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This project was funded by the EUROPEAN UNION and all included data is free of use.

SECTION 1 EXPLANATION OF THE WORK CARRIED OUT AND OVERVIEW OF THE PROGRESS

1.1 Objectives

The FUROID project focuses on developing novel engineered living materials (ELMs) and technologies enabling the formation of engineered fur, wool, and hair. In the first year of the project, we focused on the development of nanofibrous membranes, iPSC hair/wool/fur organoids, and the development of vacuum-assisted 3D printing (VAC3DP) hardware components.

Regarding specific project objectives, we have achieved the following progress:

Development of nanofibers supporting fur/wool/hair organoids with >90% viability after 14-day cultivation.

In FUROID NF/VAC3DP , we have developed protocols for nanofiber substrate production. Nanofibers were prepared and up-scaled from 12 different polymeric systems (polycaprolactone (PCL), PCL-chitosan (PCL-chit), PCL-polyethyleneoxide (PCL-PEO), PCL-cellulose acetate (PCL-CA), PCL-cellulose acetate butyrate (PCL-CAB), PCL-cellulose acetate phthalate (PCL-CAP), polyhydroxybutyrate (PHB), PHB- cellulose acetate (PHB-CA), polylactic acid (PLA), polyamide 6 (PA6), polyamide 11/polyvinyl butyral (PA11/PVB), polyvinyl alcohol (PVA)). All fibers were processed using an industrial-scale nanofiber unit with an effective nanofiber web width of 80 cm and continuous substrate deposition. The protocols for processing of fibers and analytical results are in section 1.2.2.

12 nanofiber groups were tested in vitro in combination with skin cells (fibroblasts, keratinocytes), and the testing demonstrated their viability over 8-day cultivation period in static conditions.

Key outcome from FUROID NF/VAC3DP :

SOPs for nanofiber membranes and their availability on an industrial scale. The production enables the formation of nanofibers for utilization in additional Tasks in the required width (40 cm). The completed up-scaling prevents potential issues in the future requiring a change of formulation and properties of the fiber membrane.

Progress in the next IP Protocol :

Combination with iPSC organoids and evaluation of cultivation of organoids to evaluate the target viability.

Development of continuous organoid bioprinting onto nanofiber membranes reaching cell adhesion efficacy > 70%.

In the FUROID NF/VAC3DP , we have developed a pilot VAC3DP apparatus for testing the configuration of micropatterning elements. The apparatus has an area of 17cm² and enables top/bottom patterning. Due to the lack of iPSC organoids, we have utilized alginate microbeads (details in section 1.2.3) as a replacement model with a similar shape and size to cells and organoids. The technology was tested in combination with developed nanofiber materials, and the efficacy of adhesion was evaluated. The results indicate that the adhesion/filtration efficacy of alginate particles was over 85%. The results suggest that the nanofiber membranes are efficient for the adhesion/filtration of alginate microbeads and demonstrate the functionality of VAC3DP

technology. Among key observations with implications for further development, non-swelling hydrophilic membranes show higher flux. ii. nano/microfibrous morphology is showing higher liquid flux and comparable filtration efficacy. iii. swelling nanofiber membranes with decreased efficient pore size are not promising for VAC3DP technology (i.e., PVA).

Key outcome from FUROID NF/VAC3DP :

Demonstration of efficient adhesion/filtration behavior of nanofiber membranes and proof-of-function of VAC3DP technology.

Progress in the next IP Protocol :

Nanofiber optimization will focus on increasing the liquid flux and decreasing system back-pressure (we will evaluate the hydrophilization of membranes and morphological changes). In addition, we will perform experiments in large area systems (200 x 400 mm efficient filtering area) and perform tests with iPSCs instead of alginate microbeads.

Development of PoC for vacuum printing method (VAC3DP) with spatial resolution better than 1 mm and printing speed of 10 mm/min with 40 cm width

During FUROID NF/VAC3DP , we tested different configurations of VAC3DP head assembly. We have tested the assembly either inducing the pattern from the top (supply-> micropatterning mask -> nanofiber membrane -> support textile -> vacuum source) or the bottom (supply-> nanofiber membrane -> (support textile) -> micropatterning mask -> vacuum source). Alginate microparticles were used as a test material. The results indicate optimal pattern replication in the case of top configuration (leading development stream). The bottom configuration was efficient only in the case of patterning directly to nanofiber membrane without support textile (see details in section 1.2.3).

The patterning was demonstrated on objects (square, triangle, circle, smiling emoticon) with sizes from 2mm – 6 mm. The patterns were induced by FDM 3D printed masks or PET foils structured by CO₂ laser cutting. The results show proper pattern replication and formation of features as small as 1.5 mm. The accuracy of the pattern replication was 0.1 mm. Thanks to the obtained results, it will not be necessary to use a simultaneous top/bottom (stereotactic) configuration of the system. In addition, the results show that VAC3DP is not efficient in a setup where the liquid is supplied via a needle – due to the free surface and high nanofiber porosity, the liquid filled to the surface of the fiber membrane is not transferred through the mesh effectively. The results provide a defined insight into the optimal organization of the technology, and we will progress with top and bottom micropatterning configurations.

In addition, we have demonstrated multi-layered deposition using VAC3DP technology. The laser-scanning confocal microscopy (LSCM) showed the formation of distinctive layers both layer-by-layer (one layer of particles on top of another, patterns in the z-direction of construct) and side-by-side (patterns in the xy-direction of the construct) organization.

From the technological challenges – we have observed the peeling of printed alginate beads from the surface of fibers due to the interaction between the pattern and micro-structuring mask. The mask composition needs to be further optimized to decrease the interaction with printed objects (i.e., fluoropolymer foils with increased non-foiling properties).

Demonstration of micropatterning with a feature size of 1.5 mm and accuracy of 0.1 mm and proof-of-function of VAC3DP technology. The results are very promising and indicate a high probability of achieving printing resolutions better than those proposed in DoA.

Progress in the next IP Protocol :

The composition of the patterning element will be further evaluated with novel materials (i.e., FEP foil, silicone) to decrease the peeling effect. The precision of laser cutting will be increased either by using a more sensitive laser system (picosecond lasers) or by optimization of laser settings to eliminate the melting of the membrane upon cutting. For the bottom setup of VAC3D, we will evaluate the effect of different non-woven support fabrics on the precision of the technology. However, the top approach delivers multiple advantages and will be the main developmental path for the technology. In addition, we will perform experiments in large area systems (200 x 400 mm efficient filtering area) and perform tests with iPSCs instead of alginate microbeads.

Demonstration of scalability of VAC3DP production at least 40 x 3000 cm sample of ELF (KPI).

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Integration of VAC3DP to continuous manufacturing based on a roll-to-roll production system, including maturation reactor and life-support system.

The results above show the most promising directions in continuous VAC3DP setup configuration. We have developed preliminary design constructs of the VAC3DP system with an effective width of 40 cm. The system architecture is described in section 1.2.3.

Key outcome from FUROID NF/VAC3DP :

The more refined idea of system configuration and type of micropatterning elements and preliminary design of the device in CAD software.

Progress in the next IP Protocol :

Development of an automated prototype of the VAC3DP print-head system and demonstration of printing using alginate microbeads in a continuous rewind system.

Development of design-build-test-learn (DBTL) platform based on algorithms regulating the bioprinting and maturation/growth process, including AI module.

During the FUROID NF/VAC3DP , we focused on data collection for the DBTL platform. The data from nanofiber processing conditions, composition, nanofiber membrane porosity, fiber size distribution, surface properties, liquid flux properties, adhesion efficacy and back-pressure were collected as entry data for the DBTL module. However, to create suitable quality of data, systemic experiments are needed.

Progress in the next IP Protocol :

For IP Protocol 2 we are planning to perform systemic experiments for DBTL data generation. DBTL database will be developed in the form of software, and the obtained data will be loaded into the system.

We have not yet made progress towards the remaining specific objectives during the first reporting period – the objectives are connected with Tasks scheduled later in the project:

- SOPs for developing ELF based on fur organoids forming physiologically relevant fur.
- SOPs for developing ELW based on wool organoids from sheep forming physiologically relevant wool.
- SOPs for developing ELH based on human hair organoids forming physiologically relevant hair. 4
- LCA and economic analysis of continuous skin/fur/wool production

- The key achievement in Task 2.1 was the up-scaling of nanofiber membranes to industrial-grade production necessary for scalability in the FUROID project and stability of nanofiber membrane properties.
- In task 2.1, we have developed a novel PA11/PVB composite membrane as a result of the project. PA11 is a hard-to-electrospin high-performance polymer. We have created a blend with PVB as an industrial-grade polymer, improving the quality of PA11 nanofibers. The application might be in the field of textile membranes. FUROID generated and will exploit the result.
- The key achievement in Task 2.2 was confirming the patterning effect on fiber morphology. We have created preliminary patterned nanofibers for testing in WP3.
- The key achievement in Task 2.3 was the development of emulsion nanofibers with core/shell structure and regulated release of the model molecule (TRITC-dextran). The experiment demonstrates the time-dependent release governed by core polymer molecular weight, solubility and ratio between core/shell polymer. The findings are an important baseline for further experiments with growth factors and other active molecules to stimulate iPSC-derived organoids.

Progress achieved in assigned Tasks during FUROID NF/VAC3DP :

Task 2.1: Development and biocompatibility testing of nanofibers:

Task 2.1 is focused on electrospun nanofiber preparation and the establishment of protocols as a substrate for the growing of cells and organoids. Electrospun nanofibers form a non-woven mesh of randomly aligned fibers with a diameter in hundreds of nanometers and a length in centimeters. The nanofibrous mesh shows high porosity and pore interconnection. Thanks to small pore size and high porosity, nanofibers are an ideal filtration material and serve as the crucial element in the proposed solution – vacuum-assisted 3D printing (VAC3DP). The cells and organoids with sizes ranging from 20 – 300 μm are filtered and concentrated on the surface of nanofibers (details in section 1.2.3). Simultaneously, electrospun nanofibers, thanks to their structure, mimic the structure of the extracellular matrix and support the growth of cells in quasi-3D conditions. Combining both properties makes nanofibers an ideal substrate for forming artificial tissues with living cells.

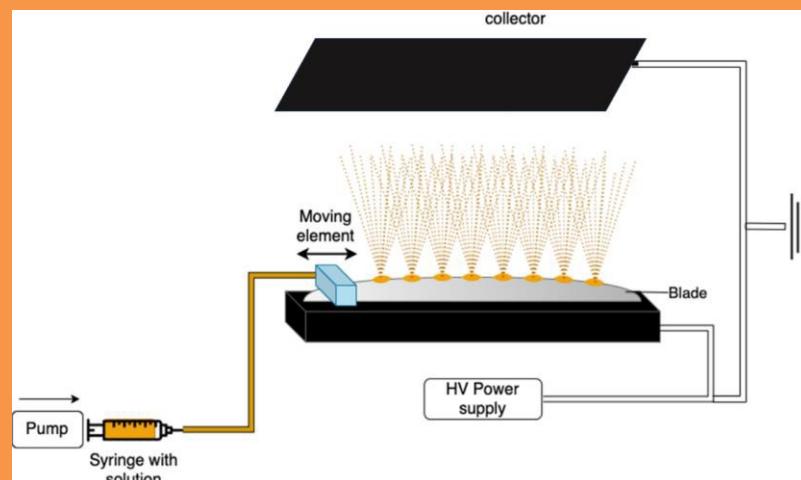


Fig: Experimental setup and configuration of needleless spinning

Target metrics:

- Industrially viable production process with > 500 m² production capacity per day and > 85% reproducibility of nanofiber diameter and mean pore size.
- Combination with support material enabling rewinding of nanofibers without damage of fiber layer.

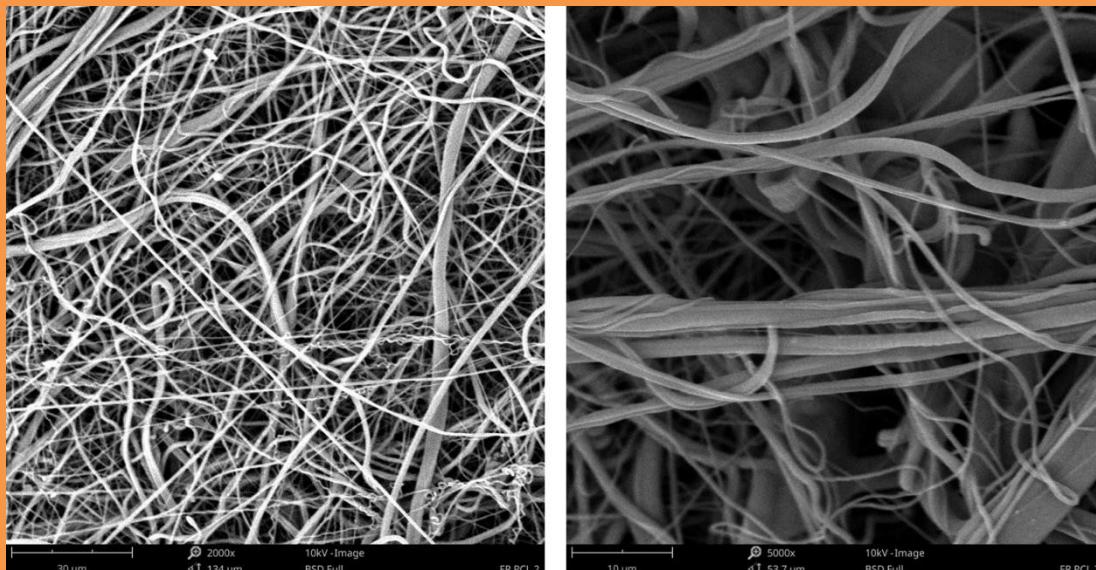
- Biocompatibility enabling > 70% viability after 14 days of cultivation for skin cells.

During FUROID NF/VAC3DP, we prepared electrospun nanofiber membranes from a selected range of polymeric materials. The experimental approach was focused on the direct creation of nanofibers using industrial-scale electrospinning technology. The testing procedure included the solubilization of a defined amount of polymer in a suitable solvent, followed by processing using electrospinning. The electrospinning setup is based on emitting electrodes and collectors connected to high-voltage power sources with opposite polarity and showing high electrical potential difference (typically 70-120 kV). The polymeric solution is delivered to the surface of the metallic emitting electrode, and upon insertion into a strong electric field, strong electrostatic forces are created, dragging the polymeric liquid toward the opposite electrode. During the transfer of the ejected polymeric droplet, due to radial trajectory and electrostatic charge, the droplet is elongated into fibers with a dramatic increase in surface-to-volume ratio. The extremely high surface results in the evaporation of solvent and deposition of dried fibers on the surface of the collector. In T2.1, we have employed novel needleless electrospinning, enabling the simultaneous formation of fibers in industrial quantity and their uniform deposition to the supporting textile. For all experiments, we have utilized polypropylene spunbond non-woven with 40gsm density.

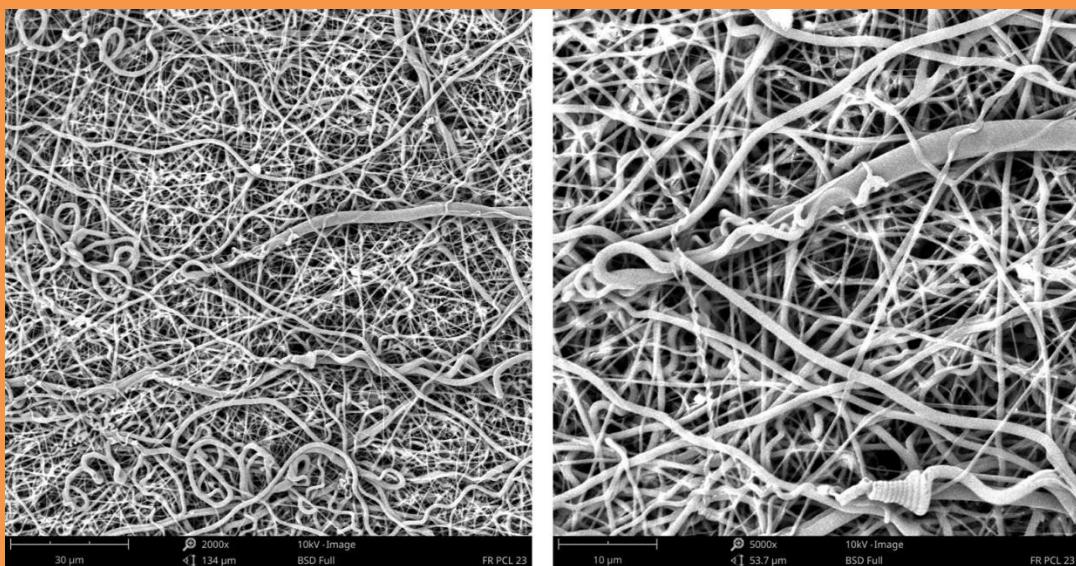
Development of nanofibers from polycaprolactone

Polycaprolactone is a synthetic biodegradable polyester that shows good cell compatibility. It is one of the most utilized polymers in tissue engineering. Protocol for PCL spinning was optimized using testing of different solvent combinations. We have tested combination of acetic acid and formic acid with acetonitrile (spinnable, higher solidification on electrode), chlorophorm/DCM (fiber thickness regulation, higher the chlorophorm and DCM content – higher fiber thickness), ethanol and methanol (thin fibers, low productivity). On the top of it, we have tested spinning of fibers from chlorophorm background with addition of ethanol, acetonitrile and methanol as co-solvents (dataset in portal tab).

To create polycaprolactone nanofibers, we have used a modified solvent system based on acetic acid and formic acid. 20% w/v polycaprolactone (MW 80,000, Sigma Aldrich) was dissolved in a mixture of acetic and formic acid (1:1) under constant stirring. During solution preparation, heat was applied, and the solution was used within 24 hours to prevent the degradation of polymeric chains in concentrated acid solution. Electrospinning experiments were performed at an industrial NUENEX electrospinning unit with a top-filled blade electrode. The experimental conditions were 30°C and < 40% RH. The voltage was set at -30 kV, +70 kV (total 100kV potential) and an emitter to collector distance of 25 cm. The fibers were deposited on a 40gsm spunbond, and the rewinding speed was 4 mm/s (14.4 m/h). For experimental trials, we have prepared two nanofiber samples from polycaprolactone. The first selected formulation was based on 15.5% PCL (w/v) dissolved in acetic acid: formic acid: chloroform (17.5 : 22.5: 5). The system resulted in nanofibers with nano/microfiber morphology. Scanning electron microscopy (SEM) showed the formation of continuous fibers with a minimal number of defects and micro/nano morphology.

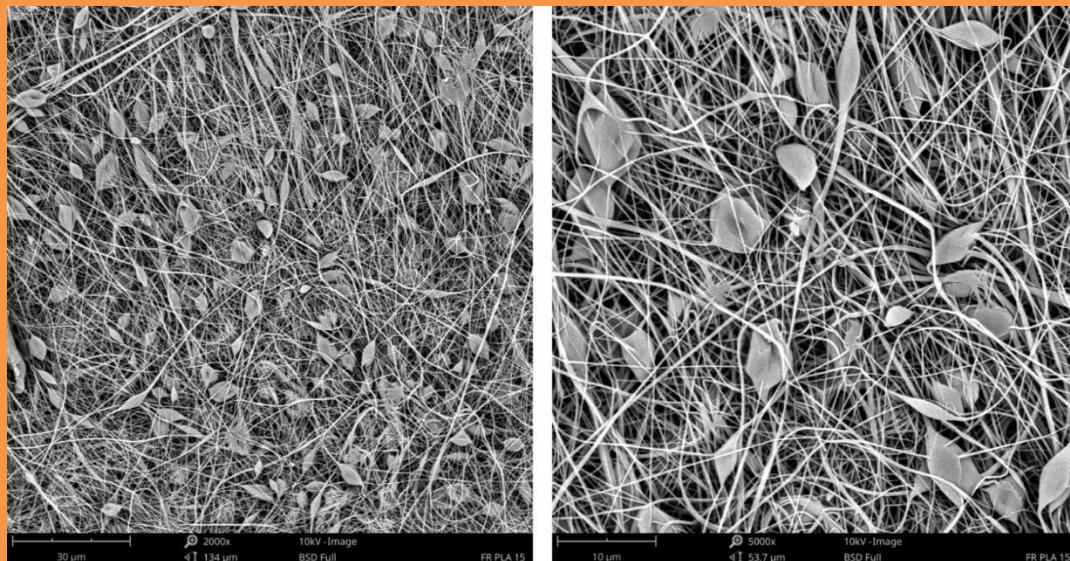


The second selected formulation was based on 14% PCL (w/v) dissolved in acetic acid: formic acid: chloroform (17.5 : 27.5: 5). The system resulted in the formation of nanofibers with nano/microfiber morphology. Scanning electron microscopy (SEM) showed the formation of continuous fibers with a minimal number of defects.



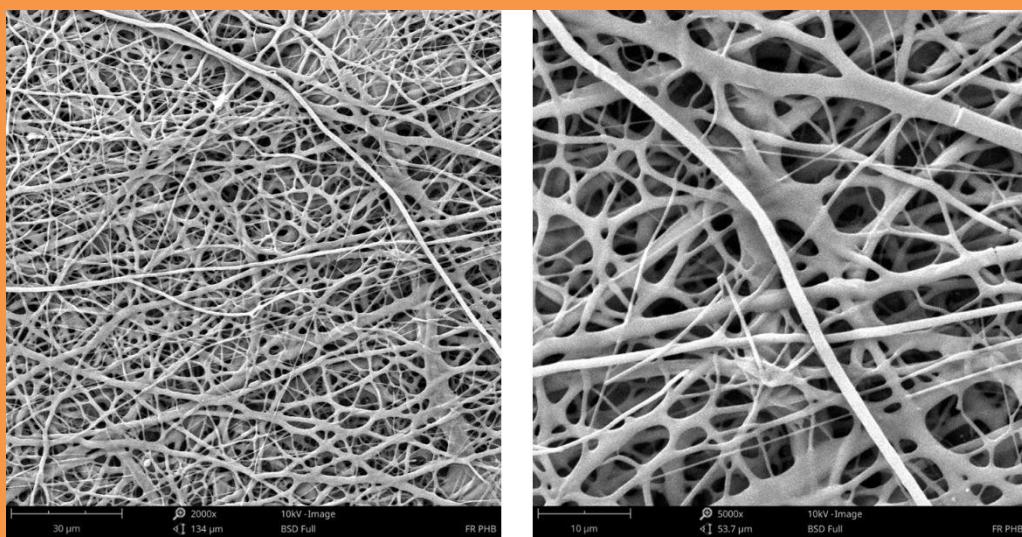
Development of nanofibers from polylactic acid

Polylactic acid is a biodegradable polyester utilized for tissue engineering applications. Compared to polycaprolactone, it shows higher crystallinity and hydrophilicity. For experimental purposes, we have used polylactic acid (15 w/v %, L105, Total) dissolved in a mixture of acetic acid, formic acid and chloroform (1:1:1). The optimization was focused on this solvent system (PLA) with pre-existing know how from needle electrode (transfer of solution) and was tested with different solvents and co-solvents (data in dataset). Electrospinning experiments were performed at an industrial scale NUENEX electrospinning unit with top-filled blade electrode. The experimental conditions were 30°C and < 40% RH. The voltage was set at -30 kV, +70 kV (total 100kV potential) and an emitter to collector distance of 25 cm. The fibers were deposited on a 40gsm spunbond, and the rewinding speed was 4 mm/s (14.4 m/h). The electron microscopy images showed the formation of beaded fibers. The beads could be caused by low polymer molecular weight.



Development of nanofibers from polyhydroxybutyrate

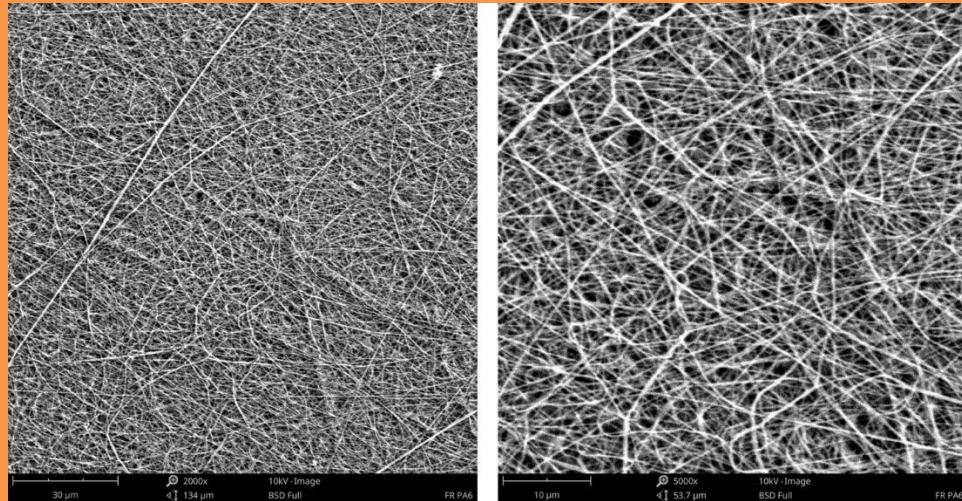
Polyhydroxybutyrate is a natural biodegradable polyester utilized for tissue engineering applications. Compared to polycaprolactone, it shows higher crystallinity and hydrophobicity. The fiber development was based on testing of different solvent systems (data in Dataset). For experimental purposes, we have used polyhydroxybutyrate (15 w/v %, Biomer) dissolved in a mixture of acetic acid, formic acid, and chloroform (1:1:1) under heating. Electrospinning experiments were performed at an industrial NUENEX electrospinning unit with a top-filled blade electrode. The experimental conditions were 30°C and < 40% RH. The voltage was set at -30 kV, +70 kV (total 100kV potential) and an emitter to collector distance of 25 cm. The fibers were deposited on a 40gsm spunbond, and the rewinding speed was 2 mm/s (7.2 m/h). The electron microscopy images showed the formation of microfibers with nanofibrous components. The fiber layers were melted, and the pore size decreased compared to polycaprolactone nanofibers.



