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Ingegneria dei Materiali

ENGINEERING OF POLYMERIC MATERIALS AND STRUCTURES FOR INNOVATIVE APPLICATION OF 3D MICRO-ADDITIVE MANUFACTURING VIA TWO-PHOTON LASER LITHOGRAPHY

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Abstract

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Two-photon laser lithography it's an innovative fabrication method which is opening exciting opportunities in many fields of research, biological one as well as in nanotechnology and industry thanks to its high spatial resolution and versatility in terms of shapes achievable. This study wants to show how a synergic use of innovative and optimized materials (as for example hydrogels) and this cut-edge technology can provide inconceivable results in many different applications.

It was found that modifying the composition of a natural gelaton based hydrogel with a more reactive material synthetic polyethylenoxide can provide a reliable fabrication of interesting structures for biological applications. Moreover, this study will show how it's possible to achieve a fast and versatile fabrication of micrometric moulds with both commercial and custom-made resists and a new multistep microprinting process.

Finally, it will be presented how hydrogels and two-photon laser lithography can become powerful tools in the development of innovative nanolasers for solid state applications.

Summary

Introduction

Some of the most important achievements in biological research nowadays, are due to the ability of recreating and studying different conditions that cells and tissues are subjected to within the human body. Reaching this aim is an extremely arduous process restrained by the complexity of the structures required as well as by the wide range of the physical properties assumed by the tissues involved. On the other hand, progress in high technology applications requires always more advanced solutions to fulfil innovative requirements and improve products rely, moving to smaller feature size also in 3D.

However, thanks to the incessant improvements in material's science and to the thrilling advances in micro and nanofabrication processes, unprecedented solutions are now available and many more possibilities are becoming reality.

This thesis wants to highlight through different practical applications how developing a synergic use of innovative smart materials (as for example hydrogels) and the cutting-edge nanofabrication method of two-photon laser lithography can provide inconceivable results not only in the biological field of research but in many others as well as in nanotechnology and industry.

Each of the four cases herein presented will pose different challenges that can be accomplished only with a specific optimization of the fabrication process and of the material's composition.

In each chapter, different solutions will be found to fulfil the requirements of the specific situation highlighting the peculiar versatility of both the two-photon laser lithography and of the materials used.

The following chapters will show how it's possible to obtain 3D microstructures made by natural materials (as for example GelMA) and how this fabrication can be improved adding a more reactive crosslinker.

Moreover, it will be possible to observe how rapid prototyping of micrometric moulds, taking advantage of two-photon laser lithography, can be helpful to understand the dependence of the cells behaviour on the shape of a biocompatible substrate.

But remarkable advances are not just limited to biological research. In chapter five, it will be presented how high-resolution lithography could develop optical devices achieving smaller and smaller coherent light sources.

A complete understanding of the two-photon lithographic process and of its own possibilities and limits wouldn't be possible without a description of the physical principles which this process is based on. For this reason, the first part of this thesis will consist of a theoretical description of the two-photon absorption and the two-photon polymerization followed by a closer overview of both the commercially available photoactive material and of other innovative alternatives.

This work of thesis wouldn't be possible without a 4 months training in two-photon laser lithography at Karlsruhe Institute of Technology.

The professor Klaus Bade's group made this period a stimulating and constructive experience and, thanks to their training, it was possible to develop the required knowledge to face the challenges that will be reported in the following chapters.

Chapter 1

Two-photon laser lithography

Achieving new and challenging results in 3D micro and nano fabrication is one of the main instrument to make breakthrough advances in many fields of basic and industrial research, whether it is health or technology. Taking advantage of non-linear multiphoton absorption, materials and fabrication's optimization, this is becoming reality using the emerging technology of two-photon laser lithography. It is of primary importance to understand physical principles behind this technique to fully exploit it and increase the benchmarks for direct laser writing.

The aim of this chapter is, therefore, to explain some of the most important physic fundamentals and limits that characterize this direct laser writing method and, finally, show some of the numerous applications in which this technology is game changing.

1.1 Multiphoton absorption

In 1931, Maria Goppert-Mayer in her doctoral dissertation described the theoretical possibility of a two-photon absorption. The main limitation that precluded the experimental validation of the theoretical hypothesis was that, due to the passage through a transition energy state, this process is achievable only with coherent light source able to provide a high intensity beam. In early 1980s, however, the development of femtosecond lasers as Ti:sapphire lasers or fiber lasers made possible to exploit Gopper-Mayer's studies and observe the two-photon absorption.

