STRUCTURE AND FUNCTION OF TRYPSIN-LOADED FIBRINOLYTIC LIPOSOMES

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CONCLUSIONS: A phospholipid-based **thermosensitive** nanocarrier has been developed, in which trypsin is attached to the inner leaflet of the bilayer shell of the liposome. The **fibrinolytic efficiency** of these liposomes is **improved under permeation-driven conditions**. Because intravascular thrombi are exposed to permeation forces, our construct could be a successful candidate as a therapeutic tool the utility of which deserves further investigation.

INTRODUCTION:

Protease encapsulation and its targeted release in thrombi may contribute to the reduction of haemorrhagic complications during thrombolysis.

OBJECTIVES:

- to prepare **sterically stabilized trypsin-loaded liposomes** (SSL_T)
- to characterize their **structure** and determine the **localization of trypsin** molecules within the vesicles
- to investigate their *in vitro* **fibrinolytic efficiency**
- and **thermosensitive** caharcter

METHODS:

Hydrogenated soybean phosphatidylcholine (**HSPC**) based SSL_T were prepared and their structure was studied by transmission electron microscopy combined with freeze fracture (**FF-TEM**), Fourier transform infrared spectroscopy (**FT-IR**) and small angle X-ray scattering (**SAXS**). Fibrinolytic activity was examined at 45, 37 or 24°C on fibrin or plasma clots with **turbidimetric** and **permeation-driven lysis assays**.

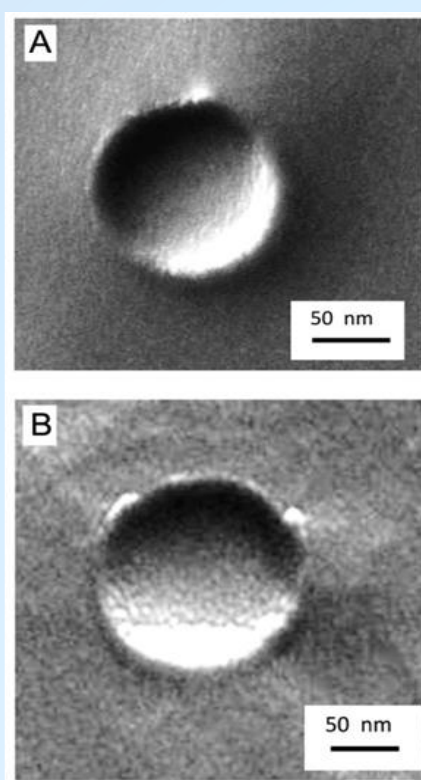

Fig. 1: The inner surface morphology of the trypsin-free (A) and trypsin-loaded vesicles (B) by freeze-fracture TEM. (A) The inner surface of a vesicle, broken through entirely, is typically smooth, but the inner surface of trypsin loaded vesicles contains closely packed grains (B).