Development of nanofibers from polyamide 6

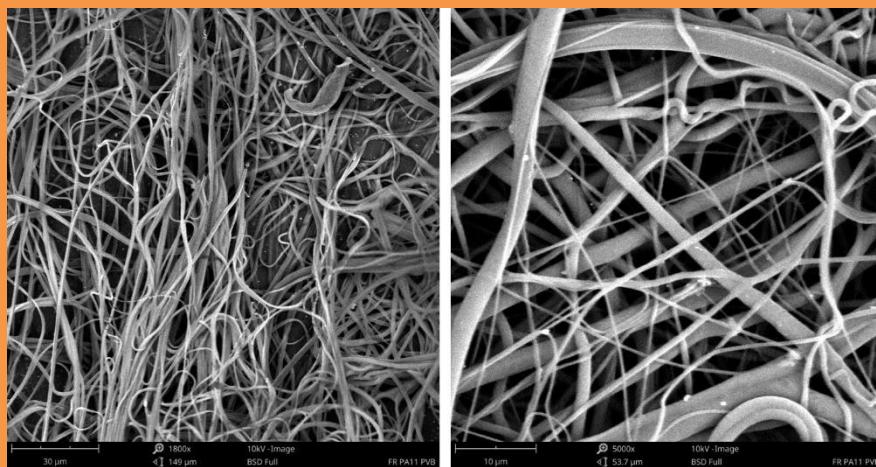
Polyamide 6 (PA6) is a synthetic polymer showing good mechanical properties and durability. In the experiment, we have utilized PA6 granules from a recycled source (Econyl). The combination with co-solvents was tested including matrix testing of concentration and solvent composition (data in Dataset). 20% PA6 stock was prepared by dissolving in acetic and formic acid (2:3). Electrospinning

experiments were performed at an industrial scale electrospinning unit with a top-filled blade electrode. The experimental conditions were 30°C and < 40% RH. The voltage was set at -28 kV, +70 kV (total 98 kV potential) and an emitter to collector distance of 25 cm. The fibers were deposited on a 40gsm spunbond, and the rewinding speed was 3 mm/s (10.4 m/h). Electrospinning was performed with 17.5 (w/v) % PA6 dissolved in a mixture of acetic acid, formic acid: chloroform (2:3:1 ratio). The formed nanofibers showed nanofibrous morphology with narrow size distribution and minimal defects.



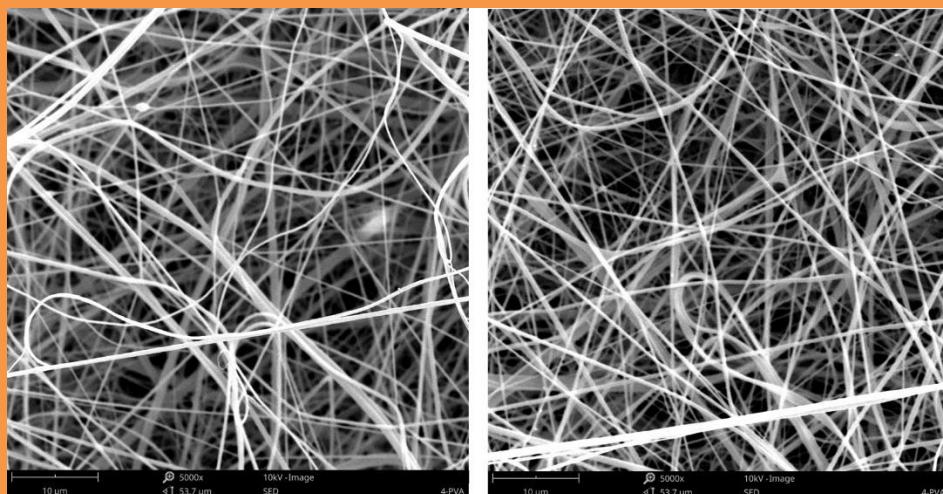
Development of nanofibers from polyamide 11/polyvinyl butyral

Polyamide 11 (PA11) is an industrial-grade polyamide with extremely high durability and mechanical properties. PA 11 is hard to electrospun material. In the experiment, we have solubilized 20% PA11 in a mixture of formic acid and dichloromethane (1:1). The standard protocol based on state-of-the-art resulted in low quality of fibers (thick microfibers, hanging fibers) and problematic spinning process (drying of solution). To increase the spinnability, we have created a blend with polyvinyl butyral (data in dataset). The final formulation for spinning contained 10% PA11 with 1% PVB dissolved in formic acid: acetic acid: dichloromethane (5: 2.5: 0.5). The experimental conditions were 30°C and < 40% RH. The voltage was set at -30 kV, +70 kV (total 100 kV potential) and an emitter to collector distance of 25 cm. The fibers were deposited on a 40gsm spunbond, and the rewinding speed was 4 mm/s (14.4 m/h). Microfibers created the formed mesh with a small quantity of nanofibrous components.



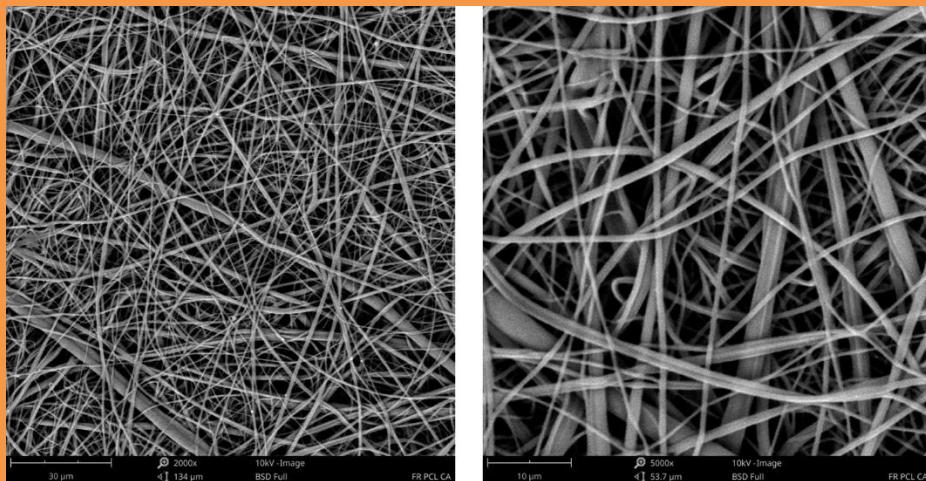
Development of nanofibers from polyvinyl alcohol

Polyvinyl alcohol (PVA) is a synthetic polymer showing water swelling properties. PVA is soluble in water, and crosslinking is required to decrease its solubility. For PVA crosslinking, we have employed crosslinking by glyoxal (a bi-functional aldehyde) catalyzed by H₃PO₄ and heat (24h, 70°C). The material was based on pre-existing protocol optimized for NUNEX electrodes. Electrospinning experiments were performed at an industrial NUEENEX electrospinning unit with bottom-filled linear electrodes. The experimental conditions were 30°C and < 40% RH. The voltage was set at -25 kV, +70 kV (total 95 kV potential) and an emitter to collector distance of 30 cm. The fibers were deposited on a 40gsm spunbond, and the rewinding speed was 4 mm/s (14.4 m/h). The formulation was based on a mixture of 6 (w/v) % PVA 5-88 and 6 (w/v) % PVA 40-88 (Poval, Kuraray) with 40,000 ppm glyoxal and 30,000 ppm H₃PO₄ dissolved in water. The experiment resulted in the formation of nanofibers without defects.



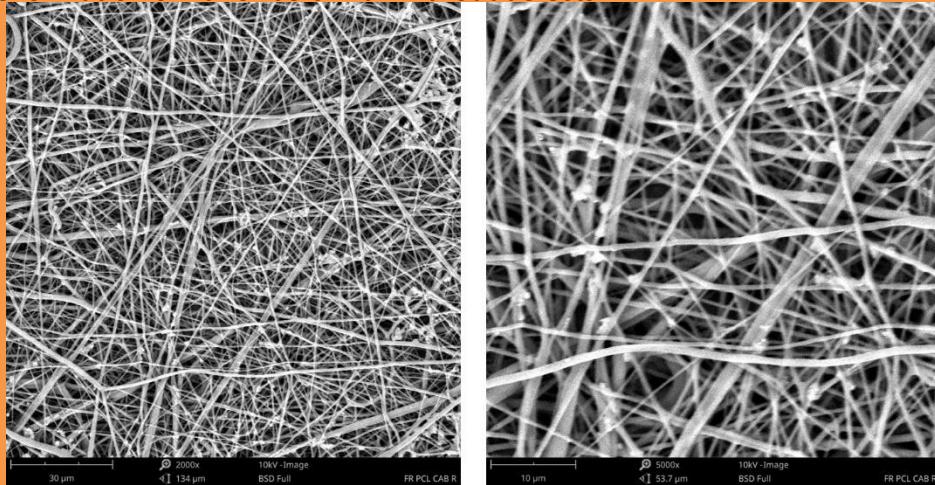
Development of blend nanofibers from polycaprolactone and cellulose acetate

Cellulose acetate (CA) is a derivative of cellulose commonly used as a scaffolding material. The cellulose acetate shows low swelling in contact with water and could be blended with PCL into nanofibers. Electrospinning experiments were performed at an industrial NUEENEX electrospinning unit with a top-filled blade electrode. Composition was optimized (variation of concentration , polymer ration and co-solvents – data in Dataset). The final experimental conditions were 30°C and < 40% RH. The voltage was set at -30 kV, +70 kV (total 100kV potential) and an emitter to collector distance of 25 cm. The fibers were deposited on a 40gsm spunbond, and the rewinding speed was 3 mm/s (10.8 m/h). The formulation was based on 10 (w/v) % PCL (80 kDa, Sigma) with 7.5 (w/v) % CA (CA-3, Eastman) dissolved in acetic acid: formic acid: chloroform (3.5: 3.5: 1). The experiment resulted in the formation of nanofibers with without defects.



Development of blend nanofibers from polycaprolactone and cellulose acetate butyrate

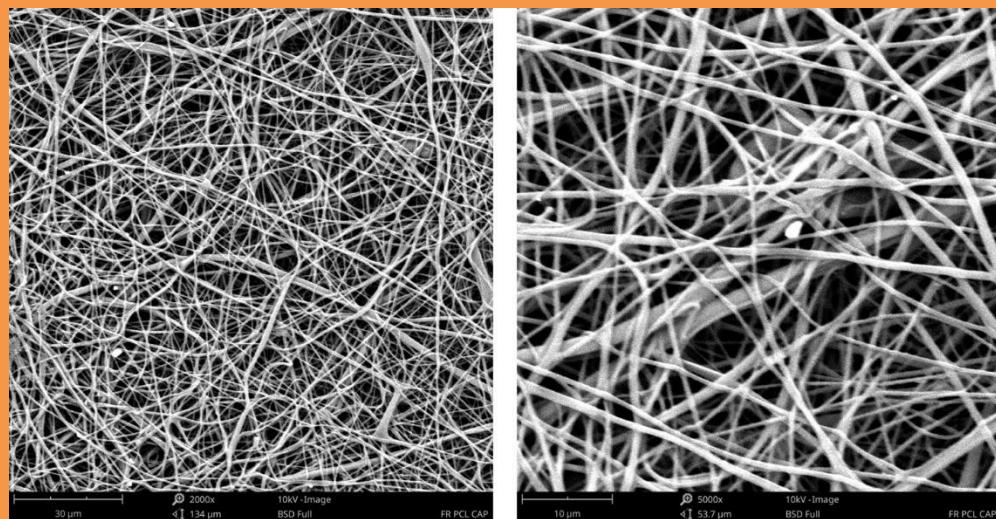
Cellulose acetate butyrate (CAB) is a cellulose derivative with higher hydrophobicity than CA. The cellulose acetate butyrate shows low swelling in contact with water and could be blended with PCL into nanofibers. Composition was optimized (variation of concentration , polymer ration and co-solvents – data in Dataset). Electrospinning experiments were performed at an industrial NUNEX electrospinning unit with a top-filled blade electrode. The experimental conditions were 30°C and < 40% RH. The voltage was set at -30 kV, +70 kV (total 100kV potential) and an emitter to collector distance of 25 cm. The fibers were deposited on a 40gsm spunbond, and the rewinding speed was 3 mm/s (10.8 m/h). The formulation was based on 10 (w/v) % PCL (80 kDa, Sigma) with 7.5 (w/v) % CAB (CAB-0.5, Eastman) dissolved in acetic acid: formic acid: chloroform (3.5: 3.5: 1). The experiment resulted in the formation of nanofibers with without defects.



Development of blend nanofibers from polycaprolactone and cellulose phthalate

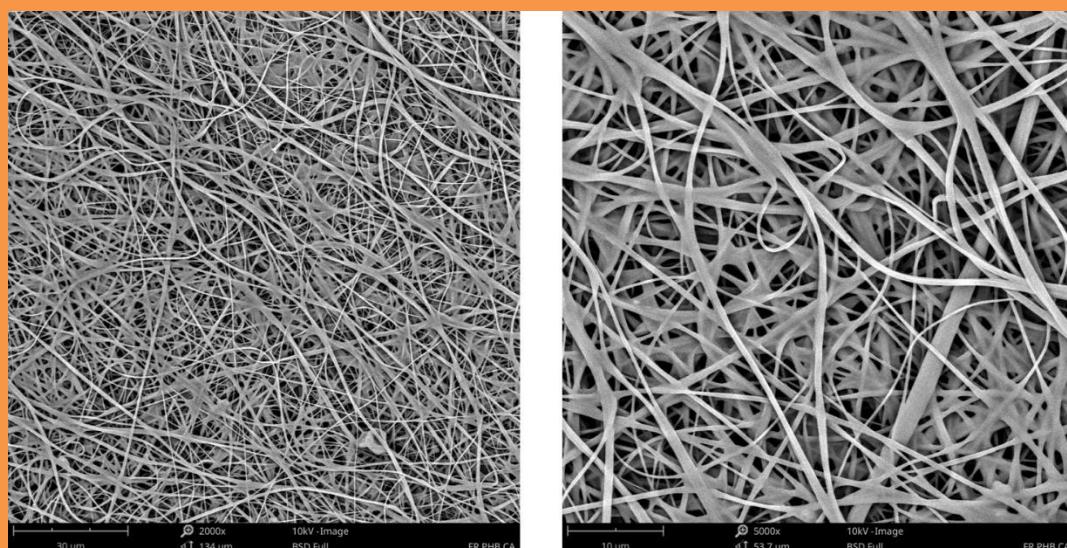
Cellulose acetate phthalate (CAP) is a cellulose derivative with higher hydrophobicity than CAB. The cellulose acetate phthalate shows no swelling in contact with water and could be blended with PCL into nanofibers. Electrospinning experiments were performed at an industrial NUNEX electrospinning unit with a top-filled blade electrode. Composition was optimized (variation of concentration , polymer ration and co-solvents – data in Dataset). The experimental conditions were 30°C and < 40% RH. The voltage was set at -30 kV, +70 kV (total 100kV potential) and an emitter to collector distance of 25 cm. The fibers were deposited on a 40gsm spunbond, and the rewinding speed was 3 mm/s (10.8 m/h). The formulation was based on 10 (w/v) % PCL (80 kDa, Sigma) with

7.5 (w/v) % CAP (CAP-0.5, Eastman) dissolved in acetic acid: formic acid: chloroform (3.5: 3.5: 1). The experiment resulted in the formation of nanofibers with without defects.



Development of nanofibers from polyhydroxy butyrate and cellulose acetate

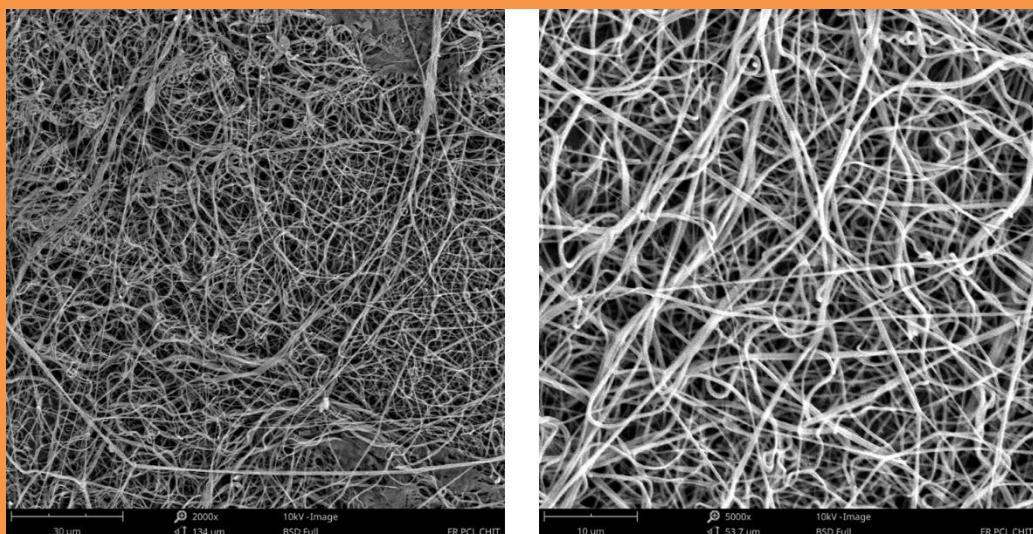
Besides blending with polycaprolactone, we have also developed blend fibers with polyhydroxybutyrate. This natural blend of polymers should show good mechanical properties combined with low swelling in water and good biocompatibility. Compared to polycaprolactone, it offers higher crystallinity and hydrophobicity. Composition was optimized (variation of concentration, polymer ration and co-solvents – data in Dataset). For final experimental purposes, we have used polyhydroxybutyrate(20 w/v %, Biomer) dissolved in a mixture of acetic acid, formic acid, and chloroform (1:1:1) under heating. Electrospinning experiments were performed at an industrial NUENEX electrospinning unit with a top-filled blade electrode. The experimental conditions were 30°C and < 40% RH. The voltage was set at -30 kV, +70 kV (total 100kV potential) and an emitter to collector distance of 25 cm. The fibers were deposited on a 40gsm spunbond, and the rewinding speed was 2 mm/s (7.2 m/h). The optimal formulation contained 15 (w/v) % PHB with 7.5 (w/v) % CA (CA-3, Eastman) dissolved in acetic acid: formic acid: chloroform (3.5 : 3.5: 1). The formed nanofibers showed fibrous morphology with decreased fiber size. However, the system showed partial melting typical for PHB, reducing the pore size.



Development of blend nanofibers from polycaprolactone and chitosan

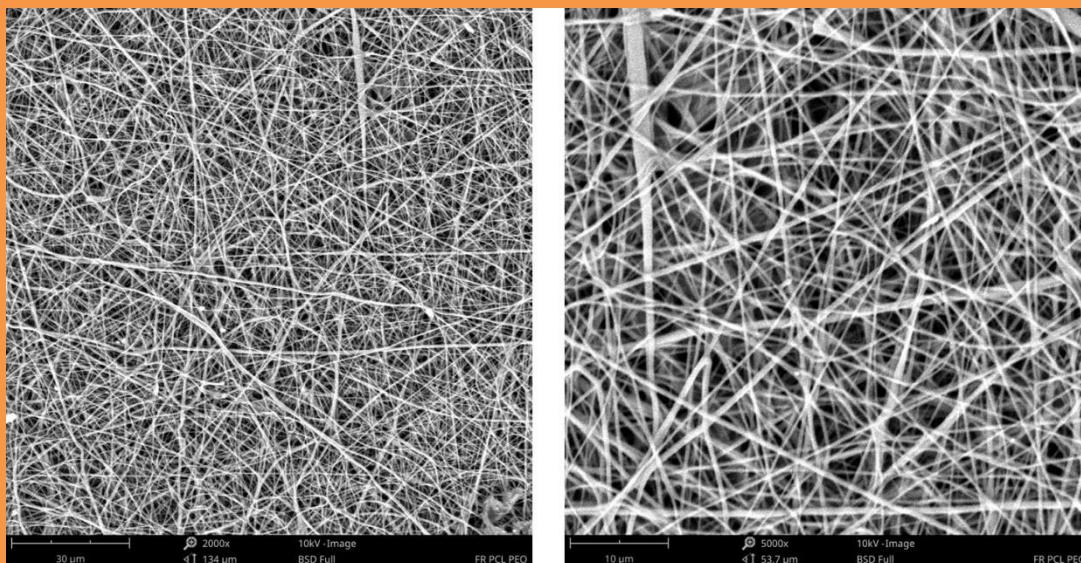
Chitosan is a natural polymer showing high biocompatibility and is typically used in tissue engineering applications. In the current experiment, we have blended chitosan with polycaprolactone. Chitosan (5 v/w %, medium molecular weight, Sigma Aldrich) was dissolved in acetic: formic acid (1:1) ratio. Fibers were created by adaptation of results from PCL-cellulose derivative experiment and applied for chitosan. Polycaprolactone (20 w/v %, 80 kDa, Sigma Aldrich) was dissolved in acetic formic acid. The final solution for spinning was composed of 11.5% PCL with 0.7% chitosan dissolved in a mixture of acetic acid, formic acid, and chloroform (3:3:1). Electrospinning experiments were performed at an industrial scale NUENEX electrospinning unit with top-filled blade electrode. The experimental conditions were 30°C and < 40% RH. The voltage was set at -30 kV, +70 kV (total 100kV potential) and an emitter to collector distance of 25 cm. The fibers were deposited on a 40gsm spunbond, and the rewinding speed was 2 mm/s (7.2 m/h).

The produced nanofibrous layer showed fibrous morphology with a minimum of non-fibrous defects.



Development of blend nanofibers from polycaprolactone and polyethylene oxide

Polyethylene oxide (PEO) is a hydrophilic synthetic polymer. In the current experiment, we have blended chitosan with polycaprolactone to decrease the hydrophobic nature of polycaprolactone. Polyethylene oxide (10 v/w %, 100 kDa, Sigma Aldrich) was dissolved in acetic: formic acid (1:1) ratio. Polycaprolactone (20 w/v %, 80 kDa, Sigma Aldrich) was dissolved in acetic formic acid. The final solution for spinning was composed of 10% PCL with 3.75% PEO dissolved in a mixture of acetic acid: formic acid: chloroform (3:3:1). Electrospinning experiments were performed at an industrial scale NUENEX electrospinning unit with top-filled blade electrode. Fibers were created by adaptation of results from PCL spinning experiment and applied for PCL/PEO blend. The final experimental conditions were 30°C and < 40% RH. The voltage was set at -30 kV, +70 kV (total 100kV potential) and an emitter to collector distance of 25 cm. The fibers were deposited on a 40gsm spunbond, and the rewinding speed was 3 mm/s (10.4 m/h). The produced nanofibrous layer showed fibrous morphology with a minimum of non-fibrous defects.



Evaluation of mean nanofiber size and pore size

The mean nanofiber size and pore size were calculated from SEM images in ImageJ software, for both variable, 200 fibers or pores was calculated. The results of the measurement are indicated in Table 1.

Table 1	Morphology	Mean fiber size	Mean pore size	Contact angle
PCL MICRO/NANO	MICRO/NANO	major: 1525 ± 523 nm, minor: 436 ± 213 nm	$21.5 \pm 10.2 \mu\text{m}^2$	$115.1 \pm 8.3^\circ$
PCL NANO/MICRO	NANO/MICRO	major: 195 ± 123 nm, minor: 650 ± 231 nm	$4.54 \pm 3.12 \mu\text{m}^2$	$111.9 \pm 4.3^\circ$
PLA	NANO	318 ± 134 nm	$2.09 \pm 1.25 \mu\text{m}^2$	$108 \pm 2.2^\circ$
PHB	MICRO	1236 ± 995 nm	$9.05 \pm 4.67 \mu\text{m}^2$	$92.8 \pm 4.9^\circ$
PA6	NANO	240 ± 32 nm	$0.4 \pm 0.12 \mu\text{m}^2$	$93.9 \pm 4.2^\circ$
PA11/PVB	MICRO/NANO	major: 1477 ± 704 nm, minor 265 ± 85 nm	$12.7 \pm 7.83 \mu\text{m}^2$	$111 \pm 5.9^\circ$
PVA	NANO	453 ± 203 nm	$35.2 \pm 9.48 \mu\text{m}^2*$	$40 \pm 3.2^\circ$
PCL/CA	NANO/MICRO	major: 679 ± 221 nm, minor 1860 ± 382 nm	$11.5 \pm 4.78 \mu\text{m}^2$	$92.8 \pm 3.4^\circ$
PCL/CAB	NANO/MICRO	major: 340 ± 112 nm, minor 1962 ± 453 nm	$10.2 \pm 5.72 \mu\text{m}^2$	$99.3 \pm 4.2^\circ$
PCL/CAP	SUB-MICRO	871 ± 431 nm	$15.3 \pm 7.34 \mu\text{m}^2$	$109.4 \pm 3.4^\circ$
PHB/CA	SUB-MICRO	838 ± 384 nm	$4.5 \pm 2.5 \mu\text{m}^2$	$110.8 \pm 3.2^\circ$
PCL/CHIT	NANO	324 ± 60.8 nm	$4.8 \pm 2.6 \mu\text{m}^2$	$107 \pm 0.9^\circ$
PCL/PEO	NANO	292 ± 131 nm	$2.3 \pm 1.2 \mu\text{m}^2$	$98.2 \pm 0.9^\circ$

*In the case of PVA, the fiber and pore diameter, upon contact with water, change due to swelling.