These new light sources, thanks to the pulse regenerative technology, have an amplification of the single pulse's energy by four orders of magnitude achievable just with an increase of the repetition rate of the laser but with no need of higher power.

Multiphoton absorption, unlike the single-photon process, relies on a non-linear dependence between energy absorbed and intensity of radiation. This dependence is described by equation (1.1).

$$\frac{dW}{dt} = 8\frac{\pi^2\omega}{n^2c^2} I^2 Im\left(\chi^{(3)}\right) \tag{1.1}$$

Where ω stands for the pulse, I stands for the intensity and Im(χ) is the imaginary part of the susceptibility tensor. This kind of absorption can be obtained in two different ways: stepwise or simultaneously. In the former, there is an actual long lifetime energy state that can be reached thanks to the first photon and the second photon can be absorbed after a while giving the energy to reach a higher energy excited state. This two-photon absorption can be treated, therefore, as two sequential single absorption processes.

The other possible absorption happens simultaneously and it can be achieved using a laser whose repetition rate has to be high enough to avoid the decay of a virtual transition energy state that has a lifetime of few femtoseconds.

The absorption can also be defined based on the energy of the two photons as degenerate and non-degenerate (figure 1.2). It is degenerate if the two photons have the same energy while is non-degenerate if their energy is different. Usually, the degenerate absorption is the one used in lithographic fabrication and it is achieved using only one light source.

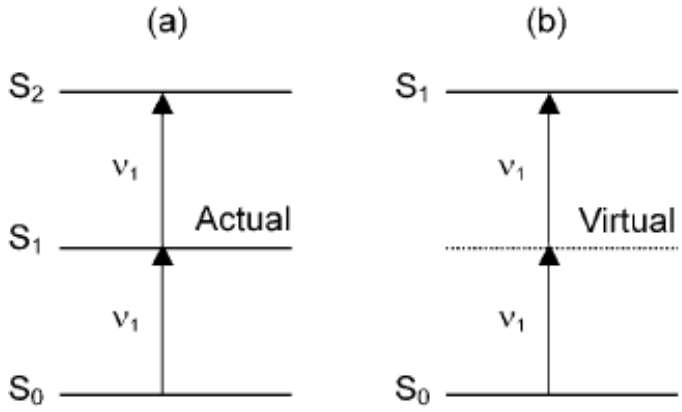

Figure 1.1 (a) stepwise absorption with an actual transition state between ground state and excited state. (b) Simultaneous absorption, the transition state is virtual and has a short lifetime

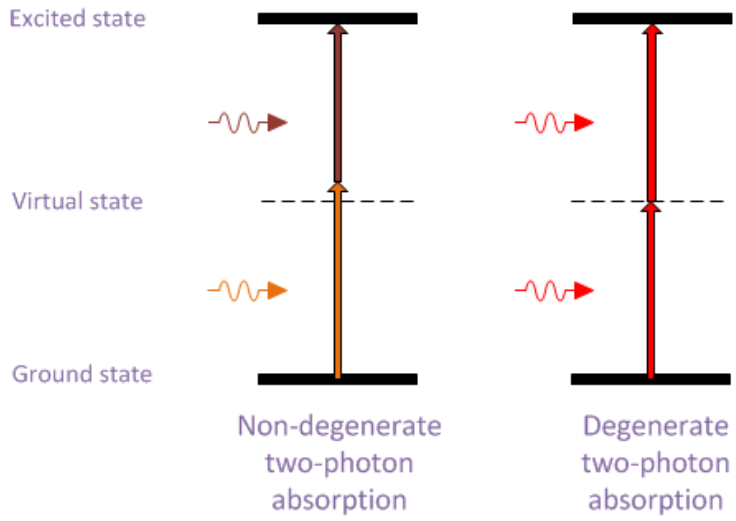

Figure 1.2 degenerative and non-degenerative absorption

1.2 Two-photon polymerization

Polymerization is a process by which a long chain is formed thanks to a chemical reaction between many single units called monomers. This reaction has to be initiated supplying a right amount of energy. This can be provided in many ways as, for example, with chemical exothermic reactions, heating up the resin or focusing a light beam (in this case it will be defined as photopolymerization).

Considering the latter case, usually, photopolymerization is driven by a UV source of light characterized by a single photon's energy high enough to overcome the energy threshold required by the chemical reaction.

