Analytical science in the Netherlands COAST

Towards the use of reaction-modulators in an integrated multi-dimensional liquid chromatography system

Bert Wouters, Bob Pirok, Niall P. Macdonald, Joan M. Cabot, Sinéad Currivan, Brett Paull, Michael C. Breadmore, Peter J. Schoenmakers

8th of March 2018, ATEurope conference

CASSS Frantisek Svec Fellowship For Innovative Studies

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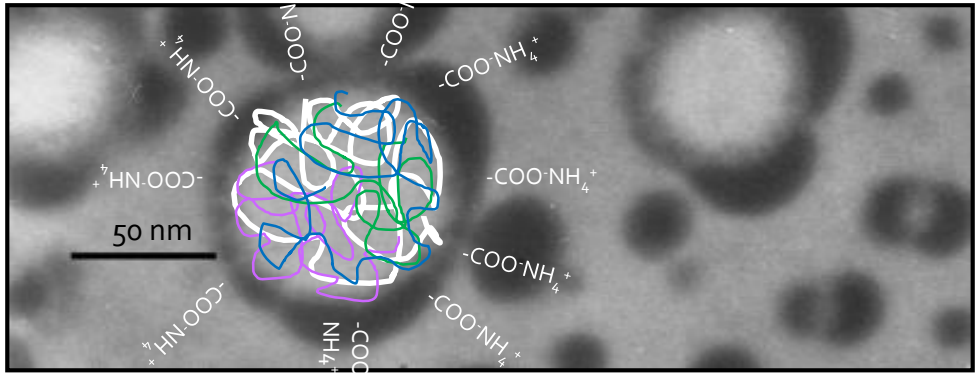
Presentation outline

1. **The MAnIAC project**
2. **Immobilized-enzyme reactors**
    1. Prototyping of polymer-based microfluidic devices
    2. Enzyme-immobillization process
    3. Proof-of-principle: offline digestion of protein samples
    4. Proof-of-principle: online digestion of polymer nanoparticles
3. **Towards 3D-printing glass microfluidic devices**

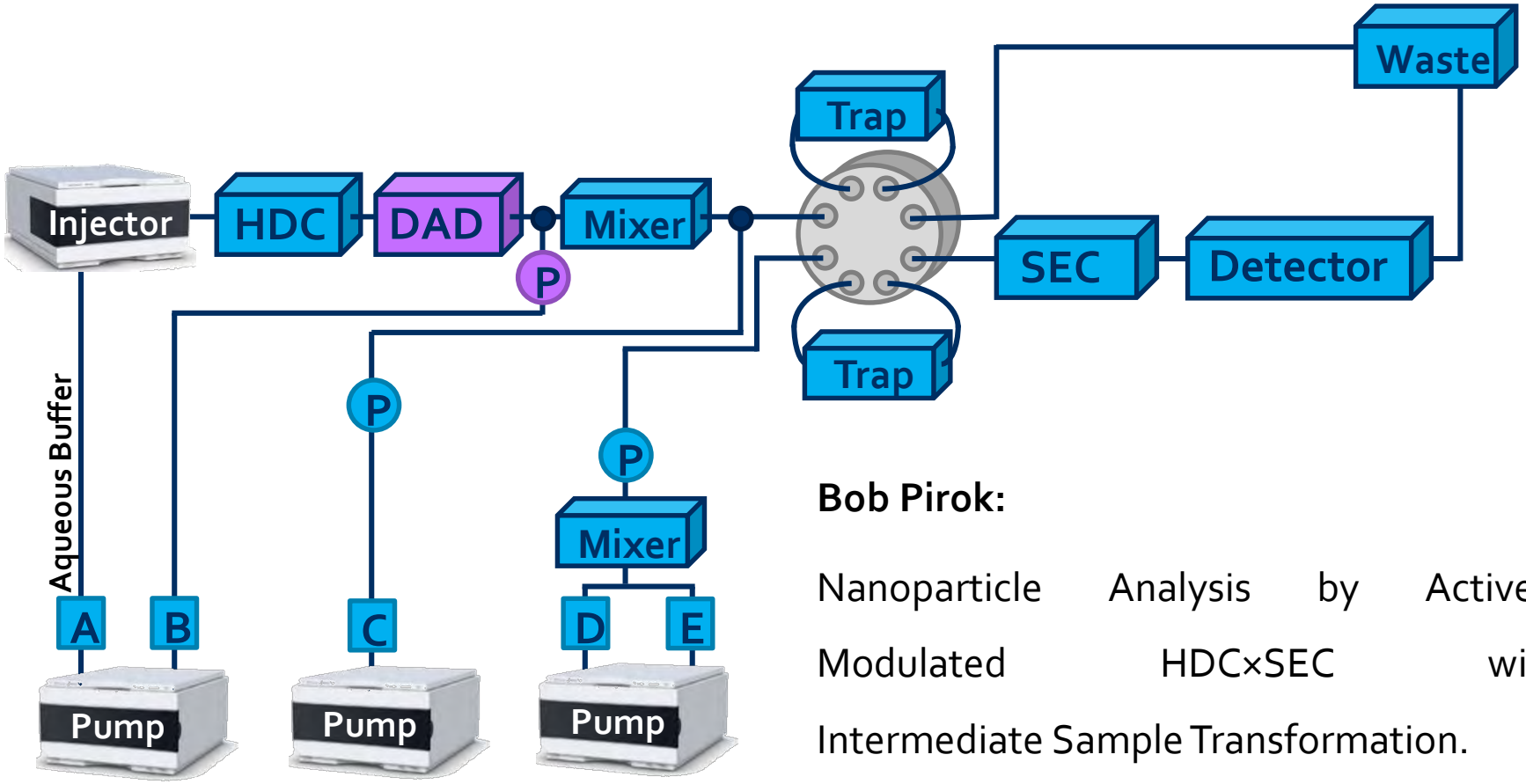
MAnIAC: Making Analytically Incompatible Approaches Compatible

- Comprehensively obtain multiple types of information on industrially-relevant samples.
- **Example**: nano-sized polymeric particles dispersed in water.



- Molecular weight distribution (**MWD**), sequence distribution (**SD**), particle size distribution (**PSD**), *etc*.

Comprehensive 2D-LC of polymeric nanoparticles

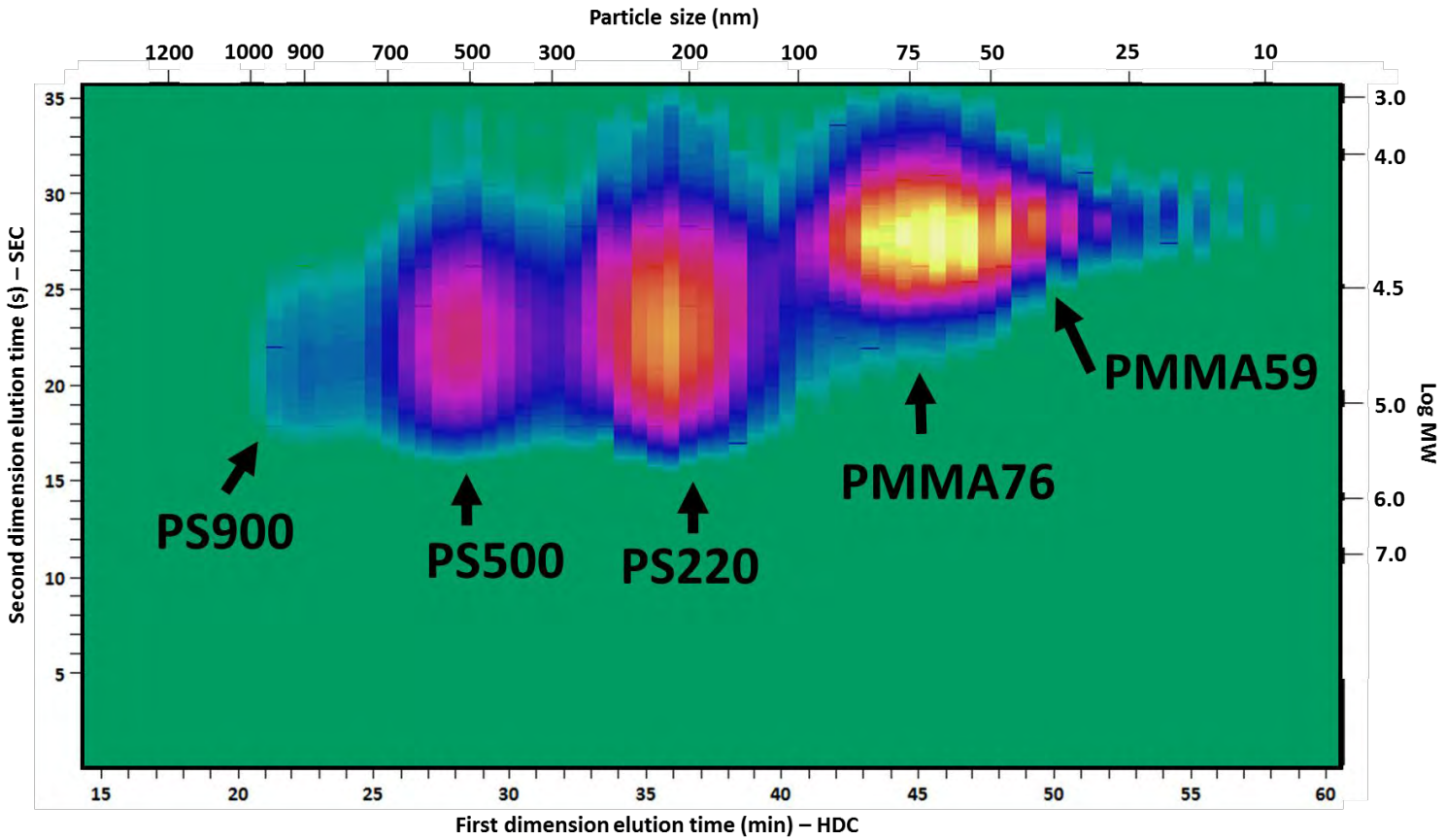


Bob Pirok:

Nanoparticle Analysis by Actively Modulated HDC×SEC with Intermediate Sample Transformation.

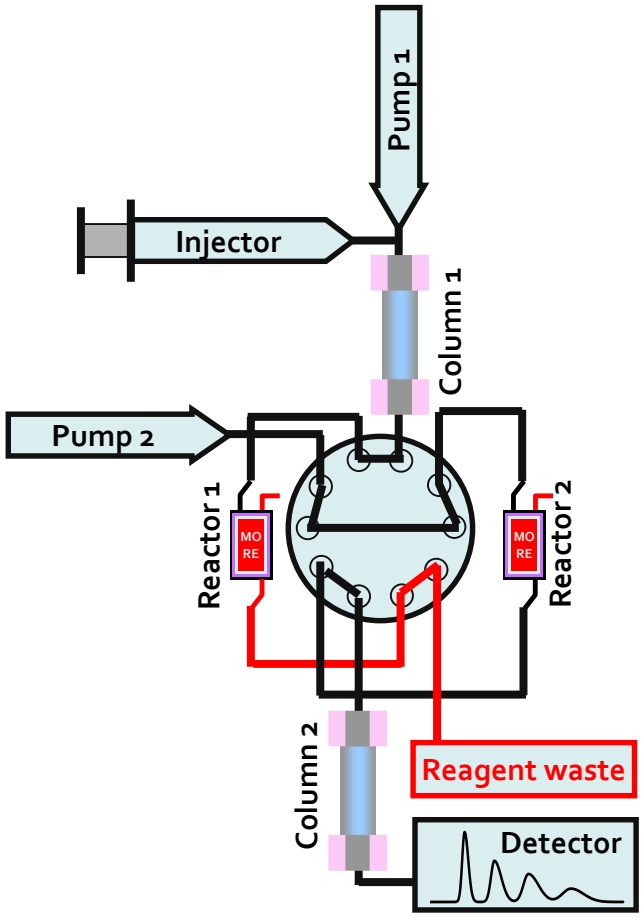
Pirok *et al.*, *Anal. Chem.* **89** (2017) p. 9167-9174.