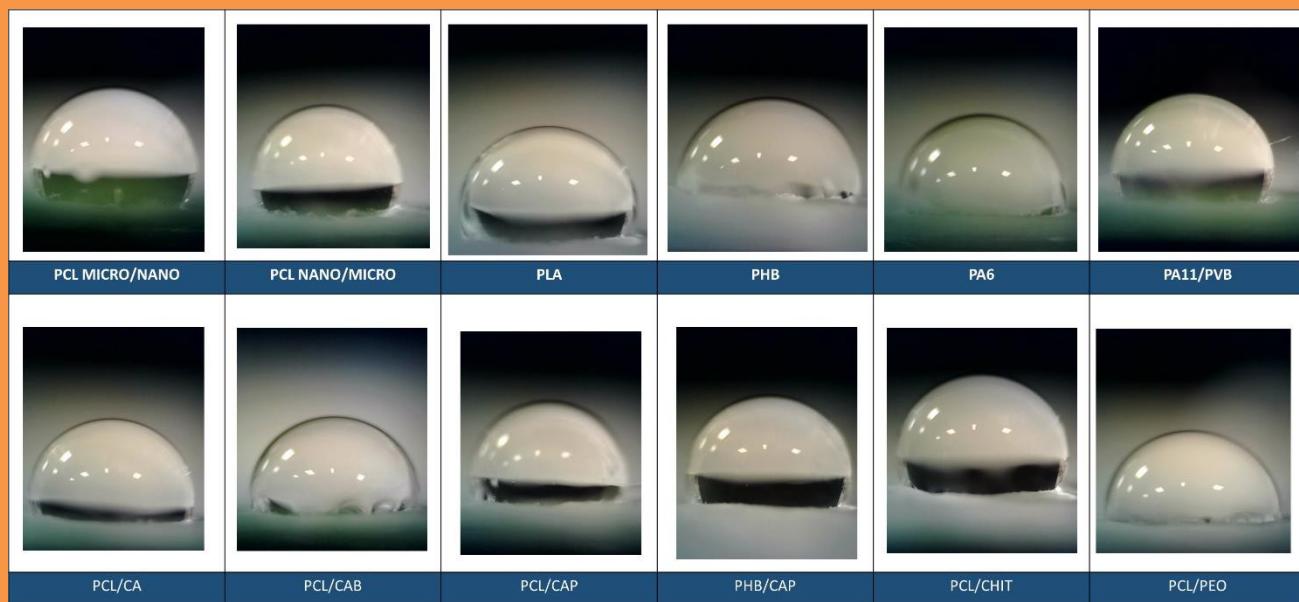
Evaluation of contact angle and wettability

The water contact angle was measured using the SeeSystems (Advex) contact angle measurement system. A drop of water ($10\mu\text{l}$) was added to the surface of the fibers, and the angle between the drop and the nanofiber surface was measured. Data are represented in Table 1.

The data indicate the hydrophobic nature of polycaprolactone, polylactic acid and PA11. The results are the following relationships:

- Hydrophobicity decreases in line of polyesters: PCL > PLA > PHB
- Hydrophobicity changes depending on cellulose additive: PCL > PCL/CAP > PCL/CAB > PCL/CA
- In case of polyamides PA6 is more hydrophilic than PA11.

The wettability data will be correlated with properties in VAC3DP pilot testing in WP3.



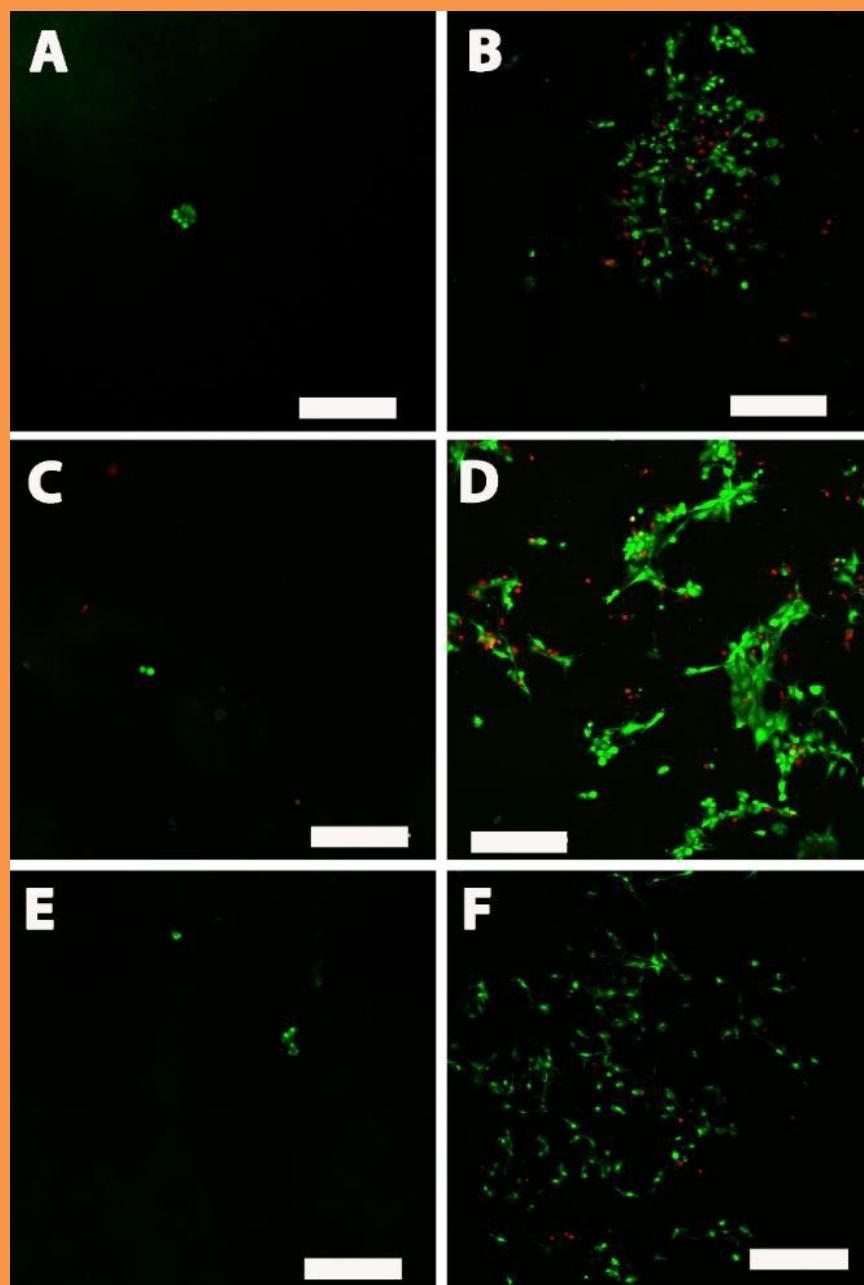
Biocompatibility evaluation of selected nanofibers – preliminary testing

To test the biocompatibility of the chosen PCL polymer with the skin cells, a preliminary *in vitro* study has been conducted. Two biocompatible and biodegradable polymers (PCL and polyvinyl alcohol (PVA)) were tested. Scaffolds were prepared by needleless electrospinning, seeded with murine skin cell lines (3T3 A31 fibroblasts, XB2 keratinocytes and melan-a melanocytes) and the cell viability and proliferation were investigated in an 8-day long experiment. The viability of the seeded cells was assessed using the MTS assay. On the days of the experiment (days 1, 3, 6, and 8), cells were incubated with the MTS reagent, and the absorbance of the media was detected at 490 nm using a multi-plate reader upon incubation. Figure shows the viability of fibroblasts on PCL and PVA. PCL promoted fibroblast viability in comparison to the PVA. A similar trend can be observed in keratinocyte viability, which shows the viability of keratinocytes seeded on the scaffolds. The viability of melanocytes was significantly promoted on the PCL scaffold in comparison to the PVA scaffold throughout the whole experiment. Proliferation of the seeded cells was assessed using the PicoGreen® assay (fluorescent dye of dsDNA). PCL scaffold was colonized with significantly more cells than PVA scaffold throughout the whole experiment. Furthermore, fibroblasts seeded on the PVA scaffold did not proliferate. The same trend as in the case of fibroblasts was observed: proliferation of the keratinocytes and fibroblasts on the PCL nanofibers was significantly more effective.



Cells seeded on the PCL and PVA nanofibrous scaffolds were visualized on the last day of the experiment (day 8). Samples were stained with BCECF (green color) to visualize living cells and propidium iodide (red color) to visualize dead cells. The results indicate good viability of fibroblasts (B) and melanocytes (E) on PCL scaffolds. In the case of keratinocytes, some dead cells were observed (D). In the case of PVA (A, C, E), cell number is much lower. However, data are consistent with Picogreen and MTS and indicate stable viability of seeded cells.

The results of the study showed PCL nanofibers as a suitable system for the stimulation of skin cells. The viability of the cells during the experiment was increasing in the case of PCL and not decreasing in the case of PVA, indicating the biocompatibility of these two types of scaffolds.



Up-scaling and production capacity

From the up-scaling perspective. Our daily target is > 500 m² production capacity. In the pilot trials, we were using 1 electrode/module (data for electrode in table bellow). The NUENEX unit can handle in utilized configuration 4 electrodes with additive function.

Polymeric solutions	1 electrode			4 electrodes		
	mm/s	m/h	m/day	mm/s	m/h	m/day
PHB, PHB/CA, PCL/CHIT	2	7.2	86.4	8	28.8	345.6
PA6, PCL/CA, PCL/CAB, PCL/CAP, PCL/PEO	3	10.8	129.6	12	43.2	518.4
PCL, PLA, PA11/PVB	4	14.4	172.8	16	57.6	691.2

Based on optimization runs during FUROID NF/VAC3DP , we have observed that PCL, PLA, PA11/PVB, PA6, PCL/CA, PCL/CAB, PCL/CAP and PCL/PEO showed productivity, enabling the production of > 500 m²/day. On

the other hand, PHB, PHB/CA and PCL/CHIT showed slower deposition, not meeting the production target.

Conclusion of progress in Task 2.1

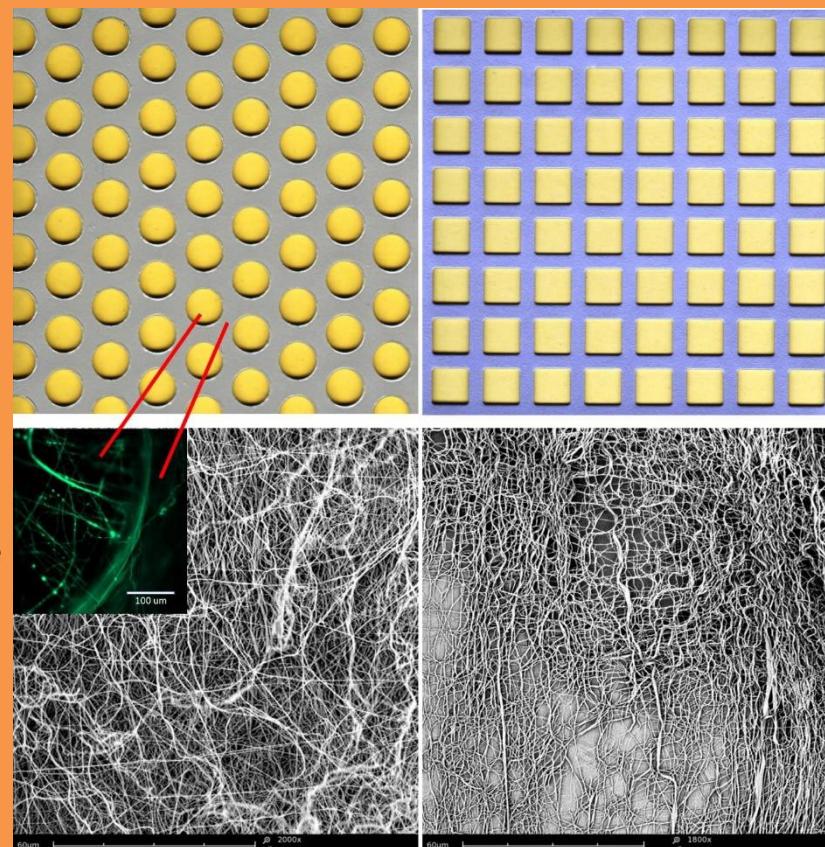
During FUROID NF/VAC3DP , we developed protocols for the industrial production of nanofibers used as support materials to build VAC3DP technology. The fibers were characterized and showed gradients of fiber distribution and wettability, enabling testing of key processing parameters in WP3. The developed systems will serve as nanofiber substrate bases for the FUROID project.

The activity in the second phase will focus on the evaluation of skin cell compatibility and adjustment of nanofiber properties.

Task 2.2: Development of patterned collectors.

The scope of research and development work in T2.2 is the development of patterned collectors for structured nanofiber creation. The collector structure determines the electric field distribution in the vicinity of the collector surface and affects the deposition of nanofibers onto the collecting substrate. Thanks to this, electrospinning could be utilized to form patterned nanofiber structures by using collectors with special patterns.

During FUROID NF/VAC3DP , we started the first experiments with a patterned collector prepared by laser cutting and utilized the finers for testing VAC3DP technology in WP3. In the first experiment, we used metallic collectors with circular patterns (3 mm gap size, circular shape, 2 mm hole distance) and square patterns (3 mm gap size, square shape, 2 mm hole distance).



Both collectors were prepared by Ar laser cutting and obtained from commercial sources. 14% PCL (w/v) dissolved in acetic acid: formic acid: chloroform (17.5: 27.5: 5). Electrospinning experiments were performed at an industrial scale electrospinning unit with a top-filled blade electrode. The experimental conditions were 30°C and < 40% RH. The voltage was set at -30 kV, +70 kV (total 100kV potential) and an emitter to collector distance of 25 cm. The nanofibers were deposited directly on the surface of the patterned collector. The results indicate that the nanofiber layer formed showed patterned deposition. In the case of circular patterns, the areas with metal resulted in the formation of thicker and denser nanofiber layers. On the other hand, the areas deposited on the gap showed lower fiber density and packing. Similarly, in the case of square gaps, the metallic areas were covered by a thicker nanofiber layer, while areas deposited on the gap showed lower nanofiber packing and looser structure. Interestingly, in the case of square gaps, the fibers were forming a network with

rectangular fiber orientation in the gap area. The developed PCL scaffolds were further used in experiments within WP3. In IP Protocol 2, we plan to further work on patterned fibers, test patterning using 3D printed patterns, and try the smallest structure to form a gap.

Target metrics:

- Development of homogenous patterned materials with > 85% reproducibility for fiber size and mean pore size for both loose and dense areas.
- Supporting of >70% cell viability after 14 days of cultivation.

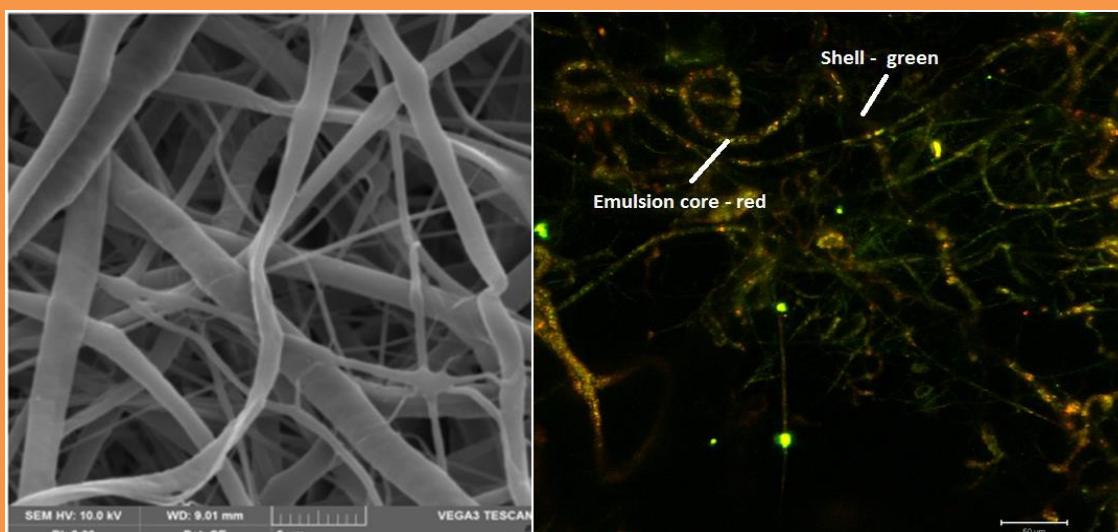
Task 2.3: Active scaffold development:

We proceeded with the development of active nanofiber scaffolds earlier than planned in the project..

Target metrics:

- Nanofiber layers with > 80% reproducibility of encapsulation efficacy and loading capacity for model growth factors across the layer (xy distribution) and different batches.
- Reproducible release profile (deviation of absolute release for 1 day, 7 day and 14 day intervals) with deviation < 10% across the layer (xy distribution) and different batches.
- Production capacity over 500 m²/day (demonstrated for model protein as cargo).

The nanofibers were formed using needleless emulsion electrospinning. To create an emulsion, we have utilized a solvent system for PCL based on chloroform with methanol and formic acid. 14% PCL (80 kDa, Sigma) was dissolved in chloroform: methanol: formic acid (5:3:2). The PCL solution was emulsified with aqueous core made of different solutions of PVA containing TRITC-dextran (70kDa, Sigma Aldrich). The coaxial nanofibers generated by emulsion spinning technology have an island in the sea morphology. In the experiment, we have produced PCL/PVA nanofibers by emulsification of aqueous PVA phase in PCL solution dissolved in chloroform: methanol: formic acid as oil phase.

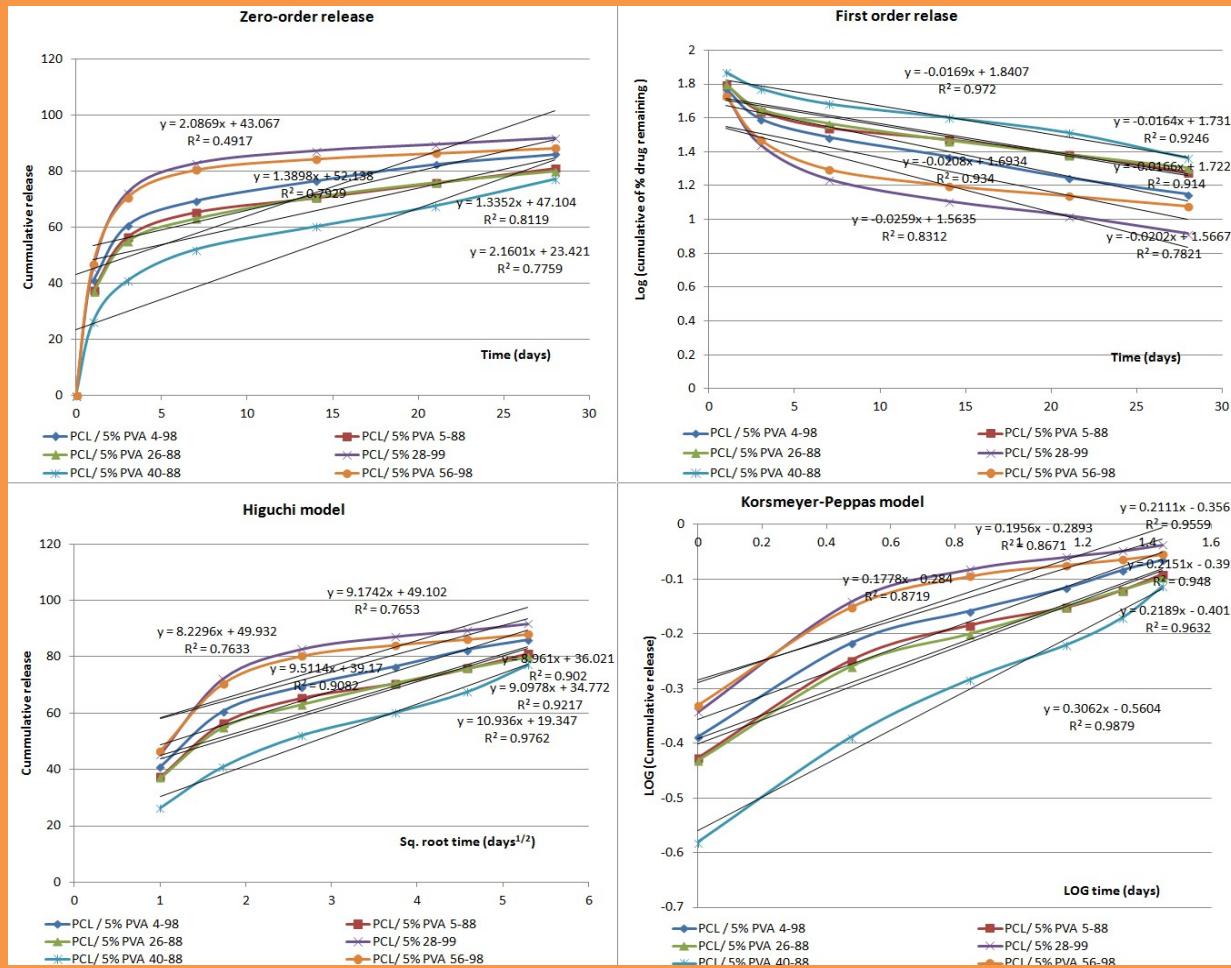


The core phase was the first modification of the experiment based on different molecular weights of PVA. The first group was performed in PVA with a de-acetylation degree of 88 or 99%, which means that the PVA contained 1% or 12% acetyl groups. In addition, the polymers had different molecular weights: 4-99, 5-88, 18-99, 26-88, 40-88 and 56-88. The first number corresponds to the viscosity of PVA under defined conditions and is proportional to molecular weight. All samples were prepared

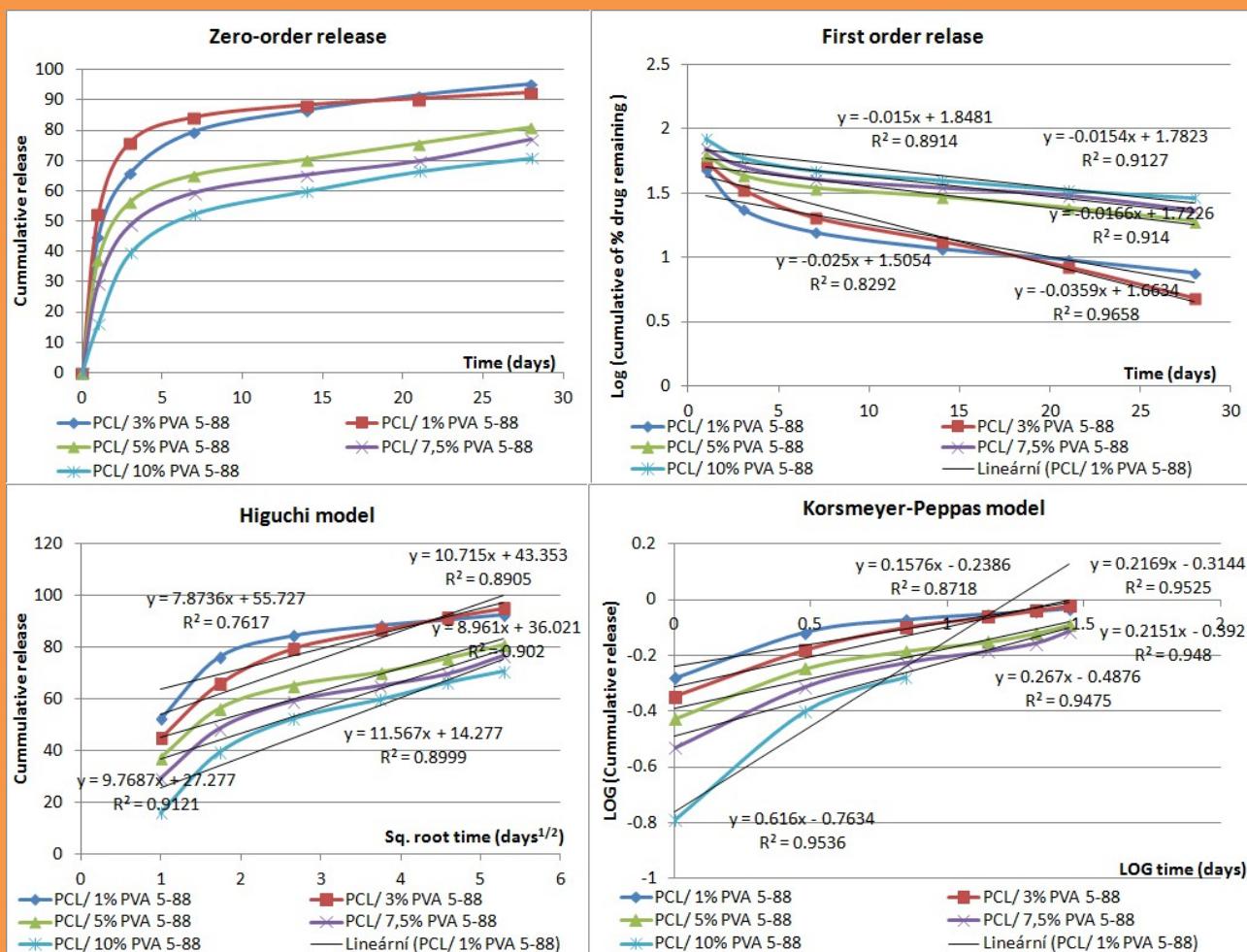
from 5% PVA (w/v). The release was performed by the standard procedure described in previous experiments on the model of TRITC-dextran with MW 70kDa.