UV curing is a well-established activation process, it's versatile, has a high activation yield and its ease of use made it the most used activation process in photopolymerization.

Lithographic techniques which relies on UV photopolymerization, however, have some serious drawbacks that limit their use in many circumstances.

The high power of the focused beam can easily damage the resist due to overheating, moreover, this polymerization has a limited spatial resolution.

One of the main advantage of the two-photon polymerization, instead, is the highest spatial resolution ever achieved with a photolithographic fabricating method. This feature relies on the non-linear behaviour of two-photon absorption.

When a laser beam pass through a homogeneous substance, every cross-section which is perpendicular to the beam direction has the same photon density only decreased by the

amount absorbed along the beam direction. If the photoactive materials react to laser excitation via a single-photon absorption, the probability of the polymerization to happen is depending only on the position along the thickness of the sample, but is non-zero in every cross-section. This means that the polymerization cannot be performed in whatever determined section inside the volume of the resist because this photoreactive material is not transparent to the wavelength beam used to start the chemical reaction.

For this reason, 3D results obtained with single-photon lithography can only be achieved due to a layer-by-layer polymerization which, sometimes, has to be aided by a mask or a mould. Tridimensionality is not an intrinsic feature of this polymerization method.

If the resist, however, is a two-photon active material, and follows a non-linear absorption process, the probability to absorb the energy carried by the laser beam is proportional to the square of the intensity. Polymerization, in this case, can happen only if high intensities are provided and this fact is due to the short time the molecule is in a transient state ready to absorb the second photon. These high intensities can be supplied only by femtosecond pulse lasers. Moreover, the energy threshold required for this polymerization to happen is localized in a small volume called voxel in which the laser is focused.

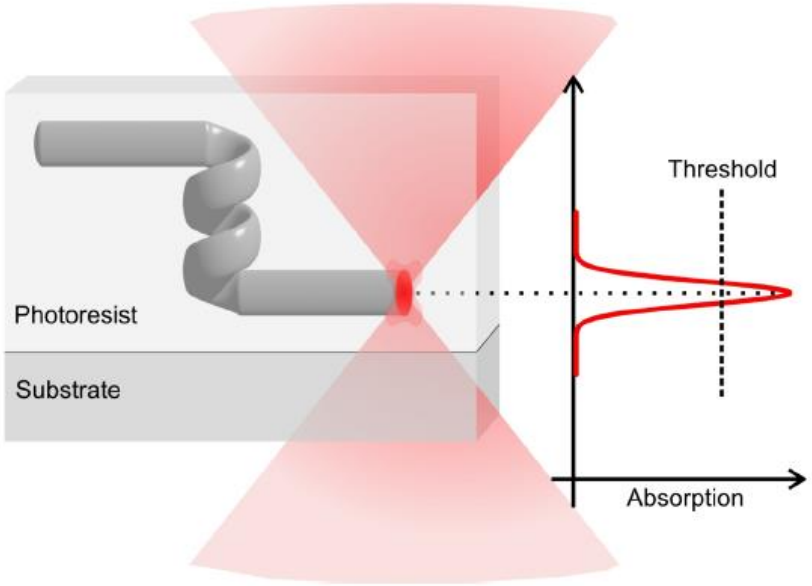

Figure 1.3 Two-photon polymerization mechanism. The voxel is represented by the red spot

This phenomenon prevents the polymerization to happen in any of the spot apart from the one decided with the focusing of the laser. 3D structures, with this polymerization method can be achieved moving the laser focus within the volume of the resist without using any

mask. Two-photon polymerization, therefore, possess an intrinsic tridimensionality and, thanks to the small volume of the voxel can reach, in principle, a spatial resolution up to 100nm. This latter aspect involves not only the non-linearity of the absorption process but requires also a suitable optimization of the photoactive material.

Energy provided via two-photon absorption, however, should not exceed a second energy threshold which will induce a laser induced breakdown of the material. This phenomenon causes microexplosions, atomization and vaporization of the resist preventing any further polymerization. Figure 1.4 describes this situation.

The polymerization mechanism followed by single-photon polymerization and two-photon polymerization, once activated by the energy, is quite similar and can be described by the following reactions [1]:

One-photon polymerization:

Initiation: $$I \xrightarrow{h\nu} I^* \rightarrow R\bullet \qquad (1.1)$$

Propagation: $$R\bullet + M \rightarrow RM\bullet \xrightarrow{M} RMM\bullet \ldots \rightarrow RM_n\bullet \qquad (1.2)$$

Termination: $$RM_n\bullet + RM_m\bullet \rightarrow RM_{m+n}R \qquad (1.3)$$

$$RM_n\bullet + RM_m\bullet \rightarrow RM_n + RM_m \qquad (1.4)$$

Where (I) stands for photoinitiator, (R•) for radical and (I*) for the intermediate state of photoinitiator.