Particle size distribution (PSD) and molecular weight distribution (MWD)



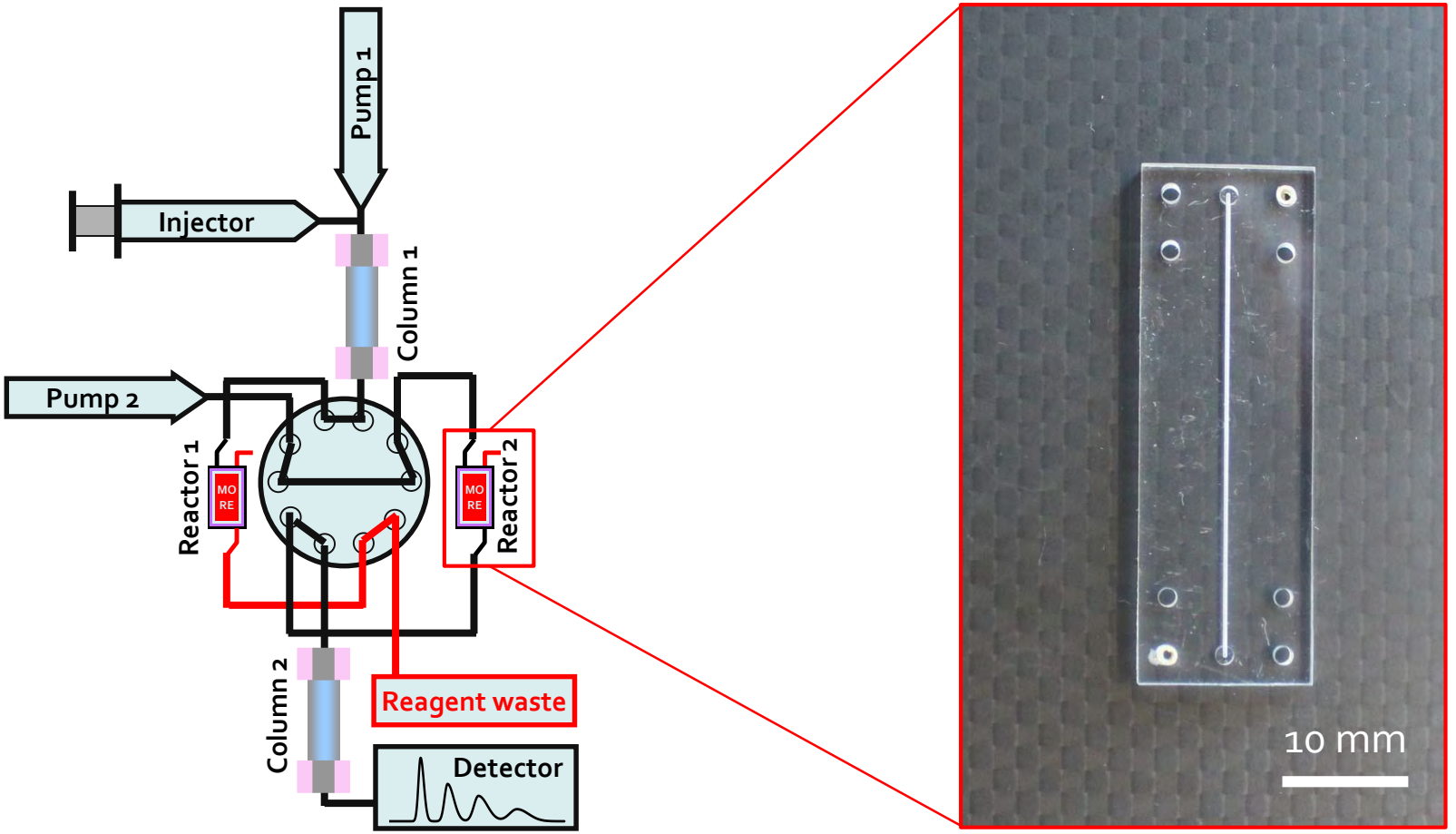
Pirok *et al.*, *Anal. Chem.* **89** (2017) p. 9167-9174.

Reaction modulators for online enzymatic degradation



- **Reaction-modulators** as an interface in a multi-dimensional liquid chromatography system.
- Specific reactions during sample transfer, *e.g.* online **enzymatic degradation** of various macromolecules .
- Insight into sequence distribution by **studying degradation products** during ^{2}D separation.
- *e.g.* Molecular Weight Distribution (**MWD**) and Sequence Distribution (**SD**) in a single 2D-LC run.

Reaction modulators for online enzymatic degradation



Why use an immobilised-enzyme reactor (IMER)?

In-solution enzymatic digestion:

Mixing proteolytic enzymes (*e.g.*, trypsin) and proteins in a typically low ratio.

Disadvantages:

- **Long digestion times** (typically multiple hours or overnight).
- Difficult to implement in LC×LC workflow.
- **Non-reusability** of the enzymes.

Immobilized-enzyme reactor (IMER):

High concentrations of enzymes immobilised in a confined space.

Advantages:

- **Degradation** in order of minutes, due to faster mass transfer and **higher enzyme-to-substrate ratios**
- **Online** implementation in LC×LC workflow and reactor can be **reused**.

Prototyping of polymer-based microfluidic devices

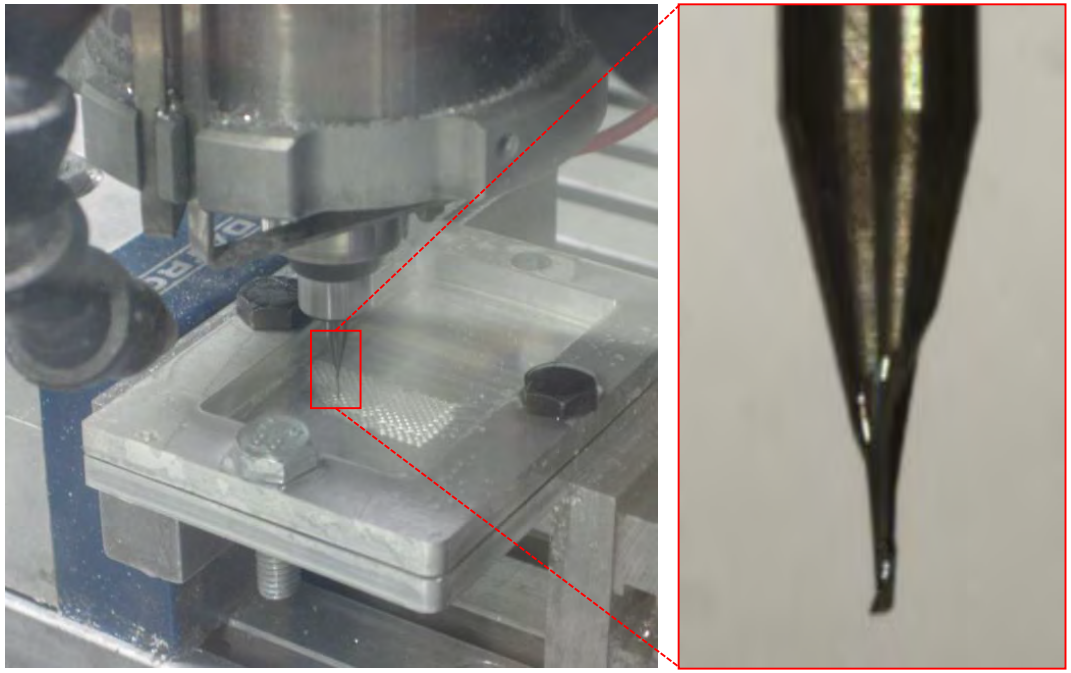
Prototyping of COC-based microfluidic devices

Substrate: cyclic olefin copolymer

- Compatibility with organic solvents and biomolecules.
- Good optical properties.
- Relatively low cost.

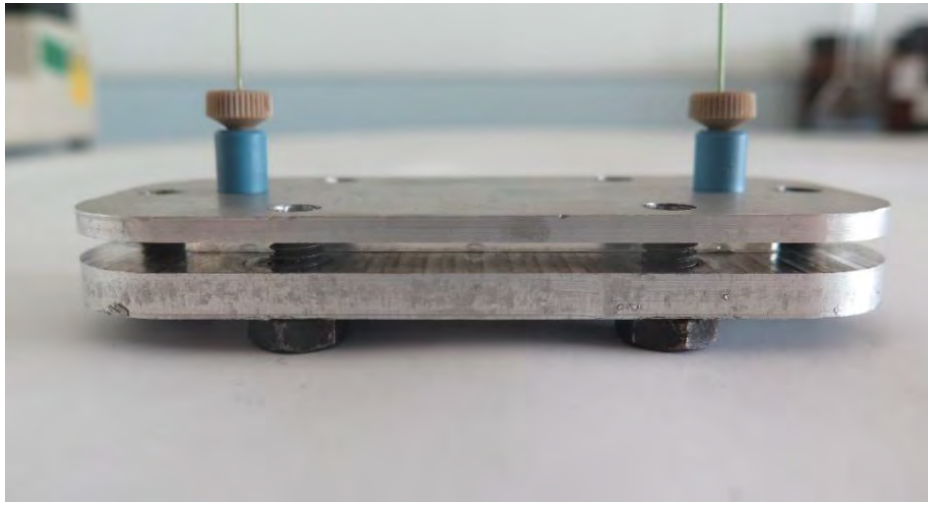
Prototyping:

- Channel dimensions ≥ 100 µm.
- Solvent-vapour-assisted bonding.



Wouters *et al.*, *J. Sep. Sci.* **38** (2015), p. 1123–1129.

First-generation microfluidic reactor for MAnIAC



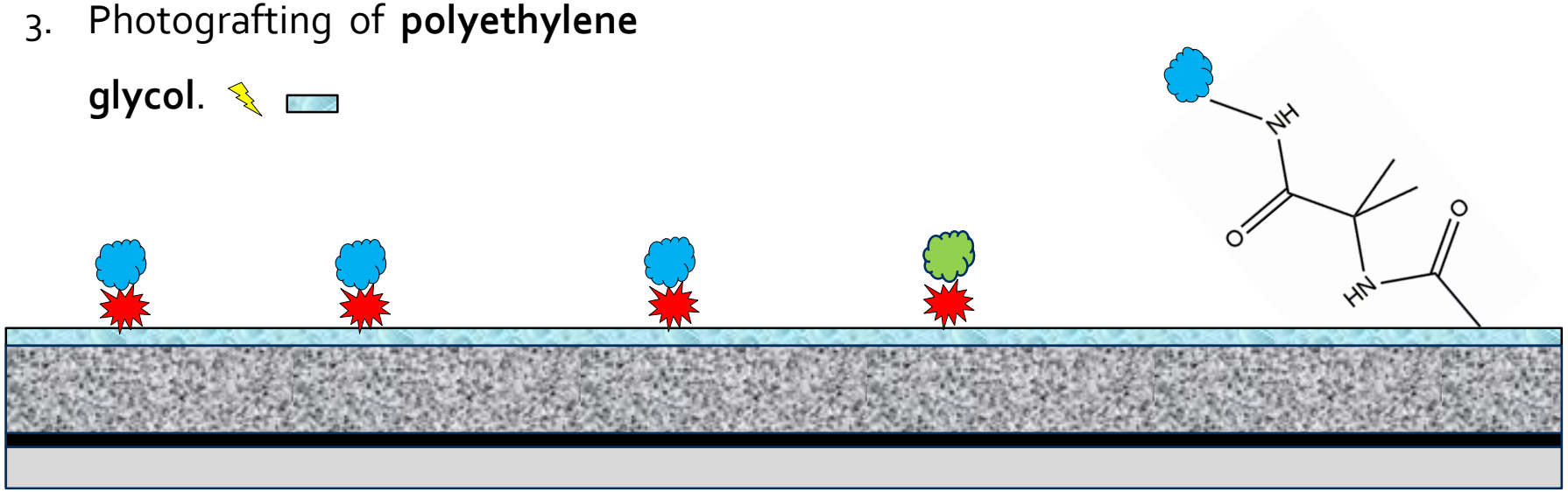
- Two layers of cyclic-olefin-copolymer bonded through solvent-vapour.
- Microchannel: 300 µm internal diameter, 60 mm length.
- Assembled chip holder consisting of two aluminum plates and six bolts.
- Connecting the chip with flat-bottom NanoPort connections.

Note: *In cooperation with Free University Brussel, Belgium.*

Enzyme-immobilization process

Enzyme-immobilisation process

1. **Pre-treatment** of COC.
2. **Polymerization** of monolithic support.
3. Photografting of **polyethylene glycol**.
4. Photografting of **vinyl azlactone.**
5. **Enzyme immobilisation**.
6. **Quenching** of azlactone groups.



Protocol adapted from Logan *et al.*, *Anal. Chem.* **79** (2007) 6592-6598.