Samples with a de-acetylation degree of 88% show less crystalline structure and should dissolve faster under room temperature. The release showed a more sustained profile with increasing PVA molecular weight (40-88<26-88<5-88). Such observation could be caused by higher viscosity and, therefore, slowed diffusion. The zero-order release model showed poor alignment (R^2 about 0.7-0.8). The diffusion-controlled release mechanism is indicated by good correlation with the first-order release model (all samples $R^2 > 0.9$), Higuchi model (all samples $R^2 > 0.9$) and Korsmeyer-Peppas model (all samples $R^2 > 0.9$). The release exponent was lower than 0.5 and, therefore, out of the model working window. The main difference in the release is observed by a higher burst in the case of lower MW of PVA and fast release in the initial release phases and then achieving the release plateau. The PVA 40-88 slope was stable for the overall release time, showing typical behavior for diffusion-regulated release.

In the case of samples with a high degree of de-acetylation, 99% of the behavior was the opposite. The high de-acetylation degree is connected with a crystalline structure and low solubility in water. The release showed the most sustained behavior in 4-99 PVA and rapid release in the cases of 28-99 and 56-99 PVA. The release mechanism showed a low correlation with all models (< 0.9). Only groups with 4-99 PVA showed diffusion controlled mechanism with correlation of First order model, Higuchi model and Korsmeyer-Peppas model (R^2 all models >0.9). The interpretation of these results is possibly connected with the crystalline nature of the samples. The combination of PVA with dextran may result in the introduction of non-homogeneity. Water molecules could easily penetrate the PVA/dextran matrix, and the dissolution is much faster. The longer polymeric chains have even lower flexibility, and therefore, the introduction of non-homogeneities is associated with faster dextran dissolution. The results of this hypothesis must be further confirmed in computational models.

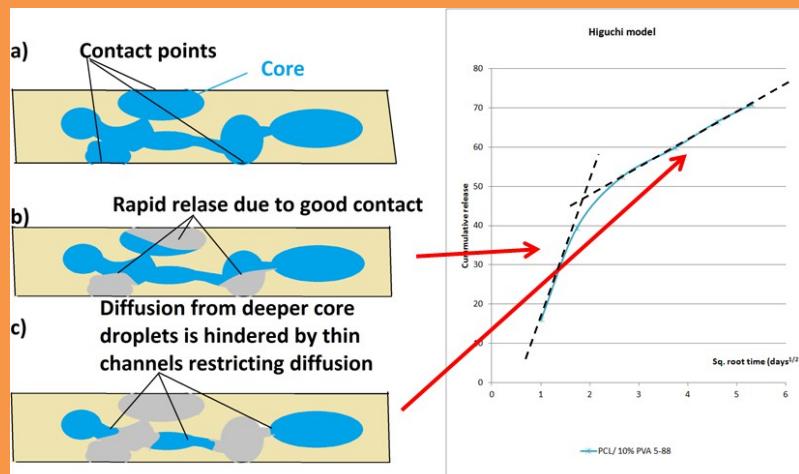


The second experimental set uses a non-crosslinked 5-88 PVA core. The 5-88 PVA had concentrations of 1%, 3%, 5%, 7.5% and 10%. The release profile of TRITC-dextran with MW 70kDa showed more sustained release with increased PVA concentration. The samples with 1% and 3% PVA showed the highest burst effect after 24 hours of incubation with aqueous media. With increasing the concentration, the burst release was lower, and the release of the samples achieved the release plateau later. The zero-order release model showed poor alignment. In the first order, the Higuchi and Korsmeyer-Peppas models showed good correlation in cases of 3%, 5% and 7.5% PVA, indicating a controlled process. In the case of 1% and 10% PVA, the release showed a correlation <0.9 . The diffusion-controlled mechanism is, therefore, the major driver; however, the results show that the other mechanisms may also play a role.



All release curves in the emulsion model are connected with the two-stage release. A slower release with different release constants follows the initial release phase. Such behavior is apparent in, for example, 10% 5-88 PVA. The reason may be connected with the non-continuous morphology of the nanofiber core. The reason may be associated with the lower availability of core droplets in deeper parts of fibers. The thin connections hinder diffusion due to high capillary forces restricting the diffusion from the pores. In this case, the diffusion coefficient is also affected. Therefore, the release becomes slower to a higher extent than caused by a longer diffusion path.

The results of the experimental study confirmed that crosslinking and changing the physical properties of the core solution is an efficient way to modifier release properties. The release is connected with the properties of incorporated drugs, and empirical release methods should identify the release pattern. The release from core/shell nanofibers examined in the study showed, in most cases, good agreement with the order release model. Diffusion-related forces drive the release. However, the system is not similar to the Korsmeyer-Peppas model of a thin film with negligible edge effects. To understand the release mechanism and develop a mathematical model for release



evaluation, further factors should be included. Among them, the effect of core morphology, prolongation of diffusion path, the effect of crystallinity, and high capillary forces in nanopores must be deeply examined.

The experiments demonstrate the possibility of controlling release properties from emulsion nanofibers by selecting the core polymer molecular weight, solubility and ratio between the core and shell of the fibers. In IP Protocol 2, we will focus on a combination of active molecules (growth factors) from the core/shell emulsion nanofibers.

Task 2.6: Development of DBTL for nanofiber production and interaction

The work on the DBTL platform was focused on collection standards for data:

- In the case of electrospinning production, parameters such as solution composition, polymer molecular weight, conductivity, viscosity at processing temperature, fiber diameter distribution, pore size distribution, weight per square meter, water contact angle and surface energy will be collected.
- In the case of VAC3DP technology, the membrane will be further characterized by pressure resistance upon filtration with latex particles with a size of 100 µm and concentration of 1 mg/ml. For this purpose, a special measurement system is under development.
- Regarding organoids/cells – their size distribution and concentration are the entry data needed for the experimental purposes.
- During VACD3DP deposition, the time of deposition, the distance between the patterning membrane and nanofiber layer, pressure drop and precision of pattern formation will be analyzed.

For IP Protocol 2, we are preparing a set of standardized experiments with selected nanofiber membranes: PCL, PCL/CA, PCL/CAB, PCL/CAP, PVA and PA6. In the case of PCL, we will also prepare a set of surface- modified fibers with a change in wettability using NaOH etching (Yaseri R. et al., 2023, DOI: 10.1038/s41598-023-36563-w). The experimental setup will include the cultivation of iPSCs on the surface of nanofibers and the evaluation of their viability. The experiments will be prepared in extended replicate counts (10 replicates per variable) to provide sufficient statistical strength. The collected data will be loaded and utilized for building the core base DBTL database.

1.2.3 Workpackage 3 – Development of biofabrication methods and roll-to-roll system.

The objective of WP3 is development of biofabrication technologies compatible with roll-to-roll deposition of skin cells on electrospun nanofibers.

Executive summary of progress during FUROID NF/VAC3DP :

During FUROID NF/VAC3DP , the experimental work was focused on the validation of VAC3DP technology (KER). We have successfully tested different configurations focusing on pattern deposition precision, scalability and the possibility to form multi-component systems. The optimal method is based on top patterning using special foils followed by full-area suction (vacuum formation). The experiments showed the formation of features with a submillimeter shape and resolution of 0.1 mm (which is better than expected in DoA). We have validated nanofiber scaffolds developed in WP2 and selected the most promising systems for further development. Moreover, we have constructed a laboratory system for testing in the next phases of the project and created the design of a system for continuous

production. Firstly we have performed testing on a substitute model (alginate microbeads).

Key achievements:

- PoC study demonstrating the possibility of creating on-demand patterns with circular, square, triangular, and complex smiling face shapes with feature sizes in the millimeter range and precision of 0.1 mm.
- Comparison of nanofiber membranes and PoC study showing their advantages in laboratory environment setting.
- Formed multicomponent systems with sequential printing in layer-by-layer deposition (layers with different particles are on top of each other) and side-by-side deposition (layers are spaced in xy direction).

Progress achieved in assigned Tasks during FUROID NF/VAC3DP :

Task 3.1: Adaptation of standard extrusion 3D bioprinting technology:

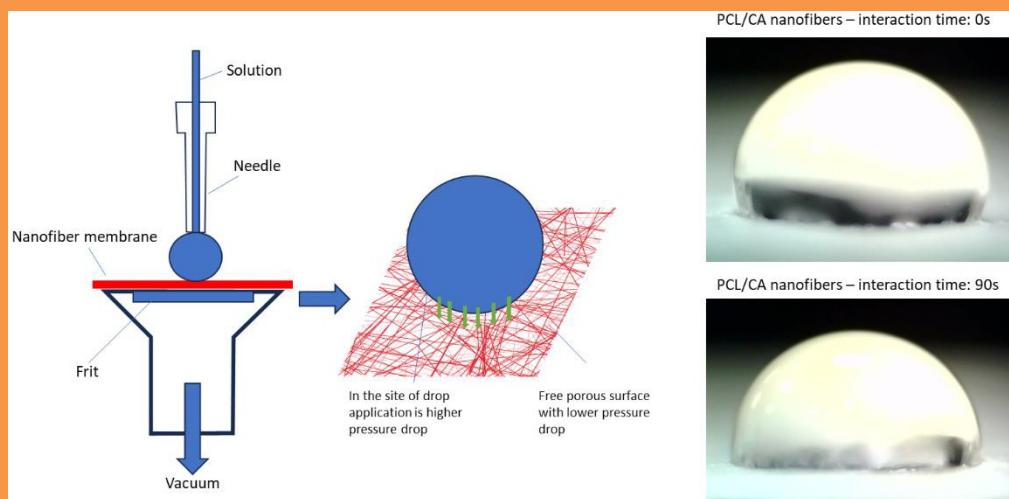
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The 3D bioprinting of organoids was not performed due to issues with GENEUS. However, it is important to state that E3DP is a backup technology for VAC3DP due to its limitations. Extrusion 3D

bioprinting is a standard method utilized to form 3D constructs. The for applicability format large-scale intended and technology developed under FUROID would be highly limited due to slow printing speed and scalability.

The experimental

work evaluated the possibility of combining needle array and vacuum suction from the bottom of the nanofibrous layer. In such a case, the cell deposition would be working on a similar principle as VAC3DP. During FUROID NF/VAC3DP , we have conducted preliminary experiments with unconvincing results. The experimental setup was based on a vacuum system equipped with a frit. On the frit surface, a nanofiber membrane with substrate was placed. Above the fiber membrane, a needle (G20 in our case) was set to deliver the solution to the fiber surface and simulate the E3DP deposition mode. The droplet, upon ejection, came into contact with a nanofibrous layer made of PCL/CA. Upon vacuum induction, the droplet should be sucked into the nanofiber membrane. However, in experiments, the suction of droplets to the nanofiber membrane was not observed after 90 interaction time.



The reason why the deposition failed is connected with nanofiber membrane properties. The nanofiber membrane shows high porosity and pore interconnection, making it ideal for our application. However, in the case of only partial covering of the nanofiber surface by a single drop, the remaining parts of the membrane are not covered and enable free airflow through the membrane, decreasing hydrostatic forces induced by vacuum application. Therefore, the drop is not soaked inside the membrane - the area with the droplet shows higher pressure resistance, and the

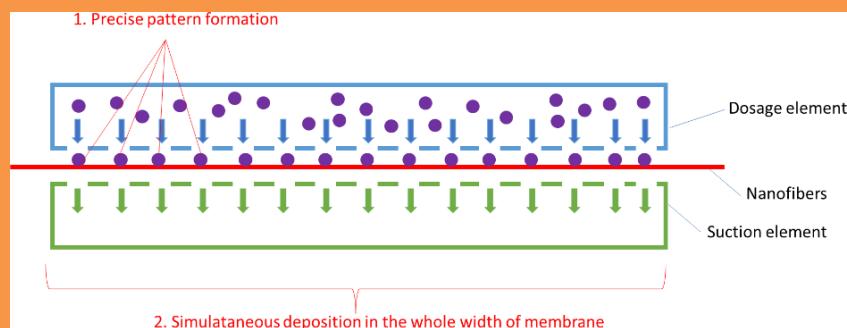
formed pressure is relaxed through the other parts of the membrane. For this reason, we will focus our efforts in IP Protocol 2 on the more promising VAC3DP technology described in Task 3.2.

In case of failure, the solution to the problem might be:

- Increased wettability of nanofibers, resulting in lower interfacial tension. The measure should help with a problem but might decrease the resolution of the printer (the spot created would be enlarged due to wetting of the surface).
- Stereotactic application/suction – decreasing of free area, which serves for relaxing pressure created by vacuum, will increase the hydrostatic forces responsible for suction. This can be achieved by reducing the frit diameter and acting of frit only in segments where the needless are placed. This solution is technically feasible – instead of using large frit and small needles, both elements should be of comparable size with a fixed position against each other. The printing would be then performed by moving the XY position of needle/suction elements compared to the nanofiber substrate. However, such technology would result in a slower printing process or the need for extensive parallelization.

Task 3.2: Development of VAC3DP (M1-M36, FUROID):

During FUROID NF/VAC3DP , we focused on the development of VAC3DP technology and the evaluation of different setups to reach optimal resolution and efficient design of technology. VAC3DP should exploit the properties of nanofibers and enable simultaneous deposition and

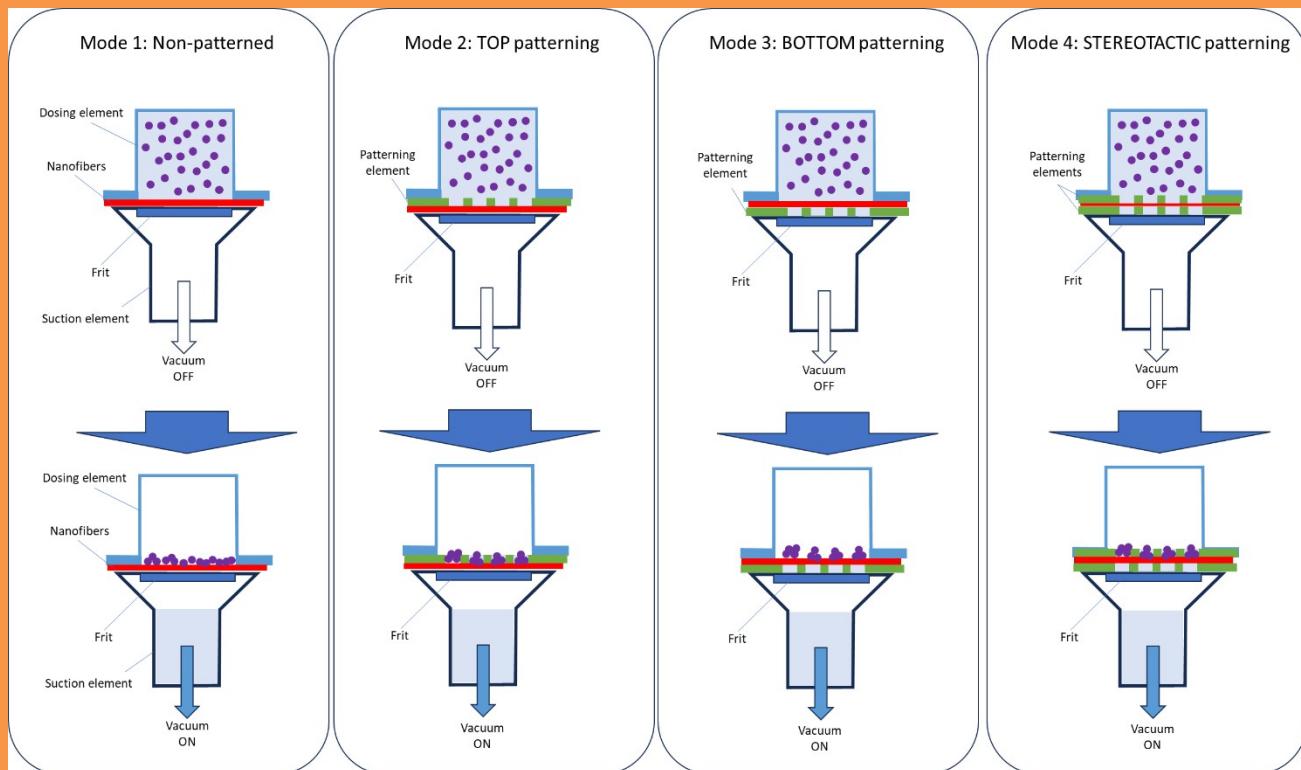


pattern creation along the full printing surface in one step. This reduces the time for creating patterns and is ideal for large-scale applications where patterning using cells is needed.

Target metrics:

- Spatial resolution of VAC3DP better than 1 mm.
- Membrane flow properties better than 3 ml/s/cm² for solution containing 10 mg/ml of test particles.
- Single layer print time bellow 10s.
- Ripping or pattern destruction bellow 1% from print area/total area.
- Support of post-seeding viability >90% for organoids.
- Organoid post seeding viability > 70% after 14 days of cultivation

From a technical perspective, there are 4 main configurations for patterning using VAC3DP:



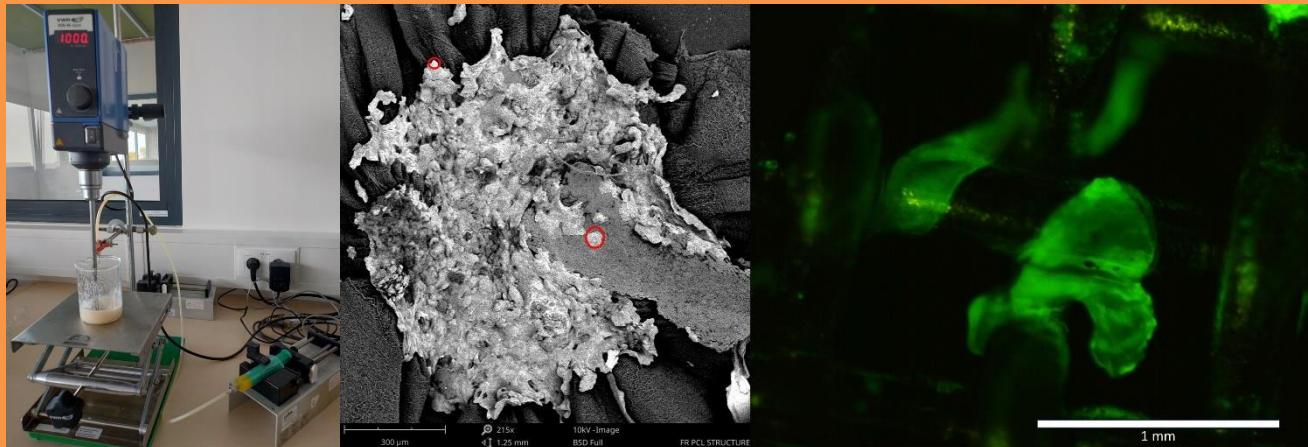
Formation of alginate microbeads as an experimental model for VAC3DP

Due to the unavailability of iPSC cells and organoids, we have utilized alginate microbeads as a replacement system for analysis of the VAC3DP method during the FUROID NF/VAC3DP . The alginate microbeads were prepared using emulsion ionotropic gelation technology previously described by Lupo B. et al. (2014, DOI: 10.1016/j.foodhyd.2013.11.003) with some modifications. Briefly, 3% sodium alginate (low viscosity, VWR) was dissolved in water, and the solution was left for complete hydration for 48 hours. The emulsion synthesis was performed using an apparatus consisting of a syringe pump (NE500, NewEra), needle (G20) and stirred collection bath (dissolver R1303, IKA). 20 ml of 3 (v/w) % alginate with 1 ml of 0.15 M calcium citrate dispersion was put into a 20 ml syringe. The solution was added at a rate of 1 ml/min to a stirring bath consisting of 40 ml of 5% Span 80 in sunflower oil. The bath was stirred at a speed of 1000 rpm. After the addition of the whole syringe content, the stirring continued for an additional 5 minutes, followed by adding 10 ml of sunflower oil with 80 µl of glacial acetic acid (dropwise, stirring 1000 rpm) to solubilize calcium citrate and inducing internal ionotropic gelation. After 15 minutes of stir time, 100 ml of 0.05 % CaCl₂ with 1% Triton-X100 was added to the mixture. The stirring continued at 2000 rpm for an additional 15 minutes. After finishing stirring, the solution was transferred to 500 ml of 0.05M CaCl₂ and was left for phase separation overnight. After phase separation, the particles were collected by filtration (Whatman filter paper) using vacuum filtration. The particles were dried at 37°C, weighted and resuspended in deionized water at 10 mg/ml. The resuspension was performed using a Turax G25 mixer (IKA) at 15,000 rpm for 60 minutes in a cooled bath.

For experimental purposes, we have created the following article types:

- AgNO₃ labeled particles containing 1% AgNO₃ relative to weight of alginate. AgNO₃ increases the contrast during SEM microscopy, increases the weight of particles (filtration efficacy testing) and enables visualization for optical microscopy (on light, the particles develop a brown-to-black color).

- FITC-dextran labeled particles containing 1 mg/ml of FITC-dextran (10,000 Da, Sigma). Particles were used for confocal microscopy.
- Rhodamin G6 labeled particles containing 0.5 mg/ml of Rhodamin G6 (Sigma). Particles were used for testing of side-by-side and layer-by-layer deposition.



Evaluation of flow properties of different types of nanofibers, filtration efficacy and back-pressure:

Nanofibers developed in T2.1 were utilized to evaluate their water flow and filtration properties. In addition, back-pressure during liquid flow was calculated (maximal pressure) to assess the properties of nanofiber mesh.

Flow properties: A filtration setup with a 17 cm² efficient filtration area was utilized. The nanofibers were assembled in Mode 1: non-patterned deposition, and 400 ml of deionized water was supplied. After vacuum activation, we measured the time until the entire water was transferred through the system in case of back pressure.

Filtration efficacy: The setup was the same as in the case of flow properties; just the fibers were treated with 20 ml of 1 mg/ml particles. A built-in tensometer monitored pressure increase. Finally, in the experiment, the supernatant passing through the membrane was collected and dried on filter paper to evaluate the filtration efficacy.

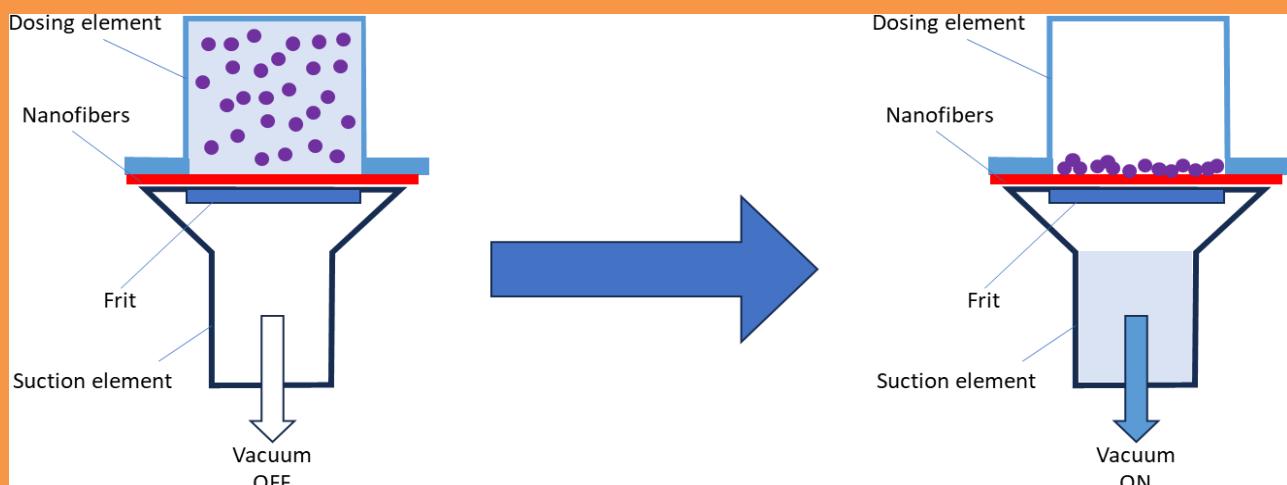
The experiment results are summarized in Table 2. Based on the flow properties of other variables; it shows that higher flow and lower back pressure are observed for more hydrophilic membranes and larger pore sizes. However, the relationship is currently not clear, and the factors interplay with each other. For instance, in the case of PA6 – despite the low fiber and pore size, the flow properties were very high. On the other hand, PCL MICRO/NANO and PA11/PVB, despite higher hydrophobicity, showed similar flow properties and back-pressure to PCL/CA. The results indicate that further work is needed to evaluate the importance of parameters and is connected with the approach proposed in Task 2.6 regarding data acquisition for the DBTL platform.