Two-photon polymerization differs from the one above for the initiation step and equation (1.1) becomes:

Initiation: $$I \xrightarrow{2h\nu} I^* \rightarrow R\bullet \qquad (1.4)$$

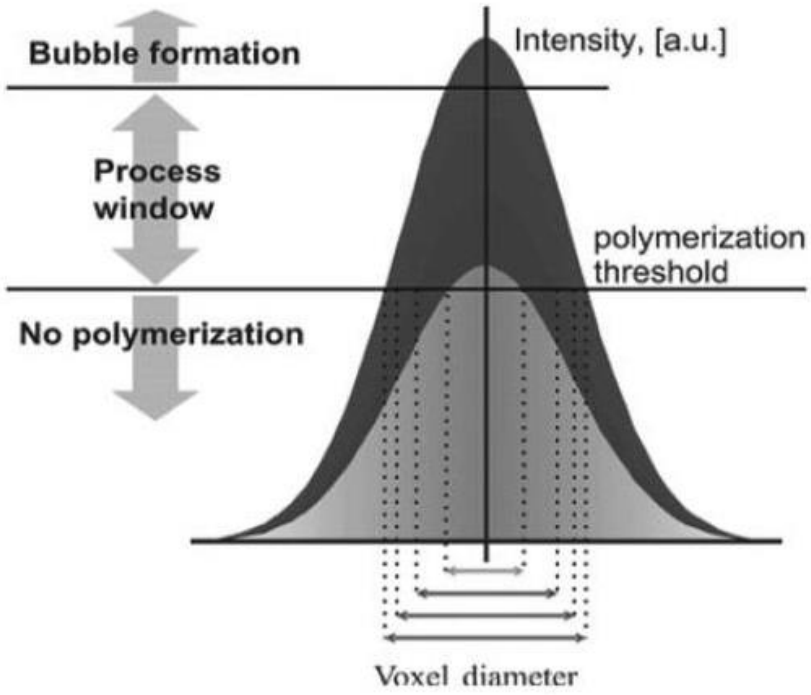

Figure 1.4 dependence of the voxel size to on the intensity of the beam

1.3 Applications

For over a decade, two-photon laser lithography caught the attention of many in research as well as in industrial field.

Being able to relies on a fabrication tool that can provide such a high spatial resolution, combined with the versatility in term of 3D structures achievable, encouraged the inventiveness and now many unprecedented applications are reality

1.3.1 Micro-optics

Optical systems rely on passive optical components like lenses, prisms and mirrors to collect, distribute or modify optical radiation.

Nowadays, an increasing use of miniaturized systems and integrated optical devices need a shrink in the dimensions of these components down to micrometric scale.

These micro-optical elements are generally realized via e-beam lithography, focused ion beam milling selective-chemical etching or UV curing. High spatial resolution direct laser writing lithography, however, has undeniable advantages over these techniques providing all degrees of freeform for the design, the highest resolution possible and high speed of rapid prototyping

Thanks to this, many groups already reported polymeric highly resolute arrays fabricated via two-photon lithography to be used in micro-optical applications as the one in figure (1.5).