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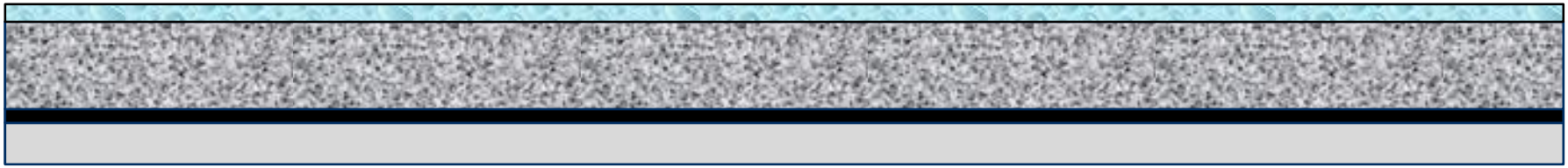

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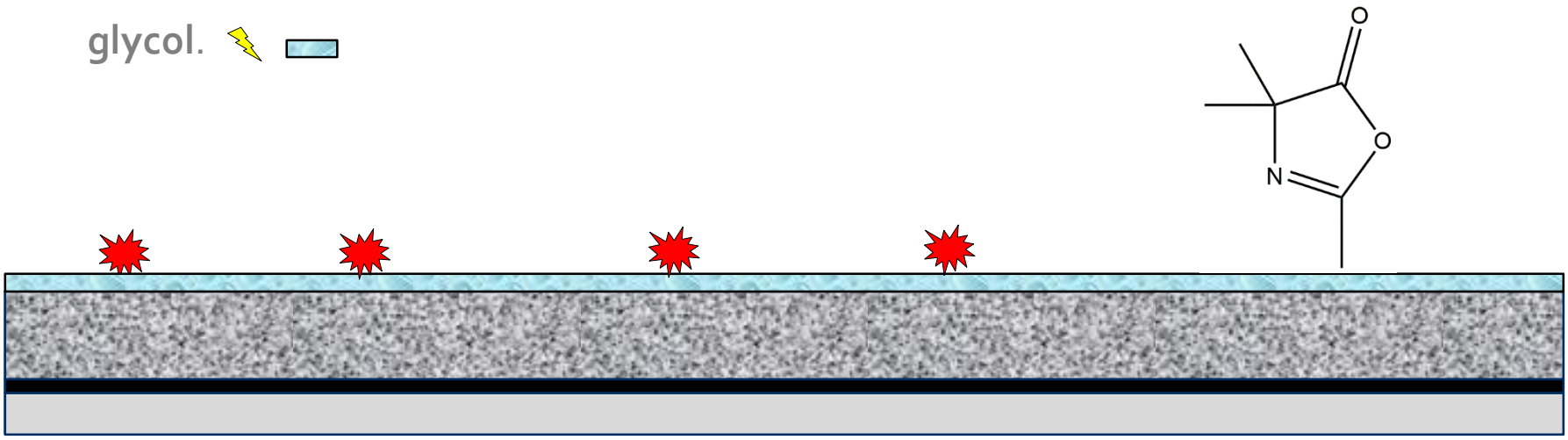

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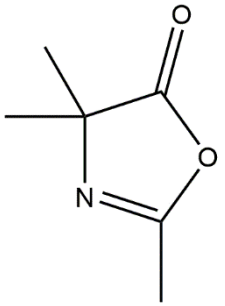

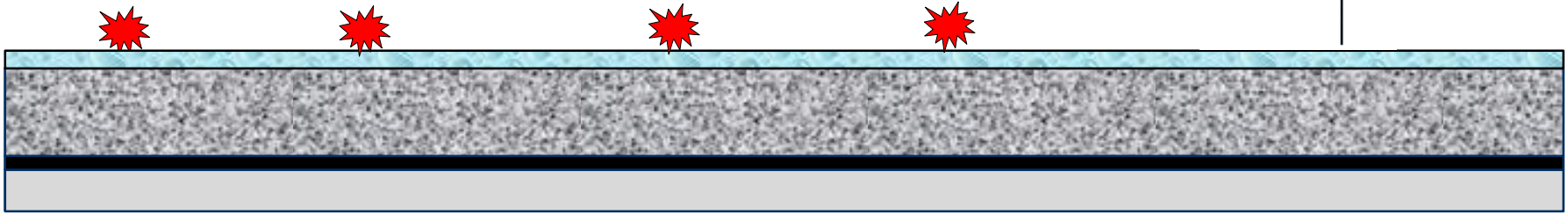

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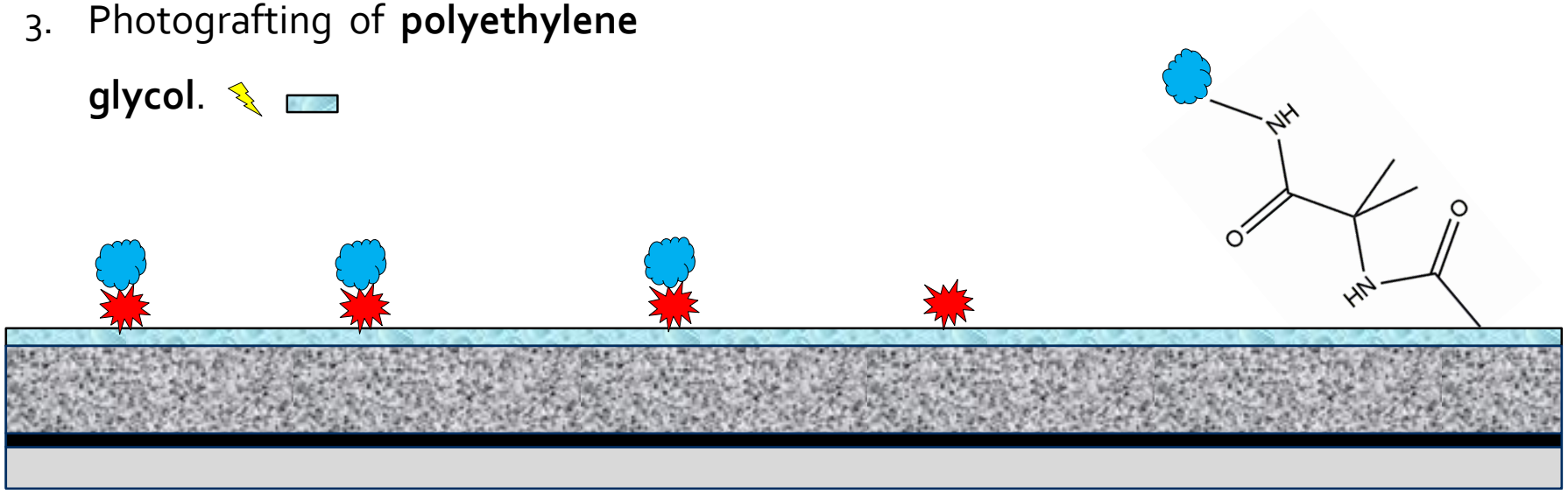

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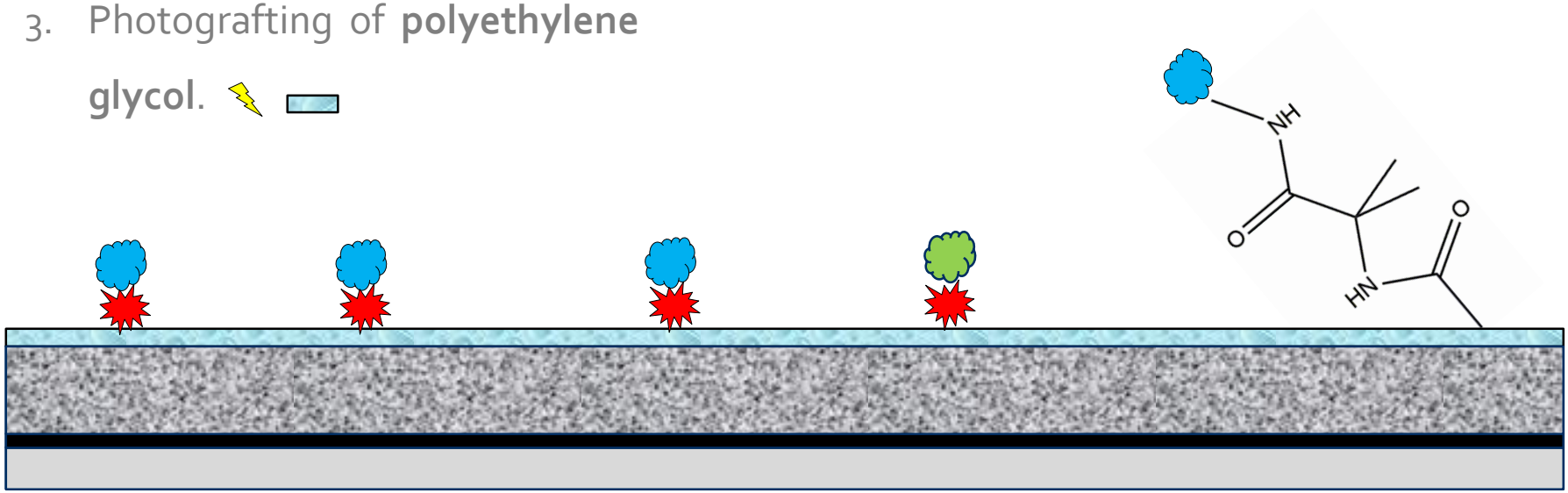


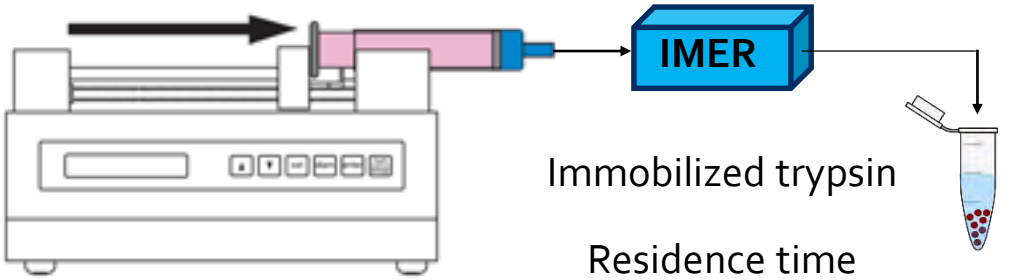
Proof-of-principle:

Offline digestion of protein samples

Proof-of-principle: Offline digestion of protein samples

IMER-facilitated protein digestion

Digestion at room temperature.



Immobilized trypsin

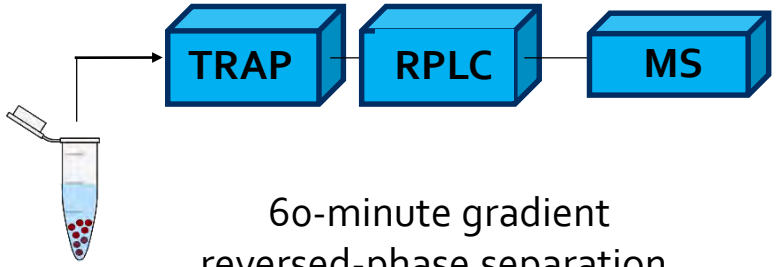
Residence time determined by flow rate.

20 µL

e.g. 100 ppm α-casein in TRIS buffer (pH = 8)

LC-MS analysis

Desalting for 10 minutes

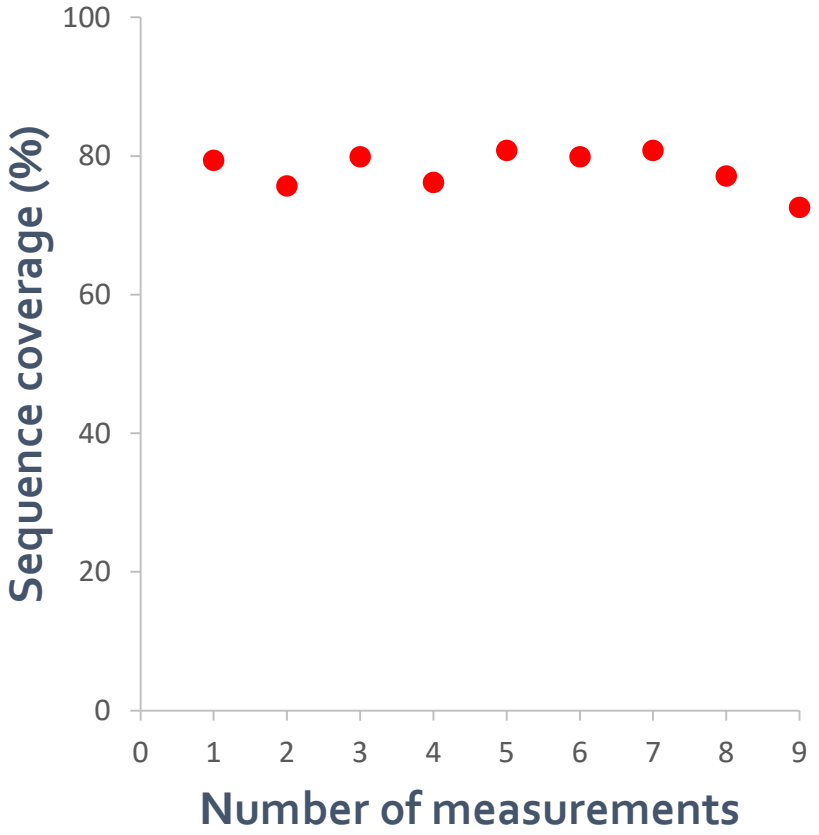


5 µL

60-minute gradient reversed-phase separation.

TripleTOF mass spectrometer.

