Corso di Dottorato di Ricerca in Scienze della Vita e dell'Ambiente, Ciclo XXXVIII

Studying fibrinogen activation into fibrin: structure and formation dynamics

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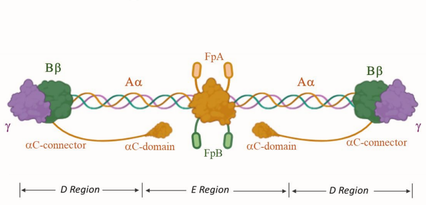
Background

The conversion of fibrinogen into insoluble fibrin and the formation of a stable clot are determinants in wound healing, tissue regeneration, and mediation of inflammatory responses that help the immune system fight invading pathogens. However, clinical evidence points to fibrin(ogen) to contribute to pathological processes that may result from altered plasma concentrations, modified structural properties, etc. Studying how environmental factors influence the properties of fibrin clots, particularly those mimicking chronic cardiovascular diseases (CVD), could help identify individuals at higher risk of thrombotic events and faster CVD progression. Therefore, our research investigates how thrombin triggers fibrinogen self-assembly under disease-relevant conditions, particularly those mimicking CVD. To better replicate physiological environments, we are also implementing a microfluidic-based system to study fibrin formation under controlled flow and shear, providing deeper insight into the molecular and mechanical aspects of clot development in health and disease.

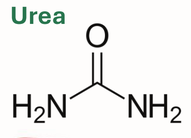
Fibrinogen



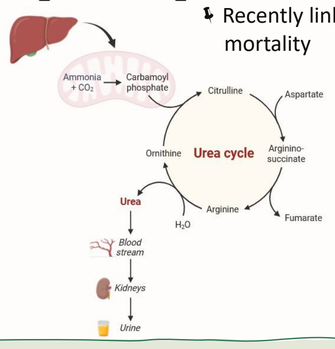
- Glycoprotein Synthesized in the liver
- Molecular Weight: 340 KDa
- Composed of three polipeptides chains (Aα, Bβ and γ) and three main regions: D-E-D
- When activated by Thrombin forms the fibrin clot

Pathogenic associated metabolites

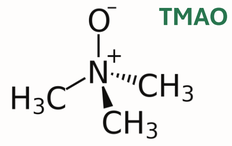
Urea

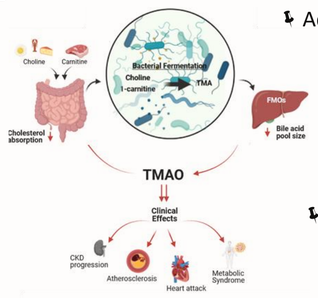


- Metabolic waste
- Produced by the liver
- Excreted by the kidneys
- Protein denaturant
- Recently linked to CVD mortality



TMAO





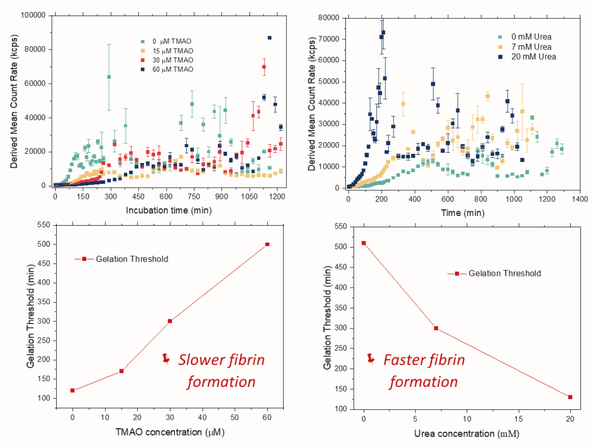
- Metabolite produced by gut microbiota
- Acts as an osmolyte, shielding cellular proteins from the denaturing effects of urea in some marine organisms
- Recently linked to CVD

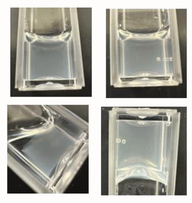
Current Results

1 *Dynamic Light Scattering (DLS)*

- *DLS measurements were performed to study the aggregation kinetics of fibrinogen under different conditions. TMAO and Urea concentrations are between physiological and pathological conditions.*

