# Summary Report

Bites from the Loxosceles genus (brown spiders) cause several clinical manifestations in mammals, especially necrotic skin degeneration. The venom of Loxosceles have a toxic component with a rare enzymatic activity, termed sphingomyelinase D (SMD). This enzyme catalyzes the conversion of sphingomyelin (SM) into ceramide-1-phosphate (Cer1P). While the enzymatic substrate SM is an integral constituent of many cell membranes, the reaction product Cer1P naturally occurs in very low concentrations. The mechanism of venom action is incompletely understood.

The main questions of the project "enzyMEmbrane" were:

1) What is the impact of the enzymatic generation of Cer1P on the lateral organization of model membranes?

2) Is lipid packing a modulator of SMD action?

3) Does SMD generate regions of different packing in cell membranes (red blood cells/adipoctes)? To what extent the aforementioned phenomena can be considered as activators of the cellular response to SMD?

Structure and dynamics of model membranes containing SM and Cer1P: We characterized different model membrane systems containing the substrate SM. We examined the phase state of pure SM-containing vesicles, i.e., lauroyl-SM (C12SM) and eggSM (differential scanning calorimetry), as well as the aforementioned samples plus SM/cholesterol- and SM/Cer1P-containing mixtures (LAURDAN generalized-polarization (GP) measurements in a fluorometer). We then studied how the phase state of these SM bilayers (in large unilamellar vesicles, LUVs) affects SMD activity and tested the impact of cholesterol as well as the reaction product Cer1P itself (enzyme kinetics study). The results show a dependence of SMD activity on the phase state of the substrate.

The lateral structure of fluorescently labeled SM-containing giant unilamellar vesicles (GUVs) was examined by laser-scanning confocal fluorescence microscopy (LSCFM) using DiIC18 probe. This provided qualitative information and showed membrane regions excluding the probe in eggSM/cholesterol/dioleoylphosphatidylcholine (DOPC) as well as C12SM/C12Cer1P mixtures. Also some experiments using two-photon excitation LAURDAN GP images were done in GUVs.

Structure and dynamics of model membranes containing SM during SMD action plus enzyme kinetics studies: we investigated the consequences of the in-situ formation of Cer1P in model membranes. Fluorescence microscopy on GUVs of C12SM, eggSM, and eggSM/cholesterol/DOPC upon SMD action show time-dependent changes of the membrane morphology linked to the phase state of the substrate. For example, macroscopic domain formation and/or vesicle shrinking accompanied by the formation of multiple membrane tubes is observed in membranes originally displaying Ld phase within less than 3 hours (Figure 1), while membranes in So phase show the formation of caps (outside curvature) more than one day after the addition of SMD. GUVs displaying macroscopic Ld/Lo phase separation exhibited a single homogenous phase upon exposure to SMD, indicating a large impact of the substrate on the supramolecular organization of the target membrane.

Structure and dynamics of cellular membranes during SMD action: Preliminary experiments with DiIC18 labelled red blood cells (RBC) using LSCFM were performed. These observations show DiIC18 excluded areas on the RBC membrane, fusion events as well as formation of tubes from the RBC surface.

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In summary, SMD alters the structure of the target membranes containing SM and the activity of the protein is sensitive to the extent of the membrane lateral packing. Some structural effects were also observed in red blood cells. Although a general mechanism of SMD action has not been elaborated yet, our findings may suggest an effect of membrane structural parameters in the action of SMD.

These results were presented at four European and three international conferences. A manuscript for publishing these results is currently in preparation.

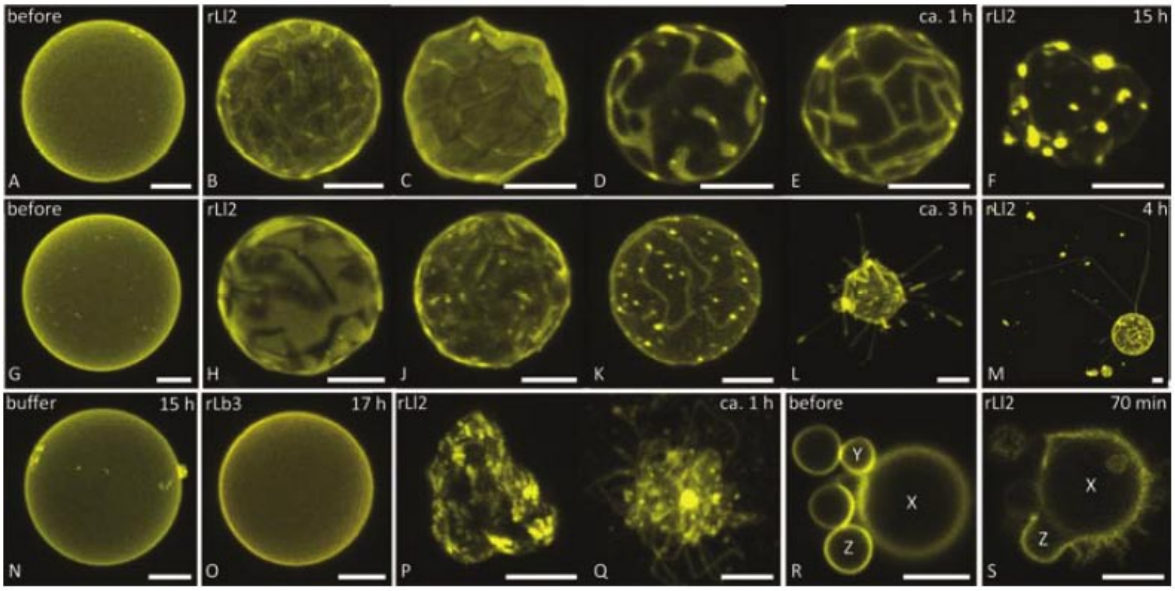


Figure 1: Images obtained by laser-scanning confocal fluorescence microscopy showing the effect of SMD activity on the morphology and fluorescent-probe distribution in giant vesicles of liquid disordered-phase SM. All giant vesicles were prepared of $C_{12}$SM incorporating 0.5 mol% Dil$C_{18}$ and 2 mol% DOPE-biotin for immobilization onto coverslips. Note the domain formation and progressive morphological changes as SMD acts on the vesicles. rLl2-recombinant SMD (isoform 2) from *Loxosceles laeta*, rLb3-recombinant SMD (inactive isoform) from *Loxosceles boneti* as control.

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