Proof-of-principle: Offline digestion of protein samples



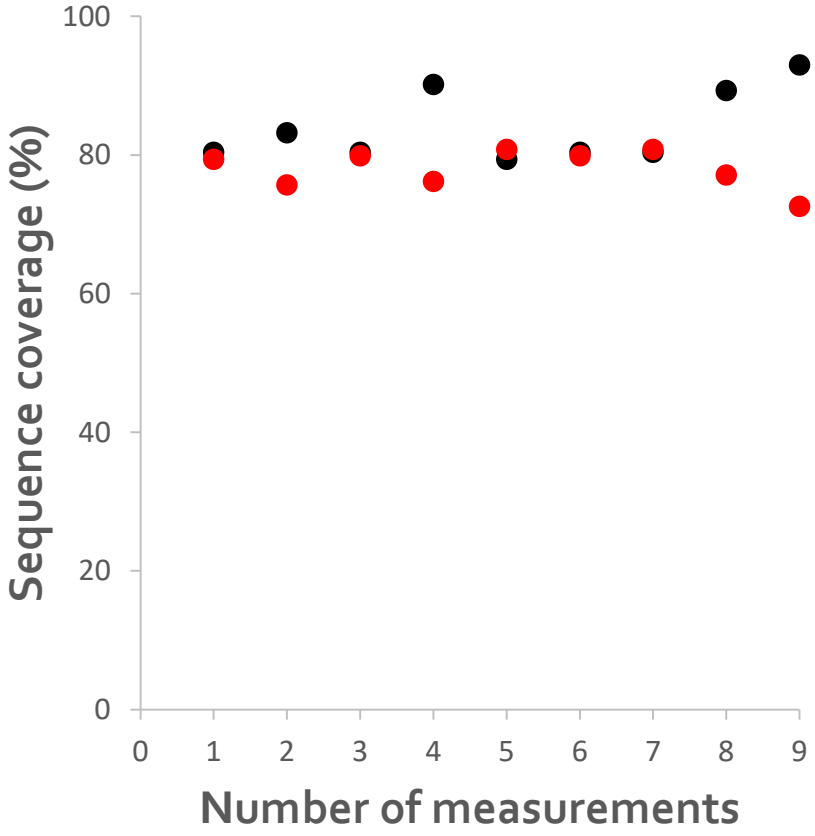
Traditional in-solution digestion:

- 18 hours, 37 °C, protein pre-treatment.
- 78.0 % average sequence coverage with RSD of 3.8 % (n=9).

IMER-facilitated digestion:

- 1 minute, room temperature, no protein pre-treatment.

Proof-of-principle: Offline digestion of protein samples



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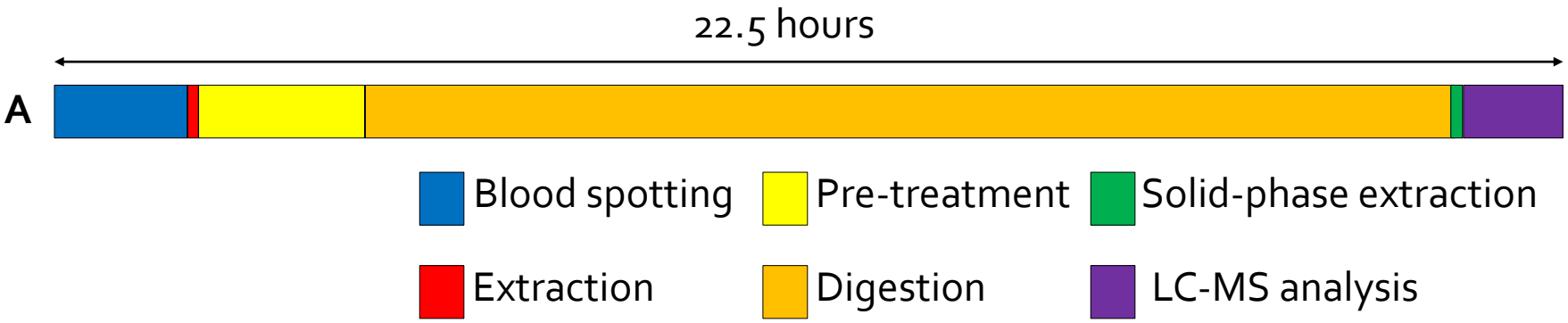
- 18 hours, 37 °C, protein pre-treatment.
- 78.0 % average sequence coverage with RSD of 3.8 % (n=9).

IMER-facilitated digestion:

- 1 minute, room temperature, no protein pre-treatment.
- 84.1 % average sequence coverage with RSD of 6.3 % (n=9).

Dried-blood-spot analysis



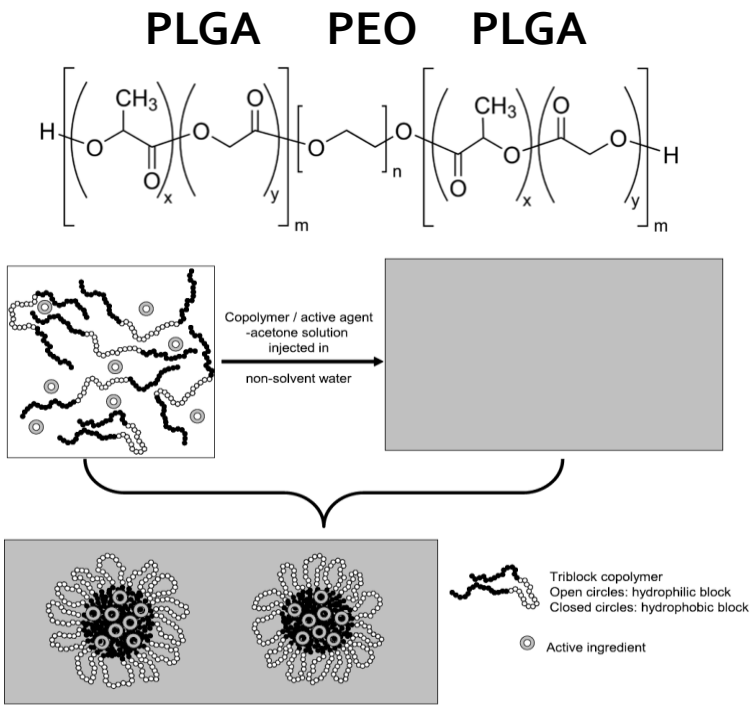
- Time needed for protein digestion reduced **from 16 hours to 5.6 minutes**.
- **Omission of protein pre-treatment step,** saving additional 2.5 hours.
- **Comparable number of protein identifications** (156 versus 142).
- **Similar trends** in terms of molecular weight and hydrophobic character.

Wouters *et al.*, *J Chrom A* **1491** (2017) 36–42.

Proof-of-principle:

Online degradation of polymeric nanoparticles

Bio-degradable triblock copolymers



- Triblock copolymers of poly(lactic-*co*-glycolic)acid (**PLGA**) and polyethylene oxide (**PEO**).
- **Nanoprecipitation process** for non-water soluble triblock copolymer micelles.
- Can be used for **drug-delivery** in human body; hydrophobic active ingredients in nanoparticle with hydrophilic outer layer.

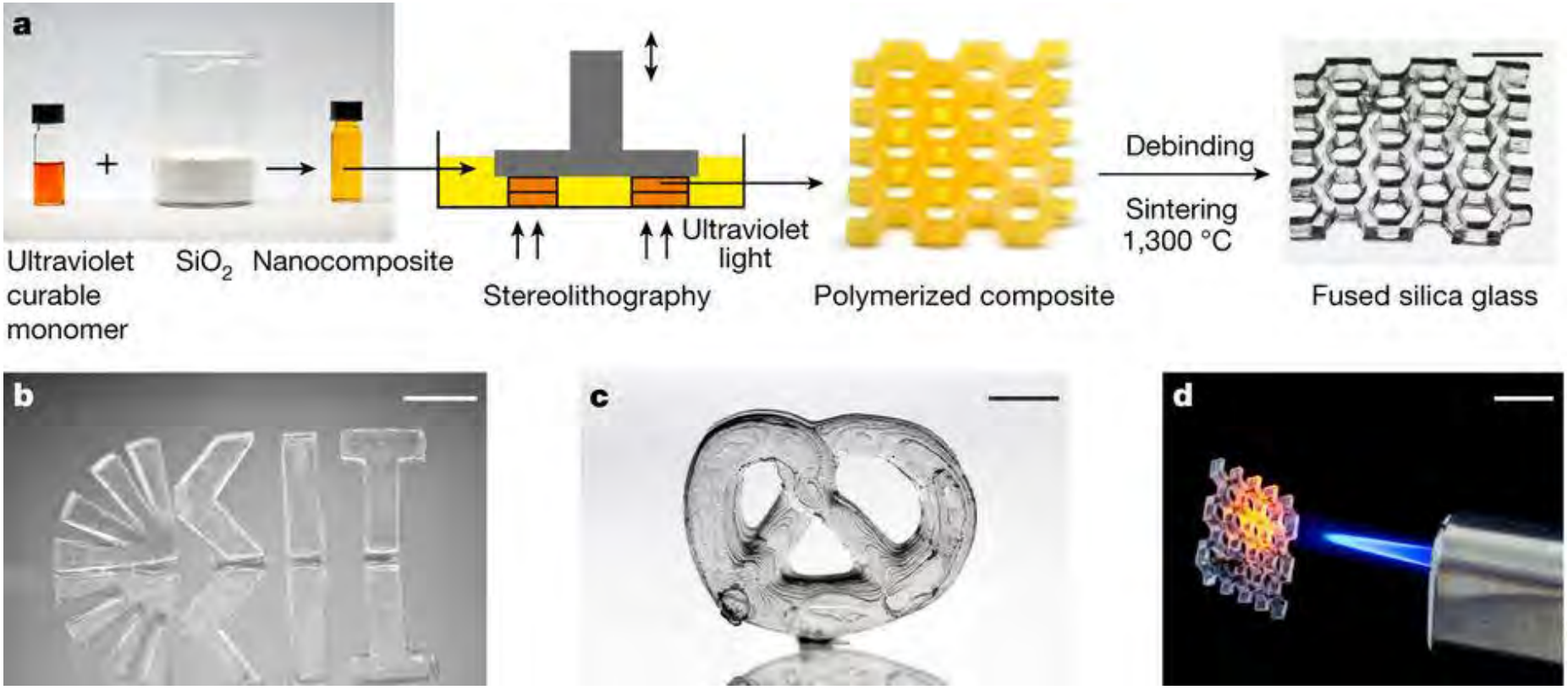
Lebouille *et al.*, *Eur. Phys. J. E* **36** (2013) 107-119.

Towards 3D printing glass microfluidic devices

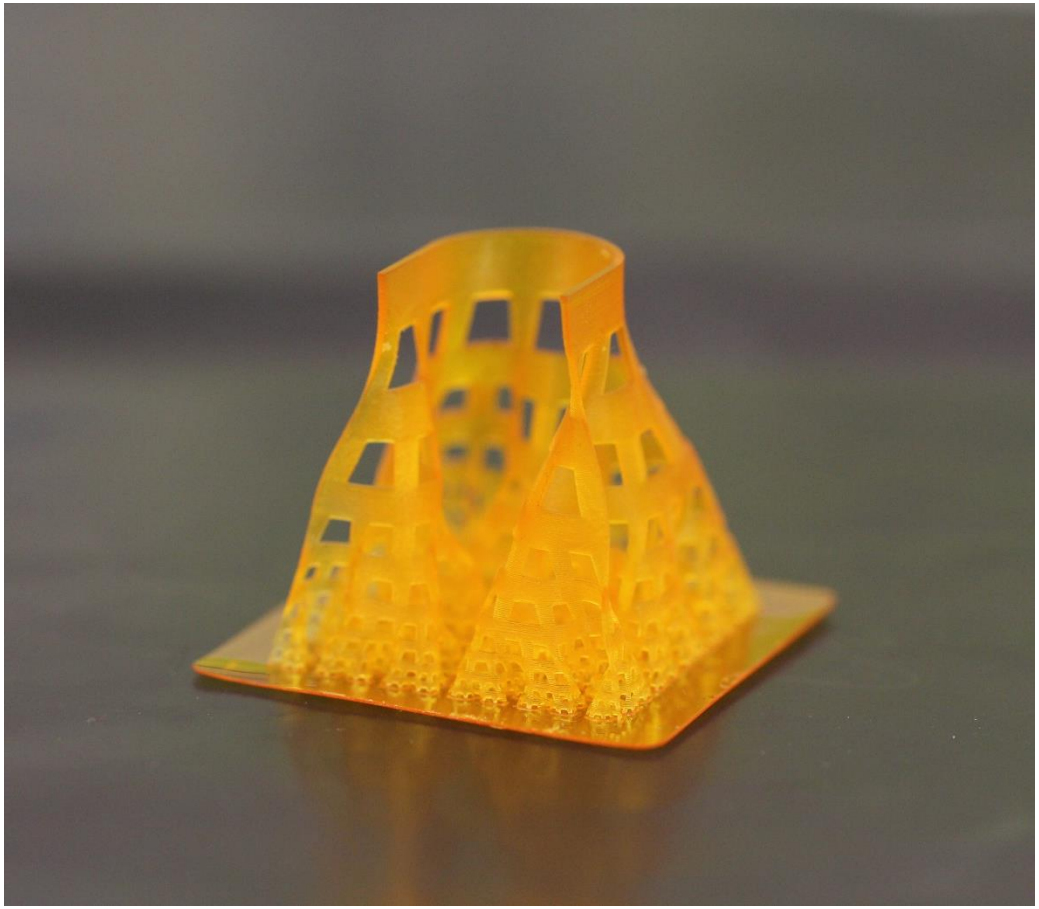
Bottlenecks for polymer-based microfluidics