Model-free analysis of the Derived Mean Count Rate

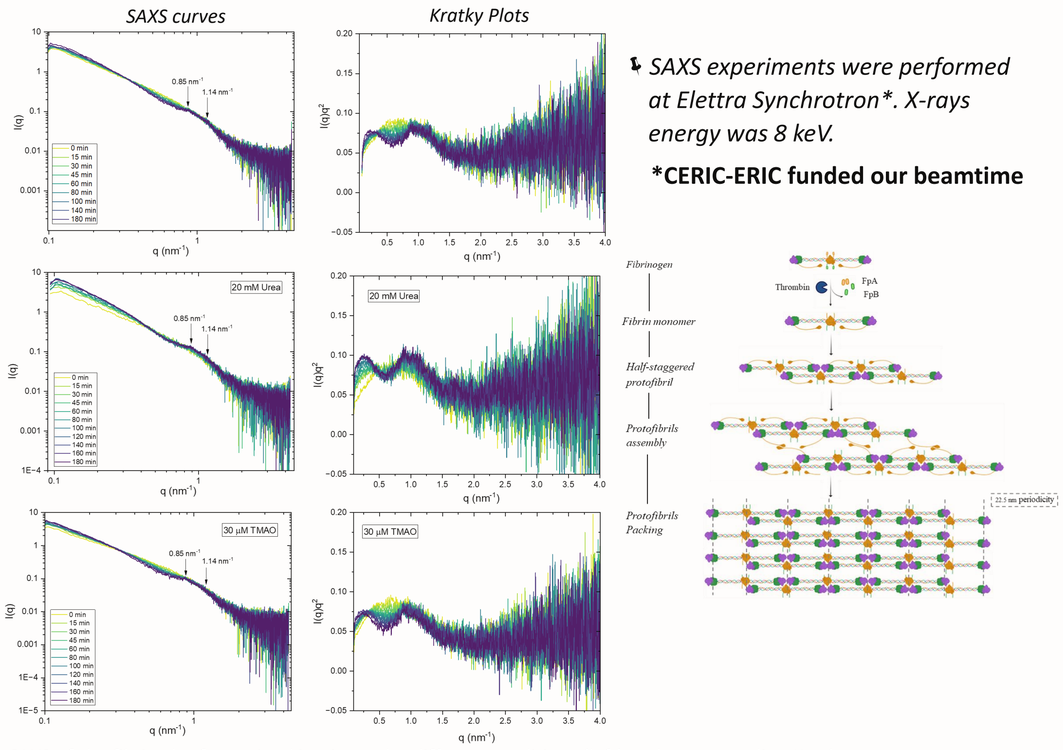




Fibrin gel after DLS measurements.

Fibrin formation kinetics in the presence of TMAO and Urea. The Gelation Thresholds were calculated from the derived mean count rate analyisis.

2 *Small Angle X-Ray Scattering (SAXS)*



- *SAXS experiments were performed at Elettra Synchrotron*. X-rays energy was 8 keV.*

***CERIC-ERIC funded our beamtime**

SAXS of the fibrin clot formation kinetics under different conditions (left) and corresponding Kratky plots (right), whose bell shapes confirm the presence of compact species.

- *The 22.5 nm periodicity of the fibrin half-staggered axial packing repeat causes the appearance of four order of reflections at q ~ 0.22 nm^{-1}, q ~ 0.57 nm^{-1}, q ~ 0.85 nm^{-1}, and q ~ 1.14 nm^{-1}. We can see just the third and fourth orders (q ~ 0.85 nm^{-1} and q ~ 1.14 nm^{-1}), indicative of the formation of a fine clot.*