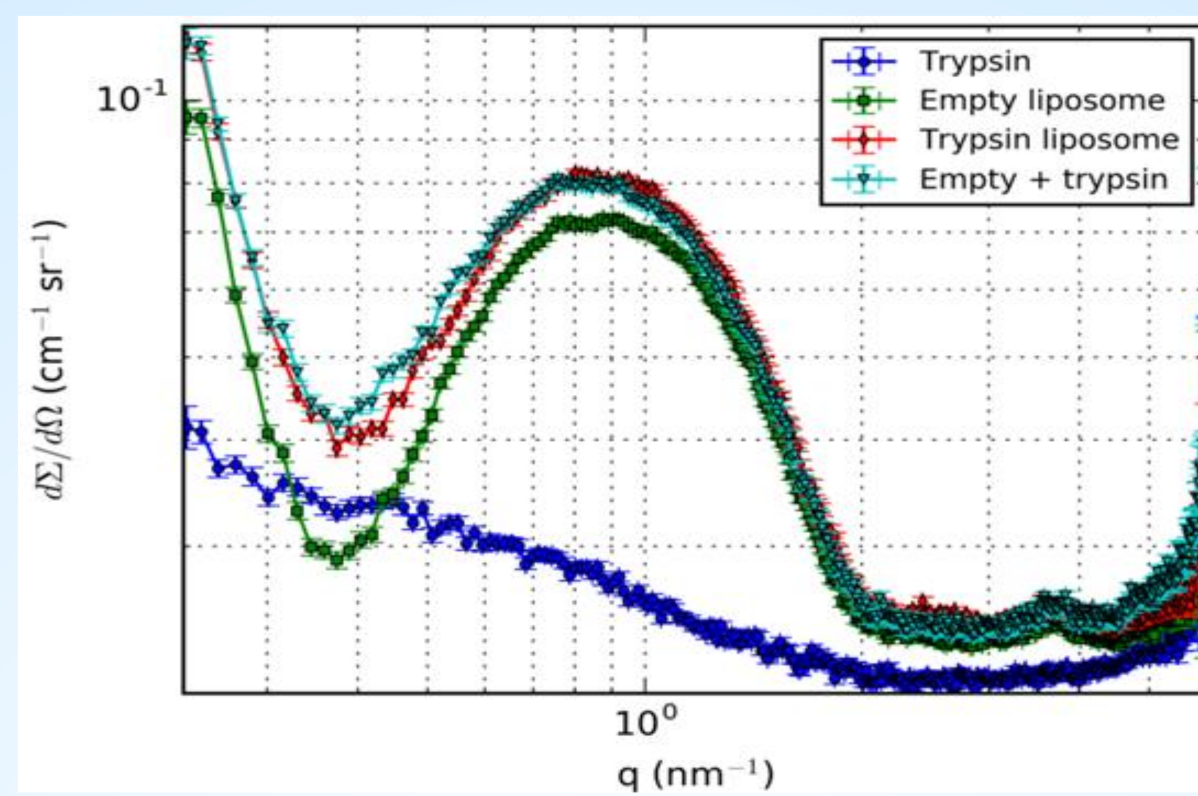

Fig. 2: The one-dimensional scattering patterns of liposomes with (red) and without (green) trypsin. The scattering of trypsin in free solution (blue) and the curve generated as a sum of the scattering of empty liposomes and solved trypsin (cyan) are also shown.

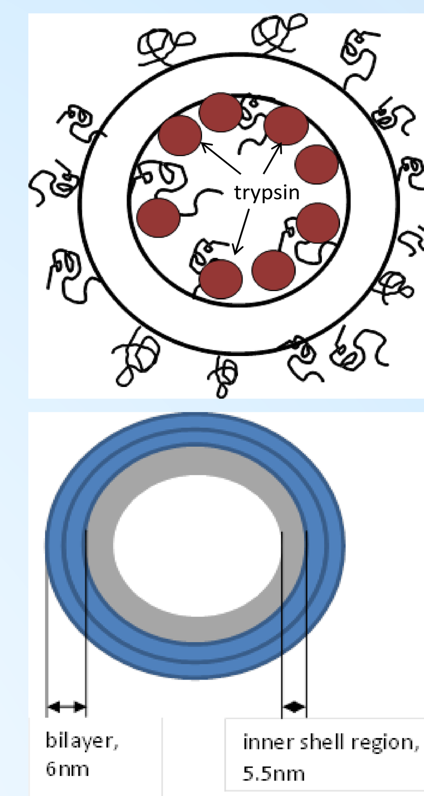

Fig. 3: Schematic illustration of trypsin loaded vesicles. Trypsin molecules localize at the inner shell molecule region of the vesicles.