- **Optical** transparency in the UV range (photografting, photo-polymerization).
- **Chemical resistance** (toluene, tetrahydrofuran, *etc*..).
- **Operating pressure** (pressure-driven liquid chromatography).
- Limited **geometries** (2 or 2.5 D, aligning of layers).
- Limited **operating temperature.**

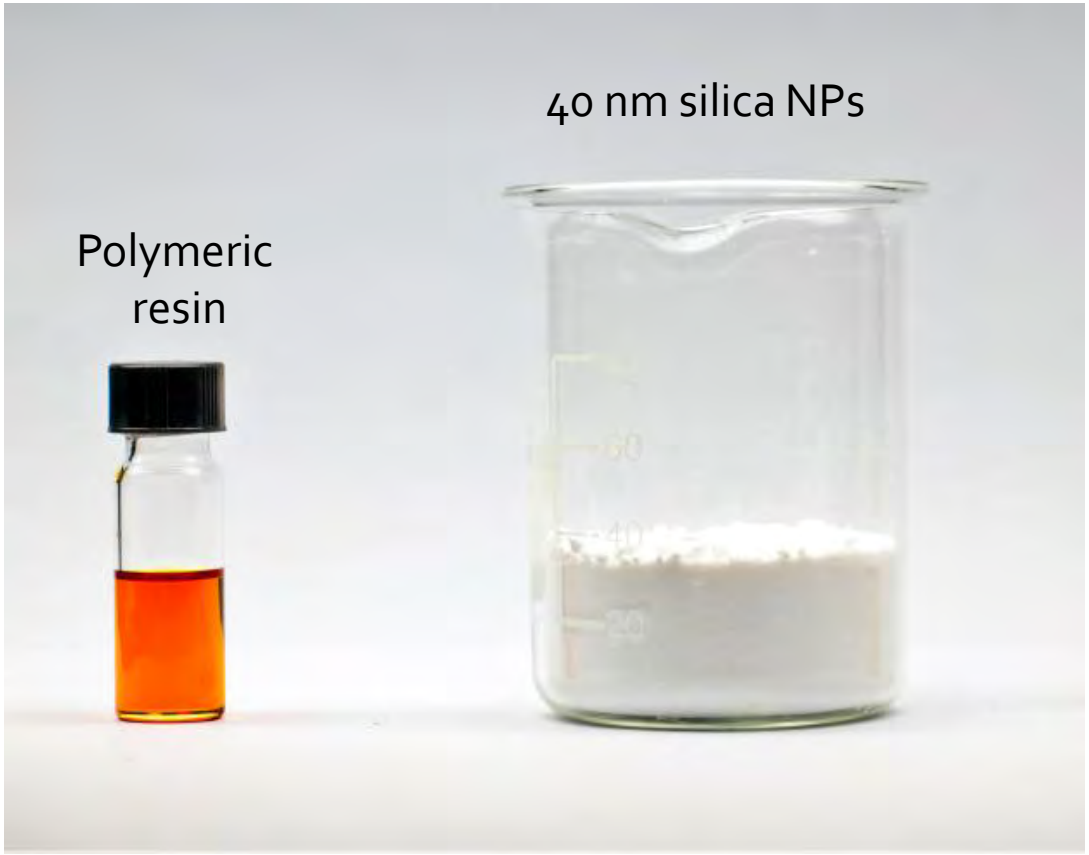
Inspiration: Letter to Nature by Rapp and co-workers



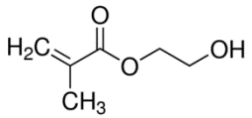
Printing with a commercially-available resin



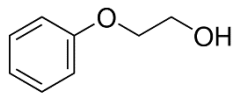
Mixing the resin



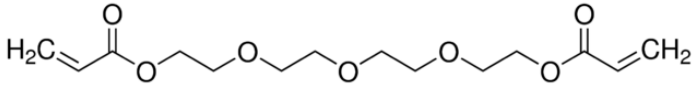
Hydroxyethyl methacrylate (HEMA)



Phenoxyethanol (POE)

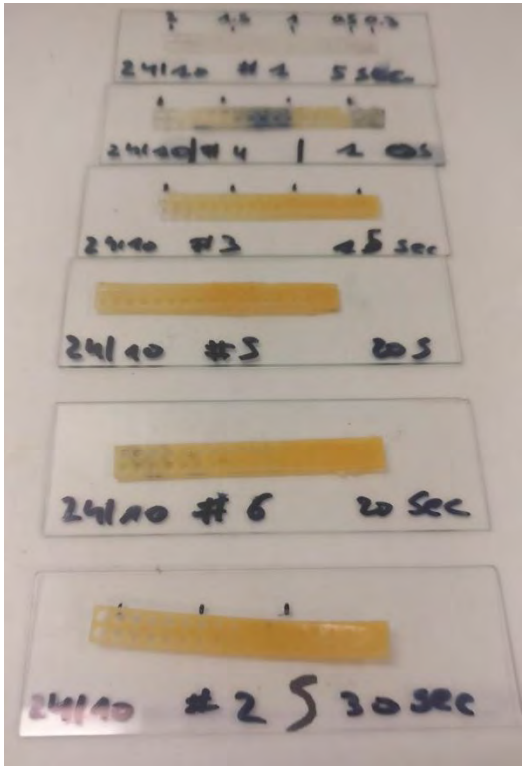


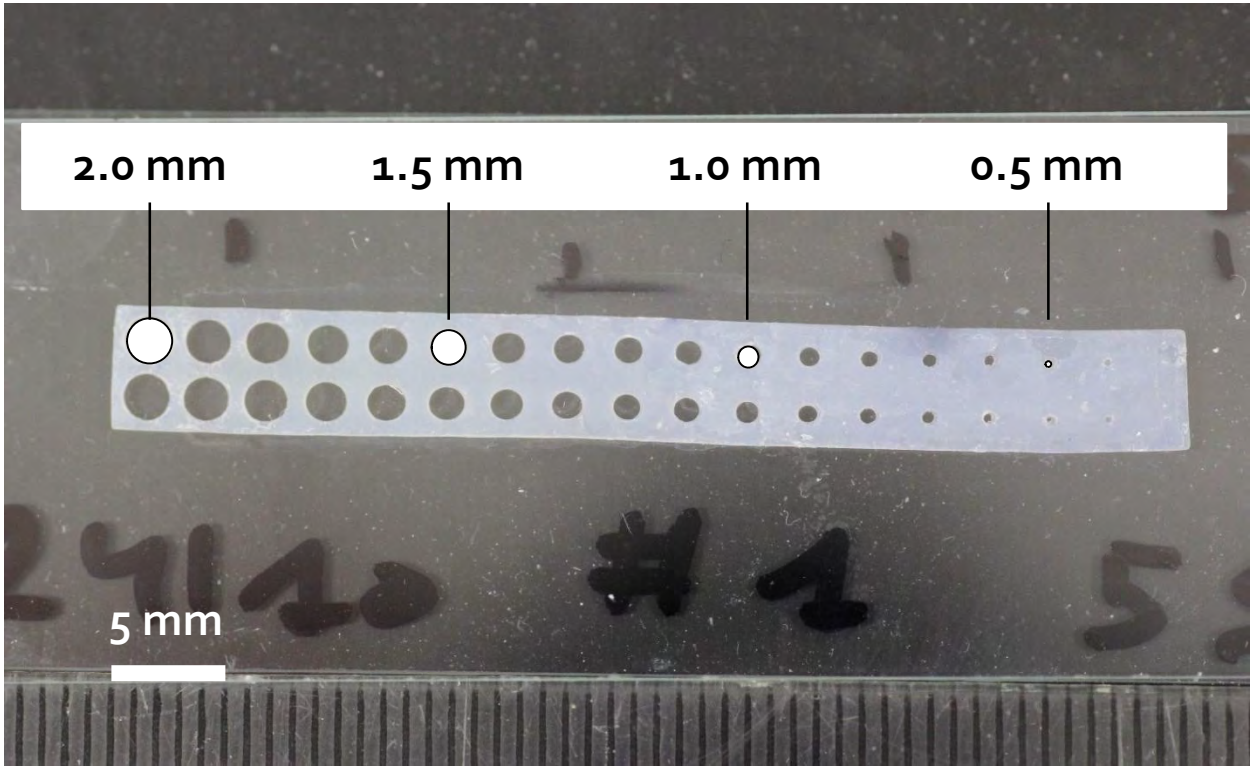
Tetra(ethylene glycol) diacrylate (TEGDA)



- Mixing with mechanical stirrer.
- Degassing of resin.

Resolution tests: vertically-orientated holes





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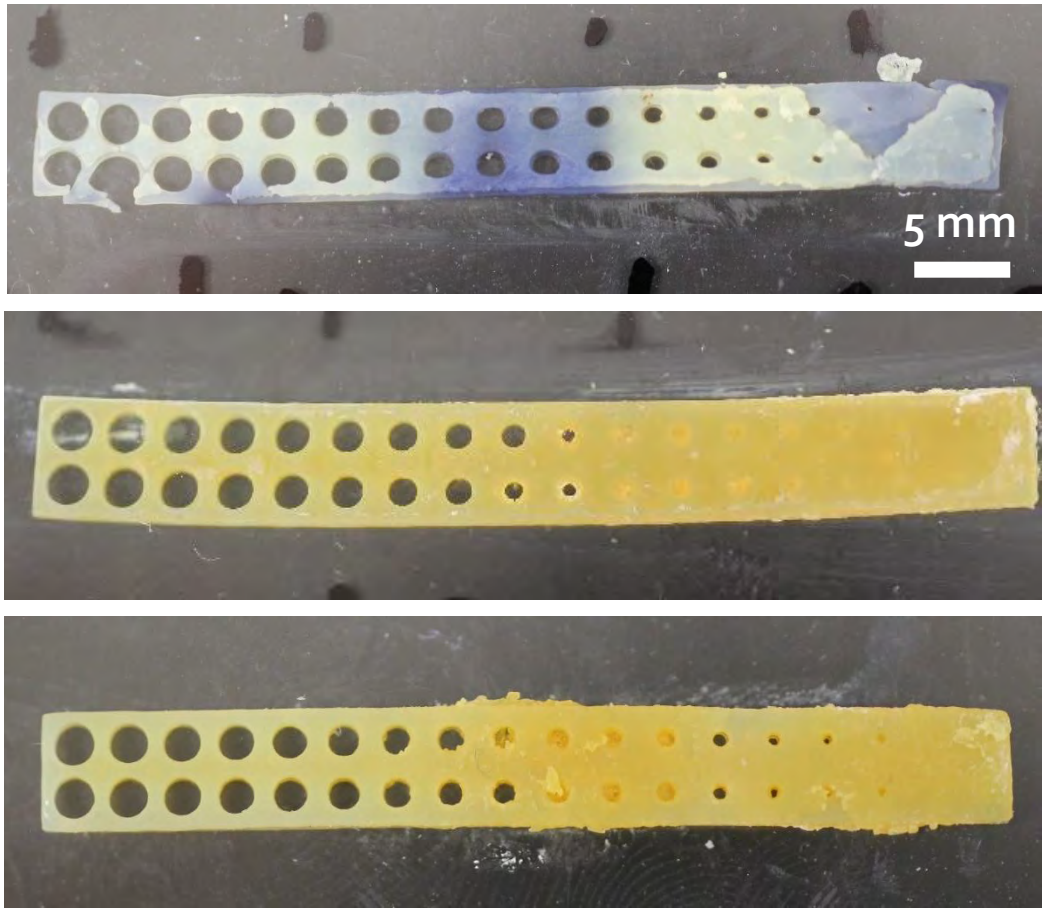
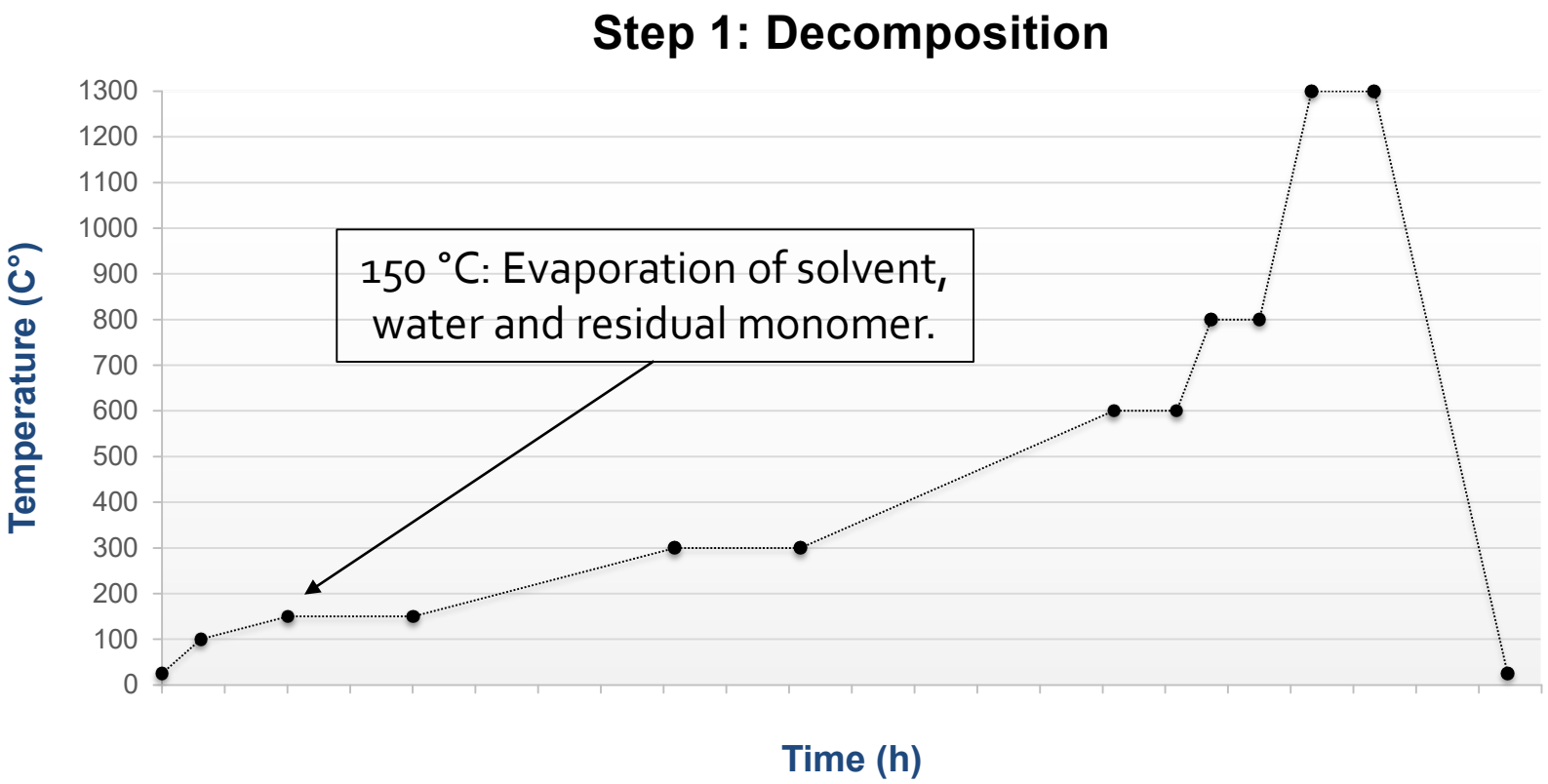


