



Project BioCombs4Nanofibers

D3.3 Report on 2PP with high resolution

Reporting period	from	01.10.2019	to	30.09.2020
Report completed and released		22.09.2020		Gerda Buchberger, Alexandra Palla-Papavlu, Irina-Alexandra Paun

1. Introduction

Overall goal: D3.3 is a public scientific report on the formation mechanisms of 2-photon polymerization (2PP) structures reporting on achievable structure sizes for the materials under investigation by partner **JKU** and **INFPLR**. It will be published on the **BioCombs4Nanofibers** website as well as on the Zenodo open data depository. According to the deliverable D5.2 Data Management Plan (DMP) - interim, it contains, among others, the design of nano- and microstructures on the calamistrum at the hindmost leg pair of cribellate spiders from partner **JKU** and the design of the technical surfaces at nano-scale, the fabrication of the technical surfaces, and the optimization of the technical surfaces related to the nanofiber adhesion and the morphological and chemical characterization of optimized 2-photon polymerized technical surfaces with different geometries and composition from partner **INFPLR**.

2. Formation Mechanisms of 2-Photon-Polymerization Structures

Microfabrication by two-photon polymerization (2PP) is a process which relies on changing the electronic state of an atom or molecule that is excited from a lower energy level to a higher electronic state through the simultaneous absorption of either two identical photons or two photons with different energies.

The probability of the two-photon absorption process has a quadratic dependence on the number of photons, and therefore femtosecond (fs) lasers with nano-Joule pulse energies are used. Fs-lasers provide a large number of photons per unit volume, and therefore it is important that the irradiated material does not show linear absorption at the working wavelength. Although the average energy is low, the peak intensity of the pulse is high enough to promote the absorption of two photons. The average laser power depends on the nature of the materials used. In addition, the laser power has to be large enough to exceed the polymerization threshold, and small enough to avoid material degradation.

Usually, in 2PP, sample preparation consists in placing a drop of photopolymer on a pre-cleaned substrate, followed by irradiation with the focused laser beam. The photopolymerization occurs only in the area of the spot in which the laser intensity exceeds the polymerization threshold. The triggered chemical reaction transforms monomers into macromolecules of repeating units. The minimum volume of solidified material created through this process around the focal point is called a 'voxel', which stands for volume element. Controlled three-dimensional microstructures can be created by scanning the laser beam or by placing the sample on a xyz-translation stage.

One of the main advantages of 2PP over conventional microfabrication processes is given by the fact that most of the lasers used for 2PP work in the near infrared range, where most curable monomers are transparent, and thus, little to no modifications on the surface or in the surrounding regions of the produced structures are noted.

Furthermore, the non-linear character of the two-photon polymerization allows a localized excitation which gives the laser direct writing (LDW) via the 2PP method a high resolution. The resolution depends mainly on the shape and size of the beam at the focal site. The two-photon polymerization configuration uses large numerical aperture optical systems, such as microscope lenses, and thus the smallest feature is limited by the numerical aperture of the microscope lens and the wavelength. Since the rate of absorption of two photons is proportional to the square of the incident beam intensity, it therefore depends on the Gaussian intensity profile of the square and the beam has a narrower waist. Therefore, due to the nonlinear nature of this process, it is possible to achieve a resolution below the diffraction limit.

This phenomenon reduces the waist of the beam by a factor of $\sqrt{2}$.

Another aspect that contributes to improving the resolution of the two-photon polymerization process is the polymerization threshold. There is a minimum power below which there is no polymerization. This usually occurs due to the presence of oxygen in the resin, which inhibits the action of the photoinitiators and therefore prevents the polymerization reaction. Hence, by adjusting the laser power close to the polymerization threshold, it is possible to create structures with a resolution well below the diffraction limit (Stokker *et al.* 2018).

3. Calamistrum as Natural Role Model

As is explained in detail in our recent publication in ACS Nano Materials (<https://dx.doi.org/10.1021/acsnm.0c00130>) (Joel *et al.* 2020), web-weaving cribellate spiders catch their prey by a capture wool consisting of nanofibers which are very sticky. The so-called calamistrum (**Figure 1** and **Figure 2**) on the hindmost (fourth) leg is thought to induce the formation of the typical puffy outer structure of the nanofibers in the thread of cribellate spiders (Joel *et al.* 2015, Joel *et al.* 2016). This suggests that the calamistrum also processes the nanofibers.

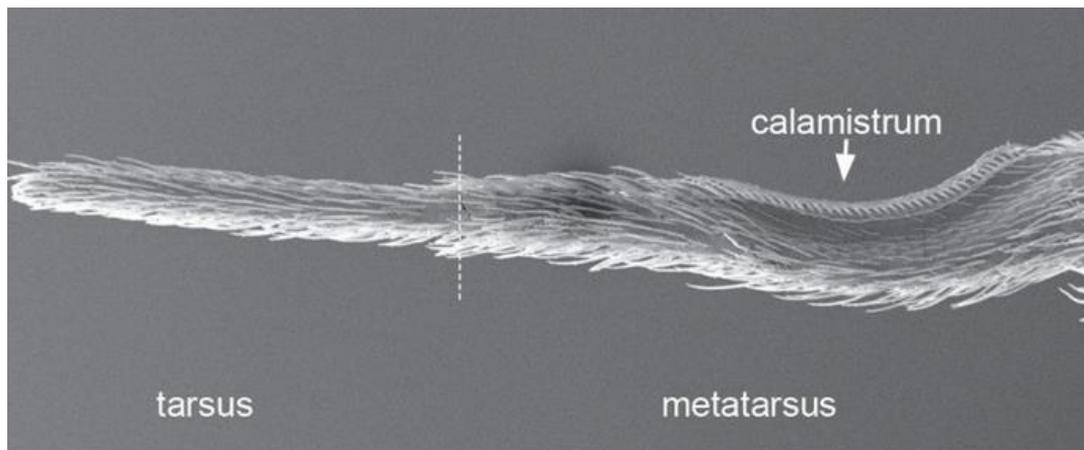


Figure 1: Location and function of calamistrum. Scanning electron microscopy (SEM) close up of the metatarsus and tarsus of the fourth leg, showing the depression where the calamistrum is situated as a specialized row of setae of the feather-legged lace-weaver (*Uloborus plumipes*). SEM. Figure adapted from (Joel *et al.* 2020) published under a Creative Commons Non-Commercial No Derivative Works (CC-BY-NC-ND) Attribution License.