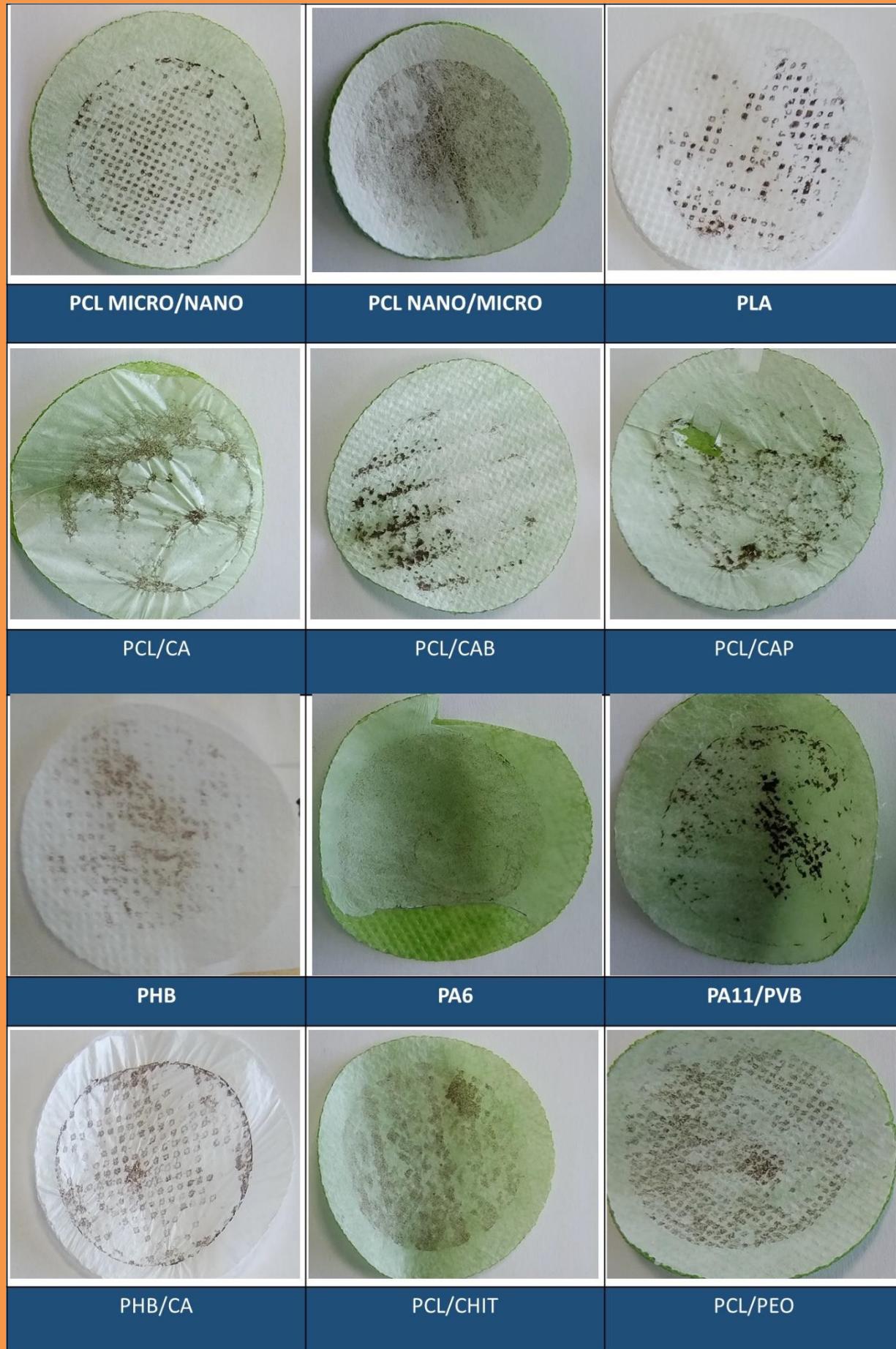
Table 2	Mean fiber size (nm)	Mean pore size (um ²)	Contact angle (°)	Flow (s)	Back pressure (bar)	Efficacy (%)
PCL MICRO/NANO	1525	21.5	115.1	9	140	98
PCL NANO/MICRO	195	4.54	111.9	15	120	96.5
PLA	318	2.09	108	13	140	96.8
PHB	1236	9.05	92.8	14	120	89.4
PA6	240	0.4	93.9	7	110	99.5
PA11/PVB	1477	12.7	111	9.5	150	87.3
PCL/CA	679	11.5	92.8	9.5	140	98.7
PCL/CAB	340	10.2	99.3	12.5	160	94.2
PCL/CAP	871	15.3	109.4	13.8	170	92.1
PHB/CA	838	4.5	110.8	12.5	170	86.5
PCL/CHIT	324	4.8	107	6.8	100	94.2
PCL/PEO	292	2.3	107	8.8	110	94.6

On the other hand, the results of filtration efficacy testing are very promising. All fibers showed filtration efficacy over 85%, indicating that reaching 70% adhesion efficacy highlighted in Objective O2 is feasible.

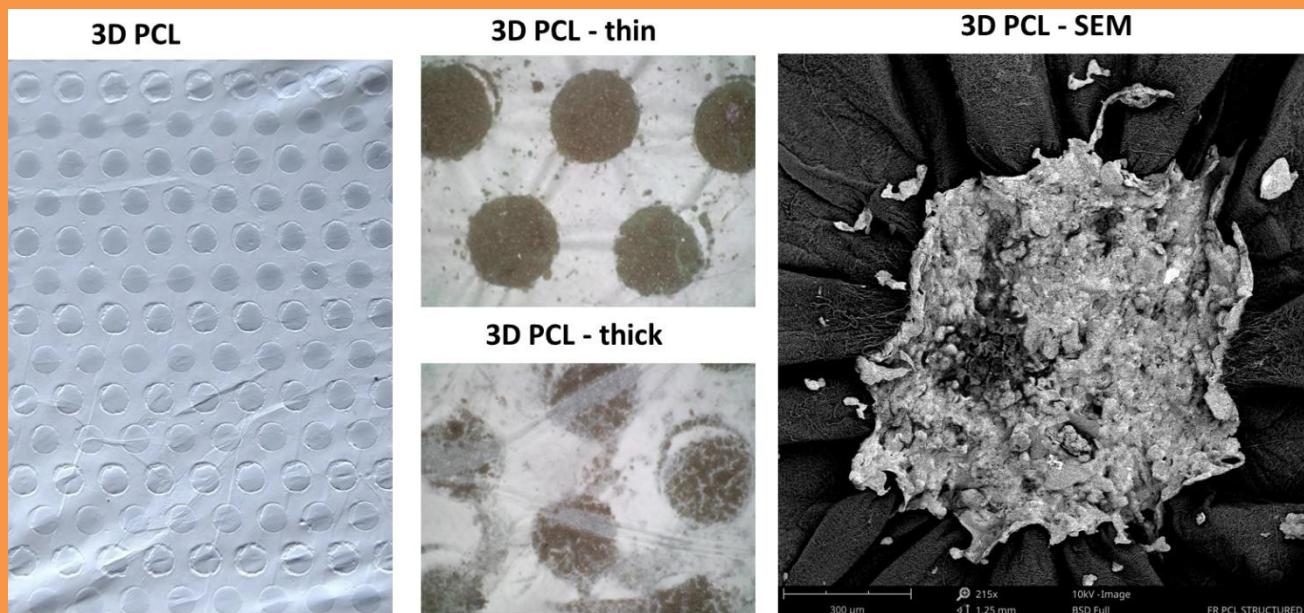
Evaluation of nanofiber effect for particle absorption in configuration Mode 1: None-patterned deposition

The methodology is based on using dosage elements without any patterning system. The protocol was based on clamping the nanofiber membrane without using any mask-creating pattern. After connecting, 10 ml of alginate particles (2 mg/ml) were poured on the surface of the membrane. Upon activation of the vacuum, the liquid was transferred through the membrane, and the alginate particles were deposited on the surface of the fibers. Analysis performed by visualization of homogeneity (visualized by AgNO₃ particles). Adsorbing particles to PCL MICRO/NANO, PLA, PHB, PHB/CA and PCL/PEO resulted in patterning induced by spunbond textile (small squares vs. pores). In the case of PCL NANO/MICRO, PA6, and PCL/CHIT, the distribution of particles was the most homogeneous, indicating that a smaller fiber diameter results in better distribution of particles and a more homogenous pattern. Finally, PCL/CAP, PCL/CAB, PCL/CA and PA11/PVB showed non-homogenous structure. The materials also offer one of the highest pressure drops and lowest permeabilities. Interestingly, there is also a very low attachment of alginate to PHB fibers after their drying.





Besides the use of standard membranes, we have evaluated deposition using Mode 1 on case-structured PCL nanofibers developed in Task 3.2. The membrane was based on structured fibers with circular patterns. Upon deposition, the particles replicated the loose (circular areas) where the fiber density was lower. Interestingly, the patterning via the use of 3D structured nanofibers was working for thin nanofibers (3 gsm) and also thick nanofibers (8 gsm). Visualization by SEM confirmed patterned deposition with a low accumulation of particles outside the circular areas.

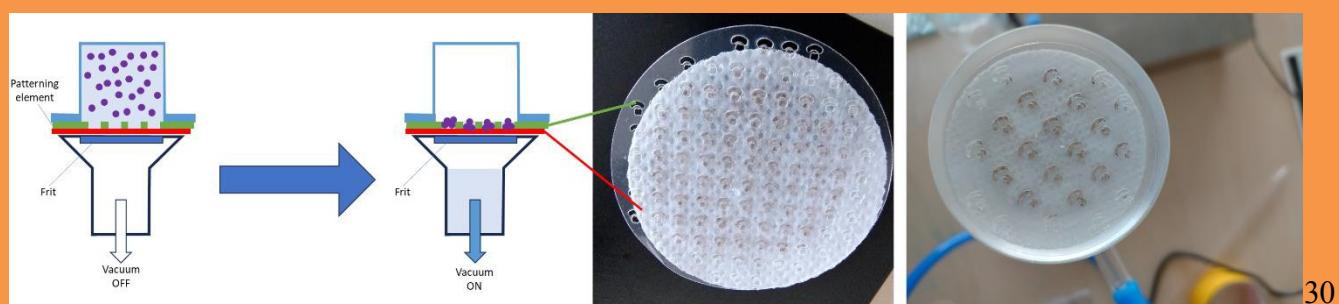


In conclusion:

- VAC3DP, without patterning elements for specific fiber types, generates patterns depending on the structure of the supporting textile. By changing the support textile texture, it would be possible to deposit patterns on the surface of fibers. The exact mechanisms factors, and robustness of the method would require further studies.
- For fibers with smaller thickness and higher wettability, the deposition mode resulted in homogenous coverage of the surface. This is especially interesting for applications requiring homogenous cell seeding (i.e., wound healing).
- Utilization of structured nanofibers (developed by special collectors – T2.2) enables patterning and creation of features on the surface of the mesh without the use of patterning elements.

Mode 2 – top functionalization using CO2-laser cut foils as a mask.

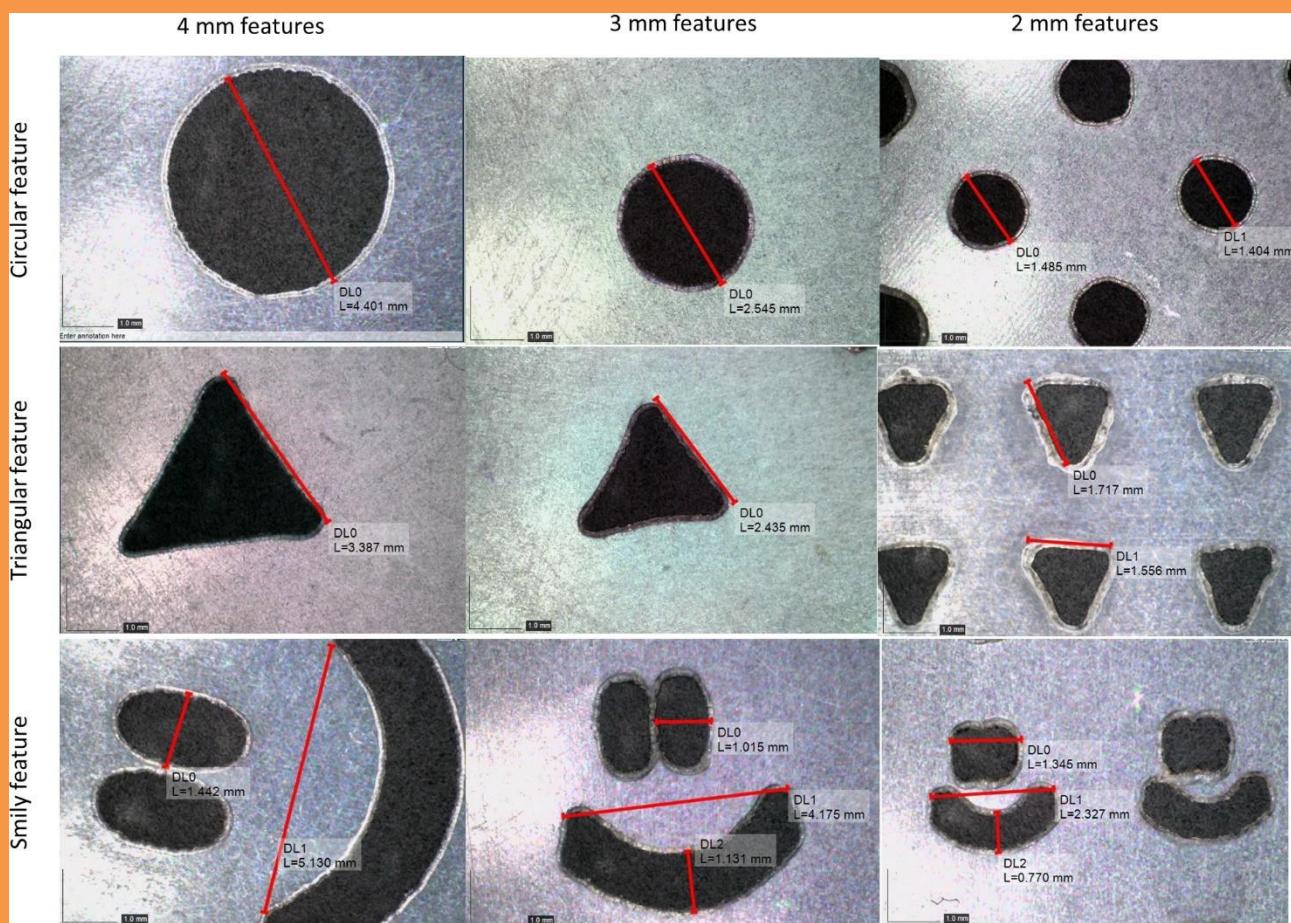
The second setup evaluated during FUROID NF/VAC3DP was patterning using masks prepared by CO₂ laser engraving. PET foil with a thickness of 50µm was used for the experiments. The scheme of the modification system is depicted in the image below. The key advantage of foil is high mechanical stability and thin surface.



CO₂ laser engraving was utilized as a method for the fabrication of features. FUROID has a standard CO₂ laser used for the general cutting of plastic materials; however, upon proper setting and low laser intensities, we could obtain features as small as 1.5mm. We have selected 3 main shapes for our patterns:

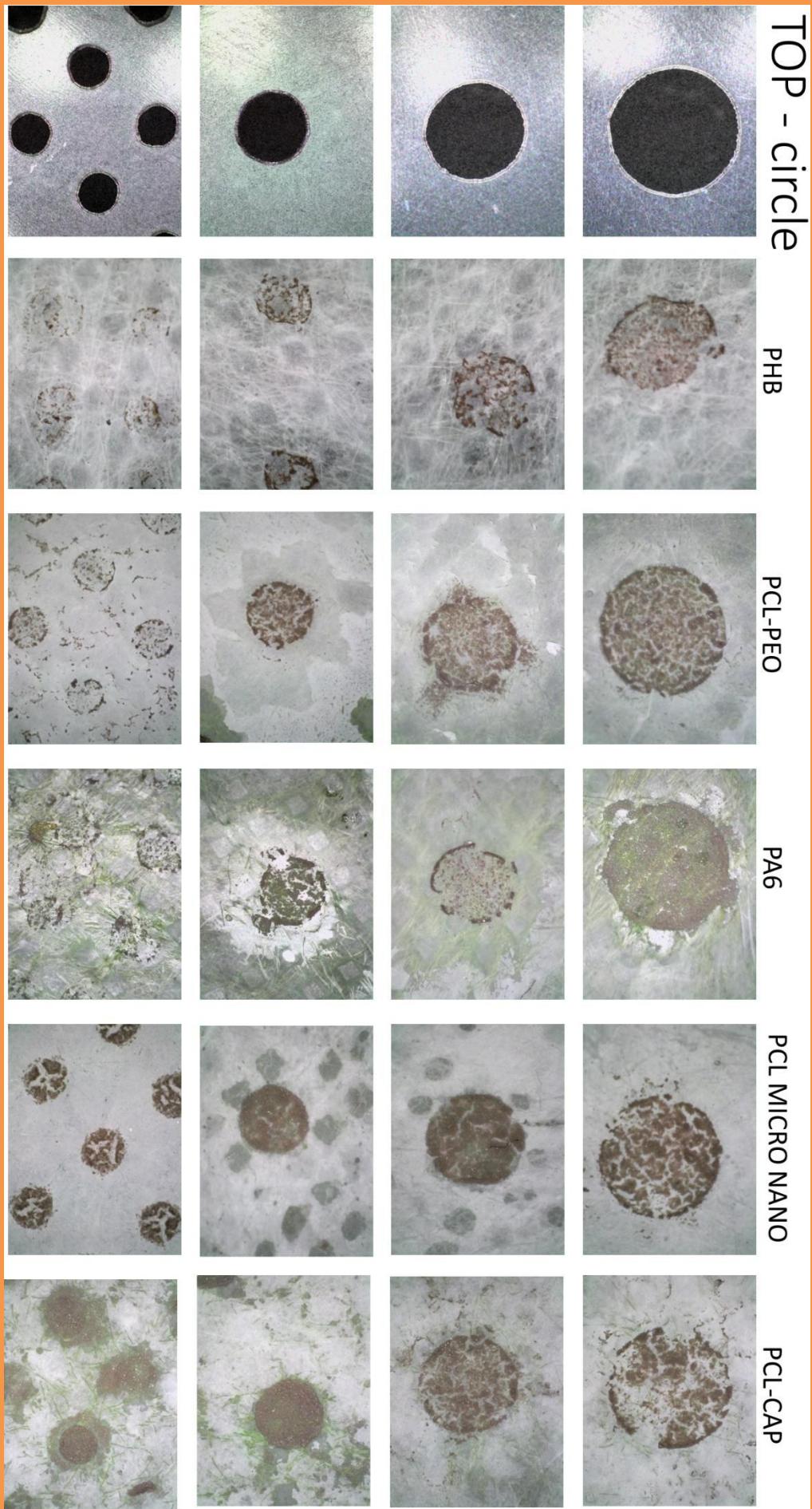
- Circular shape as a standard shape ideally suited for future masks for organoid seeding.
- Triangular shape with sharp corners to evaluate the precision of replications of thin endings in sharp corners.
- Smily pattern for evaluation of co-printing of small features with defined shape (side purpose is attractivity for dissemination/communication activities).

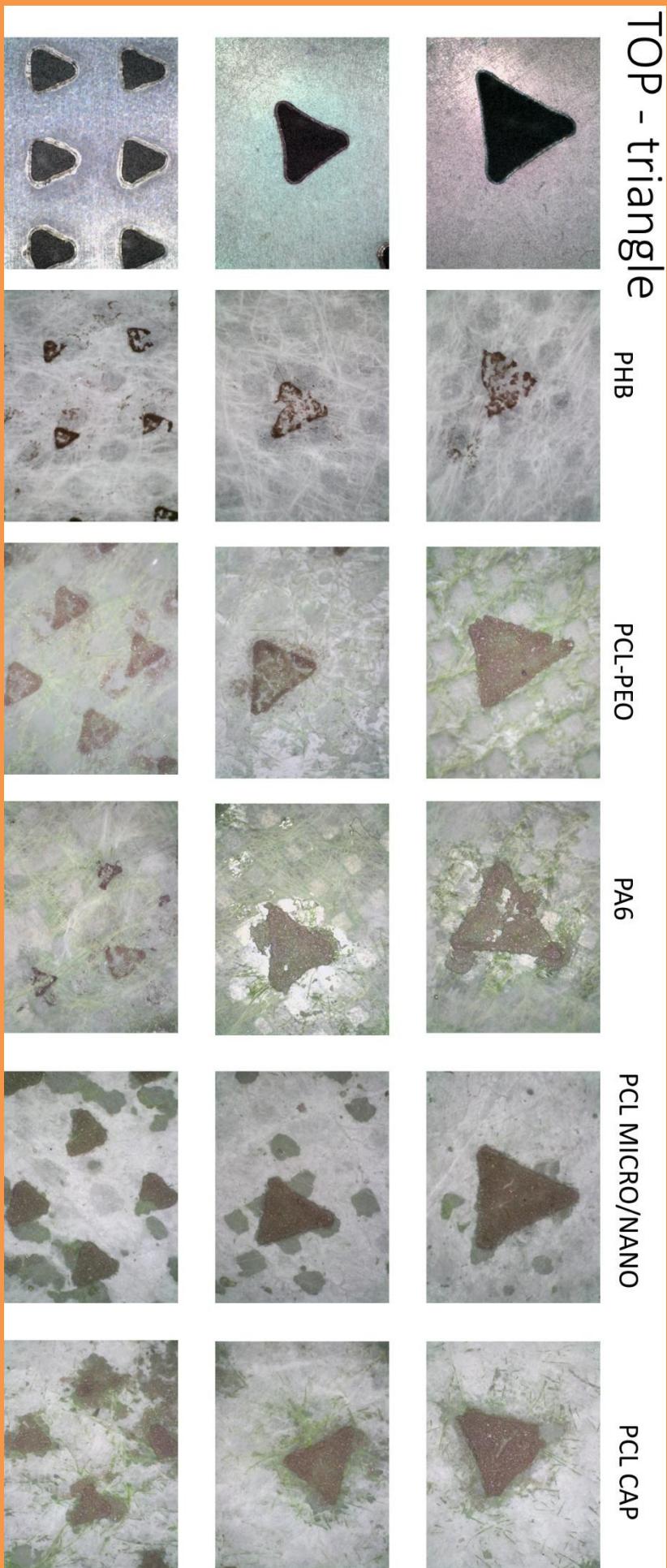
The features were imaged by DinoLite microscope with measuring function under ambient light:

