of the RBC membrane*

To date structural analyses of RBC membrane, as well as studies on the composition of RBC ghosts, have essentially been performed after detergent treatment, inducing a loss of the lipidic environment and of most of the interactions. The data deduced have led to different and increasingly sophisticated models of the RBC membrane organization [1,10–13,15], but these may contain inaccuracies due to lack of information on its native state.

In conclusion, addition of Styrene Maleic Acid (SMA) 3:1 to ghost membranes triggered an efficient and reproducible solubilization of RBC membrane proteins in detergent-free conditions. Depending on the ionic strength of the buffer (low or very low) in which ghost membranes are resuspended before solubilization, Styrene Maleic Acid Lipid Particles (SMALPs) contain RBC membrane proteins linked or not to the spectrin-based skeleton. This suggests that, depending on the conditions used for the preparation of ghosts, different protein/protein interactions and potentially distinct conformational states within multimolecular complexes can be preserved in SMALPs. Moreover, we showed that a protein of the Rh RBC membrane complex can be purified with an anti-RhD conformation-dependent antibody, suggesting that the structure of the proteins is conserved within the SMALP.

In RBCs, the major membrane protein involved in multimolecular complexes is Band 3. Published crystal structures of Band 3 have been obtained respectively for its cytosolic part [37] and for the transmembrane domain [38]. However, because of a potential interaction