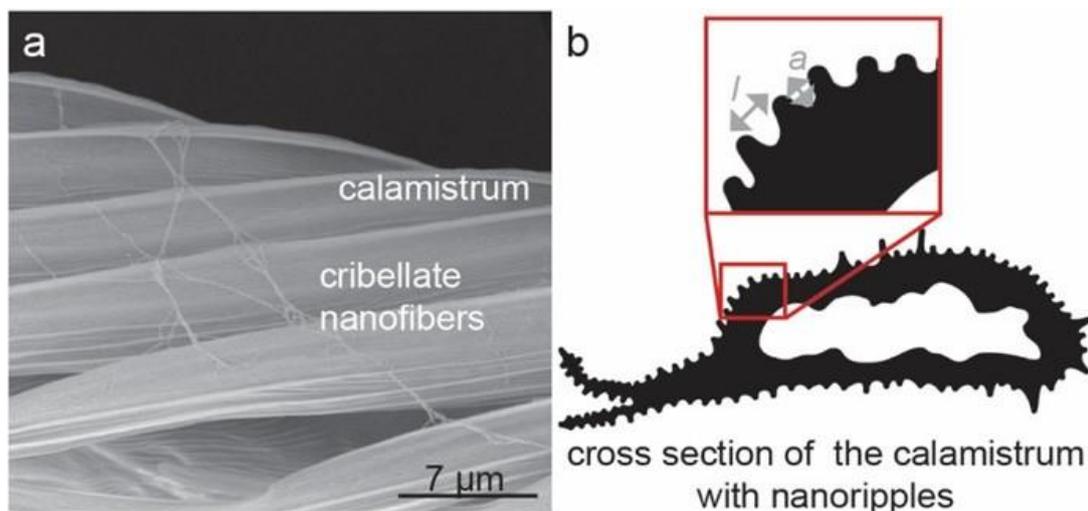


Figure 2: Detailed morphology of the Calamistrum of an exemplary cribellate spider species. a) SEM close-up of single cribellate nanofibers placed artificially over the calamistrum of a feather-legged lace-weaver (*Uloborus plumipes*). Note the surface structure of the calamistrum which is a combination of microstructures (width of approx. ten microns) with nanoripples on top (depth of $a \sim 200$ nm and spatial period l of 200 to 300 nm). b) Schematic cross section through one calamistrum seta at the region of interest. Please note, that the setae are overlapping each other, and thus not all of the calamistrum surface typically comes into contact with the nanofibers. Figure adapted from (Joel *et al.* 2020) published under a Creative Commons Non-Commercial No Derivative Works (CC-BY-NC-ND) Attribution License.

Afterwards, a detailed study of the calamistrum's morphology was performed (**Figure 2**). Therefore, both fourth legs were removed from a dead spider, dried in a descending ethanol series and ascending hexamethyldisilazane concentration series, finally letting hexamethyldisilazane evaporate. The leg was placed on a SEM-stub, using a binocular to control the orientation. Afterwards the leg was sputter coated with gold for examination in SEM. In SEM, the peak-to-peak distance was measured between the observed nanoripples covering the calamistrum (**Figure 2**). Additionally, using the data of FIB-SEM tomography (Heiss *et al.* 2018) as well as FIB-milling the depths and peak-to-peak-amplitudes of the nanostructures

covering the calamistrum were measured at the region of interest (close to the tips of the calamistrum setae). The depth was a ~ 200 nm and the peak-to-peak amplitude l was 200 to 300 nm. These nanoripples are placed on a microripple structure with a width of about 10 microns.

Preliminary investigations of the calamistra of other species showed distinct tooth-like or nap-like structures with heights of several tens of nanometers. They are placed with a distance of some hundreds of nanometers from each other (**Figure 3**). Such as on the calamistrum of the feather-legged lace-weaver these structures are placed on microstructures with width of approx. 5 - 10 microns. The functionality of these structures is still illusive and will be investigated in the following months also with the help of technical surfaces which are covered with nanostructures only, microstructures only and a combination of nano- and microstructures.

