

Detergent-free isolation of native red blood cell membrane complexes

SMALP conference, September 18th

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The logo for SMALP, featuring the letters 'SMALP' in a bold, yellow, sans-serif font. The letters are set against a blue rectangular background that is slightly wider than the text.

RBC membrane organization

- Human Red Blood Cell (RBC) membrane :

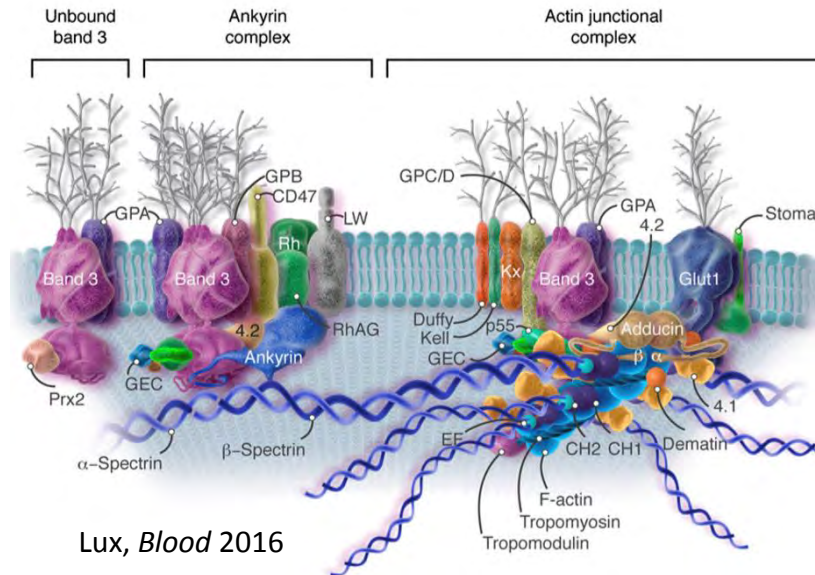
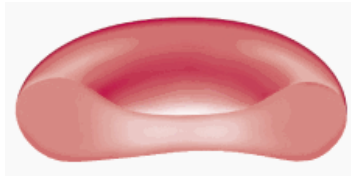
-> Proteins carrying **blood group antigens** (ABO, Rh ...)

-> **Protein network** involved in cell shape and mechanical properties

- Previous biochemical studies performed **in presence of detergent** :