- 3 minute exposure for attachment layer.
- **5 seconds** exposure for subsequent layers.

- 3 minute exposure for attachment layer.
- **30 seconds** exposure for subsequent layers.

- Inadequate post-processing.

Decomposition and sintering



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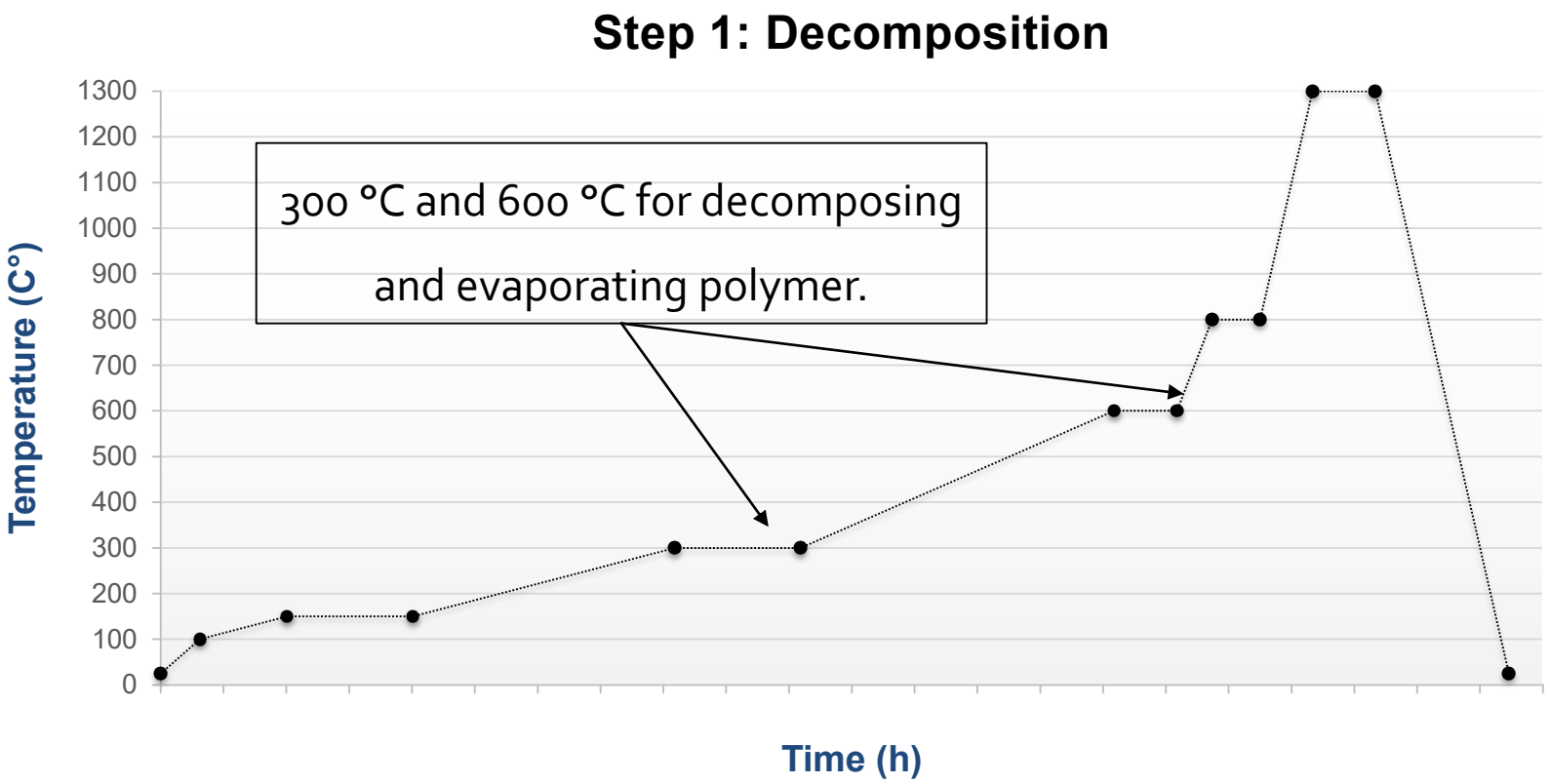

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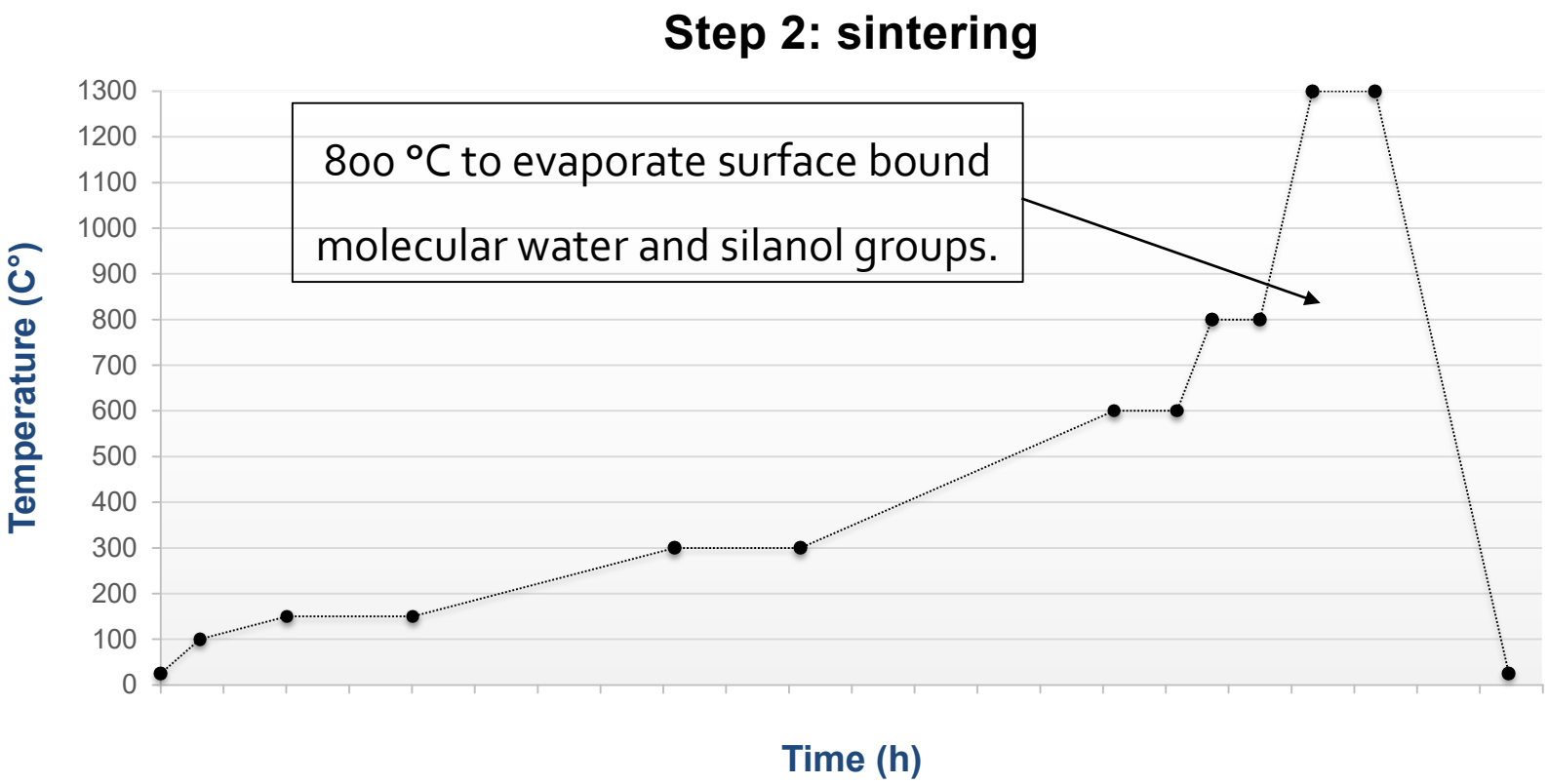