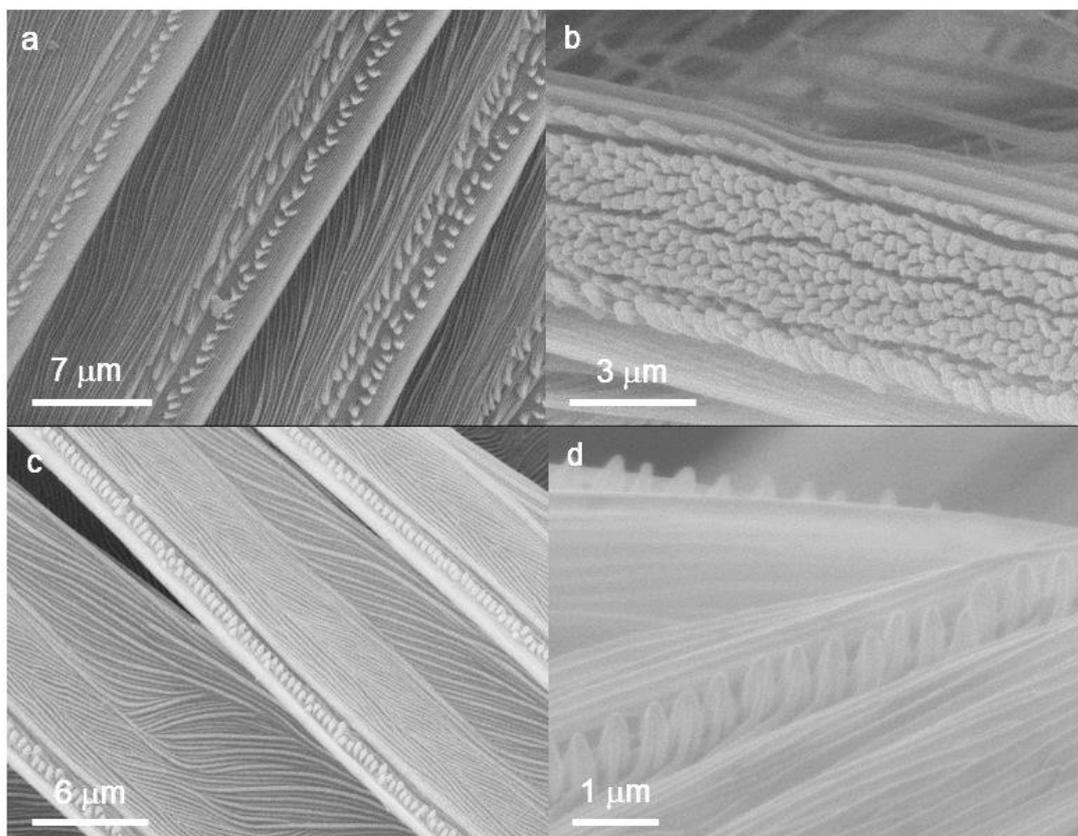


Figure 3: Calamistra of different cribellate spider species vary in their morphology; they consist of microstructures with nanoripples, tooth- and/or nap-like structures on top. (a) net-casting spider (*Avella superciliosus*), (b) ladybird spider (*Eresus walckenaeri*) and (c,d) lace-webbed spider (*Amaurobius similis*).

4. Technical Surfaces, Structure Sizes and Chemical Analysis

Using a commercial 2PP setup (Workshop of Photonics®, 515 nm, repetition rate = 1 MHz, pulse duration = 290 fs) (**Figure 4 - Figure 8**), microstructures with widths of 8 – 10 μm were fabricated; most of them were superimposed with nanoripples with spatial periods between 200 nm and some microns. Spatial periods below 400 nm were hard to achieve. A comparably high processing speed of 1 mm/s was chosen for sample fabrication because we currently aim for structured areas of 0.5 mm x 1 mm at minimum. The reason therefore is that the current adhesion measurement method described in (Joel *et al.* 2020) only works for sufficient large areas. The lengths of the microstructures with nanoripples on top range from 0.1 mm to 1 mm. The laser power was varied between 1% and 7% of the total laser power of 3.6 W – 3.8 W (measured after the focusing lens). Furthermore, glass slides of different thicknesses as well as silanized glass slides and poly(ethylene terephthalate) (PET) films of two different thicknesses and three different brands were screened as possible substrates. For direct writing on glass substrate an oil immersion objective was used (Zeiss, 63x magnification, NA 1.25) and all samples were fabricated in a pendant droplet configuration, i.e., the objective was dipped into an oil droplet and the droplet of resin hung down from the glass slide. This configuration is called transmission-type laser writing. The structures were then written at the interface between the glass slide and the resin. For direct writing on PET a dip-in configuration was applied, i.e., the microscope objective lens (Zeiss, 63x magnification, NA 1.4) was in direct contact with the resin. Several photoresists have been applied, namely pentaerythritol triacrylate (PETA) with 1 wt% of the photoinitiator irgacure (IC) 819, pure OrmoComp® (<https://www.microresist.de/en/produkt/ormocomp/> from 14.09.2020) and OrmoComp® with 1 wt% IC 819. After 2PP, sparse PETA with 1 wt% IC 819 was either cleaned with ethanol or with isopropanol; OrmoComp® and OrmoComp® with 1 wt% IC 819 were cleaned with acetone. Typical line widths and heights achieved in PETA + 1 wt% IC 819 and OrmoComp® + 1 wt% IC 819 at different laser intensities using the applied setup are given in (Buchroithner *et al.* 2020). Minimal line widths in the dip-in configuration were about 90 nm for PETA + 1 wt% IC 819 and about 110 nm for OrmoComp® + 1 wt% IC 819. Currently, the smallest structure size which has ever been achieved was 55 nm. The smallest resolution was 120 nm. To this end, STED-lithography of a PETA mixture was applied by (JKU, Wollhofen *et al.*, 2013).