3 *Confocal Microscopy (CM)*

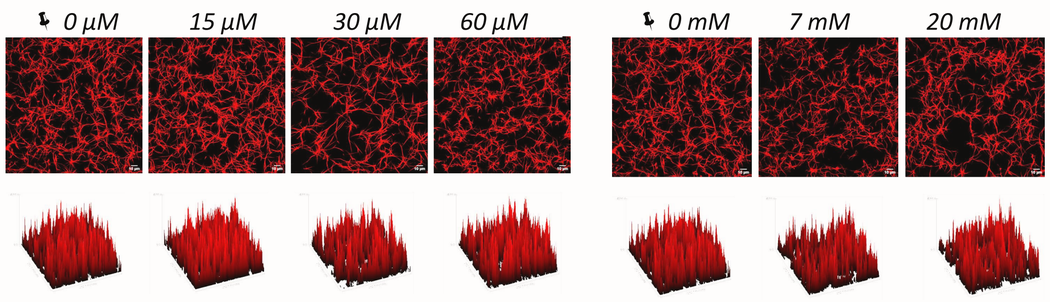
- *CM measurements were performed to study the structure of the fibrin clot under the different conditions.*

TMAO

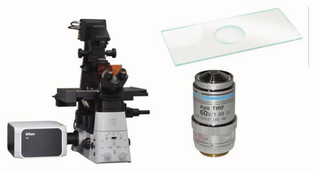
0 μM *15 μM* *30 μM* *60 μM*

Urea

0 mM *7 mM* *20 mM*



Structure of Fibrin Clot in the presence of TMAO and Urea. The images were taken at the final point of the Clot formation.



Clots were allowed to form on the concave glass slides at room temperature before measurements.

Images are a maximum Intensity projection of a Z-stack of 50 mm

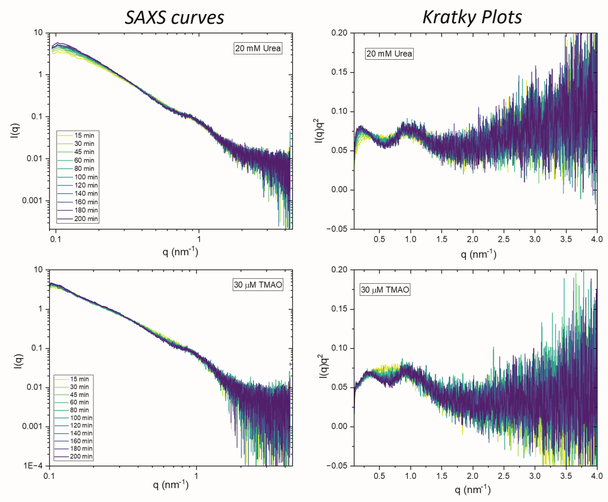
- ***No significant differences appreciated***

Microfluidic SetUp Implementation

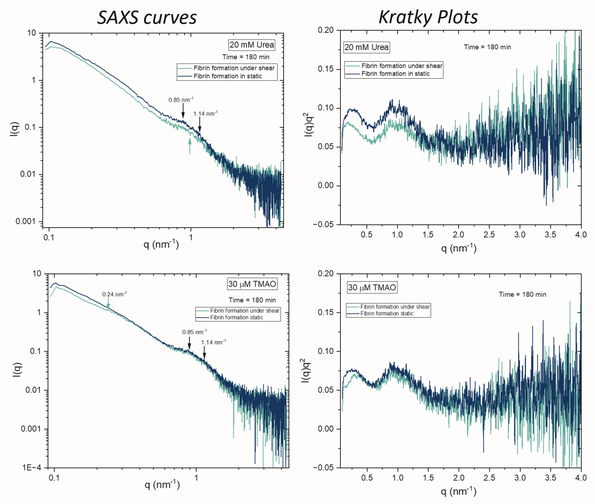
4 *Preliminary SAXS-Microfluidic Measurements*

- *Preliminary SAXS microfluidic measurements were performed at the Elettra Synchrotron in Trieste to study the effect of shear forces on the fibrin formation process. Using a syringe pump coupled to a flow-through capillary, we studied, at low shear rates, the dynamics of fibrin fiber formation in the presence of Urea and TMAO.*

