

# Cryo-EM as a tool for observing alginate-based hydrogels

**Katerina Mrazova**<sup>1,2</sup>, Anna Havlickova<sup>2</sup>, Diana Cernayova<sup>2</sup>, Kamila Hrubanova<sup>1</sup>, Petr Sedlacek<sup>2</sup>, Vladislav Krzyzaneck<sup>1</sup>

<sup>1</sup>Institute of Scientific Instruments of the CAS, v. v. i., Brno, Czech Republic, <sup>2</sup>Faculty of Chemistry, Brno University of Technology, Brno, Czech Republic

## Background

Hydrogel is an organic-based material, which finds its use in various fields ranging from well-known employment in medicine (wound treatment, scaffolds,...) to rising involvement in agriculture (superabsorbents, controlled release of fertilisers,...)[1]. While hydrogels containing chemical fertilisers provide valuable nutrients directly, the unused nutrients remain in the soil, where they accumulate, which negatively influences biodiversity, soil fertility, etc [2]. An alternative approach relies on the use of biological fertilisers (bioinoculants) in the form of plant growth-promoting bacteria (PGPRs). PGPRs positively stimulate the growth of plants using several mechanisms (phytohormone production, nitrogen fixation,...), while simultaneously reducing the growth of pathogenic microorganisms or chelating heavy metals [3]. One of the PGPRs is *Azotobacter vinelandii*, a microorganism interesting not only for its plant growth-promoting properties but also for its production of various polymers. Namely polyhydroxyalkanoates (PHAs), biopolymers praised for their properties similar to petrochemical plastics, or alginate, polysaccharide capable of forming a hydrogel. *A. vinelandii* releases alginate to form a capsule around the cells, which protects them from drying out and from other hostile environmental conditions. The production of alginate is a significant advantage of using *A. vinelandii* as bioinoculant since there is no need to add the hydrogel-forming polymers to the bacteria for encapsulation, the polymer already in the media is crosslinked and the resulting hydrogel is then processed into the final form of bioinoculant suitable for employment in agriculture [4]. This work aimed to study the morphology of hydrogel formed using different crosslinking agents (namely CaCl<sub>2</sub> and glucono-D-lactone), a step necessary to determine the most suitable crosslinker. Since alginate hydrogels are composed of polysaccharides and a substantial amount of water, chemical processing for EM could severely alter the hydrogel ultrastructure. Therefore, cryogenic fixation followed by freeze-fracture and cryo-SEM was proposed to be the most promising technique to study the polymeric net the most closely to the native state.

## Methods

Cultures of *A. vinelandii* were cross-linked using various agents (2% CaCl<sub>2</sub>, 1M GDL + 0,5M CaCO<sub>3</sub>). The resulting hydrogels were cut using a scalpel to fit into 6mm carriers for high-pressure freezing and fixed using EM ICE (Leica Microsystems). No cryoprotectant was added. Frozen samples were transferred under liquid nitrogen into a cryo-vacuum preparation chamber (ACE600 Leica Microsystems), where they underwent freeze-fracturing followed by sublimation at -95°C for 7min. Samples containing hydrogel-encapsulated bacteria were then imaged in a scanning electron microscope (Magellan 400/L, FEI) equipped with a cryo-stage, at -120 °C using a 1–2 keV electron beam.

## Results

Cryo-SEM imaging revealed cells of *A. vinelandii* containing polymeric granules, consisting of polyhydroxyalkanoate (PHA). As was previously proved, PHAs in the form of intracellular granules stay elastic even at temperatures of liquid nitrogen. They can be seen as needles sticking out of the freeze-fractured cells, as was visible also for *A. vinelandii*. Hydrogel encapsulating the cells showed different structures for both crosslinking agents. While hydrogel formed using CaCl<sub>2</sub> showed a net of individually distinguishable fibres, the gel formed by GDL showed a dense mass surrounding cells. The changes in hydrogel

ultrastructure seen in cryo-SEM support the difference in the macromorphological structure of the hydrogels visible immediately after cross-linking. Some polymer net was visible also for not crosslinked samples, possibly because of the trace concentration of  $\text{Ca}^{2+}$  ions in the cultivation media for the cells. However, the density of the net in the images as well as the overall amount of hydrogel was considerably lower. Similar results, supporting the hypothesis, were also obtained in the preliminary STEM experiments for freeze-substituted samples.

#### Conclusion

Cryo-SEM together with high-pressure freezing was proven to be a capable method for studying the structure of hydrogels. It was possible to determine the changes in the hydrogel structure based on the type of crosslinker used. Since the preliminary STEM data supported the cryo-SEM findings it is proposed to use the combination of these methods for the evaluation of other types of hydrogels.

#### Graphic:

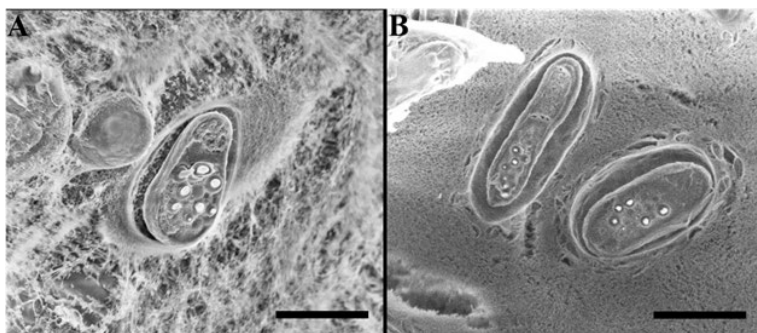


Fig.1: A. *vinelandii* cells encapsulated in the alginate-based hydrogel. Crosslinking agents used A) 2%  $\text{CaCl}_2$ , B) 1M GDL + 0,5M  $\text{CaCO}_3$ . PHB granules can be seen as needle sticking out of freeze fractured cells. Scalebar 3 $\mu\text{m}$

#### Keywords:

Hydrogel, alginate, bacteria, freeze-fracture, cryo-SEM

#### Reference:

- [1] Ahmed E. M. et al.: Journal of Advanced Research 6(2015), p. 105-121.
- [2] Bai Y.C. et al.: Microorganisms 8(2020), p. 694.
- [3] Santos M. S. et al.: AMB Express 9(2019), p. 205.
- [4] Noar J. D. et al.: Microbiology 164(2018), p. 421-436.

Acknowledgement: This work was supported by GACR (project GA23-06757S), and TACR (project TN02000020). Microscopic analysis was provided by CF ISI EM which is supported by the Czech-BioImaging large RI project (LM2023050 funded by MEYS CR).