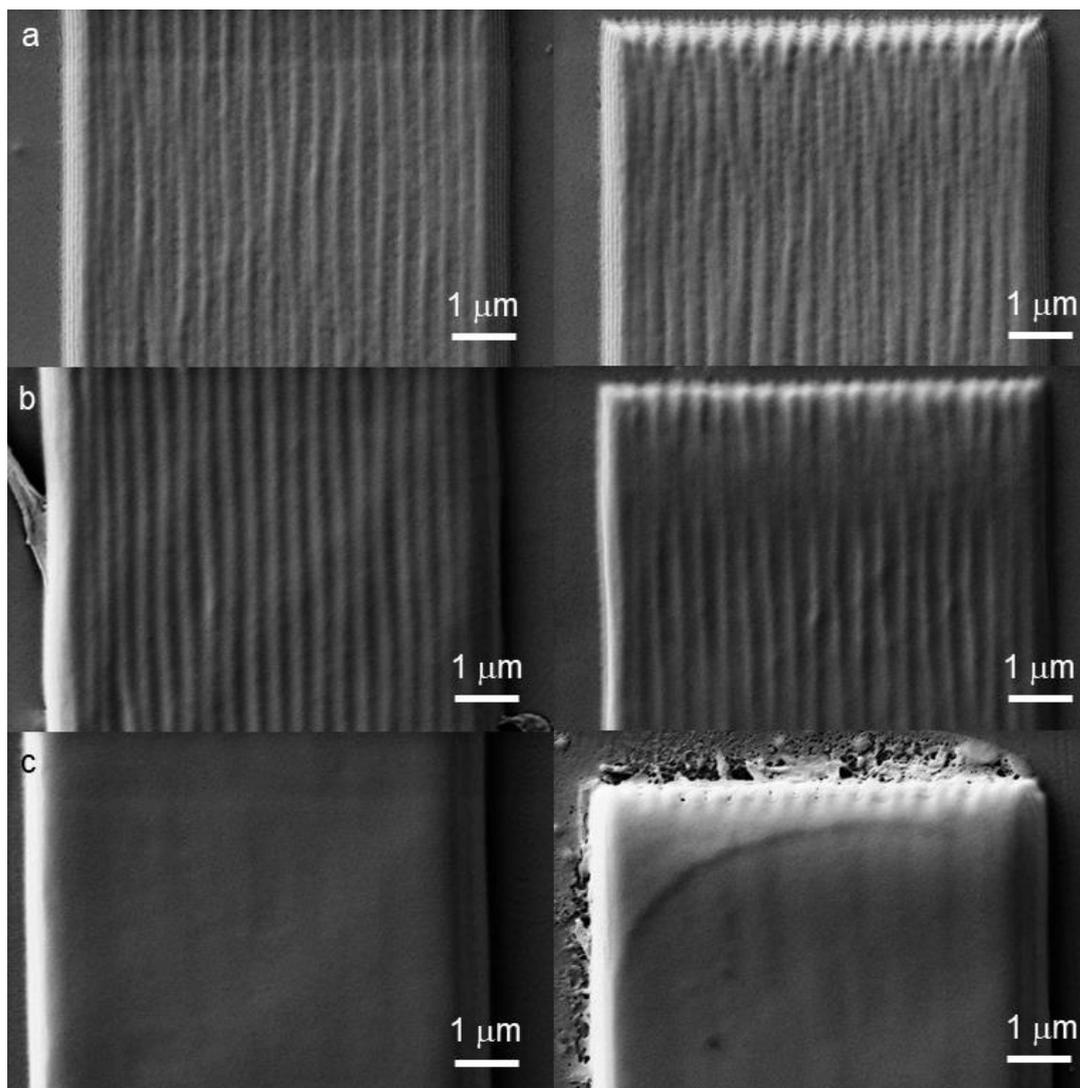


Figure 4: SEM images of exemplary microstructures superimposed by nanoripples as well as microstructures only on flexible, biaxially stretched PET films with a thickness of 50 μm . (a) PETA with 1 wt% IC 819 on a PET film; here, the spatial period was 400 nm. (b, c) Direct writing of ORMOCOMP + 1 wt% IC 819 on PET using different laser powers. In (b) microstructures with superimposed nanoripples are depicted, while (c) shows smooth microstructures only.

From the to date experiments it can be concluded that high resolution structures can be achieved more easily with PETA with 1 wt% IC 819 than with OrmoComp® and that OrmoComp® adheres to the glass slides more tightly. Silanization of glass slides seems to improve adhesion for all photoresists. PET samples were more difficult to process, since they are not as transparent and flat as glass slides (**Figures 4, 5**). However, they are flexible and can therefore be probably used in adhesion measurements according to (Joel *et al.* 2020).

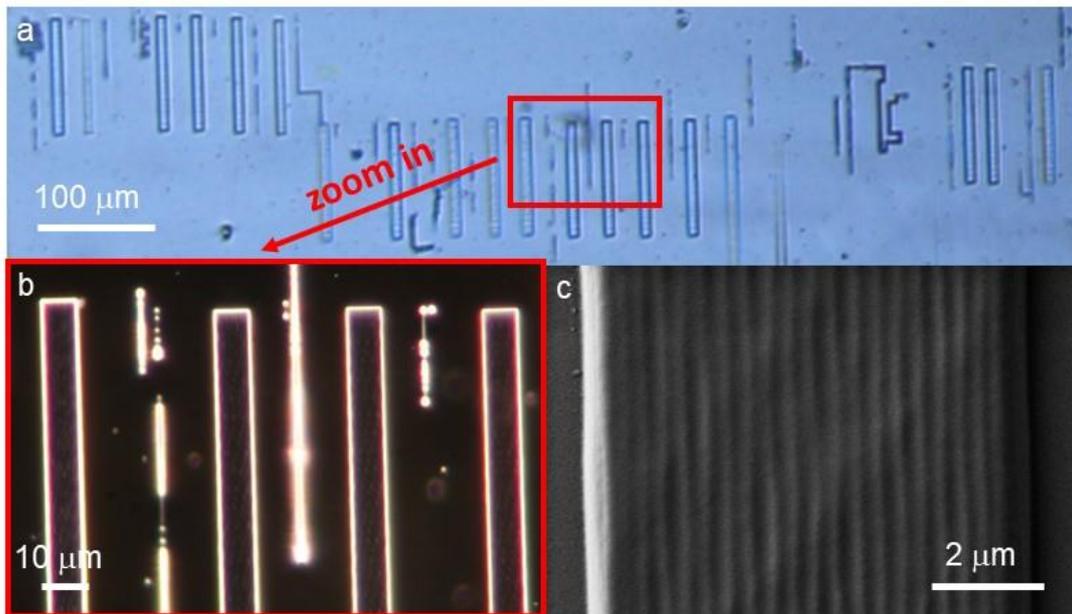


Figure 5: Microscopic images of hierarchical structures from ORMOCOMP + 1 wt% IC 819 on flexible, biaxially stretched PET films with a thickness of 50 μm . (a) Bright field and (b) dark field images of two hierarchical structures recorded by an optical microscope. (c) SEM image of left hierarchical structure from (b).