Flow rate used in every case: 50 μl/min Shear rate: 10 s^{-1}



SAXS curves of fibrin clot formation kinetics in the presence of urea and TMAO at low shear rates (left); and corresponding Kratky plots (right)

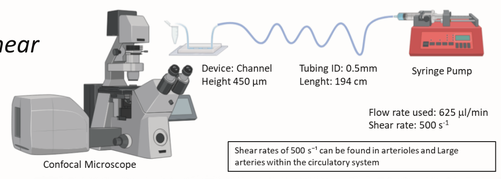


Comparison of SAXS results of the reaction performed with (green) and without (black) microfluidics. SAXS curves (left), corresponding Kratky plots (right).

- *There are slight structural differences between the fibers formed with and without microfluidics. In the case of the reaction in the presence of TMAO, we can see that the first reflection order (q ~ 0.22 nm^{-1}) is beginning to appear, indicating the formation of more compact and well-organized fibers. On the other hand, in the presence of Urea, we can see that the third and four orders of reflection (q ~ 0.85 nm^{-1} and q ~ 1.14 nm^{-1}) start to disappear, indicating the formation of more disordered fibers.*

5 *Microfluidic SetUp Implementation for Fluorescence Confocal Microscopy Measurements*

- *After preliminary SAXS-microfluidic measurements, we started to implement a microfluidic approach to study fibrin formation at more physiologically relevant shear rates using the confocal microscopy technique.*

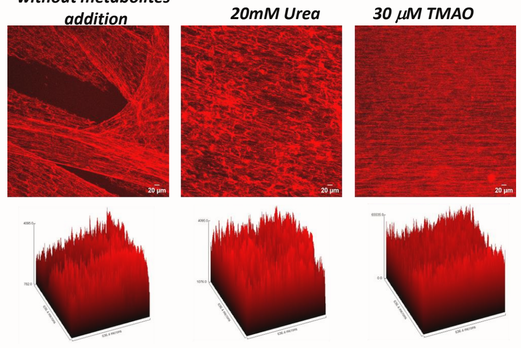


Flow rate used: 625 μl/min
Shear rate: 500 s^{-1}

Shear rates of 500 s^{-1} can be found in arterioles and Large arteries within the circulatory system

FLOW DIRECTION

Fibrin formation without metabolites' addition ***Fibrin formation with 20mM Urea*** ***Fibrin formation with 30 μM TMAO***



- ***It can be qualitatively observed that there are differences in the fibrin network structure***

Clots were formed while passing through the tubing at the desired shear rate. Images are a maximum Intensity projection of a Z-stack of 50 μm.

- *The fibrin network formed in the presence of Urea shows a higher level of disorder than that formed in the presence of TMAO, which is consistent with preliminary SAXS results.*

Structure of Fibrin Clot in the presence of TMAO and Urea.

---- Next Steps in the Microfluidic SetUp implementation ----

6 *Microfluidic Device Design and Microfabrication*

- This phase will be carried out at the lithography beamline of Elettra Synchrotron in Trieste. The device will be designed for integration into the SAXS beamline setup to enable real-time SAXS profile measurements under physiologically relevant conditions.

7 *SAXS-coupled microfluidic experiments*

- SAXS experiments using the fabricated device will be conducted at the Elettra SAXS beamline.

Take Home Message

- Both TMAO and Urea influence the kinetics of fibrin formation, but in opposite ways: TMAO slows down clot formation, whereas urea accelerates it. This observation raises the hypothesis that TMAO may counteract the effects of Urea, a phenomenon previously reported in the unfolding of model proteins, like BSA and lysozyme.
- Under static conditions, neither TMAO nor Urea appears to significantly alter the final structure of the fibrin clot. However, in the presence of shear forces, confocal microscopy reveals clear differences. Urea seems to promote the formation of more disordered fibrin fibers, while TMAO leads to a more ordered fibrin network.
- SAXS experiments conducted under static conditions confirmed the formation of a fine clot in all cases. However, even under low shear rates, subtle structural differences were observed between conditions. Further analysis of the SAXS data is needed to characterize and understand these differences.