-> Loss of complexes

-> Large number of models



RBC membrane organization

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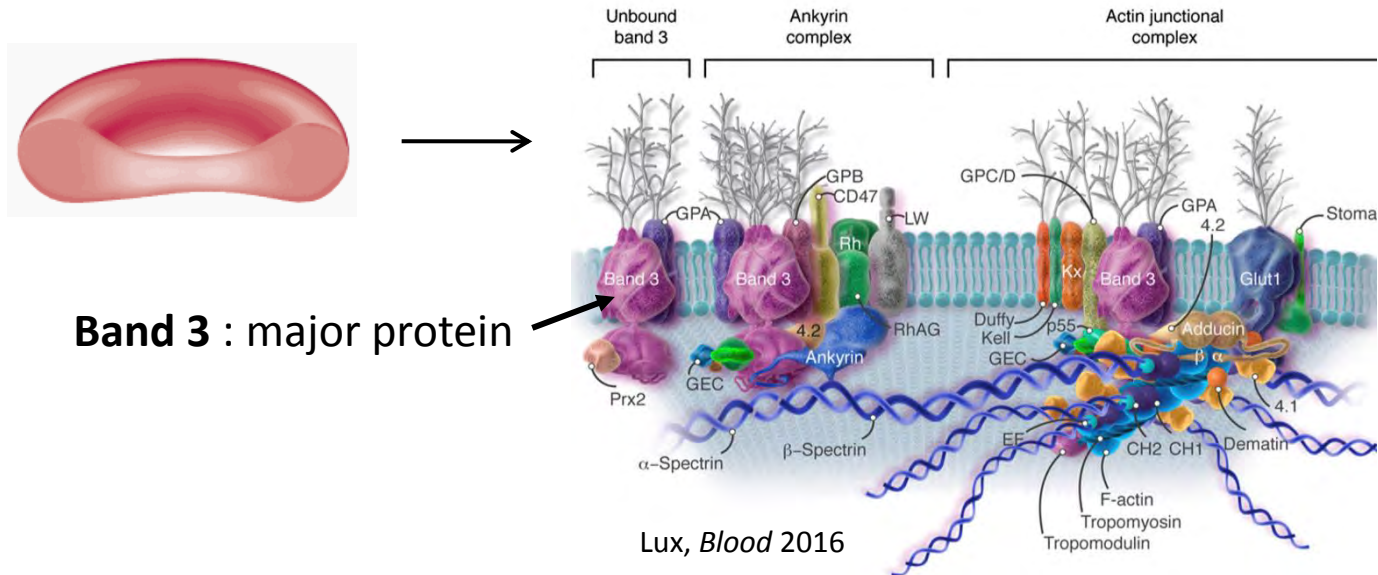
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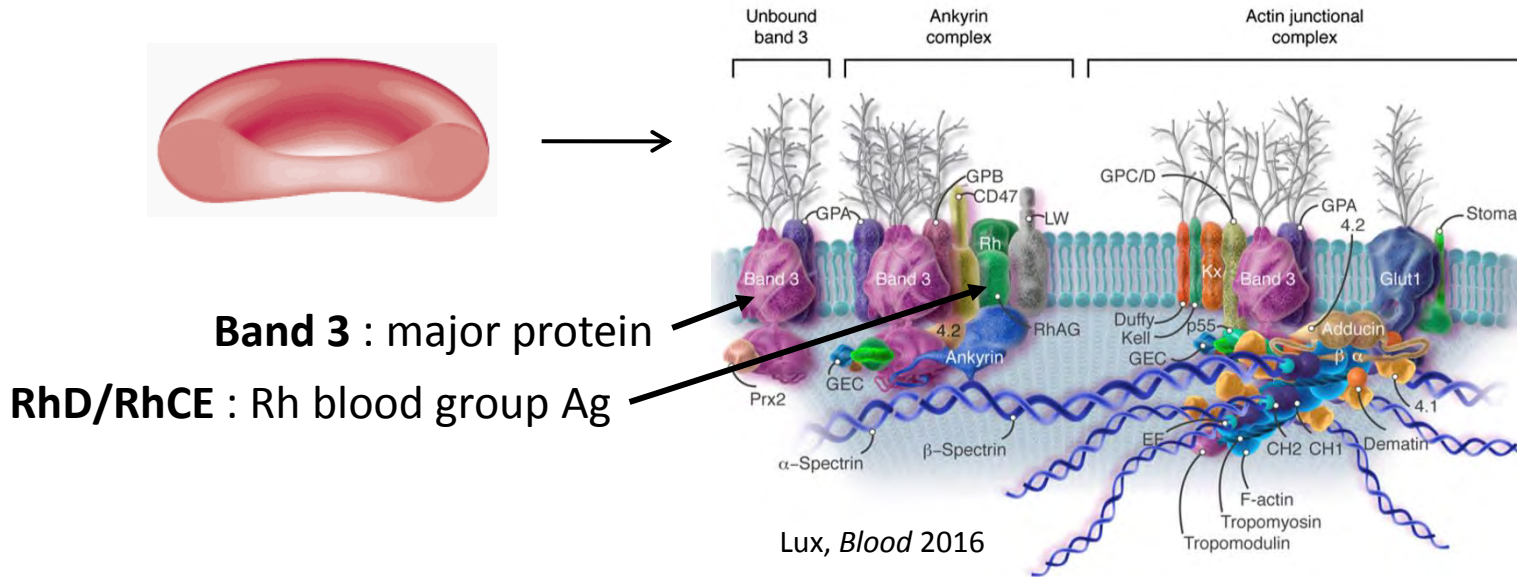
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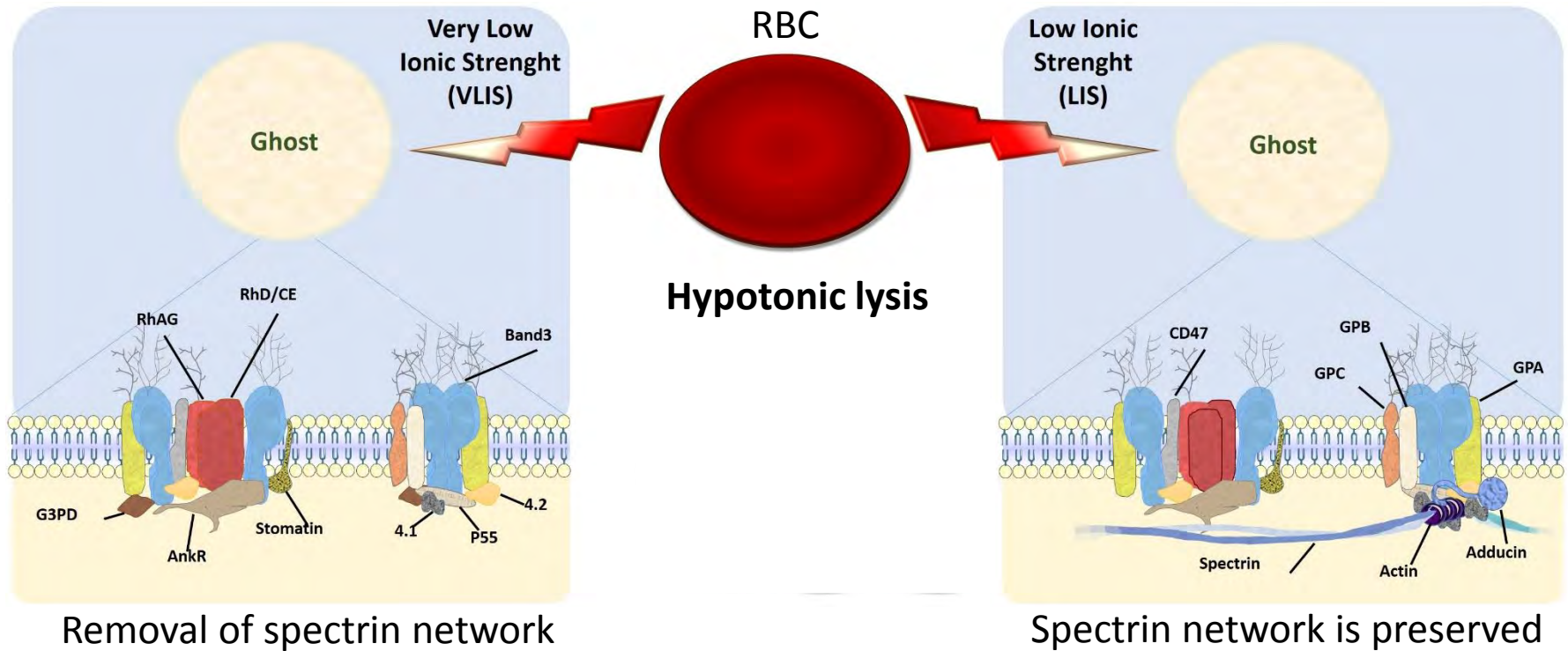
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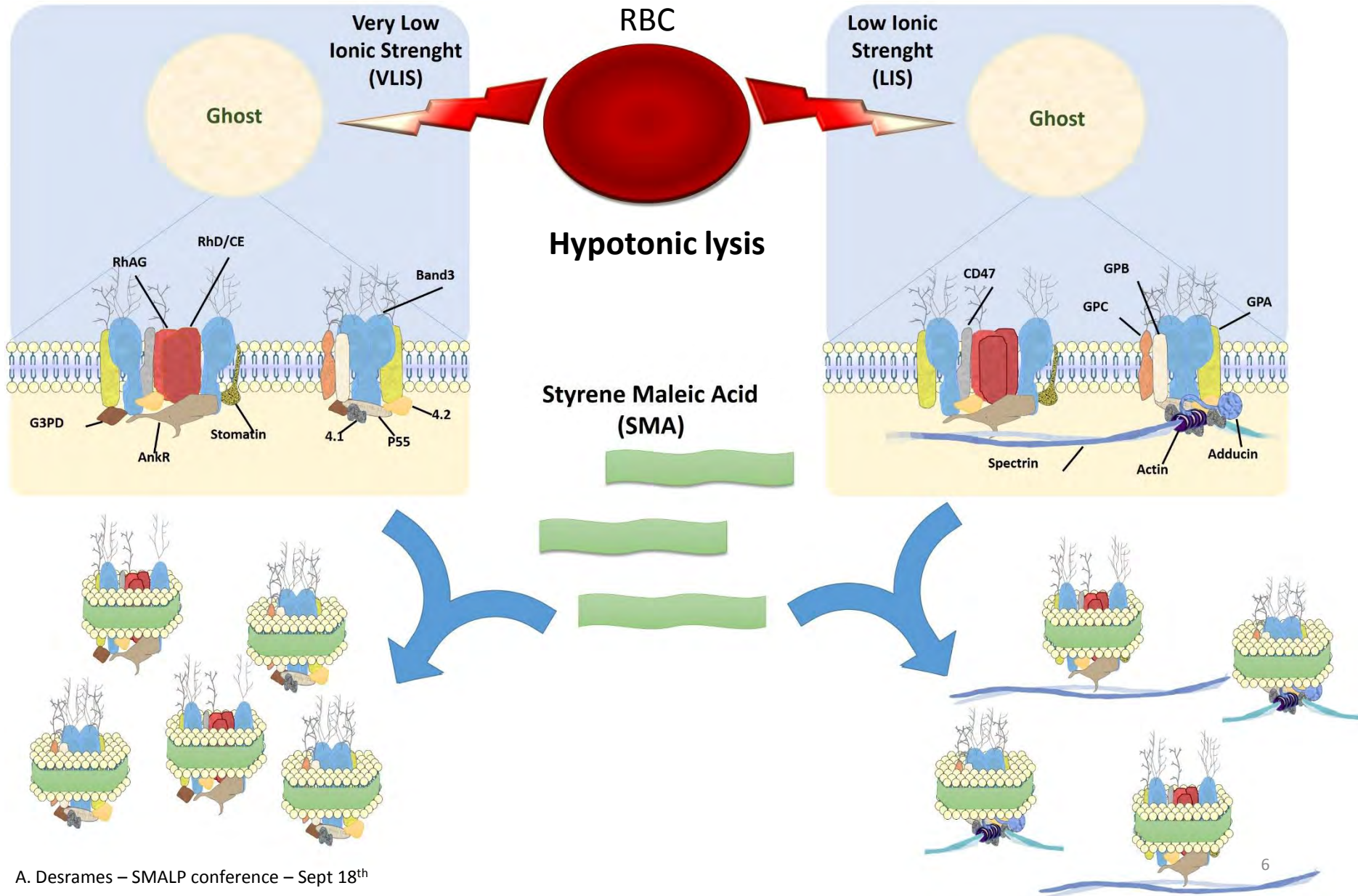