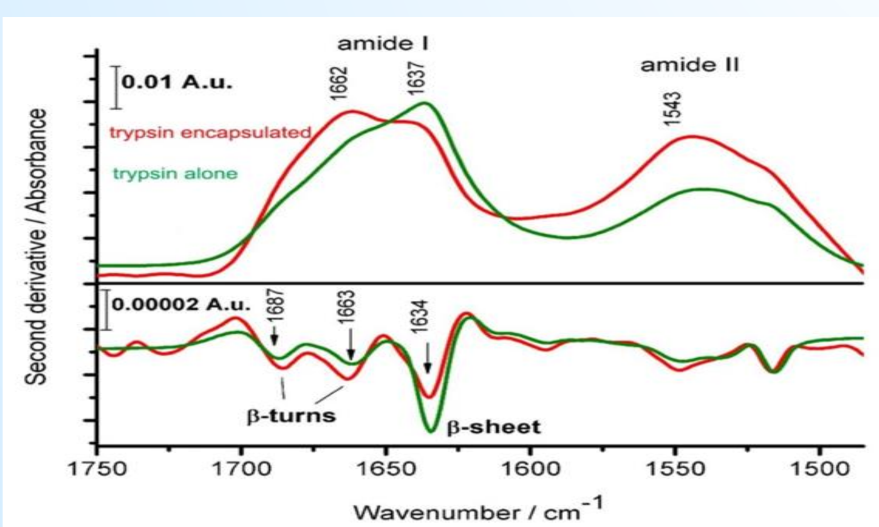

Fig. 4: Amide I spectral region of trypsin before (green) and after encapsulation (red) in liposomes. The second derivatives of the measured spectra were obtained by the Savitsky-Golay method (3rd grade polynomial, 5 smoothing points).

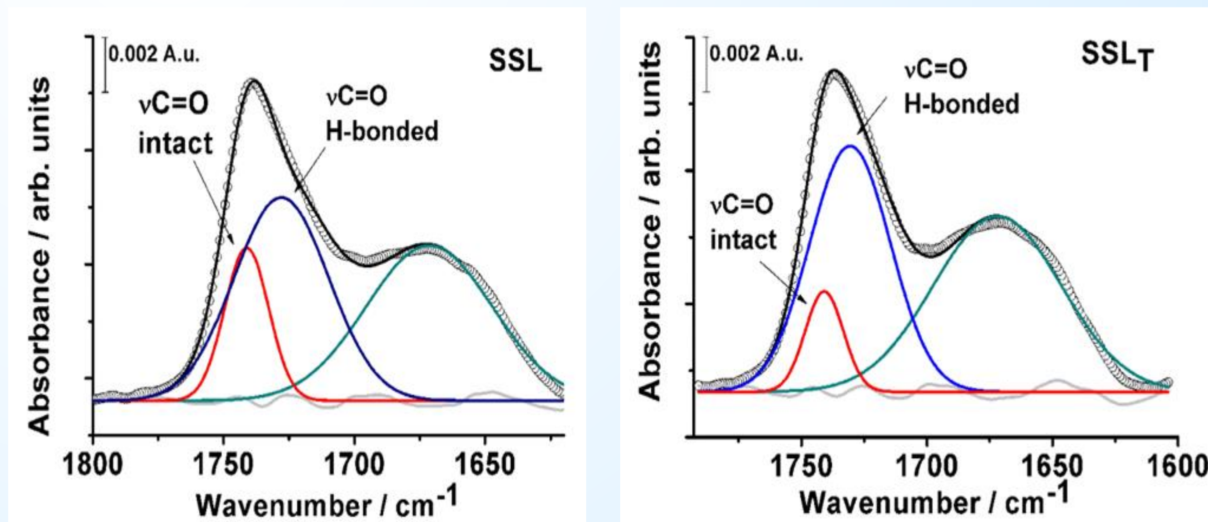

Fig. 5: Lipid ester carbonyl stretching band region for empty (SSL) and trypsin-loaded (SSL_T) liposomes. The solid black lines are the measured spectra; the blue and the red lines are the fitted bands corresponding to intact and H-bonded C=O groups, respectively. The green band component can be attributed to residual water (after subtraction) and to amide I. band positions for curve fitting using the second derivative, band shapes were approximated by Lorentzian functions. The intensities and the bandwidth of each component were optimized according to a $\chi 2$ minimization procedure. The relative contribution of each particular component was calculated from their integrated areas in the best fit. These data suggested that trypsin was located close to the lipid hydrophilic/hydrophobic interface driven by weak interaction (H-bonds).