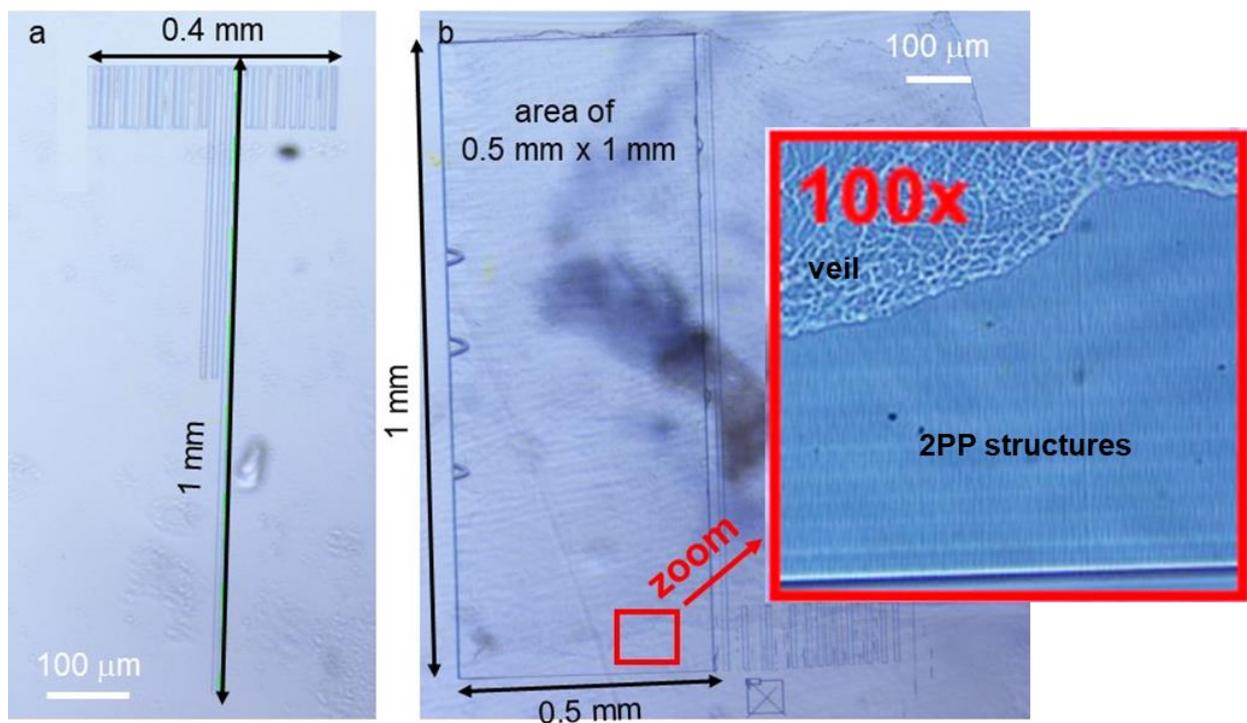


Figure 6: Microscopic images of hierarchical micro- and nanostructures from ORMOCOMP on silanized glass. (a) Bright field microscopic images of several microstructures with and without nanoripples. (b) Bright field microscopic image with an inset of a dark field microscopic image. A “veil” generated during fabrication of the area of 0.5 mm x 1 mm lays on top of the structures and is shown in an inset. Addition of 1 wt% of IC 819 was found to prevent generation of the “veil” covering the structure.

First experiments to scale up the overall structural size of OrmoComp® on glass to 0.5 mm x 1 mm resulted in a “veil” covering the structure (**Figure 6**), most probably due to unfinished polymerization. Therefore, in the next trial 1 wt% IC 819 has been added to OrmoComp®, which successfully prevented the “veil” from forming. For structures of 0.5 mm x 1 mm, reliable adhesion of the total structure onto the glass slides is challenging due to moderate tilt, unevenness and sample drift with time. Also, work on first 3D hierarchical structures, i.e., partially free-standing ones (**Figure 7** and **Figure 8**), was done; however, these are even more challenging with regard to reproducibility and reliability.

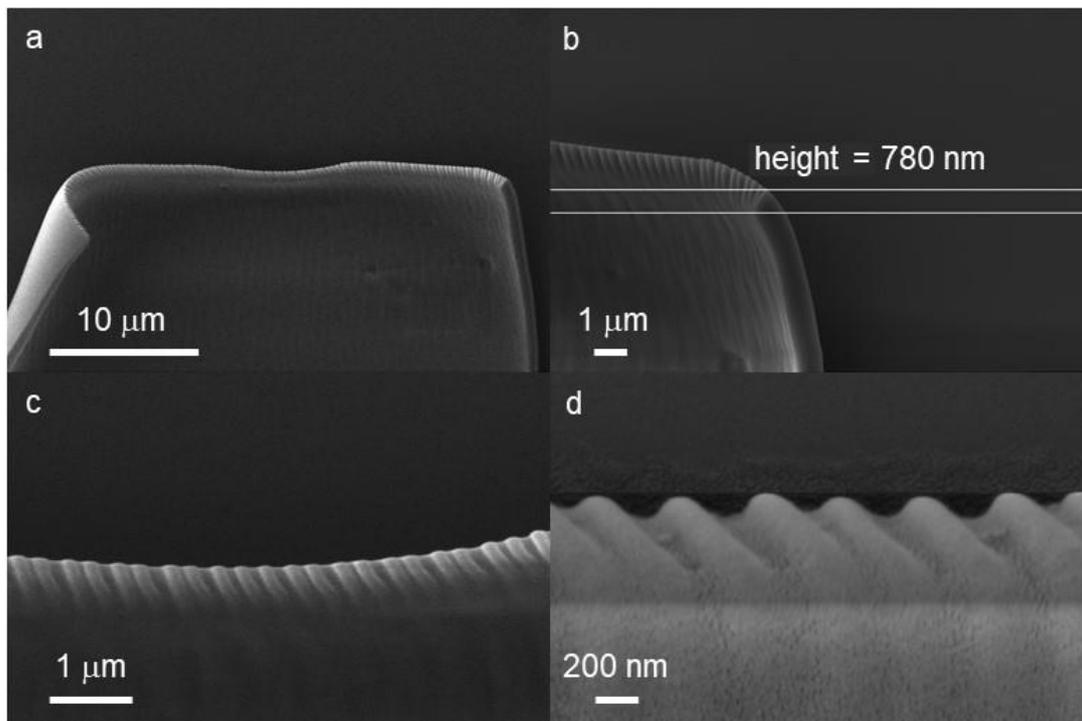


Figure 7: SEM images of first 3D hierarchical structure from PETA + 1 wt% IC 819 on glass slides; the width of the structure is approx. 50 μm. (a) Overview of the structure. (b) Thickness of the complete structure. (c, d) Details of nanoripples on top of microstructure.

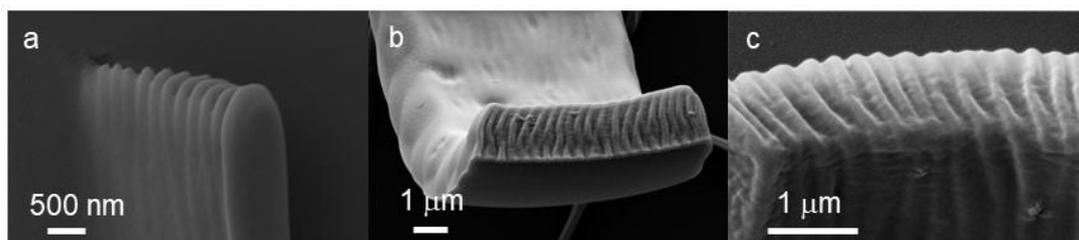


Figure 8: SEM images of 3D hierarchical structures from PETA + 1 wt% IC 819 on glass slides. (a) Staircase structure, (b, c) nanoripples on face sides of microstructures.

Below, we will show more 2-photon polymerized technical surfaces with different geometries and composition from partner **INFPLR**.