-> Study of RBCs protein complexes using SMA

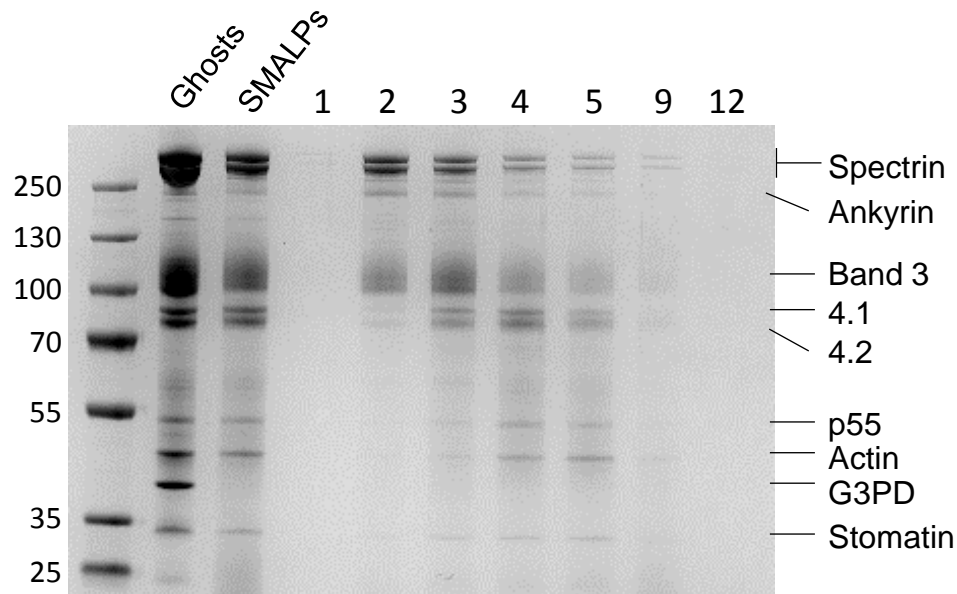
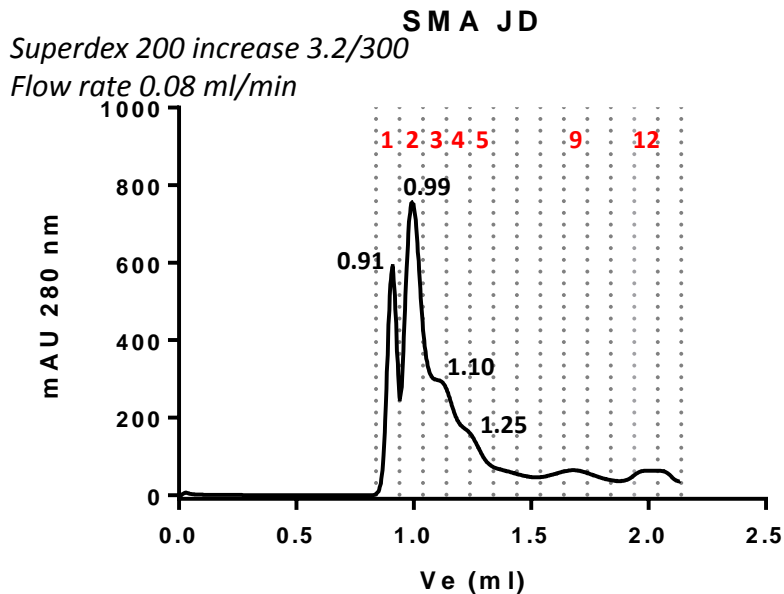
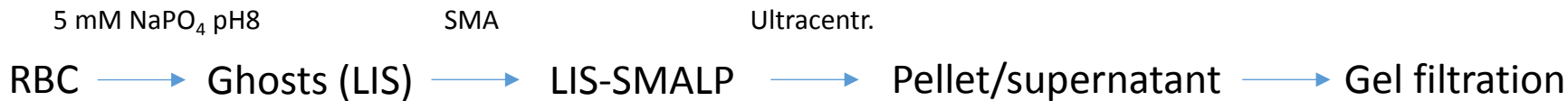
Methods



Methods

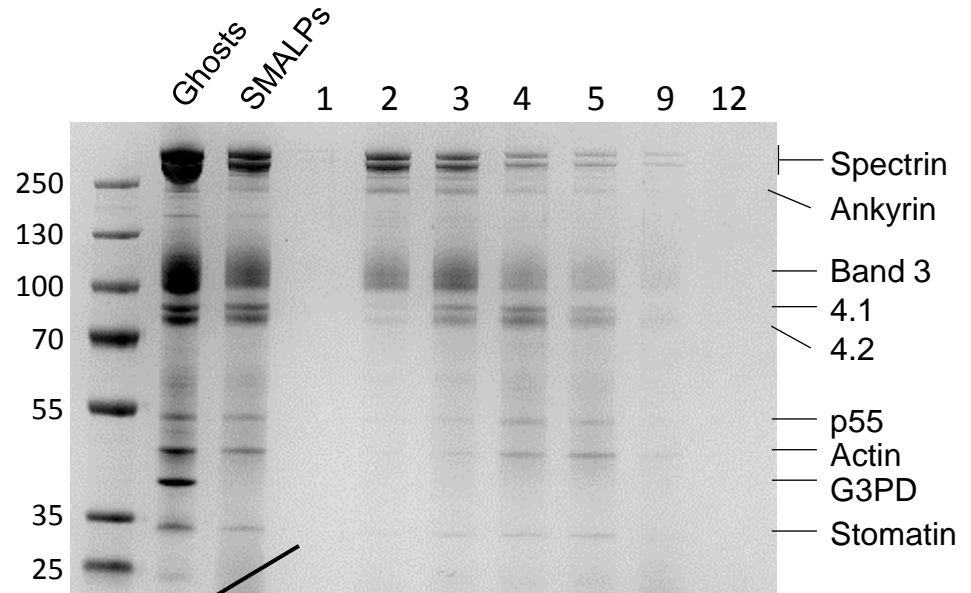
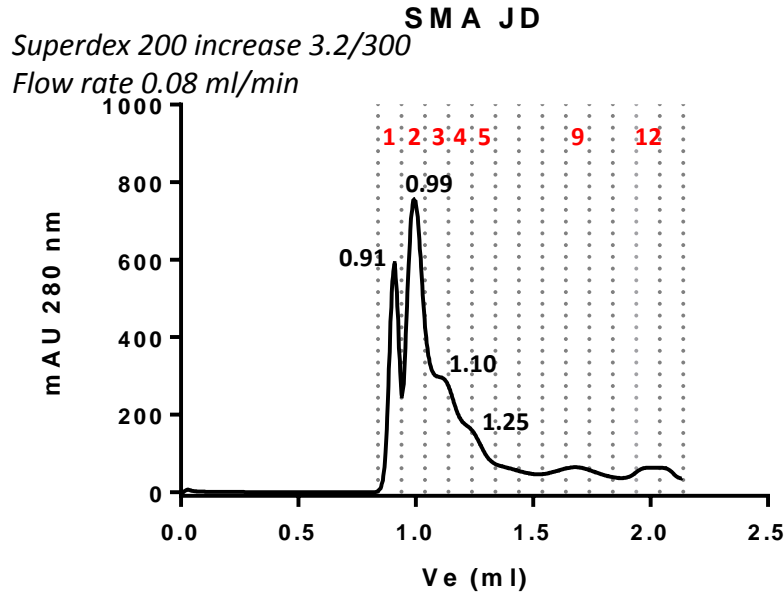
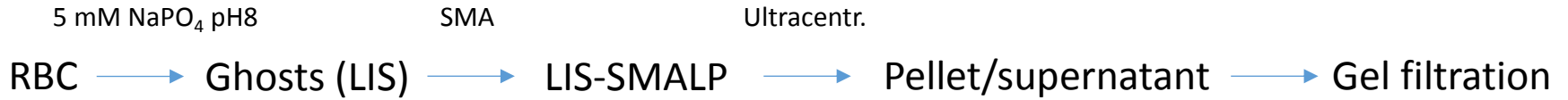