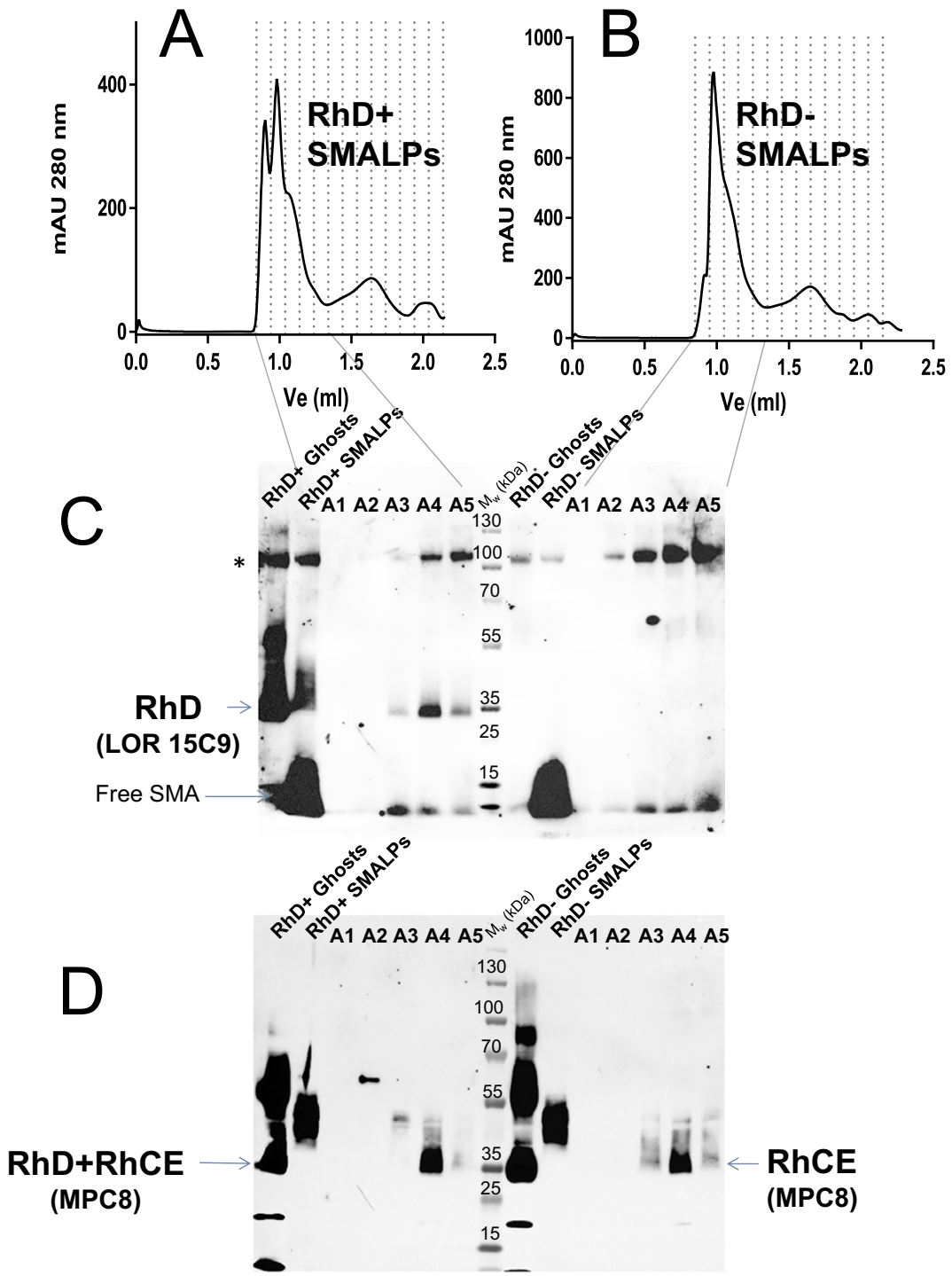


**Fig. 3.** Separation by size exclusion chromatography of SMALPs produced from ghosts derived from RhD-positive (RhD+ SMALPs) (A) and Rh-D negative (RhD− SMALPs) (B) RBCs.
Western blot analysis of fractions A1 to A5 and comparison with controls (ghosts and SMALPs), using either conformation-independent anti-RhD monoclonal antibody LOR15C9 (C) or rabbit polyclonal antibody MPC8 directed against the common C-terminal part of RhD and RhCE (D). For SMALPs, a slight shift, presumably due to free SMA which can disturb the migration, is observed. Asterisk marks an unspecific signal with LOR15C9 antibody.

between the two domains, in particular after tyrosine phosphorylation [39], it would be of particular interest to obtain an overall structure of the Band 3 dimer in a native environment [40]. The availability of isolated SMALPs containing this protein would be highly useful for a determination of the entire protein structure by cryo-Electron Microscopy, as recently described for other transmembrane proteins stabilized in SMALPs [35,41]. However, it can be assumed that purification of Band 3 in SMALPs will only be partial because of potential interacting transmembrane proteins that may be co-purified. For example, in this study, stomatin is found to be distributed in the same elution fractions as Band 3 (Fig. 2C and D) and we have previously demonstrated an interaction between stomatin and Band 3 in RBCs [42]. This suggests that, in contrast to the disrupting effect of detergent on membrane protein complexes, SMA could allow a colocalization, in the