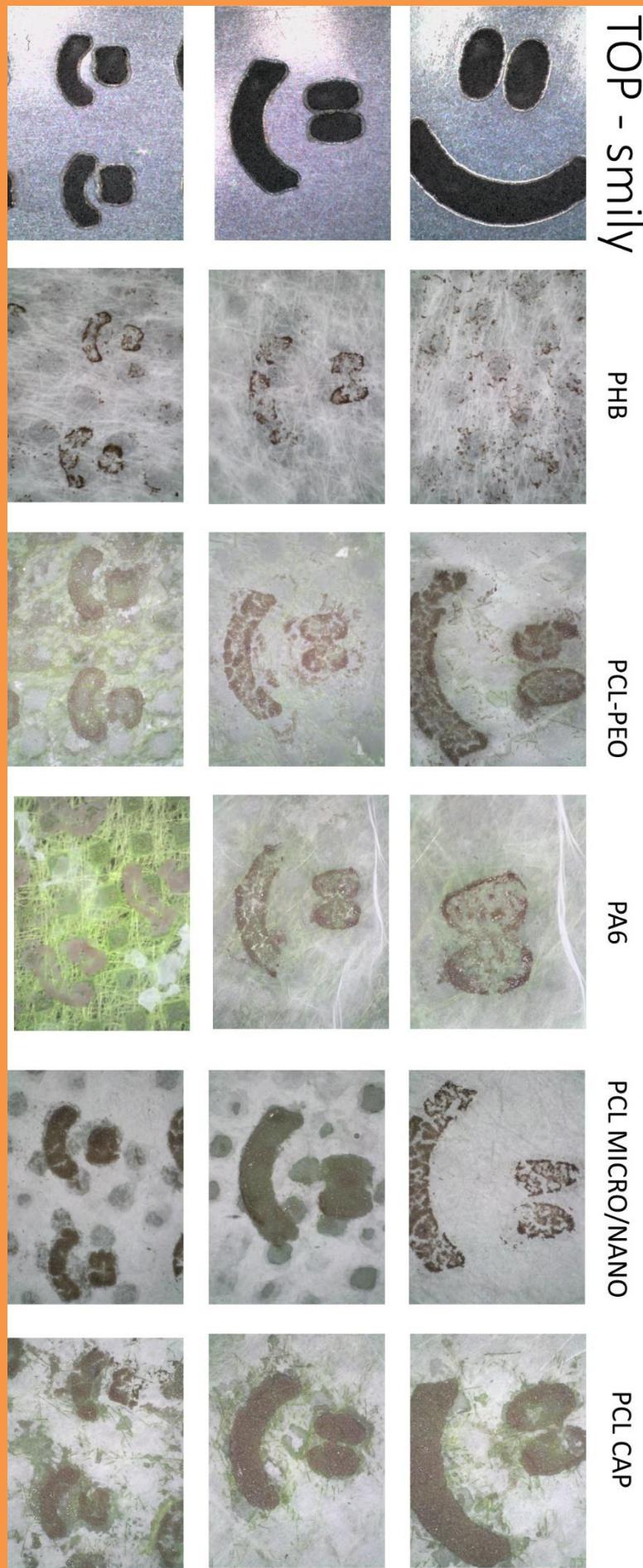


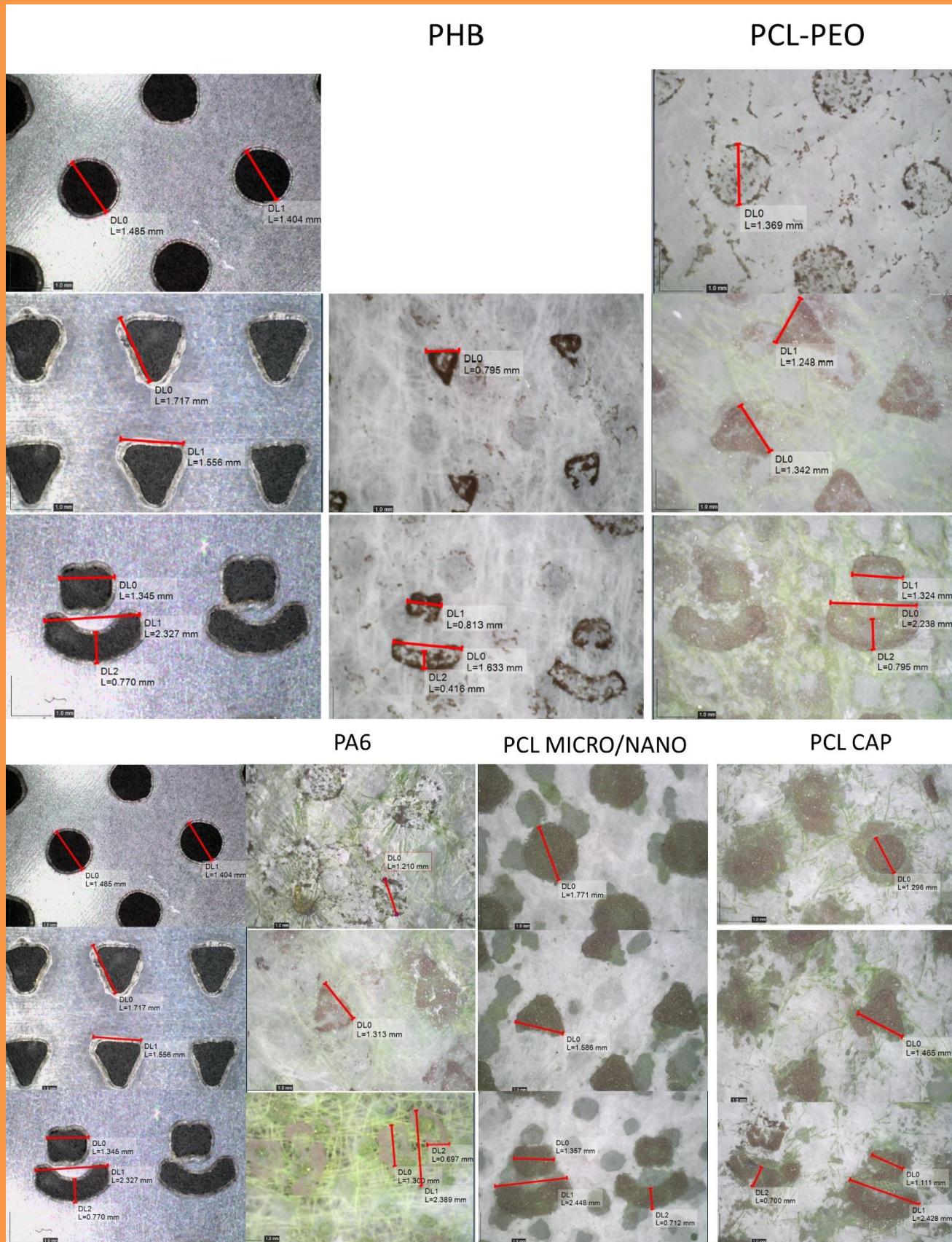
Setup was tested on 5 different nanofibrous scaffolds – PHB, PCL, PLC-PEO, PA6 and PCL-CAP. The systems are in extremes from fiber size distribution, hydrophobicity and pore size.

All fibers showed good replication of shapes, as described in the images on the next pages. From the accuracy perspective we have measured, PHB was offering the highest deviation. The reason might be in peeling off the alginate beads upon drying. For the remaining systems, the VAC3DP technology showed the ability to create patterns with sizes below 2 mm and an accuracy of 0.1 mm. The printing resolution is promising, and the technology seems very versatile and works with all tested materials except PVA (swelling and pore-clogging).





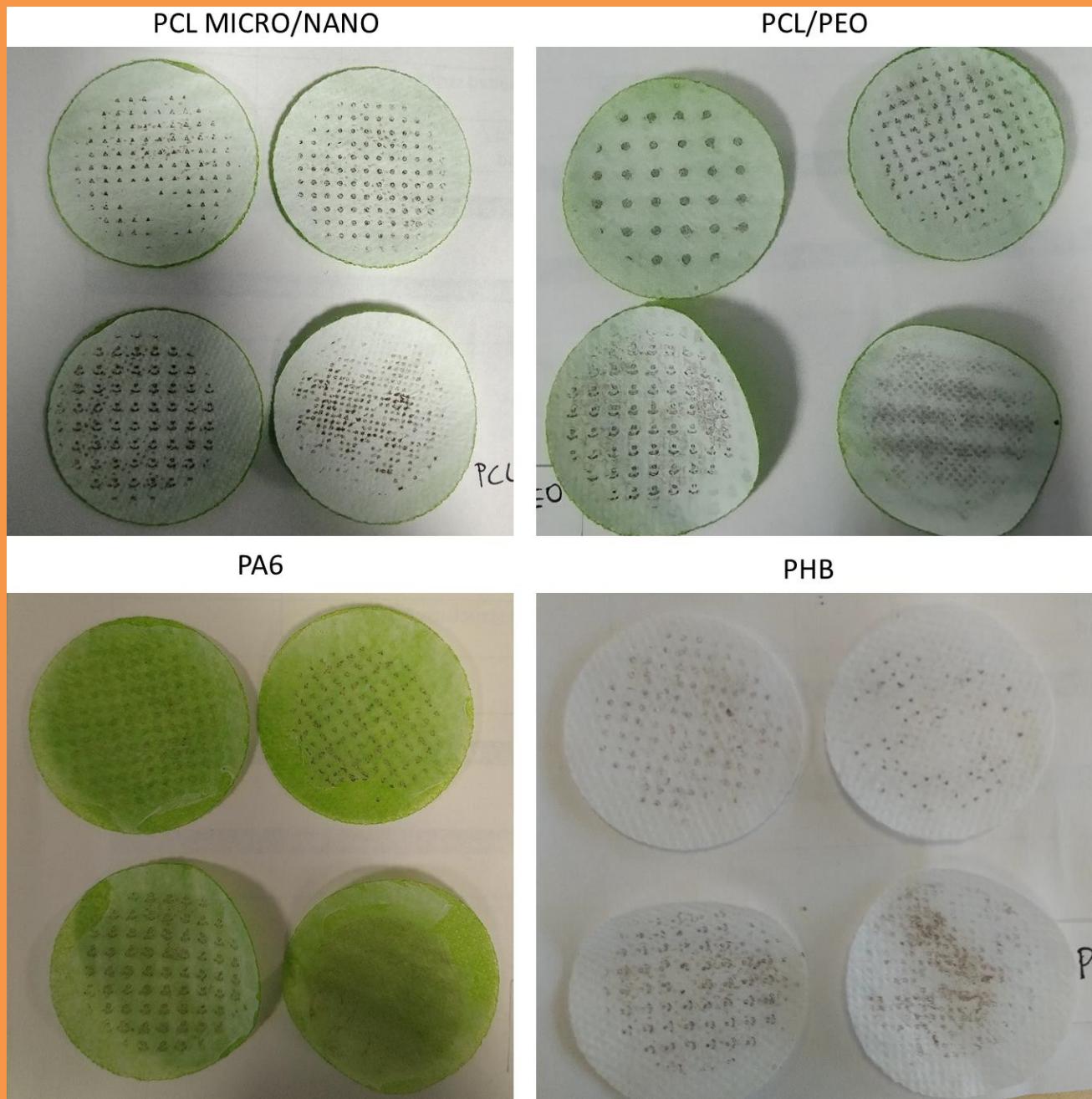




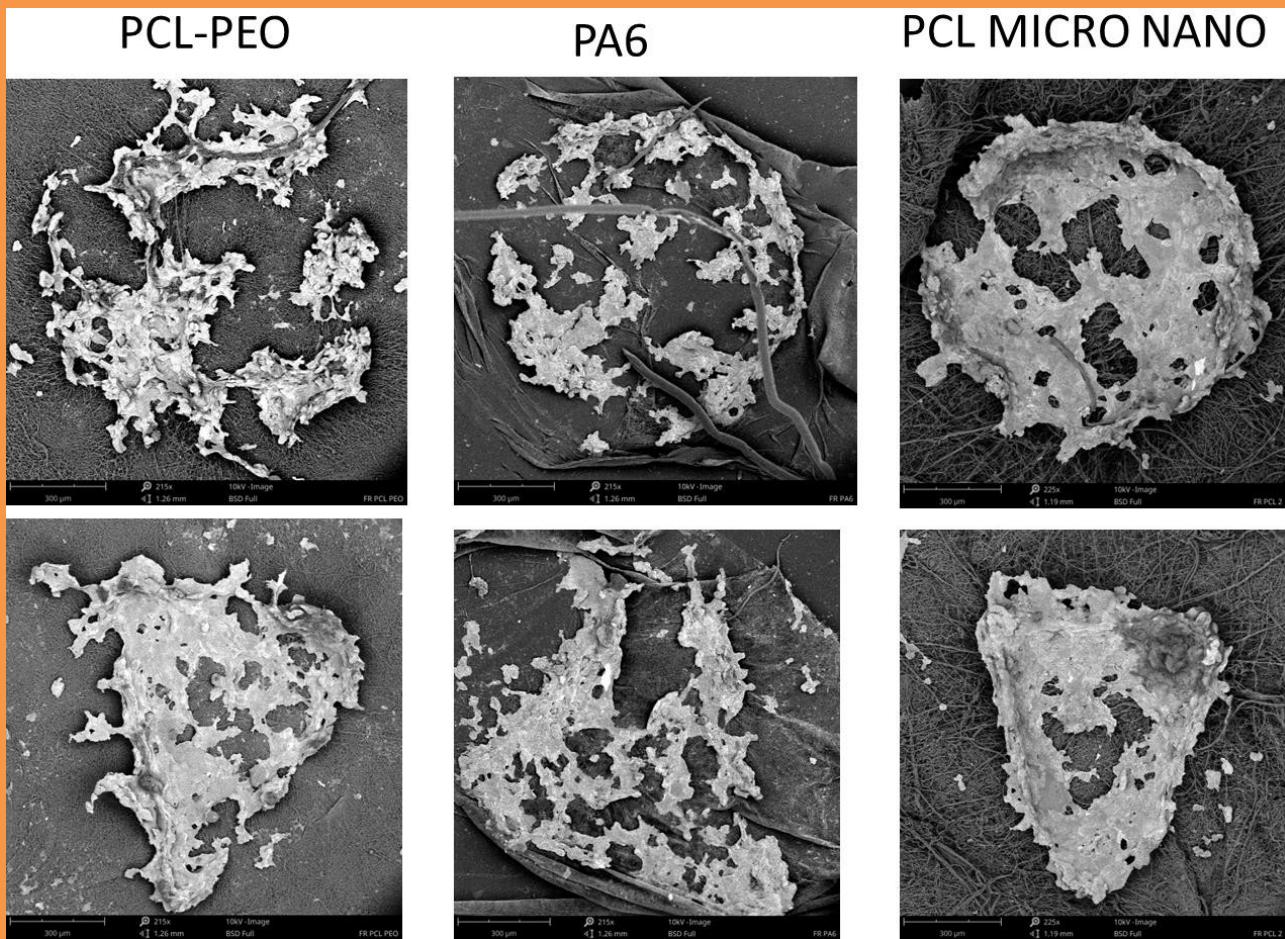
Quantitative analysis is provided in table bellow. The data show poor replication of aspect ratio (AR) in case of PHB. The reason is drying and shrinking of the material during the imaging process and sample preparation. In case of the other membranes, the replication was very precise with aspect ratios above 90%.

Shape	Foil	PHB	AR	PCL-PEO	AR	PA6	AR	PCL M/N	AR	PCL CAP	AR
Circle	1.43 0 ± 0.04 7 mm	NA	NA	1.367 ± 0.05 mm	96%	1.23 ± 0.036 mm	86%	1.75 ± 0.02 mm	122%	1.31 ± 0.06	92%
Triangle	1.59 ± 0.12 mm	0.79 ± 0.12 mm	50 %	1.39 ± 0.04 mm	87%	1.36 ± 0.07 mm	86%	1.54 ± 0.09 mm	97%	1.47 ± 0.03 mm	92%
Smily - eyes	1.34 ± 0.01 mm	0.85 ± 0.04 mm	63 %	1.34 ± 0.03 mm	100%	1.3 ± 0.01 mm	97%	1.35 ± 0.01 mm	101%	1.17 ± 0.05	87%
Smily - mouth - horizontal	2.33 ± 0.04 mm	1.64 ± 0.01 mm	70 %	2.23 ± 0.04	96%	2.29 ± 0.04 mm	98%	2.32 ± 0.12 mm	100%	2.35 ± 0.08 mm	101%
Smily - mouth vertical	0.73 ± 0.02 mm	0.43 ± 0.02 mm	59 %	0.79 ± 0.01	108%	0.69 ± 0.01 mm	95%	0.71 ± 0.01 mm	97%	0.71 ± 0.01 mm	97%

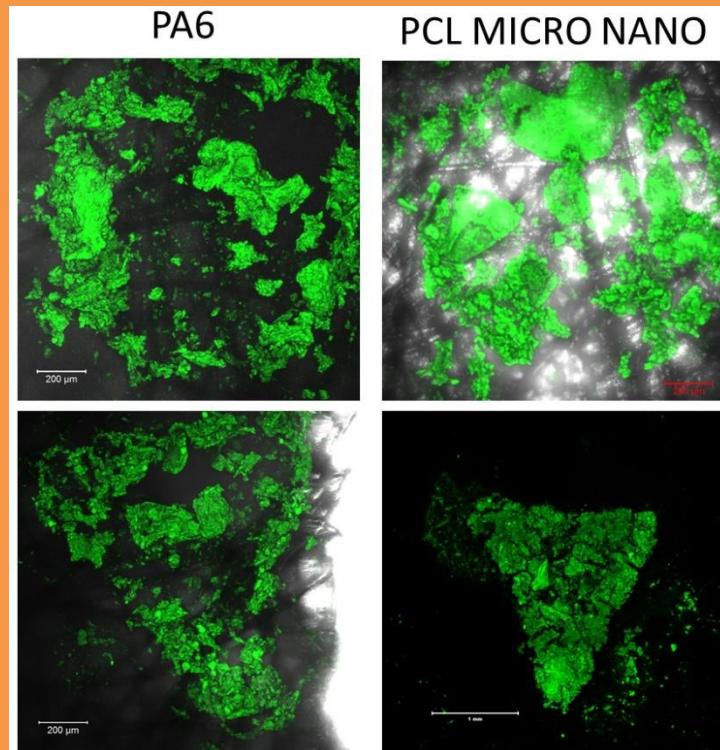
From a macroscopic point of view, the samples with the smallest patterns show nice pattern homogeneity. The right bottom sample from each group is the sample coated by Method 1. In the case of PHB, it is evident that the alginate feature peels off from the surface of the mesh. However, the resolution of printing and homogeneity seems to respect the template.



From the perspective of the sharpness of the features, we have performed SEM. It is important to note that samples for SEM need to be dehydrated before imaging – the particles, due to their hydrogel nature, showed preservation of the main feature size and shape. From the images is, visible sharp edges of the shapes.

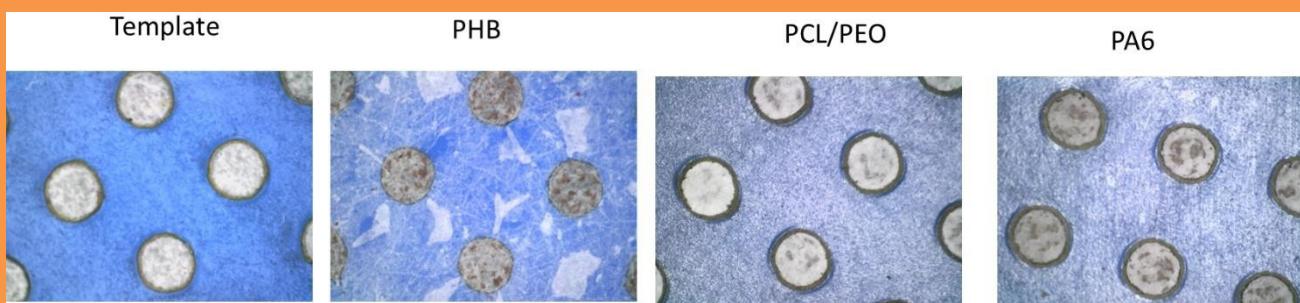
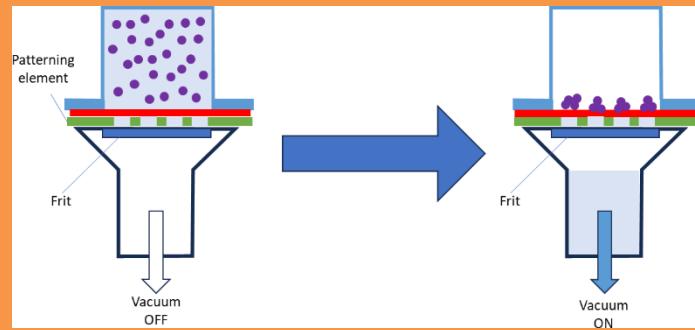


Similarly, laser-scanning confocal microscopy of FITC-dextran labeled particles shows preservation of structure and low accumulation of particles outside the pattern zone.



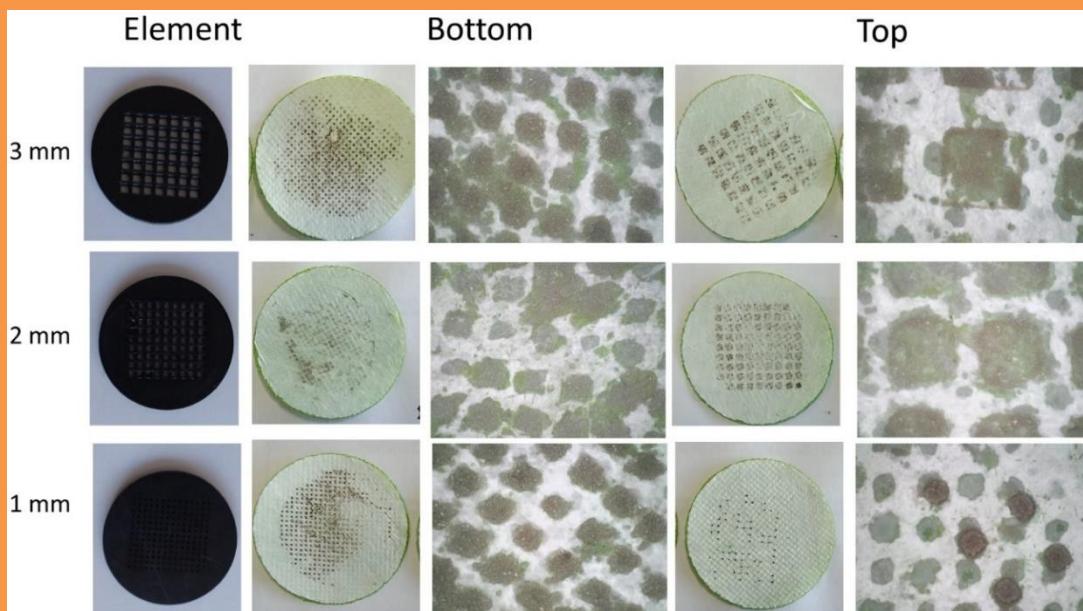
Mode 3 – bottom functionalization using patterned foils structured by CO2 laser engraving.

Applying a PET foil mask from the bottom on nanofiber membranes with support textile resulted in low pattern replication. Therefore, as an alternative, we have prepared a system, which is based on a foil with an adhesion layer directly in contact with nanofiber membranes. Perhaps the adhesive foil replaces the support textile. The experiments were performed in Mode 3 with the bottom application of the patterning system. The results showed good pattern replication. However, compared with TOP modification (Mode 2) using foil, the technology has lower practical benefits.



Utilization of 3D printed elements for patterning purposes

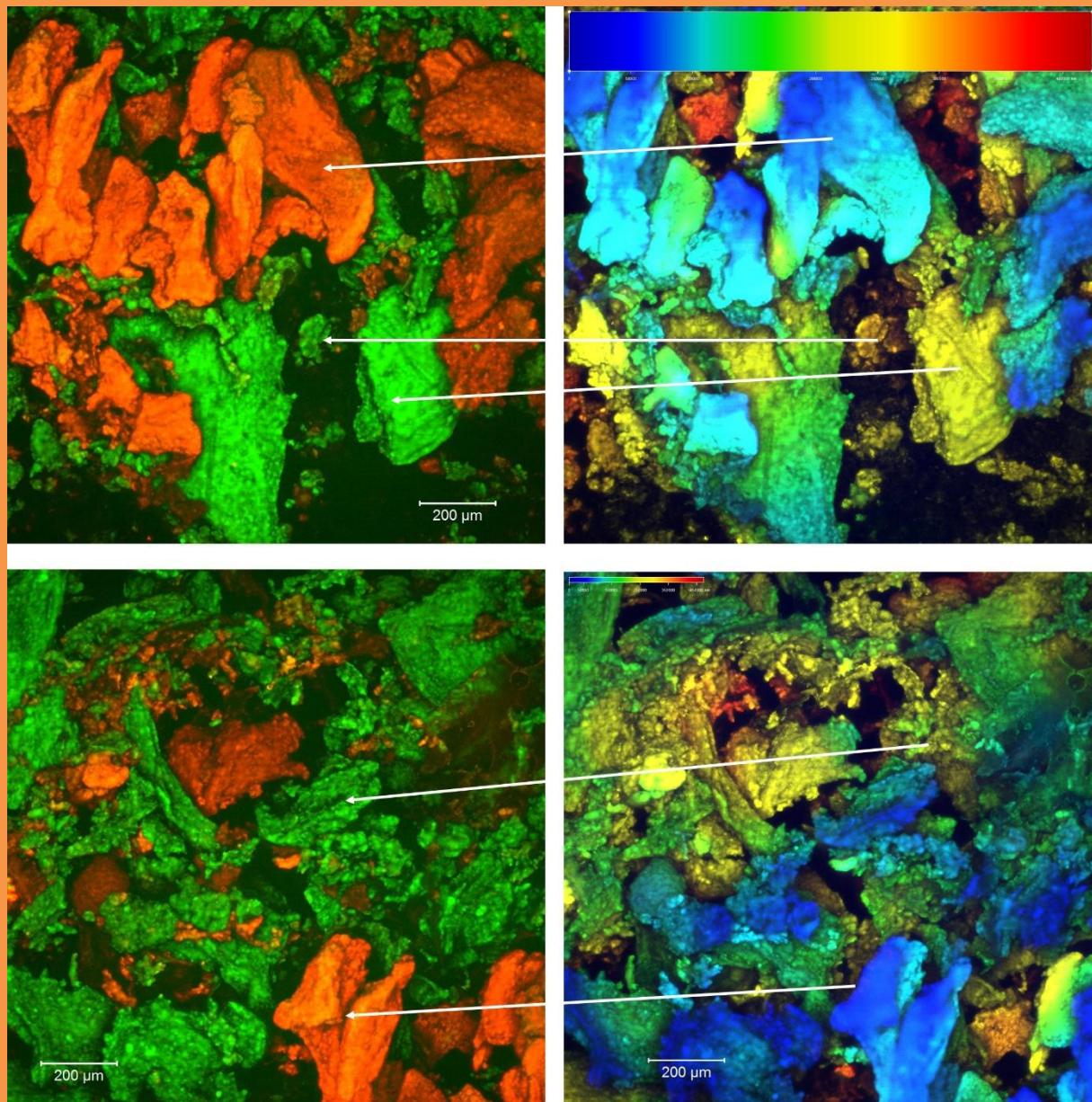
Besides the utilization of CO₂ laser cut forms, we have also evaluated the possibility of using FDM 3D printed patterning elements as an alternative. 3D printed elements were prepared from PET and consisted of a square array with a thickness of 3 mm and a square size ranging from 1 mm to 3 mm. We have tested the patterns in both TOP (Mode 2) and bottom (Mode 3) configurations. The results showed that the patterns were replicated, especially when using the top elements. The key issue observed was connected with the peeling of the formed feature of higher thickness upon the exchange of elements. 3D printed elements, whoever lacked the precision of laser printed elements and, are a secondary option for further use in the project.