RBC membrane solubilization by SMA



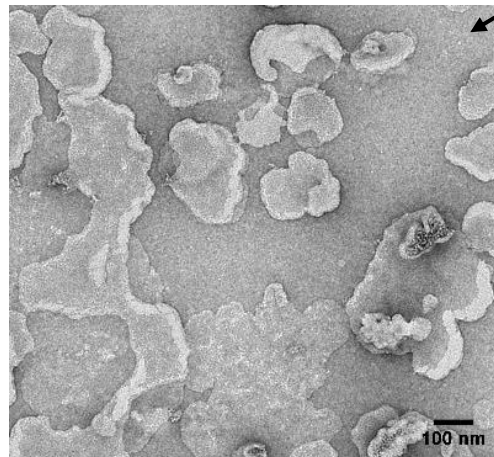
Instant Blue staining

RBC membrane solubilization by SMA



Instant Blue staining

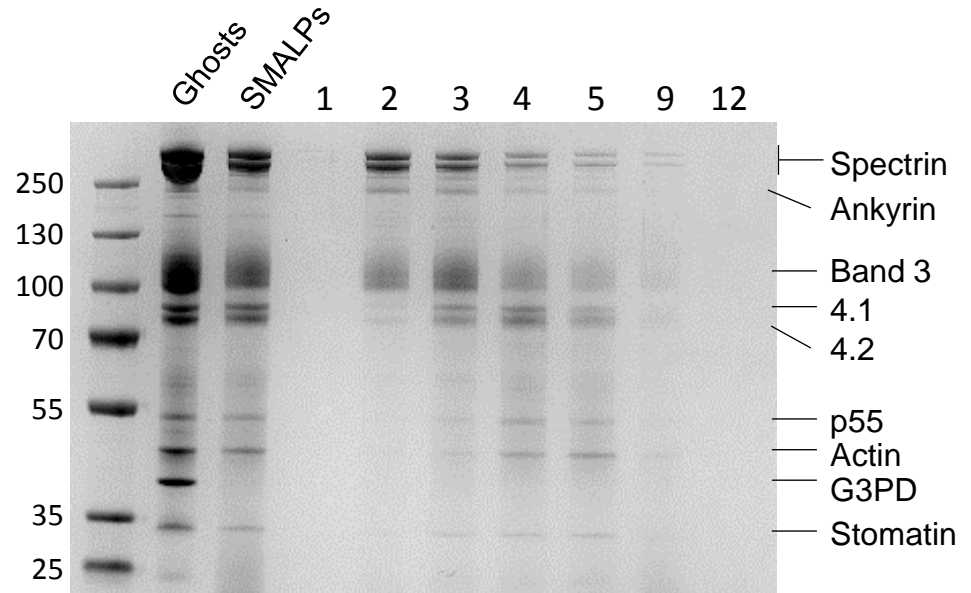
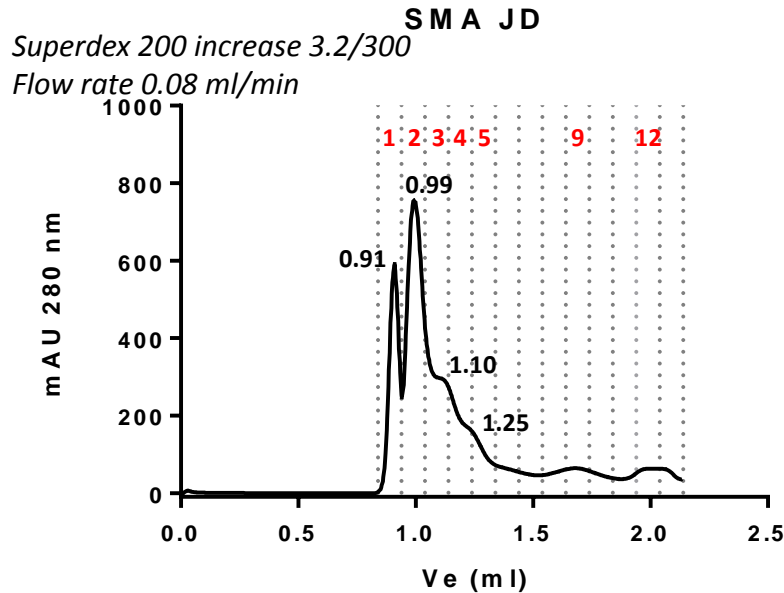
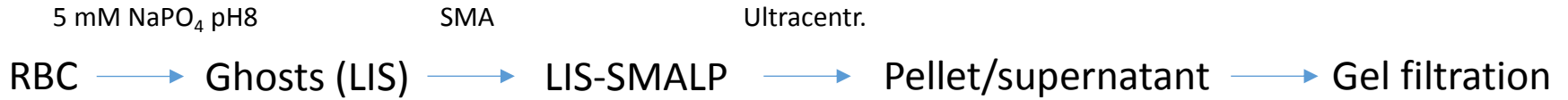
Fraction 1 : no protein
 Excessive SMA ?
 Pieces of membranes ?



Negative staining electron microscopy, 30 000X

(JM Verbavatz, ImagoSeine platform, Jacques Monod Institute)

RBC membrane solubilization by SMA



Fraction 1 : no protein

Fractions **2-3** enriched in **spectrin and Band 3**

Fractions **3-4-5** enriched in proteins **4.1 and 4.2**

Same profile with VLIS-SMALPs

-> What about protein conformation ?

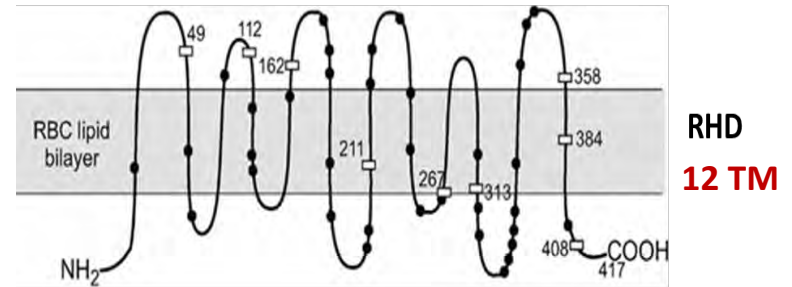
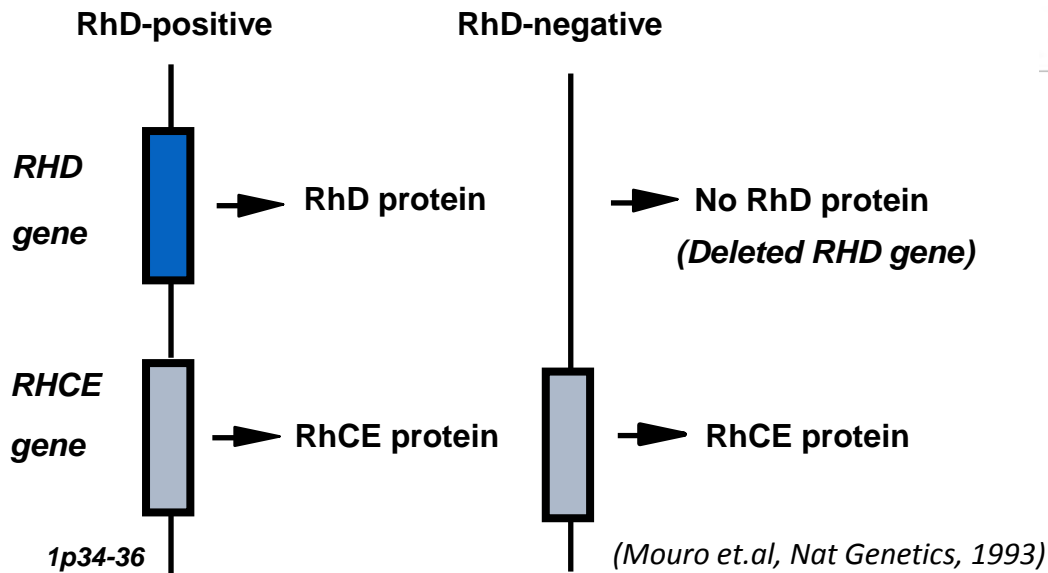
SMALPs-RhD interaction with anti-RhD antibodies

Rh blood group system : 54 antigens carried by protein RhD or RhCE

D is the Rh **antigen most involved in allo-immunisation** (pregnancy, transfusion)

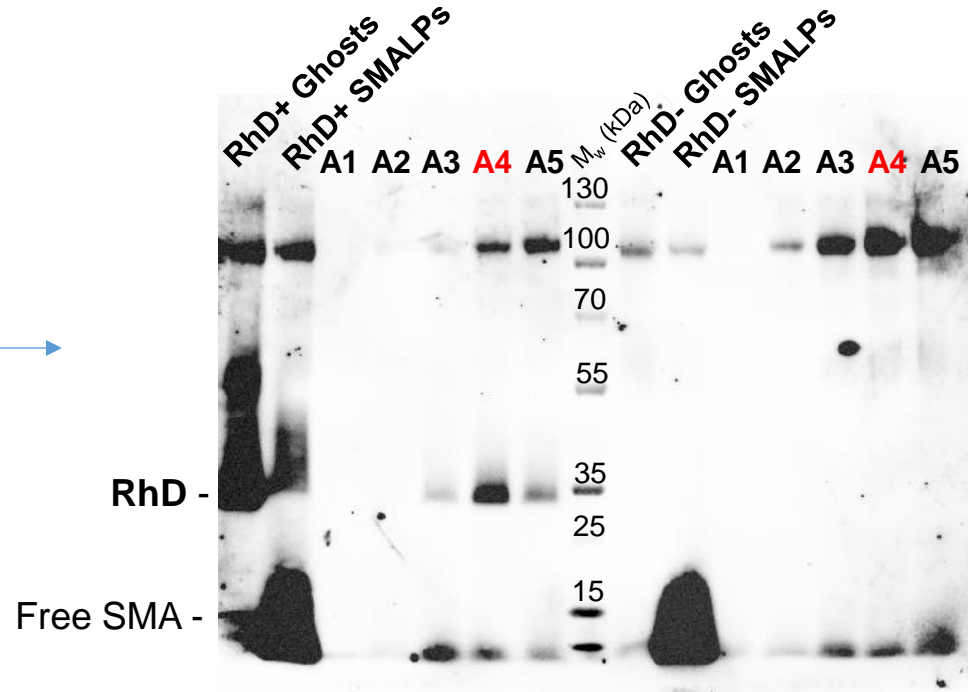
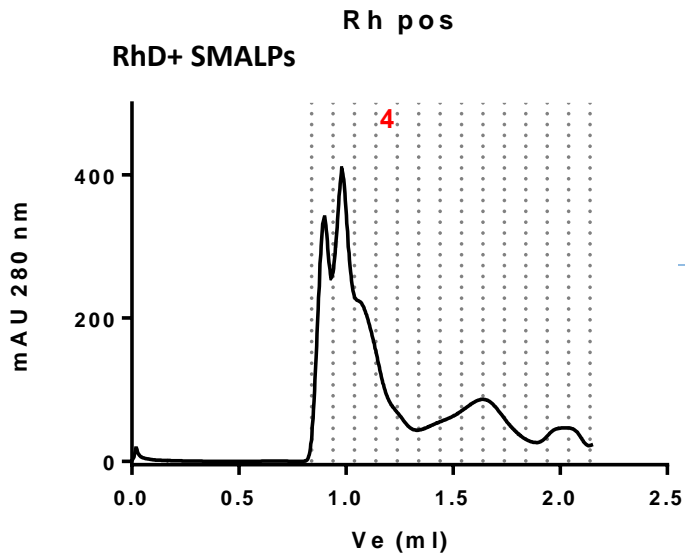
-> Several anti-D antibodies available in the lab