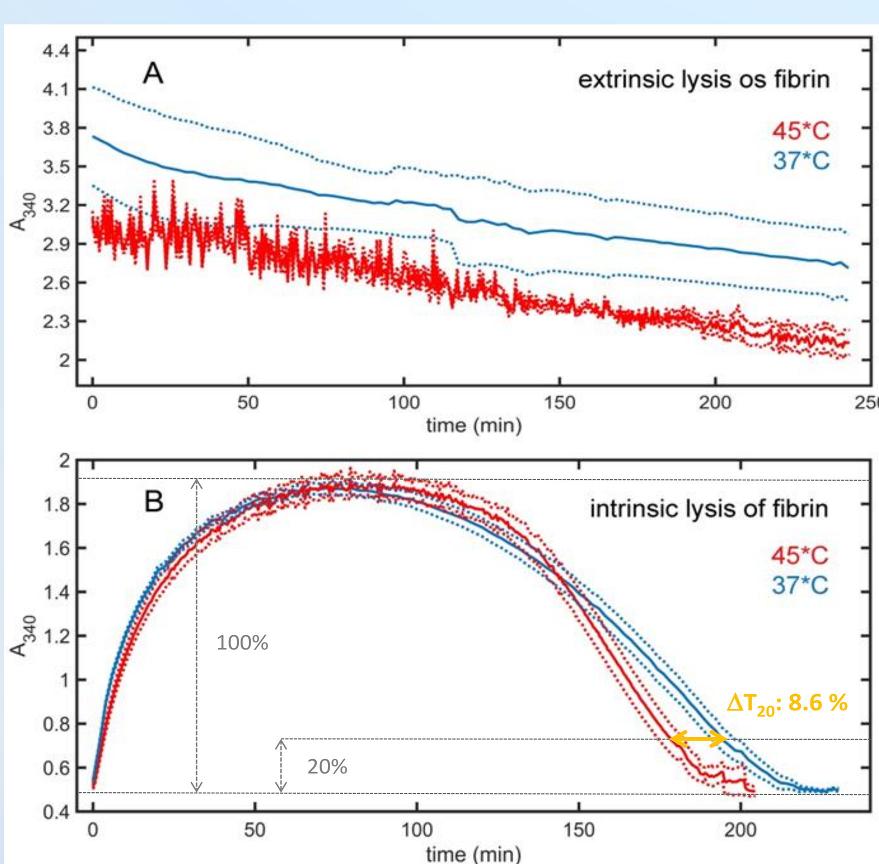

Fig. 6: Turbidimetric lysis of fibrin by SSL_T layered on the clot surface or homogeneously dispersed in the clots. The liposomal solution was layered over pre-formed clots (A) or added to fibrinogen before the clotting phase (B) and thereafter formation and/or dissolution of fibrin were followed as changes in absorbance at 340 nm shown as mean (solid lines) +/- SEM (dotted lines) of three measurements. Red and blue colours indicate clot lysis at 45°C and 37°C, respectively.