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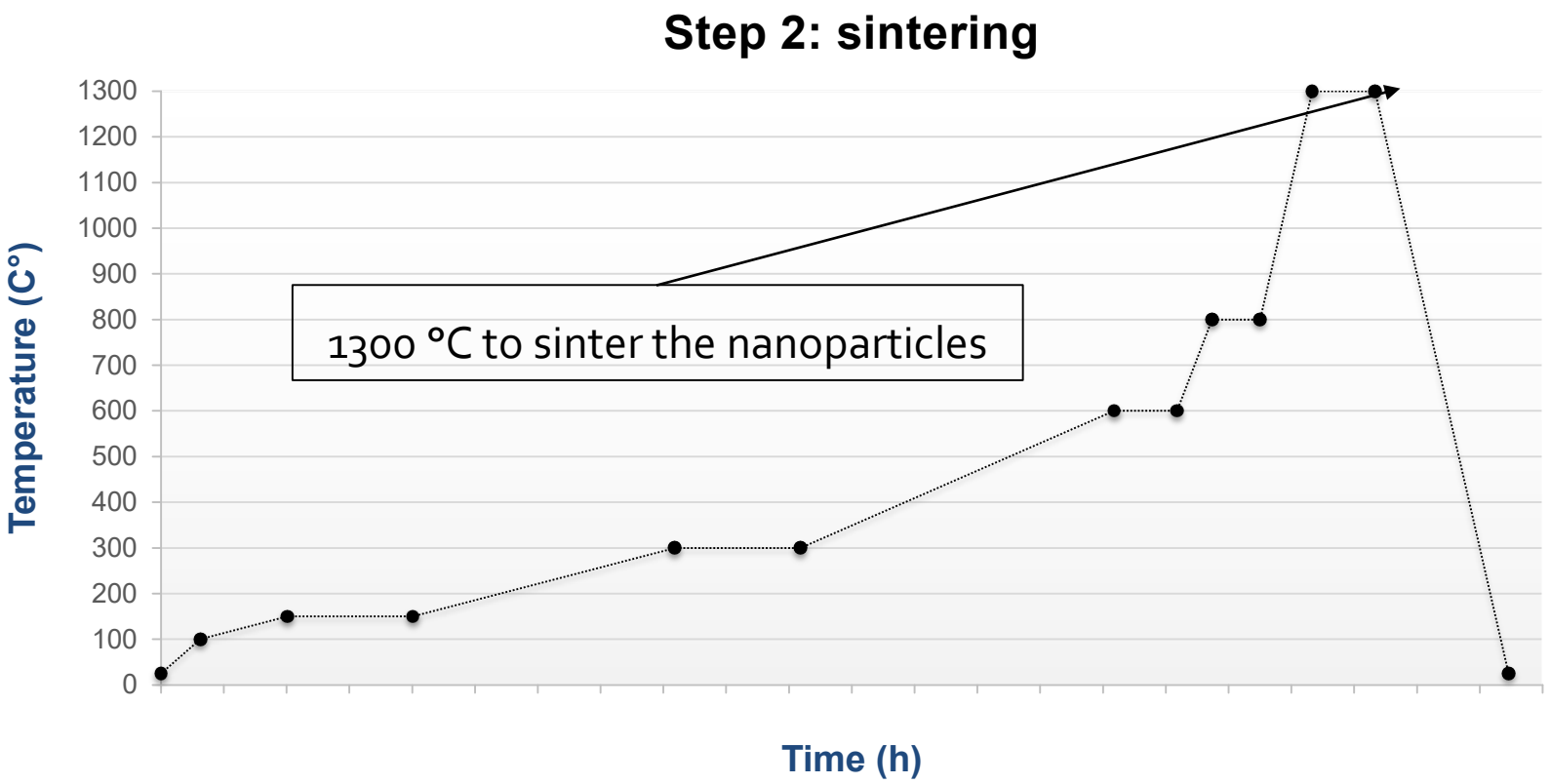
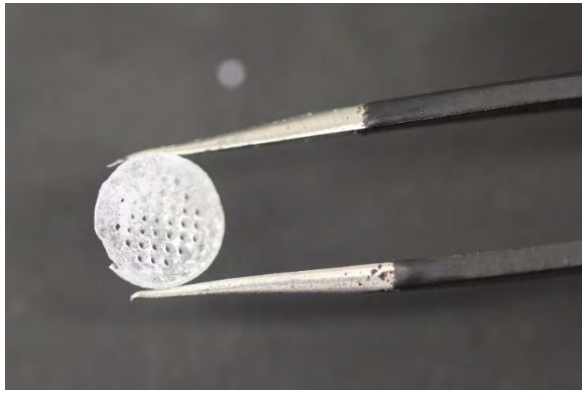
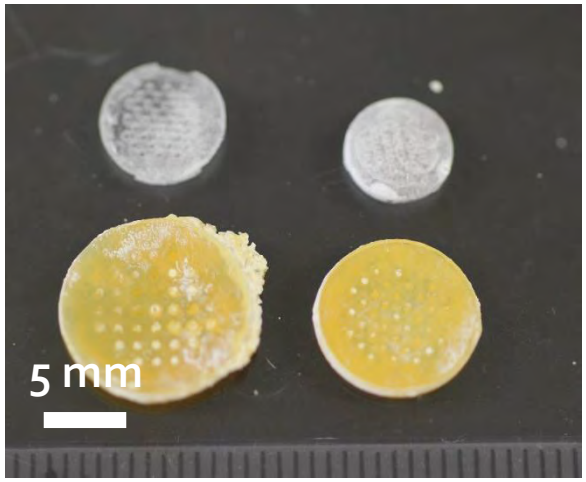
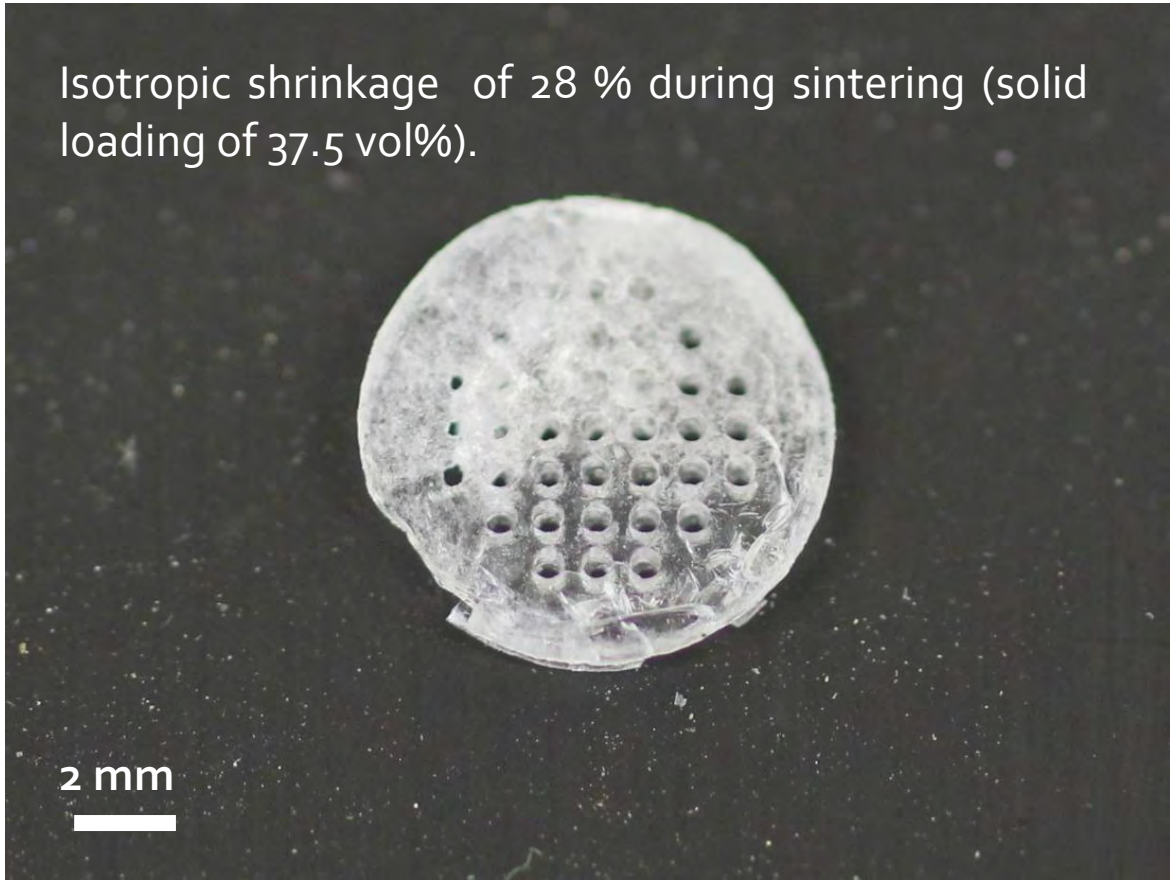