RhD+/RhD- genotypes and phenotypes



1/ SMALP-RhD immunopurification

RhD detection in gel filtration fractions :



Anti-RhD LOR15C9

(Apoil et.al, Br J Haematol. 1997)

- RhD is mainly detected in **fraction 4** -> **selected for IP**
- Gel filtration profile from RhD- is similar -> fraction 4 taken as negative control for IP

Ab LOR15C9 reacts non specifically with free SMA

1/ SMALP-RhD immunopurification

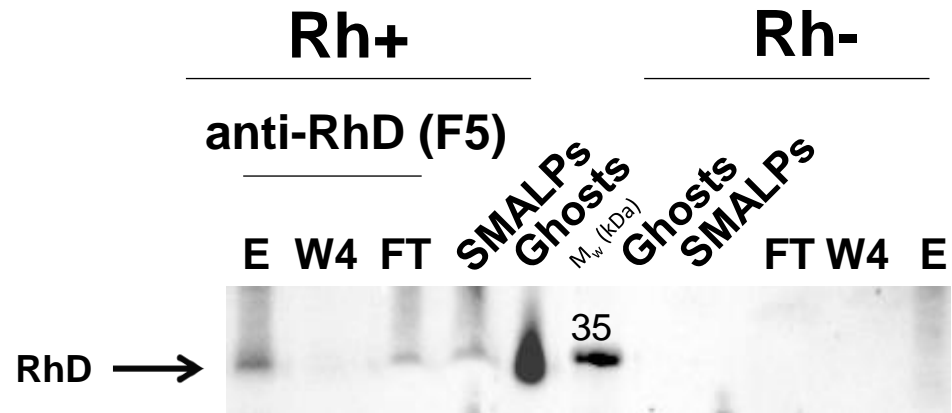
Immunopurification with monoclonal Ab F5, **conformation-dependent** (Goossens et.al, JI methods, 1987)

Ab bound on **protein A sepharose** then pre-cleared SMALPs are added

Washes in SMA Buffer

Elution with Laemmli buffer

Detection on WB with Ab LOR15C9, **conformation-independent**



RhD is partially retained by and eluted from the F5-resin

1/ SMALP-RhD immunopurification

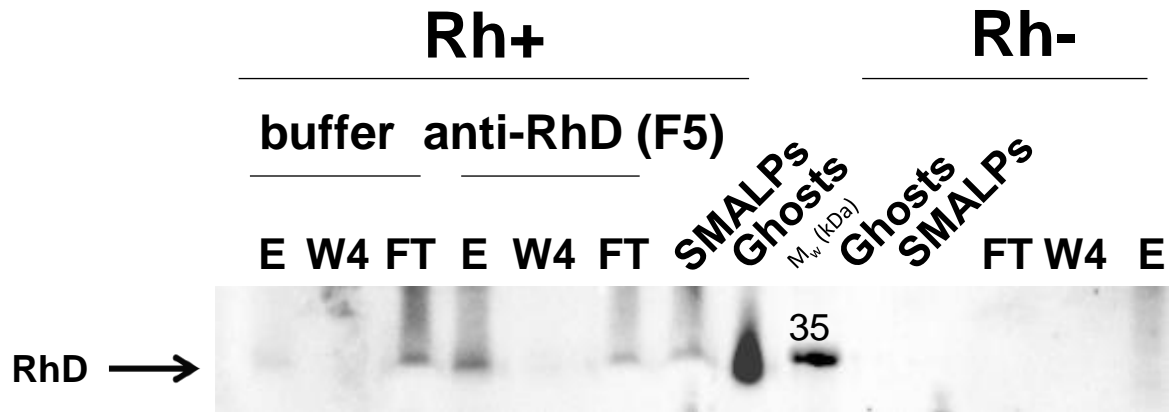
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Washes in SMA Buffer

Elution with Laemmli buffer

Detection on WB with Ab LOR15C9, **conformation-independent**



RhD is partially retained by and eluted from the F5-resin

RhD is not detected in eluate from resin alone

RhD conformation is conserved in SMALPs

2/ Microscale Thermophoresis

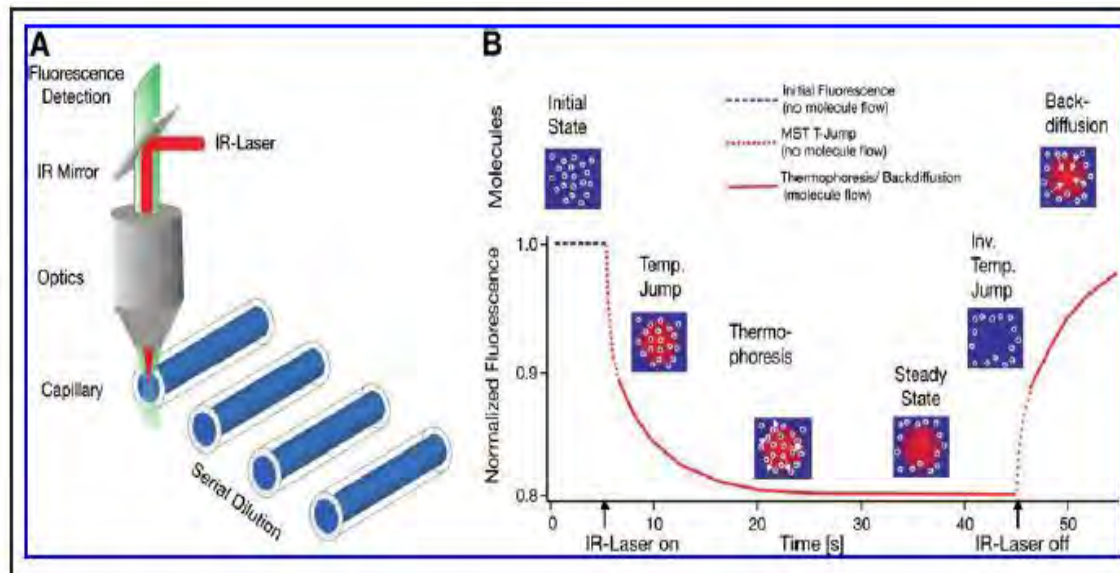
Protein-ligand interaction method, based on complexes mobility in a thermic gradient

Size of complexes (sample in presence or absence of ligand) impacts on their mobility

TECHNOLOGY REVIEW

Molecular Interaction Studies Using Microscale Thermophoresis

Moran Jerabek-Willemsen,^{1,2} Christoph J. Wienken,¹
Dieter Braun,¹ Philipp Baaske,^{1,2} and Stefan Duhr^{1,2}



Samples (analyte or ligand) must be fluorescent

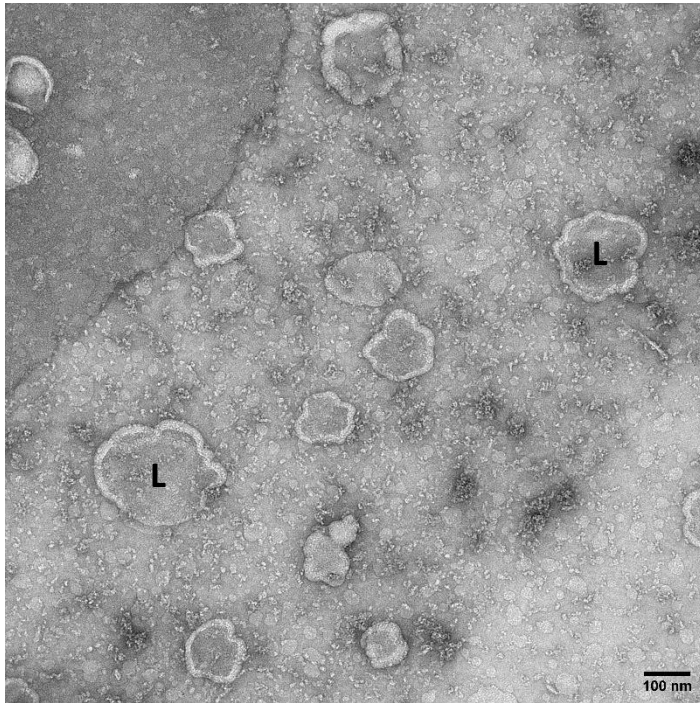
Method recently used with SMALPs (*Bada-Juarez et.al, BBA 2020*)

What about RBCs-SMALPs ?