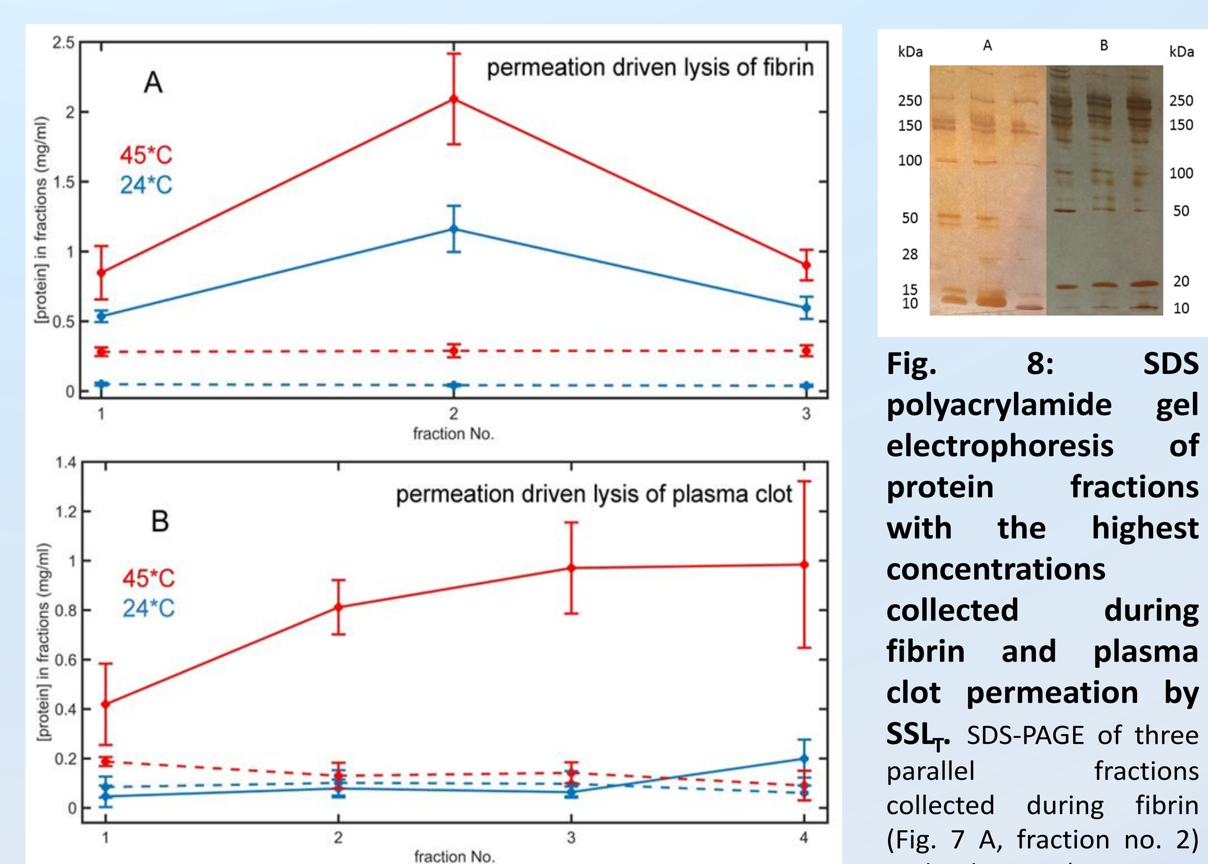

Fig. 7: Permeation-driven lysis of fibrin and plasma clots. SSL_T (solid lines) or HBS (dashed lines) was layered to the surface of fibrin (A) or plasma (B) clots, thereafter a constant hydrostatic pressure was maintained over the clots and the eluted fluid was collected in fractions of 50 μl each, in which the protein content was measured and is shown as mean+/-SEM, n=3. Red and blue colours indicate clot lysis at 45°C and 24°C, respectively.

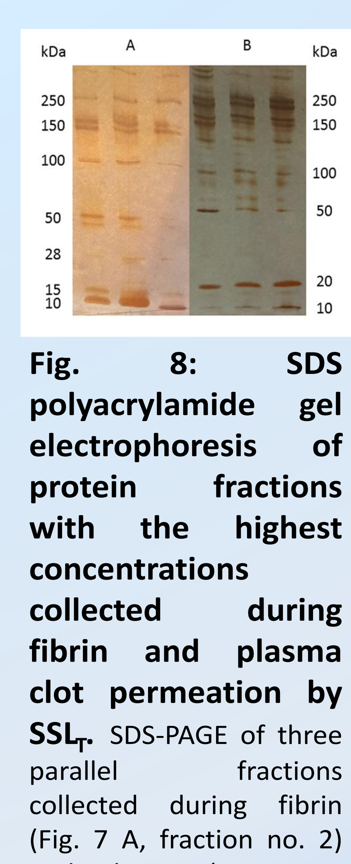

Fig. 8: SDS polyacrylamide gel electrophoresis of protein fractions with the highest concentrations collected during fibrin and plasma clot permeation by SSL_T. SDS-PAGE of three parallel fractions collected during fibrin (Fig. 7 A, fraction no. 2) and plasma (Fig. 7 B, fraction no. 3) clot permeation with SSL_T was performed on 4-15% gradient gels under nonreducing conditions. Plasmin-like fibrin degradation pattern was observed in both cases.

RESULTS:

- Trypsin is attached to the **inner surface of vesicles** (SAXS, FF-TEM) (Fig. 1-3) close to the hydrophilic/hydrophobic lipid interface (FT-IR). (Fig. 4-5)
- The **thermosensitivity** of SSL_T was evidenced by enhanced „intrinsic fibrinolysis" at 45°C: time to reduce the maximal turbidity to 20% decreased by 8.6% compared to 37°C (Fig. 6 B) and fibrin degradation product concentration in the permeation lysis assay was 2-5 fold higher than at 24°C (Fig. 7).
- SSL_T exerted its fibrinolytic action on fibrin clots under both static and dynamic conditions (Fig. 6 A,B; 7 A), whereas **plasma clot dissolution** was observed only in the **permeation-driven assay** (Fig. 7 B).

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