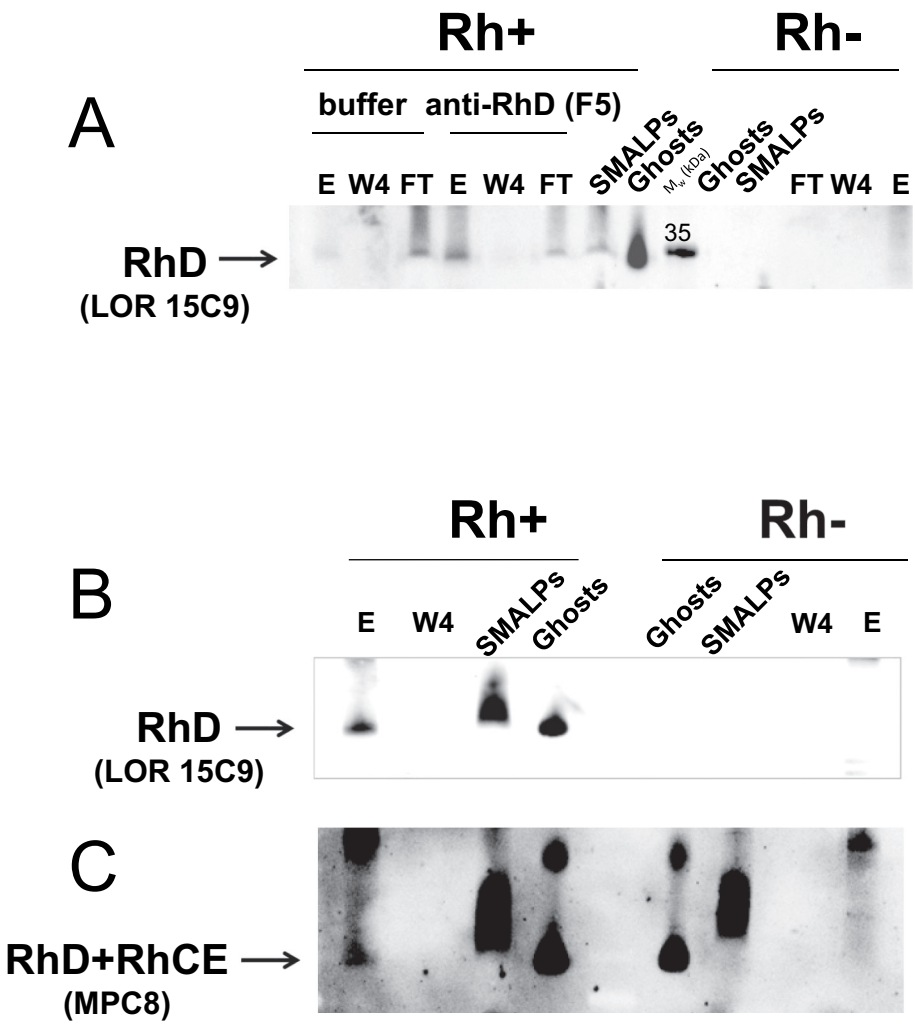


**Fig. 4.** Immunoprecipitation of SMALPs containing RhD proteins using conformation-dependent anti-RhD human monoclonal antibody F5. Elution fraction (E), last wash (W4), the flow through (FT) were analyzed by western blot using conformation-independent anti-RhD human monoclonal antibody LORC15C9, showing a band with an apparent molecular weight of 35 kDa. Negative controls were buffer instead of F5 with RhD-positive SMALPs (left) and antibody F5 incubated with SMALPs prepared from RhD-negative instead of RhD-positive RBCs (right) (A).
Comparison by immunoblot for RhD positive and RhD negative SMALPS of F5 immunoprecipitates and controls: reactivity with conformation-independent anti-RhD human monoclonal antibody LORC15C9 (B) and rabbit polyclonal antibody MPC8 against the common C-terminal part of RhD and RhCE (C). Only from RhD positive SMALPS is RhD protein immunoprecipitated by F5.

same SMALPs, of integral membrane proteins which belong to a same multimolecular complex.

Thus, detergent-free solubilization of RBC ghost membranes opens new perspectives for RBC membrane study i) since RBC membranes can be easily prepared in large quantities and because their protein composition is well known, with a simple and characteristic pattern in Coomassie staining, they can provide an excellent model system to test and compare the solubilization efficiency of new copolymers ii) after immunopurification, the 3D structure of different RBC membrane proteins can be determined by cryoEM in native conditions iii) the SMALP system could provide alternative approaches for the screening of alloantibodies against members of antigenic complexes such as, but not limited to, Rh blood group antigens: instead of RBCs, stable and soluble particles i.e. proteins embedded in SMALPS could be used, and tested using conformation-dependent antibodies by MicroScale Thermophoresis iv) our study can be extended to patients exhibiting either RBC pathologies (sickle cell disease, spherocytosis, stomatocytosis, …) or RBC infections (*Plasmodium falciparum*, bacterial hemolysin alpha, …) in order to characterize, by mass spectrometry, abnormalities in membrane complexes in association with possible modifications of proteins interactions or of lipid composition.

## Author contributions

Alexandra Desrames: Methodology, Investigation, Visualization. Sandrine Genetet: Investigation, Visualization. Païline Delcourt: Investigation, Visualization. Dominique Goossens: Conceptualization, Methodology, Investigation, Writing- review &editing. Isabelle Mouro-Chanteloup: Supervision, Conceptualization, Methodology, Investigation, Writing- review &editing.

## Transparency document

The Transparency document associated this article can be found, in online version.

## Declaration of competing interest

We declare no competing interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamem.2019.183126.

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