2/ Microscale Thermophoresis

Samples must be homogenous for MST (advice from MST platform members)

Ghosts -> VLIS-SMALPs -> GF -> RhD+ fraction :



Heterogenous material in samples :

- Liposomes (L)
- Nanodiscs of various sizes (10-50 nm diameter)

Negative staining electron microscopy, 30 000X

(JM Verbavatz, ImagoSeine platform, Jacques Monod Institute)

A. Desrames – SMALP conference – Sept 18th – unpublished data

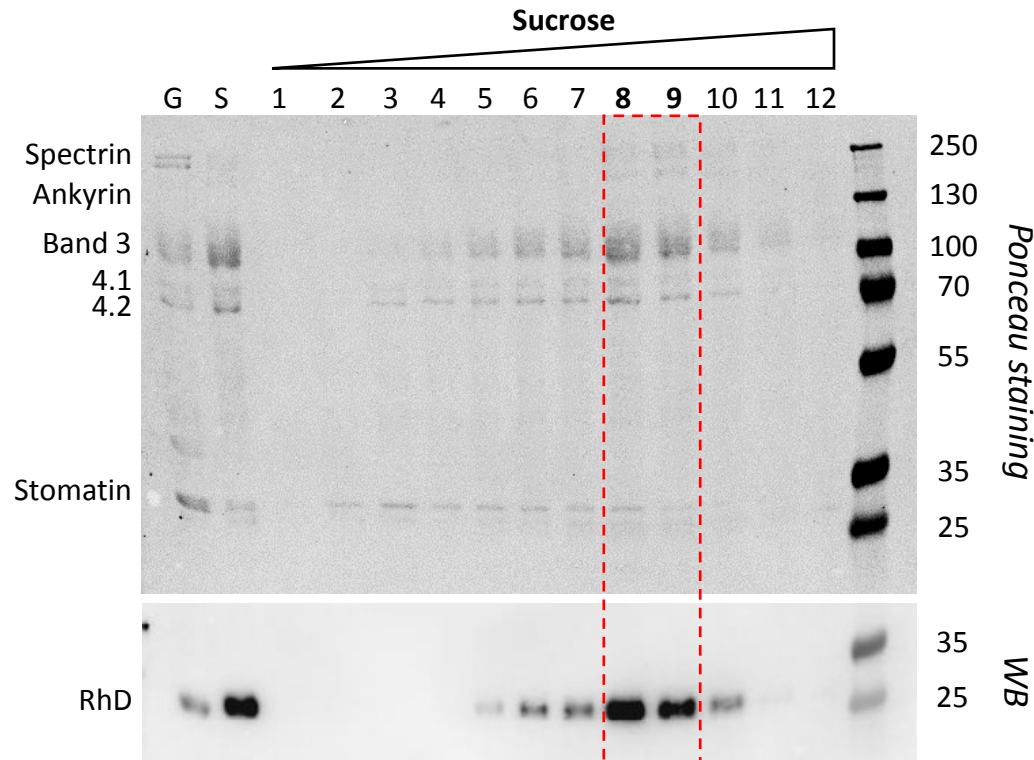
-> First isolation step using sucrose gradient ultracentrifugation

2/ Microscale Thermophoresis – Sample preparation

SMALPs loaded on a sucrose gradient (2,5 – 5 – 10 – 30%)

(B Raynal, Molecular Biophysics Platform, Pasteur Institute)

Western blot anti RhD :



RhD is mostly detected in fractions 8-9

Band 3, 4.1, 4.2 and **stomatin** are also present in these fractions

A. Desrames – SMALP conference – Sept 18th – unpublished data

-> Second isolation step using gel filtration

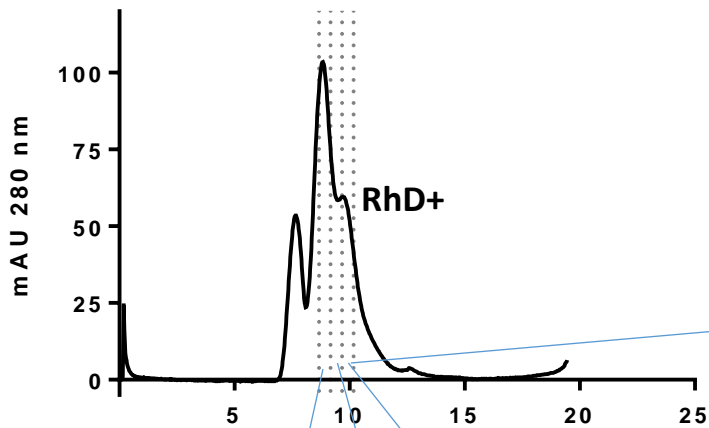
2/ Microscale Thermophoresis – Sample preparation

RhD + fractions loaded on GF column :