Sintered glass pieces

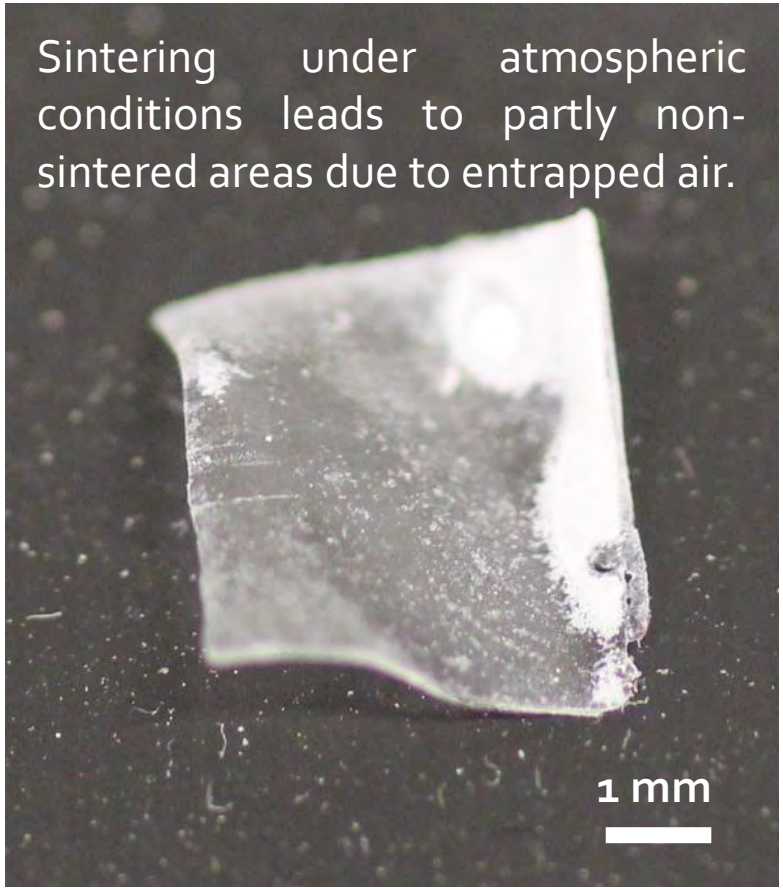


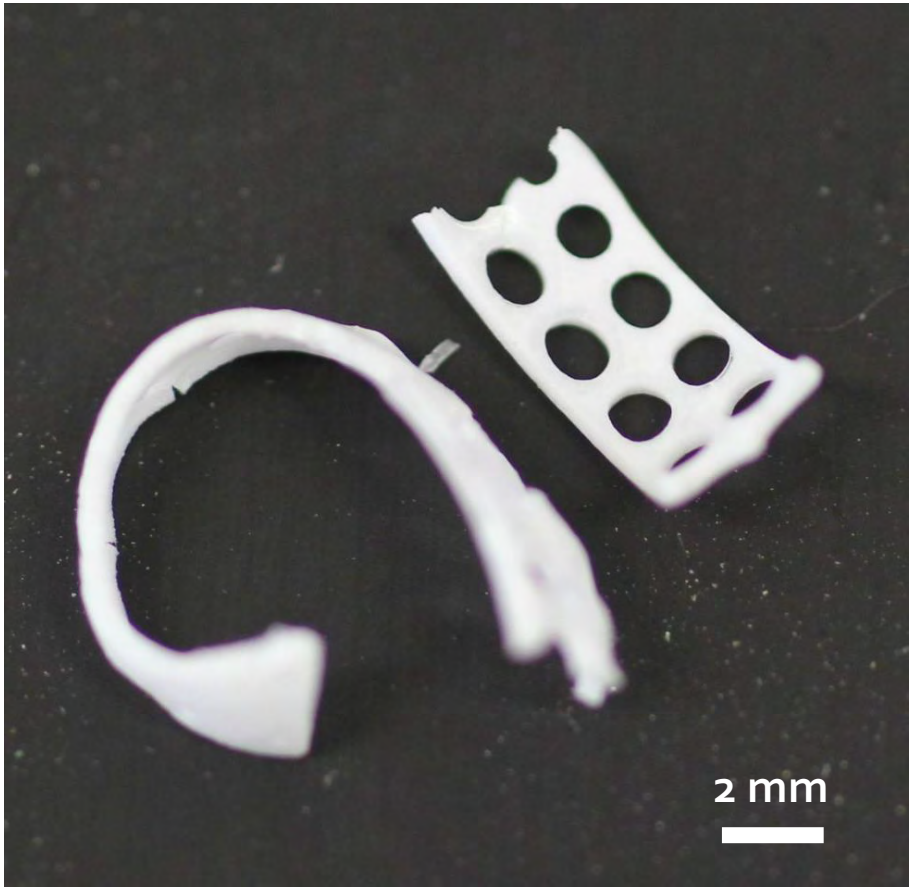




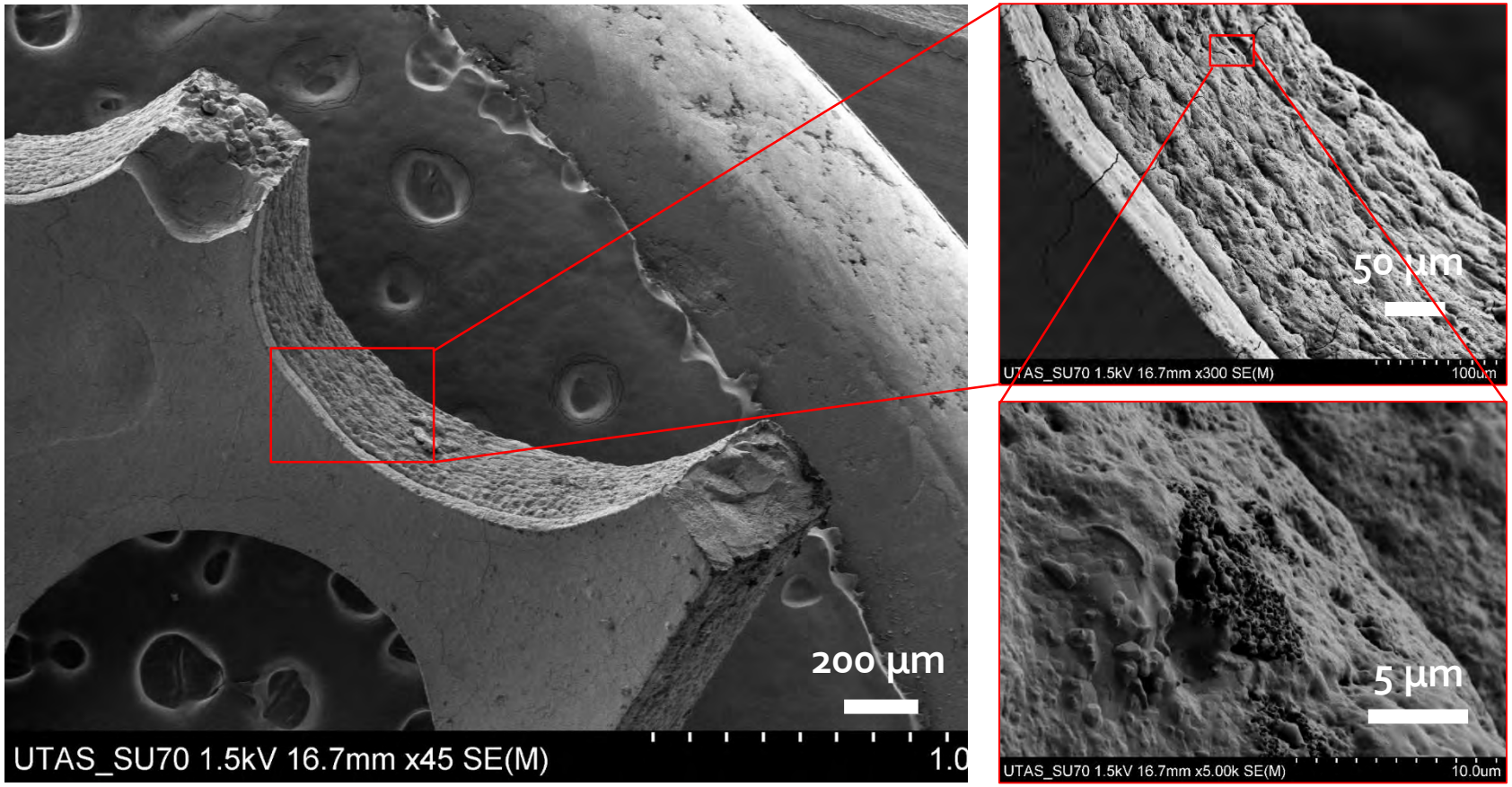
Sintering under atmospheric conditions

Sintering under atmospheric conditions leads to partly non-sintered areas due to entrapped air.

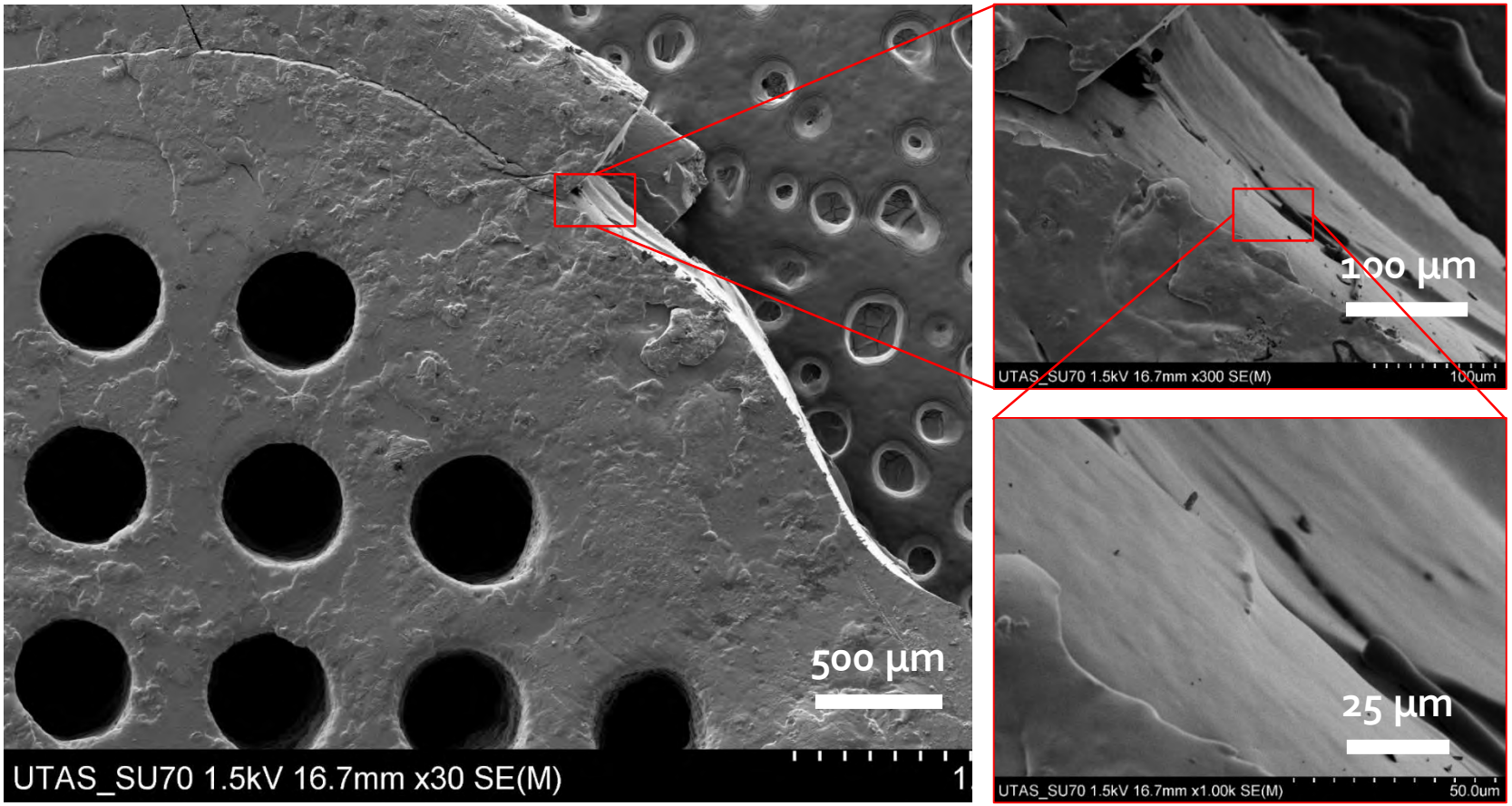




Scanning electron microscopy: layers

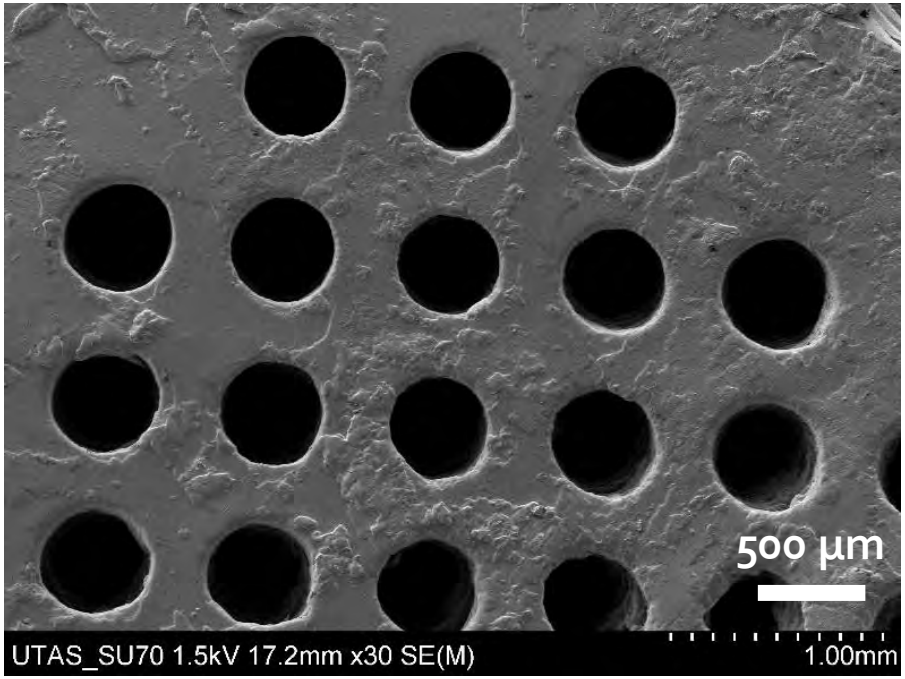


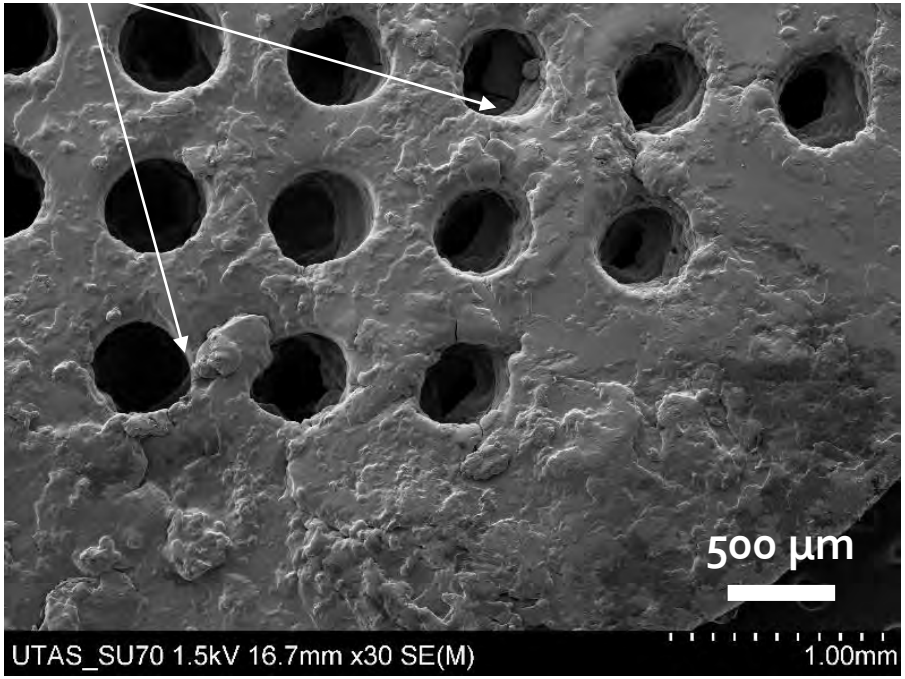
Scanning electron microscopy: smooth surfaces



Scanning electron microscopy: artefacts on surface

Insufficient removal of polymer after printing leads to artefacts after sintering.





Challenges and bottlenecks

Preparation:

- Difficult to mix enough nanoparticles into resin, always some loss during transfer.
- Working with nanoparticles tricky, difficult to clean, potential health risks.

Printing:

- Printing is difficult and slow due to viscosity and need for long exposure; limited resolution for now (down to 400-500 µm ID holes).
- Resin gets more viscous during printing, repeatability issues.

Debinding and sintering:

- Sintering under atmospheric conditions: trapped air, glass opaque. Need for vacuum.
- Pieces very fragile, some break in oven. Macro- and micro-cracks appear.

Summary

- Aim to **comprehensively obtain multiple types of information** in a single 2D-LC run**,** for instance Molecular Weight Distribution (**MWD**) and Sequence Distribution (**SD**) of polymer nanoparticles.
- Developed a **microfluidic platform with generic enzyme-immobilization strategy**.
- Established proof-of-principle for IMER with **offline protein digestion** and applied this to analysis of dried-blood-spots. Preliminary results for **enzymatic degradation of polymer nanoparticles**.
- Exploring use of **3D-printing fused-silica glass** as an prototyping method alternative to micromilling.

Future perspectives

- Ovens have been purchased for new **3D-printed glass** microfluidic devices.
- Extending the microfluidic platform to include **mixer** and IMER, as an interface between analytical processes.
- **Extending the range of applications** to various macromolecules, *e.g.* various polyesters, protein samples, lignin.
- Implementing **online immobilised-enzyme microfluidic reactors** in a two-dimensional liquid chromatography system.

Acknowledgements

University of Amsterdam

- Sander Fokker, Rocio Garmendia, Dionysis Soulis, Bob Pirok, Dr. Irena Dapic, Prof. Garry L. Corthals, Prof. Peter J. Schoenmakers

Free University Brussel

- Dr. Sam Wouters, Prof. Sebastiaan Eeltink, Prof. Gert Desmet

University of Leicester

- Thalassa Valkenburg

DSM Coating Resins

- Prof. Ron Peters

Micronit Microfluidics

- Dr. Maciej Skolimowski, Dr. Marko Blom

University of Tasmania

- Dr. Niall McDonald, Dr. Joan M. Cabot, Dr. Sinead Currivan, Prof. Brett Paull, Prof. Michael C. Breadmore