In the first trials at INFPLR we tried to optimize the laser power and scanning speed in order to obtain a geometry/dimension control of the written structures as well as a high spatial resolution. The setup used to write the structures is based on the Photonic Professional 3D Lithography system Nanoscribe GmbH.

For laser fabrication, we used 2 types of commercially available IP-photoresists, from Nanoscribe GmbH: IP-L 780 and IP-Dip. Both solutions are liquid, UV-curable negative photoresists, particularly optimized for two-photon absorption laser direct writing.

IP-L 780 is used to obtain highest laser writing resolution for 3D lithography using the aforementioned system. IP-Dip is similar, but with a higher refractive index and optimized for immersion-based, constant aberration laser writing. Both can be used to fabricate nanostructures with lateral spatial features as low as ~100 nm for two-dimensional structures, and 300 nm for three-dimensional structures, albeit with lowered reproducibility. The reason for lower reproducibility is that, apart from the laser writing step, sample development also influences the shape and size of the resulting structures.

IP-L 780 is used for transmission-type laser writing, i.e., the laser beam first propagates through the substrate. As such, microstructure height is limited (depending on the geometry), as polymerized material affects the transverse laser profile (polymerized material has a higher refractive index than the photoresist). IP-Dip is used with the microscope objective immersed in the photoresist itself, and therefore has no height limit from the point of view of the laser transverse profile (maximum height is determined by the mechanical strength of the resulting polymer and the geometry of the structure). In both cases (IP-L 780 and IP-Dip), the substrate must have good evenness and roughness, and a different refractive index than the photoresist, for focusing purposes. IP-Dip can be used to fabricate microstructures on opaque substrates. IP-L780 and IP-Dip have been used in various tissue engineering experiments and are reportedly biocompatible (Paun *et al.* 2018). However, the manufacturer (Nanoscribe) offers a standardized (ISO 10993-5 / USP 87) version of IP-type photoresist that is biocompatible and non-cytotoxic (IP-Visio). Development is usually done through immersion in PGMEA for ~15 minutes (immersion time can vary depending on the geometry of the structure).

Laser writing is mostly dependent on the energy dose delivered to the photoresist, i.e., energy per unit volume. In other words, laser power and writing speed are correlated and can compensate one another, i.e., faster writing speeds require higher laser powers to obtain the same polymerization degree and voxel size. However, this correlation is limited by the polymerization time interval of the material itself. On one hand, if the laser fluence is too low, the photo-chemical reaction is not initiated and therefore no polymerization is obtained. On the other hand, if the laser fluence is too high, microexplosions can form and affect the laser writing process (i.e., the solvent quickly evaporates, forming a gas bubble inside the liquid photoresist, which damages neighboring structures and affects the laser beam profile).

We used writing speeds between 80 - 100 $\mu\text{m/s}$, and laser powers between 25 - 35 mW for IP-L 780, using a 63x NA = 0.75 microscope objective, and 10 - 15 mW for IP-Dip, using a 100x NA = 1.3 microscope objective. Higher writing speeds can be used to obtain similar structures, but it will also increase fluid movements and lower the positioning accuracy of the translation stage, which can result in geometric defects of structures.

In our experiments, we have used IP-780 to fabricate structures on 170 μm thick BK7 glass substrates, 125 μm , 250 μm and 500 μm thick acetate sheets and 250 μm thick polyethylene terephthalate (PET) sheets. As mentioned previously, IP-780 is used for transmission type laser writing.

Results indicate that pillars can be obtained on glass substrates, but with low reproducibility due to the depth of focus of the 63x microscope objective, i.e.,: aspect ratio is too high and

pillars collapse during development step. Structures have also been obtained on acetate sheets, but with an even lower reproducibility, exacerbated by the low evenness of the acetate substrate and poor polymer adherence. Structures have not been obtained on PET sheets, due to the fact that the laser spot is highly affected by the propagation through the substrate and polymerization does not occur. IP-Dip photoresist has been used to fabricate structures on 500 μm thick SiO_2 glass substrates. Results indicate good structural integrity and high reproducibility over large areas (millimeter scale).

The tests for pillar fabrication on a glass substrate are presented in **Figure 9**. Results indicate a good reproducibility of the focusing steps. Before development, all pillars were standing. Pillars are laid down on the substrate following the development process, determined by the surface tension of the evaporating solvent and aided by a high aspect ratio.

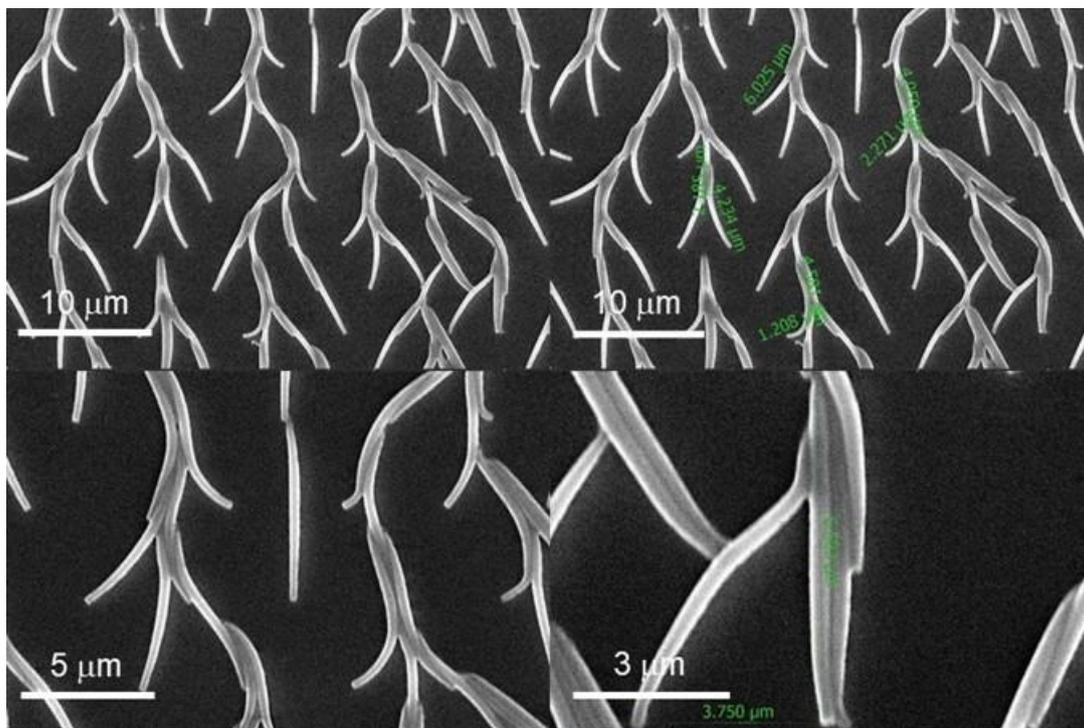


Figure 9: SEM micrographs showing fabrication test results for pillars using IP-L photoresist and a 170 μm thick BK7 glass substrate taken at different magnifications.