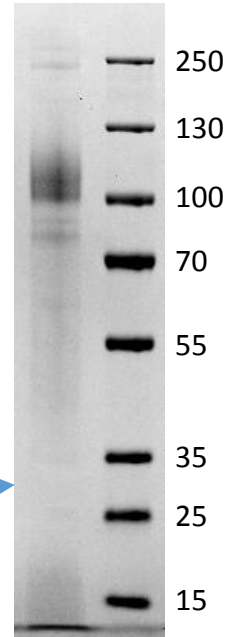
Superdex 200 increase 10/300

Flow rate 0.4 ml/min

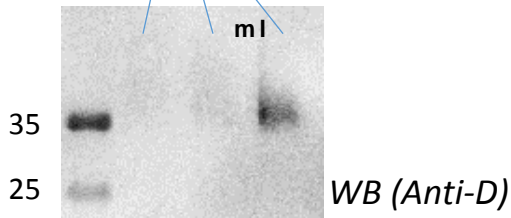
Rh pos F8



Coomassie staining



RhD+ GF fraction also contains
Band 3, 4.1 and 4.2 proteins



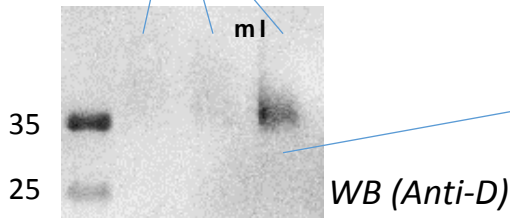
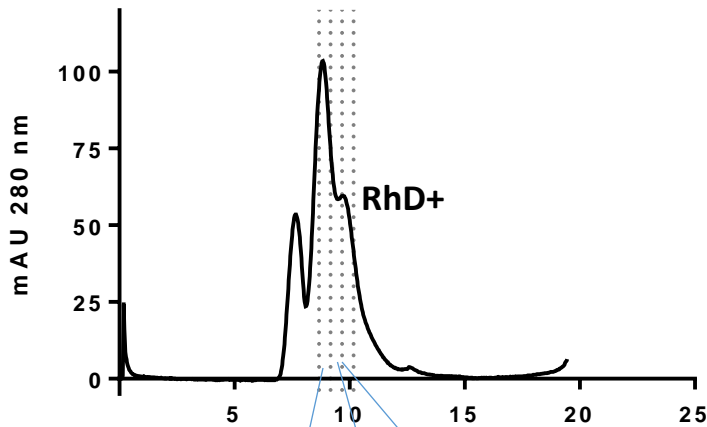
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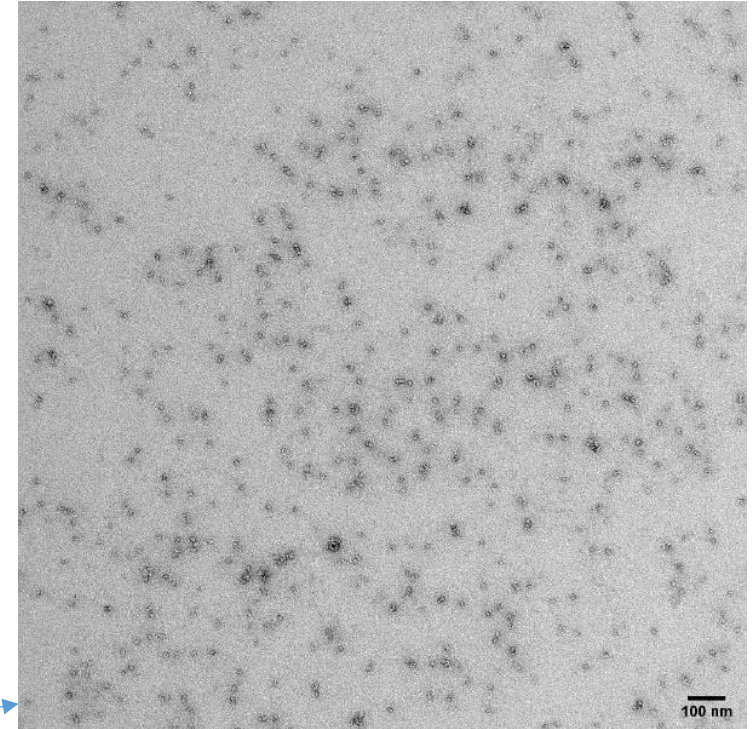
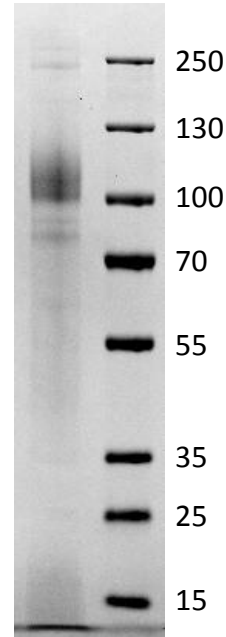
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Coomassie staining



Negative staining electron microscopy, 23 000X
(JM Verbavatz, ImagoSeine platform, Jacques Monod Institute)

Monodisperse nanodiscs, mean diameter = 12.7 nm (*ImageJ software*)

-> Sample quality is acceptable for MST

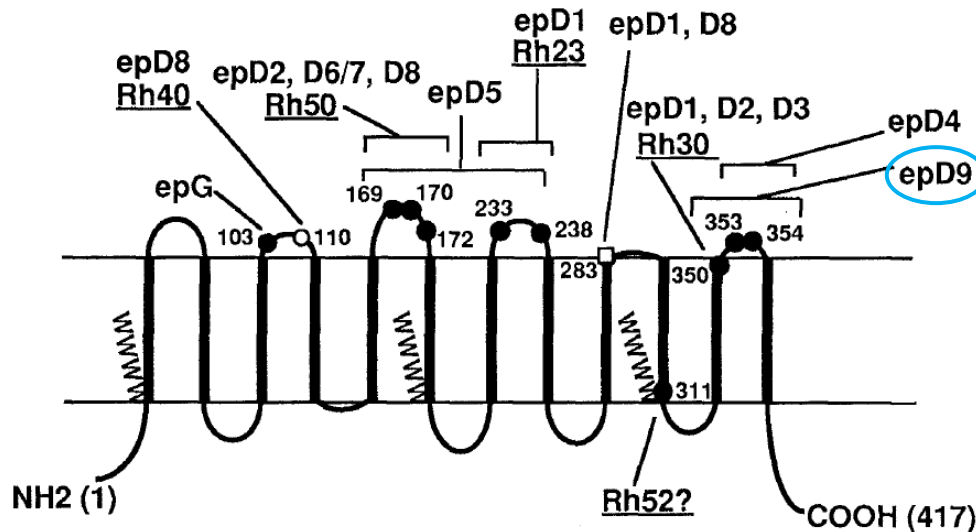
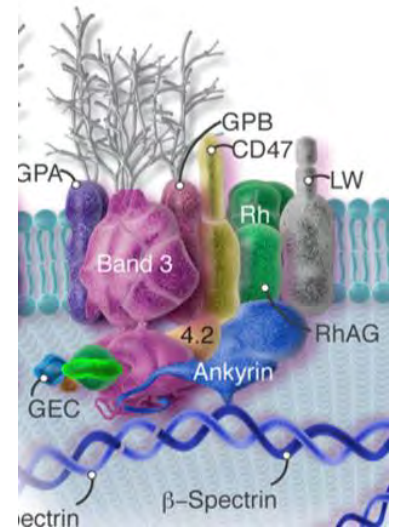
3/ Microscale Thermophoresis – Results

SMALPs fluorescent labelling : **eosin-5-maleimide (EMA)**, $\lambda_{exc} = 524 \text{ nm}$

EMA binds to Band 3 and RhD proteins (same complex)

RhD-SMALPs incubated with EMA then washed by Amicon

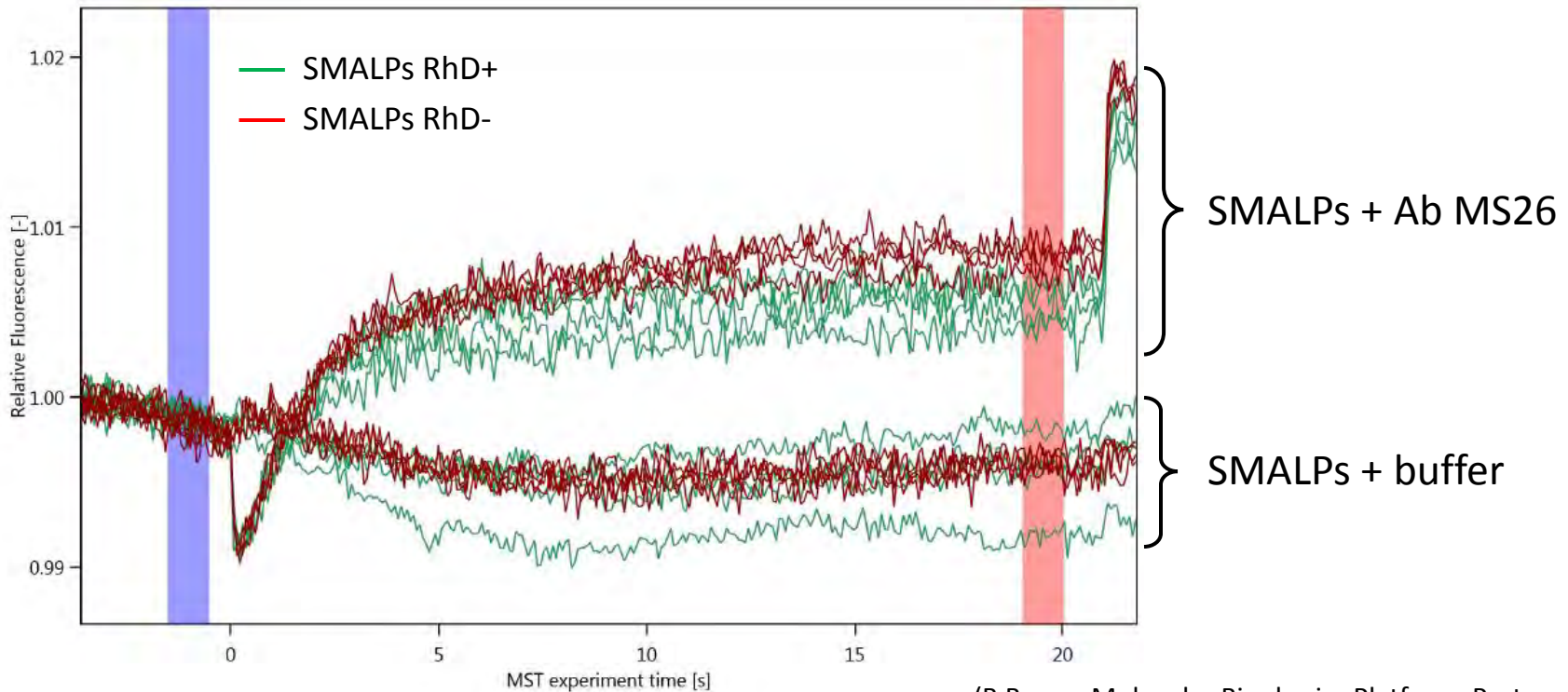
Monoclonal Ab MS26 : epitope **D9**



3/ Microscale Thermophoresis – Results

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(B Baron, Molecular Biophysics Platform, Pasteur Institute)