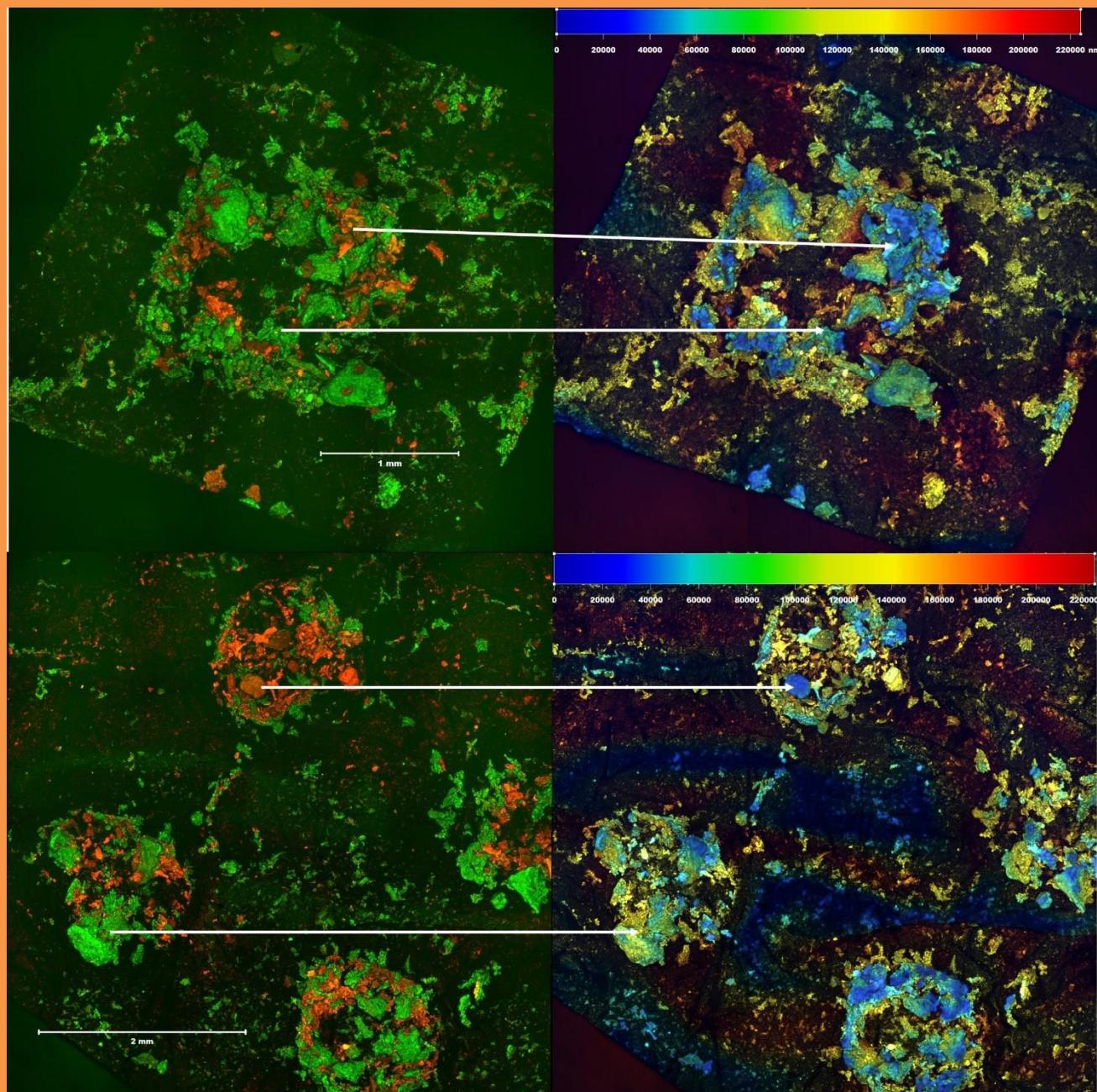
Multi-component systems: Layer-by-Layer (LBL) and Side-by-Side (SBS)

Finally, we have evaluated the possibility of forming multi-component systems using the VAC3DP technology.

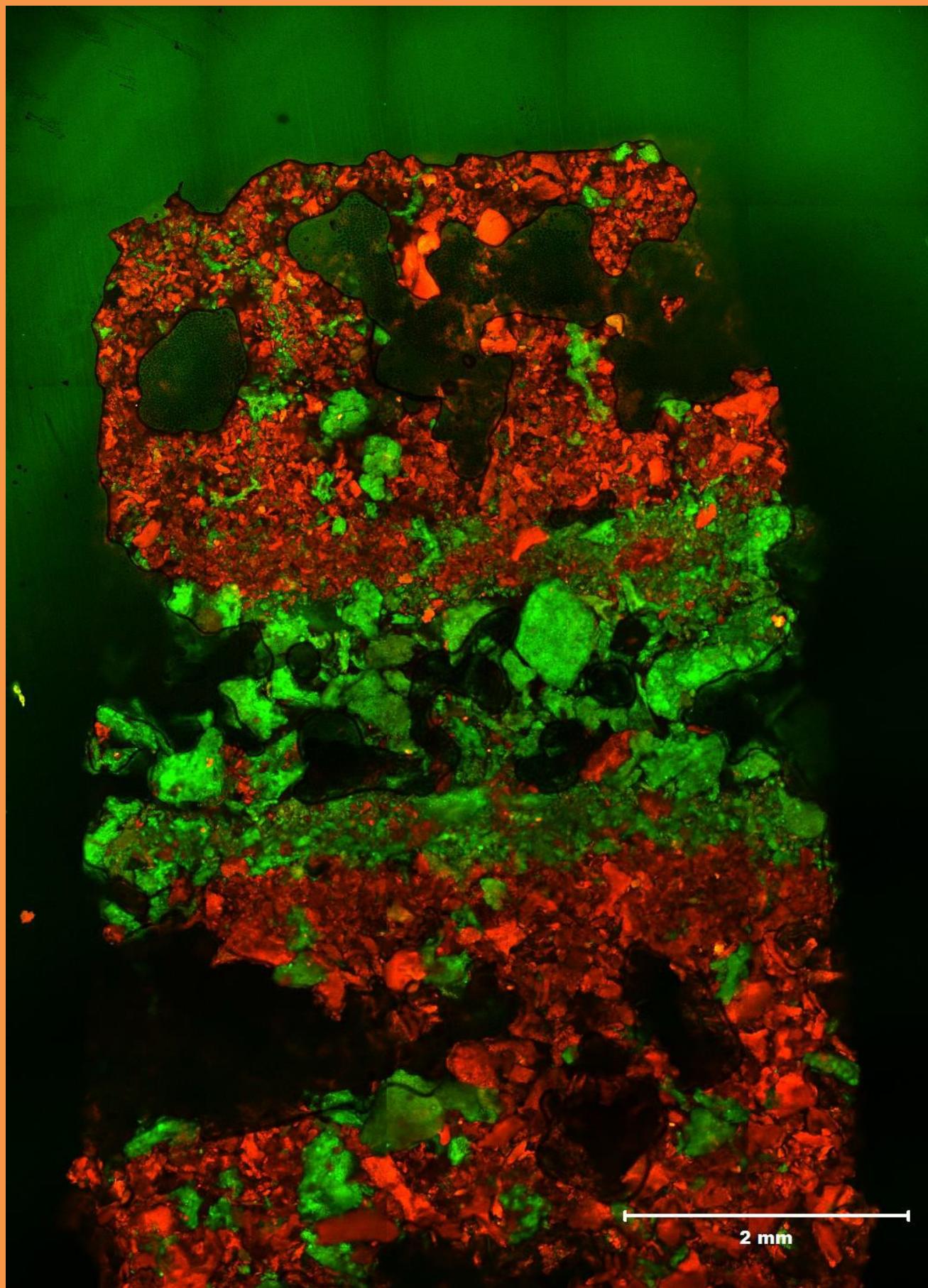
Layer-by-layer deposition was performed using 3D printed patterning elements in top configuration. The nanofibers from PCL were initially modified using 10 ml of a solution containing 2 mg/ml alginate microbeads labeled by FITC-dextran (green die, 1 mg/ml) followed by the addition of 10 ml of a solution containing 2 mg/ml alginate microbeads labeled by rhodamine G6 (red die, 0.5 mg/ml). Confocal microscopy (Zeiss LSM880 Airyscan) was used to obtain z-stack images. Images showed 2 particle populations belonging to two particle sets used. The color-coded visualization indicating the depth of particles showed that the green particles are located in deeper layers corresponding with the modification protocol. However, in some cases, we can observe some level of mixing probably caused by the distribution of particles during dosage and the re-suspension and mixing. Therefore, it is highly probable that for layer-by-layer systems, fixation by hydrogel elements would be necessary to maintain the layer organization and minimize fluctuation.



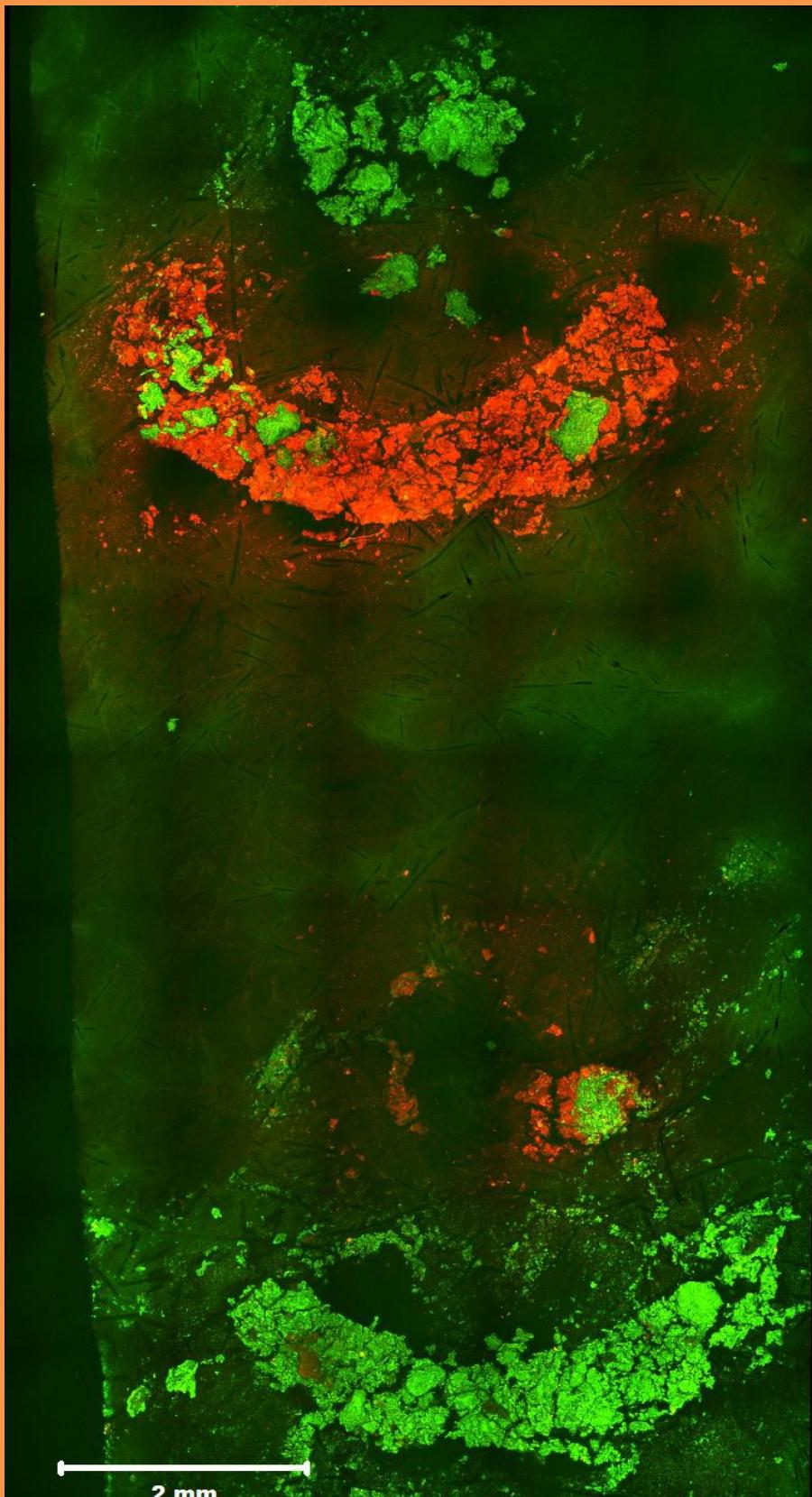
Similarly, we have tested LBL patterning using PET foil-based patterning elements in the same setup. The results were analyzed using confocal microscopy, showing the formation of LBL patterns.



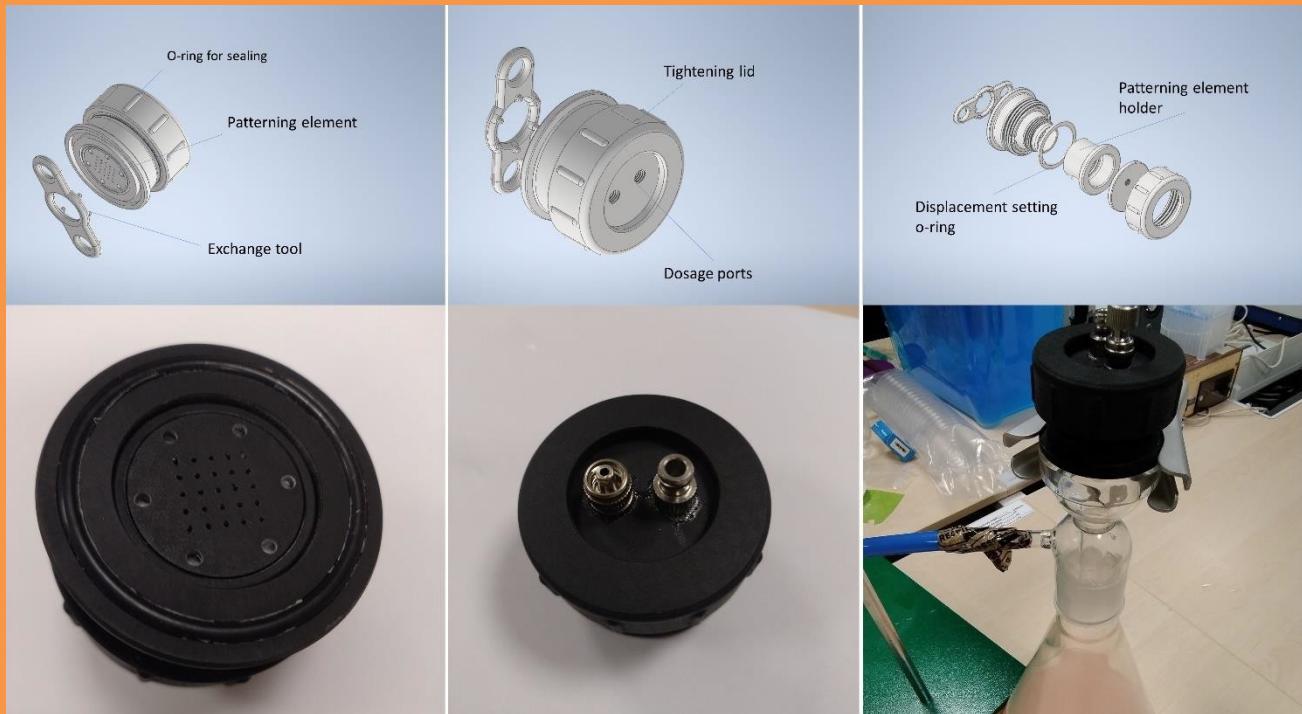
Regarding side-by-side (SBS) multi-component systems, we have utilized a set of CO₂ foils with complementary patterns. In the first case, a solution with the first type of particles (rhodamine G6) was used to print straight lines with defined gaps. In the second step, elements with complimentary lines filling the gaps were applied with FITC-dextran labeled particles. The result was tested on a confocal microscope. Imaging was performed in z-stack with scanning wider area (pattern of 8 x 4 images). The results indicate the successful formation of patterns. However, due to manual manipulation and positioning, the layers are in some spaces; the results demonstrate the SBS patterning capability of the technology.



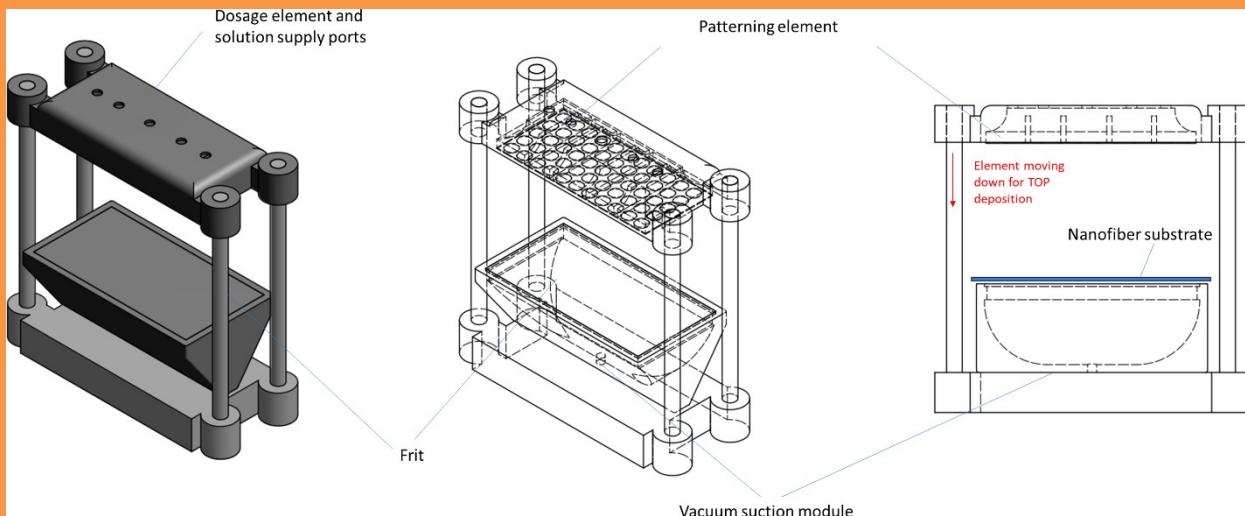
Finally, we prepared a smiley pattern with the exchange of color in the parts of the pattern – one smiley had green eyes and a red mouth, and the other had red eyes and a green mouth. The pattern with a size below 1 mm was printed, and the data indicate that even such small elements could be printed. However, as in the case of the first SBS example, automatic positioning will help to deliver further precision to the technology.



For experimental purposes, we have developed a 3D printed holder of foils to define displacement between foil and nanofiber layer. The prototype was 3D printed (Anycubic Photon M3 Max) using a proprietary resin formulation. The device will be used for prototyping purposes.



The insights gained during FUROID NF/VAC3DP are refining the concept for continuous VAC3DP. In IP Protocol 2, we will focus on building hardware and testing functionality.



Conclusions and future outlook

The work performed during FUROID NF/VAC3DP demonstrates potential of VAC3DP technology. Based on obtained results we consider the technology as main KER of the FUROID project with transversal applicability to fields such as medical devices, textiles, tissue engineering and cultured meat development.

In IP Protocol 2, we aim to focus on the testing of technology iPSCs and iPSC-derived organoids. In addition, we will focus on the formation of multi-layered systems and their combination with hydrogels (including photocrosslinking).

From a technical perspective:

- Nanofibers with different properties will be developed and systemically tested to decrease pressure drop and increase liquid flux through the fibers.
- Foil material will be optimized – during testing, we observed interaction between microbeads and foil, resulting in peeling of the printed pattern. As a solution, we will evaluate silicon molded systems and non-adhesive foils (i.e., based on fluoropolymer), ensuring low ripping and peeling of structures.
- System for laboratory-scale and industrial scale will be developed and tested. The system will be combined with a roll-to-roll system developed in Task 3.3. In the first phase, we will experiment with alginate microbeads.

Task 3.3: Development of roll-to-roll system including maturation reactors: During FUROID NF/VAC3DP, we did not execute direct work regarding Task 3.3. The work will commence with the development of a roll-to-roll system as indicated in the future outlook for Task 2.2. In next stage we will integrate the bioreactor systems and sensing system (in IP Protocol 2 especially design and primary prototypes not integrated to the roll-to-roll technology).

1.2.4 Executive summary of progress in Deliverables

The project undergoes serious management issues due to the non-performance of BIOFABICS. Due to this, the set of deliverables is delayed.

During FUROID NF/VAC3DP , the technology for wool, fur, and hair production per se was not advanced. The technological advancement in nanofiber membranes and VAC3DP are indirect components that fulfill these expected impacts. However, it is important to state that due to the non-performance of GENEUS Biotech, we have not made progress in developing organoids. Therefore, the progress (especially from the technical side) lags behind the plan.

Expected impact described in DoA:	Relevance of impact after FUROID NF/VAC3DP :
Industrialized process for wool production	<p>Development in the wool production market was not changed during FUROID NF/VAC3DP , and animal farming, with all the drawbacks (environmental, chemical pollution and ethical issues), is the only manufacturing technology for natural wool – the proposed impacts are still valid bases for business models in fur production.</p> <p>The wool market size is estimated at 36 billion EUR. The wool price fluctuates between 5.5-10 EUR/kg from conventional sources. From the price level, it is evident that our exploitation efforts will be focused on the tag “cruelty-free natural wool,” and the effort in IP Protocol 2 will be focused on building awareness about the technology in luxury and responsible fashion areas instead of commodity markets.</p>
Industrialized process for fur production	<p>Development in the fur production market was not changed during FUROID NF/VAC3DP , and animal farming, with all the drawbacks (environmental, chemical pollution and ethical issues), is the only manufacturing technology for natural wool – the proposed impacts are still valid bases for business models in fur production.</p> <p>The key drivers in the market towards alternative approaches are the ban (complete or partial) of animal farming for fur effective in most EU countries combined with negative user perception of natural fur use in the fashion industry. The mink price shows a significant drop and is sold for around ~ 25 EUR/m², and rabbit pelt for ~65 EUR/m² from conventional sources. From the price level, it is evident that our exploitation efforts will be focused on the tag “cruelty-free natural fur,” and the effort in IP Protocol 2 will be focused on building awareness about the technology in luxury and responsible fashion areas instead of commodity markets.</p>

	The exploitation strategy is discussed in Section 1.3.5.
Alopecia treatment using hair organoids	<p>The economic potential in allogeneic transplants of hair follicles remains similar to the state in DoA. Current technologies are based on autologous transplants of hair follicles.</p> <p>The market size is estimated at 5 billion EUR, and growth is expected in 2023-2032 (20% CAGR). The technology of culturing hair follicles is transformative from a market point of view for autologous and allogenic hair follicles.</p> <p>Regarding alopecia treatment, the technology needs evaluation regarding regulatory compliance with EMA guidelines. Since alopecia is a non-life-threatening disease, the use of iPSCs might face regulatory issues that need to be examined during IP Protocol 2.</p>

Impact importance in a broader context:

The connection to other impacts accounts especially relation to the social impacts of the project in the fields of cruelty-free production and environmental impacts as described in Societal aspects below.

Deviations:

Despite lagging in the progress in WP1, we are unaware of risks decreasing the potential to reach the specific impact or deviation in the market strategy. The potential future deviation may arise from the status of EMA on the application of iPSC-based therapies for alopecia treatment. If the status is negative, we must re-formulate the exploitation pathway for human hair follicle ELM and seek alternative application routes.

In addition, the development of the technologies resulted in new economic opportunities (detailed in D5.3 Plan for dissemination and exploitation including communication activities:

Impact of FUROID on wool, fur and hair production	
Novel application areas identified:	Relevance of potential impacts and development under the FUROID project
In vitro hair models	<p>The field of hair loss and alopecia treatment currently employs a series of products in cosmetics and pharmaceuticals. However, the</p> <p>The utilization of FUROID hair models for in vitro testing is aligned with the development of hair constructs for hair follicle transplantation and does not require changes in the actual experimental plan. The models might be a</p>

	<p>byproduct of developed technology with a faster pathway to market.</p> <p>The innovation scoring (D5.3 section 3.2.2) showed high technology readiness after the project. From an economic point of view, the technology will compete with reconstructed skin models (i.e., MatTek EpiDerm FT – skin model without hair) with a price per well of around 25 euros. FUROID project can deliver a real hair model replacing human testing at a competitive price.</p> <p>The commercialization and market entry for in vitro models will be much easier compared to MedTech or Pharma markets with no requirements for clinical trials.</p> <p>The market adoption will be fostered by the need for alternative models to animals due to the ban on animal testing for cosmetic products in the EU, US, and China as key cosmetic markets.</p>
Wound healing	<p>The application of VAC3DP technology and closed-loop manufacturing platforms in wound healing was already mentioned in the DoA.</p> <p>The developed technology will be compatible with skin cells. However, this application is outside the scope of the FUROID project and will be subject to further analysis under another project scheme (for instance, the EIC Transition project). The technology should work especially with autologous human cells and/or platelet-rich plasma for a more straightforward application.</p> <p>Preparedness for application at the end of the project will be low and will require subsequent research and development.</p>
Bio-artificial leather	<p>The application of VAC3DP technology and closed-loop manufacturing platforms in wound healing was already mentioned in the DoA.</p> <p>The technology might be adapted for combination with skin cells or mycelium for non-animal leather. The technology is out of the scope of the current project and might valorize the developed hardware/software technology in</p>

	<p>the future. The market application will be exploited in a separate project scheme.</p> <p>Preparedness for application at the end of the project will be low and will require subsequent research and development.</p>
Cultured meat	<p>The field of cultured meat is a highly relevant topic, and developed technology might be utilized in the final phases of cultured meat product production (formation of multi-layered patterned structures – condensation to meat-like cell sheets).</p> <p>The application is out of the scope of the FUROID project. Preparedness for application at the end of the project will be low and will require subsequent research and development.</p>

Regarding deviations, the project is undergoing management issues with GENEUS Biotech as a non-performing partner. Therefore, the development of organoid technologies is lagging behind the initial plan. The consortium is performing changes in composition and DoA not affecting the primary goals of the project, and the progress in associated objectives and impacts should be aligned with timeline during IP Protocol 2.