In order to avoid pillar collapse, a lower aspect ratio is required. However, shorter pillars determine a lower reproducibility over large areas due to lower accuracy of the focusing step. Therefore, the sample setup was changed to a dip-in setup, where the microscope objective is immersed in the liquid photoresist. For this sample setup we used IP-Dip photoresist (Nanoscribe GmbH) and SiO_2 substrates, in order to have a higher refractive index between the photoresist and the substrate, therefore obtaining higher accuracy focusing. The microscope objective was changed to a 100x, NA = 1.3 objective, with a working distance of 170 μm . This setup offers superior accuracy and resolution, but also involves a direct interaction between the microscope objective and the liquid photoresist. Sample movements using this setup determined supplementary fluid movements which may affect high resolution structures. As such, lower laser writing velocities were employed (80 $\mu\text{m}/\text{s}$). However, a higher resolving power also lowers the voxel volume, i.e., the voxel is approximately 1 μm in diameter and 2 μm tall.

Results are presented in **Figure 10**. Pillars have a high positioning accuracy and high reproducibility over large areas (4 mm x 4 mm). There are no visible deformations determined by the developing process. Pillar diameters are approximately 0.8 μm , while the distance between their centers was kept to 4 μm .

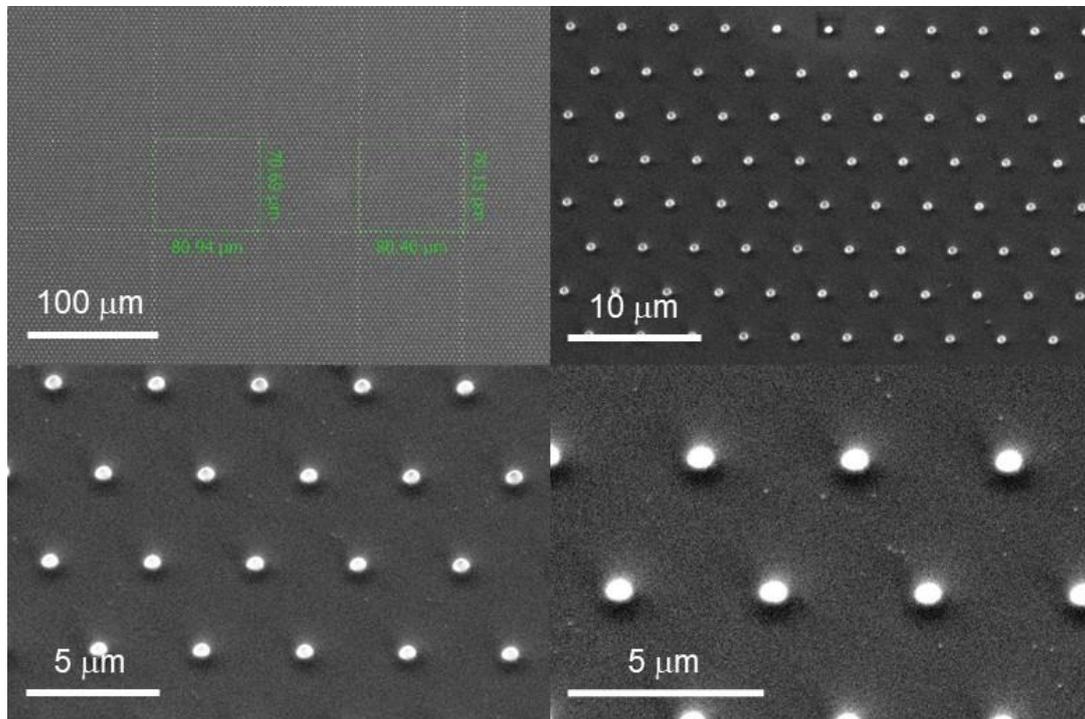
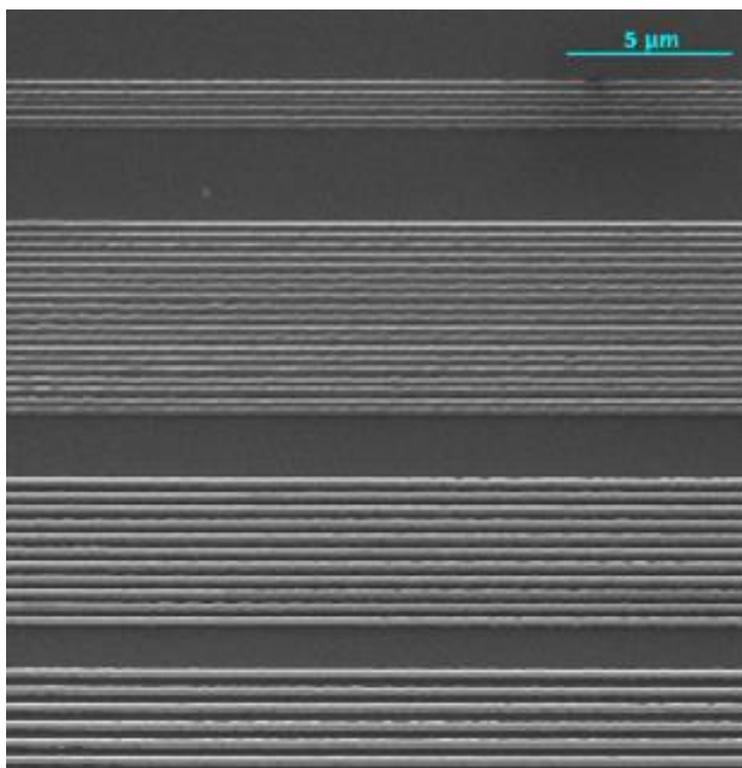


Figure 10: SEM micrographs showing fabrication test results for pillars using IP-Dip photoresist and a 500 μm thick SiO_2 glass substrate taken at different magnifications.