SMALPs do not diffuse in the thermic gradient
No difference between RhD+ and RhD- samples

A. Desrames – SMALP conference – Sept 18th – unpublished data

-> MST is not suited for RhD-SMALPs analysis

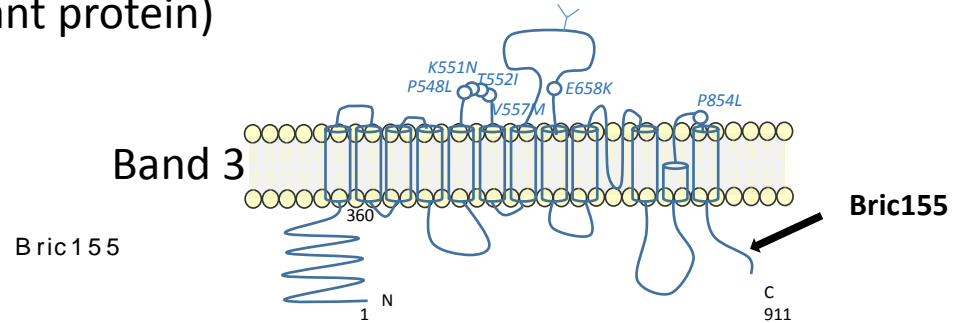
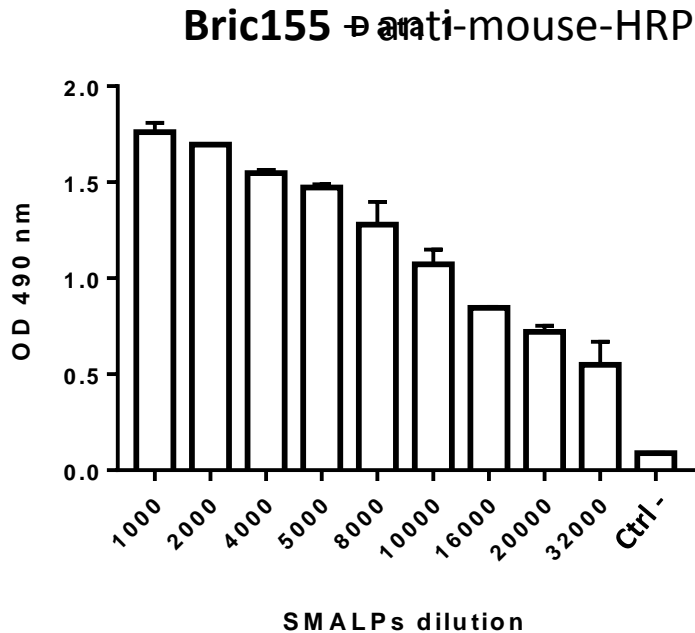
4/ ELISA assays – Preliminary results

First ELISA assays with SMALPs described very recently (*Ayub et. al, BBA July 2020*)

SMALPs prepared for MST were used for ELISA

First trials with anti-Band 3 Ab (most abundant protein)

-> Bric155, linear epitope



- Ctrl- : no SMALP, Ab background
- Strong **dose-dependant signal**

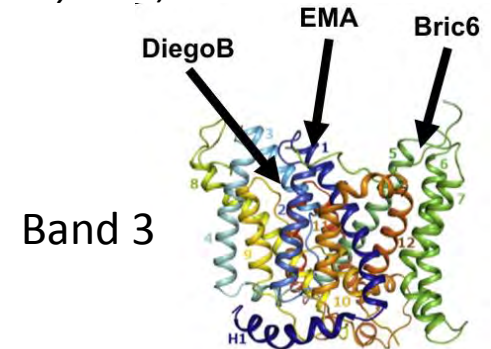
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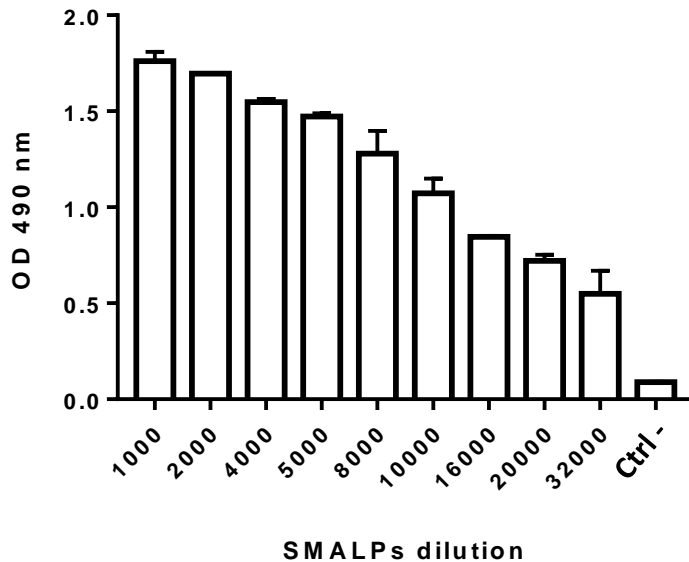
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- > Bric155, linear epitope
- > Bric6, conformation-dependent



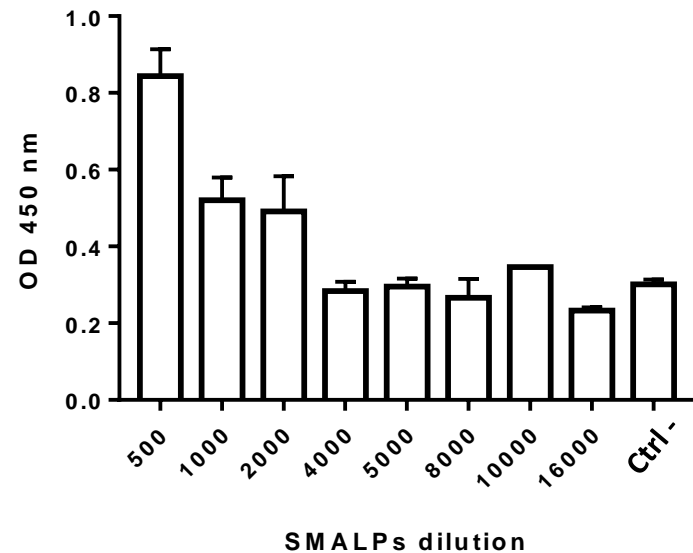
Bric155 + anti-mouse-HRP

Bric155



Bric6 + anti-mouse-HRP

OD



- Ctrl- : no SMALP, Ab background
- Strong **dose-dependant signal**

- High Ab background
- Weak **dose-dependant signal**

Conclusions

SMALPs can be generated from **RBC membrane**

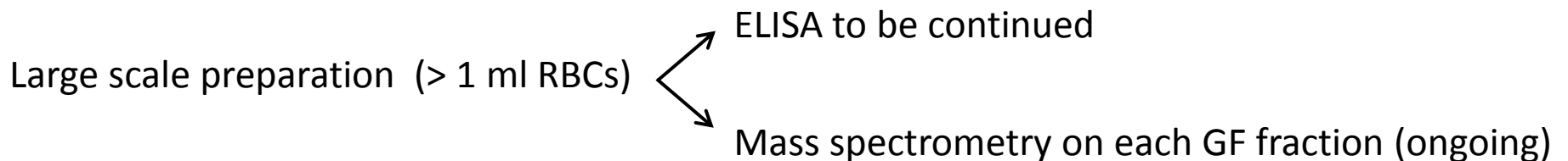
Most proteins observed in ghosts are detected in SMALPs

RhD can be purified by IP with **conformation-dependent antibody**

Semi-purification by sucrose gradient followed by GF leads to **homogenous nanodisc samples**

RBC-SMALPs can be used in ELISA assays but not in MST

Perspectives



Small scale preparation (200 μ l RBCs): study of pathological RBCs (genetic or infectious diseases)

Thank you !

INSERM U1134/INTS/Université de Paris, Paris, France

Sandrine Genetet

Païline Delcourt

Dominique Goossens

Isabelle Mouro-Chanteloup



ImagoSeine platform, Jacques Monod Institute, Paris, France

Jean-Marc Verbavatz



Molecular biophysics platform, Pasteur Institute, Paris, France

Patrick England

Bertrand Raynal

Bruno Baron

