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

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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:

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

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 2D 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 $\geq 100$ µm.
- Solvent-vapour-assisted bonding.

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.

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[Diagram of enzyme immobilisation process]
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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.

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

**LC-MS analysis**
- Desalting for 10 minutes
- 60-minute gradient reversed-phase separation.
- TripleTOF mass spectrometer.

**Diagram:**
- IMER
- TRAP
- RPLC
- MS
- 20 µL
- 5 µL
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

**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.
- 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.

Towards 3D printing glass microfluidic devices
Bottlenecks for polymer-based microfluidics

- **Optical transparency** in the UV range (photografting, photopolymerization).
- **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

- Mixing with mechanical stirrer.
- Degassing of resin.

Polymeric resin

40 nm silica NPs

Hydroxyethyl methacrylate (HEMA)

Phenoxyethanol (POE)

Tetra(ethylene glycol) diacrylate (TEGDA)
Resolution tests: vertically-orientated holes
Resolution tests: vertically-orientated holes

- 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

Step 1: Decomposition

150 °C: Evaporation of solvent, water and residual monomer.
Decomposition and sintering

Step 1: Decomposition

300 °C and 600 °C for decomposing and evaporating polymer.
Decomposition and sintering

**Step 2: sintering**

800 °C to evaporate surface bound molecular water and silanol groups.
Decomposition and sintering

Step 2: sintering

1300 °C to sinter the nanoparticles
Sintered glass pieces

Isotropic shrinkage of 28% during sintering (solid loading of 37.5 vol%).
Sintering under atmospheric conditions leads to partly nonsintered areas due to entrapped air.
Scanning electron microscopy: layers

- Layers: 200 µm, 50 µm, 5 µm
Scanning electron microscopy: smooth surfaces
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.
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.
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