Furthermore, at **INFLPR** we have the possibility to fabricate polymeric periodic surface nanostructures, similar to LIPSS, by using laser direct writing via two photon polymerization. The line widths can reach sizes below 1 μm , with similar or above 1 μm heights.

These nanostructures show significantly better periodicity and greater control in respect to their shape and size. One main disadvantage, however, is the initial fabrication time, which can be significantly higher. This is due to the direct laser writing methodology, in which each line is fabricated sequentially, rather than through stochastic laser-matter interaction, as is the case for LIPSS. By “initial fabrication time”, we want to emphasize that, once optimized, a geometry transfer methodology (stamping / printing) can be developed. As such, the nanostructures are fabricated only once, in order to produce a “master” that can be further used to replicate the nanostructures. Preliminary results are shown in **Figure 11**.

In addition, regarding the chemical characterization, as the monomers used are commercially available, it is known that their polymerization results in an unique compound, with known structure and chemical composition.



Figures 11: Preliminary results of a “master”, fabricated by laser direct writing via two photon polymerization that can be further used to replicate the nanostructures.

5. References

(Buchroithner *et al.* 2020) Buchroithner, B.; Hartmann, D.; Mayr, S.; *et al.* . Biomimetic combs as antiadhesive tools to manipulate nanofibers. *Nanoscale Adv.* 2020, 2, 2422–2488, DOI: 10.1039/D0NA00154F

(Joel *et al.* 2020) Joel, A.-C.; Meyer, M.; Heitz, J.; Heiss, A.; Park, D.; Adamova, H.; Baumgartner, W. Biomimetic combs as antiadhesive tools to manipulate nanofibers. *ACS Appl. Nano Mater.* 2020, 3 (4), 3395–3401, DOI: 10.1021/acsnm.0c00130

(Heiss *et al.* 2018) Heiss, A.; Park, D.; Joel, A.-C. The calamistrum of the feather-legged spider *Uloborus plumipes* investigated by focused ion beam and scanning electron microscopy (FIB–SEM) tomography. *Microsc. Microanal.* 2018, 24 (2), 139–146, DOI: 10.1017/S1431927618000132

(Paun *et al.* 2018) Paun, I.A.; Popescu, R.C.; Mustaciosu, C.C.; *et al.* Laser-direct writing by two-photon polymerization of 3D honeycomb-like structures for bone regeneration. *Biofabrication.* 2018; 10(2):025009, DOI:10.1088/1758-5090/aaa718

(Stokker *et al.* 2018) Stokker-Cheregi, F.; Palla-Papavlu, A.; Paun, I.A.; Lippert, T.; Dinescu, M.; Chapter Laser Structuring of Soft Materials: Laser-Induced Forward Transfer and Two-Photon Polymerization in *Advances in the Application of Lasers in Materials Science, Springer Nature* (2018)

(Joel *et al.* 2016) Joel, A.-C.; Scholz, I.; Orth, L.; Kappel, P.; Baumgartner, W. Morphological adaptation of the calamistrum to the cribellate spinning process in *Deinopoidae* (*Uloboridae*, *Deinopidae*). *R. Soc. Open Sci.* 2016, 3 (2), 150617, DOI: 10.1098/rsos.150617

(Joel *et al.* 2015) Joel, A.-C.; Kappel, P.; Adamova, H.; Baumgartner, W.; Scholz, I. Cribellate thread production in spiders: Complex processing of nano-fibers into a functional capture thread. *Arthropod Struct. Dev.* 2015, 44 (6), 568–573, DOI: 10.1016/j.asd.2015.07.003

(Wollhofen *et al.* 2013) Wollhofen, R.; Katzmann, J.; Hrelescu, C.; Jacak, J.; Klar, T.A. 120 nm resolution and 55 nm structure size in STED-lithography. *Optics Express* 2013, 21(9), 1083-10840, DOI: 10.1109/CLEOE-IQEC.2013.6801550

6. Evaluation of Goals and Resulting Actions

Manufacturing a wide range of different technical surfaces by two photon polymerization (2PP) at JKU and INFPLR (**Figure 4 - Figure 11**), we followed several strategies to unravel the functionality of the various structures found on calamistra of spiders (**Figure 3**). We fabricated nanostructures only, namely naps as well as ripples, which have similar dimensions as the ones found on calamistra (**Figure 10** and **Figure 11**). Furthermore, we generated microstructures only to sort out which role they play in adhesion independently from the nanostructures (**Figure 4c**). As a more complex bioinspired model system (**Figures 1, 2**), we superposed flat microstructures with nanoripples (**Figure 4a and b - Figure 6**). Furthermore, we did first experiments on 3D hierarchical micro- and nanostructures which will mimic the natural role model in the most precise way (**Figure 7** and **Figure 8**).

In first proof-of-principle experiments, we demonstrated that areas of 0.5 mm x 1 mm (**Figure 6**) or even 4 x 4 mm (**Figure 10**) can be covered by biomimetic 2PP structures and that biomimetic 2PP structures can be written onto flexible PET foils (**Figures 4, 5**).

All goals described in the introduction are still valid and will be pursued further in the course of the project. As the overall aim is to study the interaction of the bio-inspired surfaces with natural and artificial nanofibers, the next step will be to measure the adhesive properties of the structures fabricated by 2PP. To this end, there is a need for structures written on flexible substrates (like PET foils) and for structured areas with minimal sizes of 0.5 mm x 1 mm, on one hand. On the other hand, adhesion measurement methods and setups have to be developed further for their usage together with hard samples as well as with samples of sizes of only several μm^2 . Alternatively, hard substrates with 2PP structures could be used as masters for replication processes resulting in soft samples.

This report has been published as a public report (PDF) entitled “Report on 2PP with high resolution” in the dissemination section of the website of the **BioCombs4Nanofibers** project (<http://biocombs4nanofibers.eu>) as well as on the Zenodo open data depository.

This report is part of the project **BioCombs4Nanofibers** that has received funding from the European Union’s Horizon 2020 research and innovation programme under grant agreement No 862016.