1.3.3 Impact on citizens and society:

Environmental impact of FUROID	
Status and deviations	
The environmental impacts of FUROID are associated with LCA analysis of the technology and cannot be quantified in the current stage of the project. Based on the relevance analysis for proposed impacts, we do not see any deviations after FUROID NF/VAC3DP .	
Expected impact described in DoA:	Relevance of impact after FUROID NF/VAC3DP :
Decreased emissions compared to standard wool and fur production: The use of toxic chemicals and high environmental footprint is connected especially with wool production due to its worldwide consumption and methane emissions by sheep.	Developing wool and fur production with lower emissions than the current industrial technology is essential for positive market entry of the “bio-engineered wool.” To meet the environmental goals, we have oriented our efforts in FUROID NF/VAC3DP on utilizing polymers and solvents with a low environmental footprint in the production process. For instance, we have eliminated or decreased toxic solvents such as dimethylformamide, fluorinated and chlorinated solvents. Moreover, the PA6 nanofibers utilized in the project are from recycled PA6.
Reduction of animal use:	
Status and deviations	
Reduction of animal use for wool and fur production and replacement by industrial animal-free production process is the key goal of the FUROID project. The development in FUROID NF/VAC3DP does not change the relevance of the goal and expected impact.	

Expected impact described in DoA:	Relevance of impact after FUROID NF/VAC3DP :
Replacement of animals used in wool and fur production	<p>The project aims to demonstrate wool and fur production using FUROID technology. The development in FUROID NF/VAC3DP was focused on the validation of key parts of the technology.</p> <p>Due to the delay in WP1, we had not yet evaluated the combination of nanofibers with iPSC organoids using VAC3DP technology. However, the results from testing using alginate microbeads indicate that the technology will enable the formation of fur and wool and the impacts for their commercialization until 2030 are realistic.</p>
Protection of endangered animals:	
Status and deviations	
<p>Due to a delay in organoid development, the experiments regarding the traceability system have not been tested yet. Similarly, the project is focused on animal species which does not belong to endangered animals. Translating technology to endangered animals is mainly connected to the availability of proper iPSC cell lines, knowledge of genome sequences and molecular similarities in the physiology of fur follicle formation.</p>	
<p>Formed fur follicles from endangered animals should be compatible with the technology; however, reaching such systems in the current setting is not realistic. Therefore, FUROID will create enabling tools for exotic animals, and the technology for forming pelts should be universal. However, the practical development of materials from endangered animals is subject to further scientific advances.</p>	
Expected impact described in DoA:	Relevance of impact after FUROID NF/VAC3DP :
Gene-encoded traceability system.	<p>During FUROID NF/VAC3DP , we have not performed any steps toward the gene-encoded system in iPSCs. However, it is important to note that the gene encoding of specific iPSC lines is manageable, and every gene modification creates new barcode systems as a target for particular probes.</p>
Strengthening the ELM position in Europe:	
Status and deviations	
<p>The work performed in the project during FUROID NF/VAC3DP resulted in 2 results – the transformation of VAC3DP technology to a validated laboratory prototype stage and the development of a novel high-performance PA11/PVB nanofiber membrane. The first innovation was created by the joint cooperation of GENEUS SAS and FUROID, increasing the bonds between partners from Portugal and the Czech Republic.</p>	

From the portfolio perspective, during FUROID NF/VAC3DP, we integrated our team into the ELM Portfolio and actively participated in meetings/events organized by the portfolio. In addition, we intend to intensify the cooperation with partners and propose cooperation on VAC3DP technology as a novel enabling tool in the ELM market segment. Specifically, VAC3DP technology might be interesting for LoopOFFun project development of living air filters, NextSkins for living skin products and Prism- LT for cultured meat applications.

1.3.4 Impact on young participants and early-stage researchers:

FUROID project employs research and development tasks for young researchers (Continuous Project Reporting, tab Researchers). During the project, a special focus was placed on expanding young researchers' careers. For instance, Elcin Toren from FUROID is a researcher responsible for developing VAC3DP technology. During FUROID NF/VAC3DP, she was directly involved in RnD activities, experimental design and analysis. The activity helps her build research and management skills and will help her obtain novel results (publications, patents) important for her future career.

1.3.5 Identification of Key Results

The project results obtained so far are described in the Continuous Project Reporting tab Results.

In addition, we have performed an analysis of Key Exploitable Results (KERs) for FUROID NF/ Engineered fur and wool

Description:

The engineered fur/wool product is based on a combination of the nanofibrous membrane with fur or wool follicles. The follicles are based on cultured cells and formed de novo – the follicles are not from animal origin per se. The fur/wool forms a confluent layer resembling native fur/wool.

USPs

- Non-animal fur/wool from non-artificial source – cruelty-free product
- Natural structure of fur/wool follicles with their properties (insulation etc.)
- The width of the product is not limited by animal size.

Application area:

Fashion/textile industry as cruelty-free replacement of fur/wool from native sources. Currently, we will focus on rabbits and sheep – in the future, we will expand to exotic/endangered animals.

Key stakeholders:

Big luxury textile/fashion brands – LVMH, Gucci, Chanel, Versace, Michael Kors, Ralph Lauren, Prada.

Innovation score:

	Price per product	Market size	Readiness after project	Total
Fur	5	1	3	3
Wool	1	3	2	1.7

Engineered hair

Description:

The engineered hair product is based on a combination of nanofibrous membranes with hair follicles. The method can be used for maturation and increased expansion of follicles. The follicles, after expansion, are harvested and re-operated. The key problem is the regulatory status of stem cells or iPSC-based follicles for reoperation. The alternative application is an in vitro model for hair biology, pharmacology and cosmetics.

USPs

- High concentration of follicles and cells
- Oriented growth of follicles before implantation
- The tissue might be used for testing drugs in combination with the immune system.

Application area:

Hair therapy is a primary application area. The application is expected for autologous follicle development or low-immunogenicity follicles.

Secondary application as a model for in vitro hair biology research cosmetic and pharmaceutical testing.

Key stakeholders:

Hair replacement – medical providers of hair transplantation.

In vitro model – big pharma companies (Pfizer, Merck, Bayer, AstraZeneca)

Innovation score:

	Price per product	Market size	Readiness after project	Total
Hair transplant	3	3	2	2.7
In vitro hair model	2	2	5	3

VAC3DP technology**Description:**

Vacuum-assisted 3D printing is the enabling technology developed within the project. The technology enables rapid micropatterned deposition of cells/particles to nanofiber substrate using vacuum. Nanofibers serve as a filter material and support for cellular growth and maturation. The project will result in pre-industrial scale hardware enabling the processing of 40cm wide rolls. The hardware will be in finished device format and can be utilized to form wool, fur and hair ELMs. However, the technology could be expanded for further applications.

USPs

- High concentration of particles or cells.
- Resolution better than 1 mm.
- On-demand particle/cell patterns.
- Combination of multiple cell/particle populations in a single run.
- Continuous roll-to-roll process.

Application area:**Hardware:**

Hardware may be sold to end users for their development purposes – the business model would be providing the device and supplying the nanofiber material and shape templates as a paid refill option.

Specific applications:

- Wound healing - burns – formation of skin cell constructs for treatment of large area burns.
- Wound healing – non-healing wounds – formation of platelet-enriched wound dressings with autologous growth factors.
- Artificial leather – formation of patterned skin deposits for exotic leather formation
- Mycelium-based leather – printing of fungal cells to form patterned mycelium.
- Cultured meat printing technology - formation of meat/fat constructs for the native-like meeting texture.

Key stakeholders:

The stakeholder structure depends on the specific application:

- Wound healing – medtech companies – Covidien, Hartman, J&J
- Leather - LVMH, Gucci, Chanel, Versace, Michael Kors, Ralph Lauren, Prada
- Cultured meat – Mosa Meat, Good Meat, Unilever

Innovation score:

	Price per product	Market size	Readiness after project	Total
Hardware	2	1	5	2.7
Wound healing	3	3	2	2.7
Leather	2	2	2	2
Cultured meat	1	3	2	2
In vitro hair model	2	2	5	3

Bioreactor technology**Description:**

Bioreactors will be integrated with the VAC3DP device. The bioreactors will be based on perfusion flow and nutrient supply. The unique feature will be based on material cultivation as a sheet. The bioreactor bed will be based on chambre with efficient culture chambre volume and shape, enabling follicle maturation and growth.

USPs

- Cultivation in the form of rolls.
- Optimized shape for low media consumption.
- Build-in perfusion system enabling the Exchange of nutrients and waste products.

Application area:**Hardware:**

Hardware will be used alone or in combination with the VAC3DP device.

Specific applications:

The connected to VAC3DP for the moment:

- Wound healing – non-healing wounds – formation of platelet-enriched wound dressings with autologous growth factors.
- Artificial leather – formation of patterned skin deposits for exotic leather formation
- Mycelium-based leather – printing of fungal cells to form patterned mycelium.
- Cultured meat printing technology - formation of meat/fat constructs for the native-like texture of meat.

Key stakeholders:

The stakeholder structure depends on the specific application:

- Wound healing – medtech companies – Covidien, Hartman, J&J
- Leather - LVMH, Gucci, Chanel, Versace, Michael Kors, Ralph Lauren, Prada
- Cultured meat – Mosa Meat, Good Meat, Unilever

Innovation score:

	Price per product	Market size	Readiness after project	Total
Hardware	2	1	5	2.7
Wound healing	3	3	2	2.7

Leather	2	2	2	2
Cultured meat	1	3	2	2

4. EIC Specific Activities: exploitation/regulatory services

HORIZON, EIC AND EMA FREE SERVICES:

Members of FUROID have not utilized any of the free services during FUROID NF/VAC3DP . During IP Protocol 2, we plan to use EMA services for consultation on the iPSC-based hair follicle technology. In addition, we plan to utilize the EC service for IPR help to ensure proper protection of VAC3DP technology.

EIC transition application is planned with the topic of wound healing. The application will further expand the applicability of VAC3DP technology. The application will involve GENEUS SAS and FUROID from the FUROID consortium. The technical and business objective will be the miniaturization of the technology for medical device class I/II utilized to form cell sheets and platelet sheets directly on the operator's bench or standard bio-bank environment. The technology will enable the formation of cell sheets without the support of a scaffold and will speed up the tissue maturation process.

5. ELMs portfolio activities

1. Technology Portfolio activities

The information about Technology Portfolio activities is included in Deliverable D7.3.

2. Environment and Sustainability Portfolio Activities

Executive Summary

A) Contribution from the Environment and Sustainability WG

The first task for the Environment & Sustainability workgroup, as listed in the Portfolio strategic plan, was to provide a **Utility Mapping to specific environmental and sustainability issues being targeted (explicitly/implicitly) within each project domain**. This utility mapping is now complete. It can be found hereafter.

Project	Issue 1	Issue 2	Issue 3	Issue...n	Sector	Existing solutions	ELM Approach	ELM value proposition in comparison to existing
Biorobot-Minheart	Medical drug development in heart sector requires increased effort and resources due to lack of highly representative model system #1	Reduction of animal testing as a possible model system for drug development	Using the model system to optimize dosage and delivery reduces the overall quantities of drug production and its resource consumption		Biomedical, pharmaceutical	Animal testing, machine learning, immortalised cell lines.	Creating ELM model system for human heart to facilitate medical drug development	Less resource intensive, more precise and accurate method hence less failure rates in drug discovery process and consequently lower chance of losing all R&D done until that point, no animal testing involved.
	The development and fabrication of conventional environmental sensor requires processes based on high energy consumption #2	ELM approach to environmental sensing would reduce non-biodegradable waste in contrast to standard sensor technology	Lack of good cardiac sensitive testing for environmental toxicants		Environmental, Sensors/technology	Animal cells that don't fully represent the human cells and immortalised cell lines that have very poor sensitivity to toxicants	Creating ELM Biorobot to sense environmental conditions and give corresponding feedback	Human relevant response. Biodegradable, removing the need for external stimulus allows for greater flexibility compared to previous soft bio-robots
FUNGATE RIA	Maternal environmental Site contamination #1	new material approaches reducing reliance on fossil fuel based and/or extractive approaches in the production of materials for construction	energy saving across production (embodied energy) operational energy use and end-of-life energy requirement		Built environment	Excavation and removal. Soil vapour extraction (SVE). In-situ chemical oxidation. Solidification/stabilisation. Bioremediation.	Bioremediation using living fungi	Technology fitting within existing bioremediation approaches. Greater control of design could improve deployability and effectiveness.
	Maternal environmental Build-up of carbon dioxide through carbon emissions #2	new material approaches reducing reliance on fossil fuel based and/or extractive approaches in the production of materials for construction	energy saving across production (embodied energy) operational energy use and end-of-life energy requirement		Built environment	Green roofs and living walls. Carbon-negative materials. Carbon capture and storage (CCS) in production of materials. Reclaimed and recycled material use. Carbon sequestering materials.	Carbon sequestration performed by ELM skin as building surface	Additional technology within the carbon sequestering materials, potentially targeting additional areas/surfaces of buildings.
Furoid	Maternal environmental Animal cruelty is a main issue in this field, fur and wool breeding animals are extremely cruel and inhumane treatments and processes, held under conditions not appropriate for the species #1	High GHG emissions at farm	extensive and toxic processing of raw material, from tanning to coloration	social inequalities and labor exploitation because of pricing pressures across the complete vertical supply chain	Biomaterials for fashion, textile and automotive applications	Plantbased and (oil based) synthetic polymers	Creating a cruelty free scalable FUROID and wool material	enhanced (comparative) properties, no limitations in coloration and patterning, no tanning or chemical coloration required, as pigmentation can be introduced in culture free and with inseparable anti counterfeit measures.
	Maternal environmental There's no effective treatment against alopecia, the prevalence is extremely high, however since it's considered an inherited genetic condition alopecia is often not perceived as a medical condition #2	chemical treatments are not lasting also they cause severe side effects from Diabetes Mellitus TYPE 3 to impotency. No toxicological relevant test model exists.	Conventional interventions such as hair transplantsations are limited by the amount of available donor hair which is not receptive to DHT (dihydrotestosterone) up regulation	Even transplanted hair is often damaged by rejection (white scarring) or affected from apoptosis because of insufficient conditions in the reception/transplantation area	Biomedicocal, personalised tissue engineering, toxicity, cosmetics assays	Conventional hair transplantation and DHT blockers with grave side effects- cosmetic assays	Bioprinting of allogeneic hair follicles and scalable testing models with reduced batch variability	Scalable and stratified human hair follicles with all necessary appendices (such as psi muscles, formed hair shaft, formed DP and hair bulge, usable for huge compounds libraries and toxicology testing
Loop of Fun	Maternal environmental Producing living wood composites eliminates reliance on a product of petrochemistry and are sustainable and recyclable materials with desired properties #1	No			Wood composite	Particle boards using petrochemicals like urea formaldehyde	Wood composite materials using engineered living fungi	without using petrochemicals, material stiffness is controllable by load and light. Materials are sustainable and recyclable
	Maternal environmental Producing filters with tunable porosity is key in resource-saving lightweight materials #2	Self-renewal of air filters is a key property to maintain sustainable material resources			Bioremediation/Volatile organic compound removal	sensing is done manually with lab equipment. Porosity cannot autonomously be adjusted at clogging sites.	Filter materials using engineered living fungi and bacteria	It prolongs a filter lifetime by closed-loop anti-clogging control. Engineered living filter materials autonomously sense and adapt porosity. It autonomously increases porosity in clogged regions.
Next Skins	Maternal environmental Skin care and therapeutic products are often (partly) derived from renewable resources. A challenge is to find an alternative that generate less non-biodegradable waste. #1	sense-respond abilities of living skin raw materials, challenging to achieve with easily available elements	energy required for operation of sense-respond technology		Biomedical	sensing is done with lab equipment, sample must be taken at each timepoint	bacterial cellulose membrane hosting yeast cells that sense pathogens and therapeutically respond	continuous sensing and responsiveness is an intrinsic quality of the ELM
	Maternal environmental Strong and tough materials for everyday use often made from fossil fuels. A challenge is to find an alternative production that generate less non-biodegradable waste. #2	end-of-life energy requirement.			Everyday product, civil/automotive/aerospace/etc. industry	recycled plastics, bioplastics, bio-based materials	biomineralized bacterial cellulose with sensing and self-repair functions	less energy to produce (harnesses growing biological systems) and uses renewable sources as feeding elements self-repair quality increases lifetime of product
PRISM-LT	Maternal environmental Biodegradable and Renewable materials. Mycelium made from living organisms, which can self-reproduce, regenerate, and are inherently biodegradable. This contrasts with many traditional materials that can contribute to pollution and waste #1	The project is focused on using 3D bioprinting for the creation of ELMs. This additive manufacturing process minimizes waste as it only uses the amount of material needed, potentially reducing the subtractive manufacturing processes that remove material to shape the final product.	In the context of food production, ELMs can be developed to create plant-based or lab-grown alternatives to meat, which could reduce the environmental footprint of food production. Meat production is a major contributor to greenhouse gas emissions, water use, and land use, and alternatives could help mitigate these impacts.		In the field of tissue engineering, ELMs could lead to more efficient production of tissues for medical applications, potentially reducing waste associated with traditional tissue culturing methods.	Healthcare/Tissue engineering / food manufacturing	PRISM's ELM approach uses 3D bioprinting and engineered helper cells to create biolinks for the controlled production of live tissues, with potential applications in both medical tissue engineering and food production.	PRISM's ELM approach offers the potential for highly customizable, scalable, and sustainable production of living tissues, providing more precise control over cell differentiation, reducing dependency on traditional tissue sourcing or synthetic materials, and introducing environmentally friendly alternatives to conventional manufacturing and food production methods.
	Maternal environmental The development and adoption of ELMs could potentially reduce environmental pollution and waste related to traditional manufacturing materials. #2				ELMs have the potential to perform functions under normal environmental conditions without the need for high energy input, as they can grow and self-repair. In contrast, traditional materials often require high energy input for manufacturing and repair.			

Next, the Environment & Sustainability workgroup now focuses on its first objective, which is to **assess the environmental impact and sustainability of ELMs and provide a fact-based argument for policy makers and investors of the positive contribution of ELMs**. A first step in that direction is to organise online workshops on standardization and life-cycle analysis (LCA) to provide the necessary knowledge to carry out a brainstorming session over environmental analysis strategy. Three online workshops have been planned and given by the Horizon Standardization Booster (HSbooster):

Workshop #1 : Standards and how to influence standardization using ELMs relevant standards/committees as examples

Date: 19th of September 2023 (time 10:30-11:30)

Workshop #2 : CE-marking products for the European market

Date: 9th of October 2023 (time 14:00-15:00)

Workshop #3 : Life Cycle Assessment standards

This workshop starts with an introduction on LCA by Mirko Busto (LCA expert part of Prism-LT project), followed by a workshop on LCA standards given by HS Booster.

Date: 30th of October 2023 (time 10:00-11:30)

B) Specific contribution from the members of the project

Members of FUROID project did not actively participated in activities within FUROID NF/VAC3DP .Project representatives passively attended organized webinars organized by the ELM Portfolio workgroups.

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SECTION 2: OPEN SCIENCE

Open-science practices are described in deliverable D5.2 Data Management Plan in section 3 FAIR data. The envisaged research and development tasks generate data adhering to FAIR principles. The data obtained in the project will be primarily deposited in a protected cloud repository opened for all partners within the FUROID project. Upon the data use, the data will become either:

1. Data linked to KERs with strategic meaning for protection of know-how. Data will not be made available so as not to jeopardize the exploitability of the project results. The data with this flag are subject to confidentiality, and all project partners need to ensure their proper protection. The embargo period for data is based on specific conditions required to exploit project results. The subset of data may become part of a “trade secret” and will not be provided openly.
2. Data linked to publications. Data connected with planned publication will not be provided openly before publication. Upon publication, the data will be deposited together with publication using OpenAire, Zenodo and EUDAT B2BSHARE. The embargo period for data will be 3 months to re-evaluate the conflict with “trade secret” information.
3. Opened data for immediate sharing. Images, photographs, infographics and schemes not containing sensitive information will be published online on the opened SharePoint database or EUDAT B2BSHARE.

Specifically we are expecting the following scheme for data generated in the project per WP:

WP	Task	Data generated	Category	Expected opened release	Key partner
Work package 1	Task 1.1: Isolation of skin cells from animal sources and generation of iPSCs	Cultivation protocols	1	Trade secret	GENEUS
		iPSC cell lines	1	Trade secret	GENEUS
	Task 1.2: Development of hair, wool and fur follicles	Cultivation protocols	1	Trade secret	GENEUS
		Gain-of function genes identified	1	Trade secret	GENEUS
	Task 1.3: Genetic manipulation of the cell lines	Relation of genes vs. their effect of fur/wool/hair production in vitro	2	Upon publication	GENEUS

Work package 2	Task 2.1: Development and biocompatibility testing of nanofibers	Nanofiber spinning protocols – not intended for commercialization	2	Upon publication	GENEUS
		Nanofiber spinning protocols – intended for commercialization	1	Trade secret	GENEUS
		Biocompatibility of nanofibers	2	Upon publication	GENEUS
	Task 2.2: Development of patterned collectors	Nanofiber morphology vs. collector design	2	Upon publication	GENEUS
		Belt-collector system	1	Upon IP protection	GENEUS
		Cultivation data – nanofiber patterning vs. cell growth	2	Upon publication	GENEUS
	Task 2.3: Active scaffold development	Active scaffolds with release mechanism governed by core/shell structure	2	Upon publication	GENEUS
		Specific formulations supporting differentiation of organoids or physiological effects with commercial importance	1	Trade secret	GENEUS
		Specific formulations supporting differentiation of organoids or physiological effects with commercial importance	2	Upon publication	GENEUS
	Task 2.4: Development of on-demand degradable nanofiber layers	Nanofiber layers with on-demand degradation – commercially important formulations	1	Trade secret	GENEUS
		Nanofiber layers with on-demand degradation – without commercially importance	2	Upon publication	GENEUS
	Task 2.5: Development of waterproof-coatings on bioengineered ELW and ELF	Nanofiber layers with water repellence – commercially important formulations	1	Trade secret	GENEUS
		Nanofiber layers with water repellence – without commercially importance	2	Upon publication	GENEUS

	Task 2.6: Development of DBTL for nanofiber production and interaction	DBTL protocols and software	2	Upon publication	FUROID
Work packa ge 3	Task 3.1: Adaptation of standard extrusion 3D bioprinting technology (E3DP)	Bioprinted constructs on nanofibrous mesh	2	Upon publication	FUROID
	Task 3.2: Development of VAC3DP	Design of laboratory prototypes of VAC3DP	1	Upon IPR protection and publication	FUROID
		Validation results of laboratory prototypes	1	Upon IPR protection and publication	FUROID
		Multilayered hydrogel constructs including crosslinking	1	Upon IPR protection and publication	FUROID
		Up-scaled prototypes and final hardware	1	Trade secret	FUROID
		Validation of up- scaled prototypes	2	Upon IPR protection and publication	FUROID
	Task 3.3: Development of roll-to-roll system including maturation reactors	Bioreactor designs and their verification - without commercial importance	2	Upon publication	GENEUS
		Bioreactor designs and their verification – with commercial importance	1	Trade secret	GENEUS
		Protocols for DBTL platform	2	Upon publication	GENEUS
Wo rkp ack age 4	Task 4.1: Wool and fur production technology	Protocols for formation of commercially viable ELF and ELW	1	Trade secret	GENEUS/
		Validation studies	2	Upon publication	GENEUS
	Task 4.2: Living human hair	Protocols for formation of commercially viable ELH	1	Trade secret	GENEUS

production and PoC	Validation studies	2	Upon publication	GENEUS
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The published and openly provided data will be permitted for re-use under standard conditions enabling their non-commercial use without the approval of consortium members. The consortium will permit the commercial use of data in most cases except for use violating traditional ethical principles, IP generated or precluding exploitation of project results.

The data re-useable after the project are especially:

- Protocols for the combination of organoids with nanofiber matrices (GENEUS).
- Protocols for preparation of nanofibers with specific properties (ALL).
- Designs of prototypes of VAC3DP devices (ALL)
- DBTL platform regarding the selection of nanofiber/organoid properties and prediction of required experimental values (ALL).

The systems will be provided in open form to enable re-use by researchers and developers. However, the extent of data provided will be dependent on the final exploitable results and exploitation strategy. Our strategy will be to provide the data as soon as possible. For instance, if polycaprolactone nanofibers are selected as the primary system for exploitation, we will make available data for polylactic acid nanofibers and additional nanofiber types not directly involved in the exploitation of results. The other prerequisite for such an opening is proper IP protection of the technology, limiting the loss of IP rights to the technology after data provision.