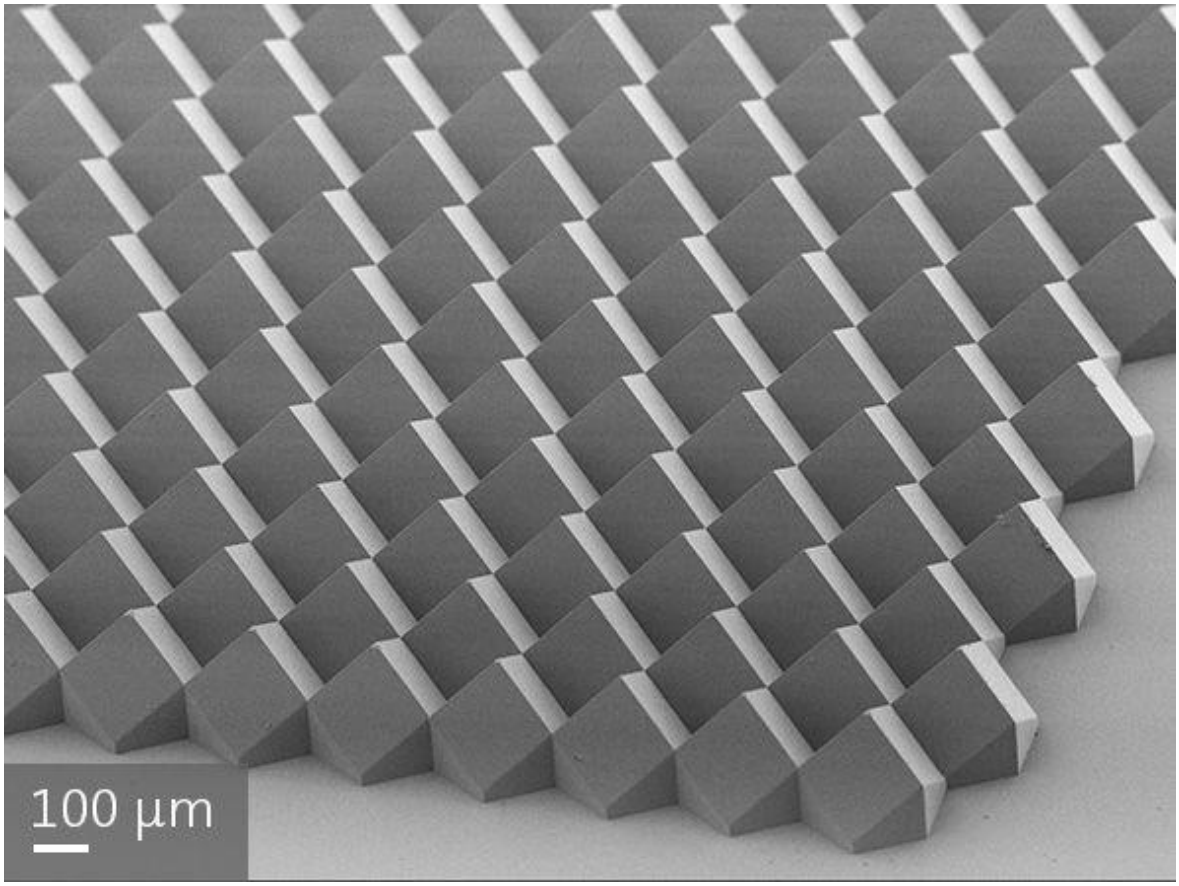

Figure 1.5 refractive optic consisting of repetitive micro cornercubes

1.3.2 Photonic

Photonic crystal are optical media which has a periodic modulation of the refractive index. They can be natural or artificial and they can be further divided depending on the geometry in one-dimensional, two-dimensional or three-dimensional.

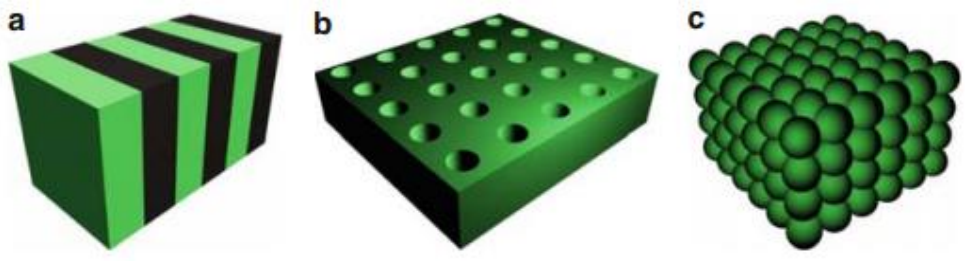

Figure 1.6 one-dimensional, two-dimensional and three-dimensional photonic crystal

These periodical structures generally have a geometrical configuration which correspond to a type of crystal lattice of solid state crystals.

While one-dimensional photonic crystals have very low numbers of configuration, two-dimensional crystal can have an infinite number of configurations if the shape of the single element can be varied. However usually square or hexagonal shapes are used so the number of lattice is limited. Three-dimensional crystals geometries can be varied in many different ways, but some of the fabrication methods can't achieve some of them. Two-photon laser lithography thanks its intrinsic tridimensionality allows a limitless design of 3D crystals giving the possibility to tune the optical properties. Some example of 3D structures obtained with nanoscribe can be seen in figure 1.7.

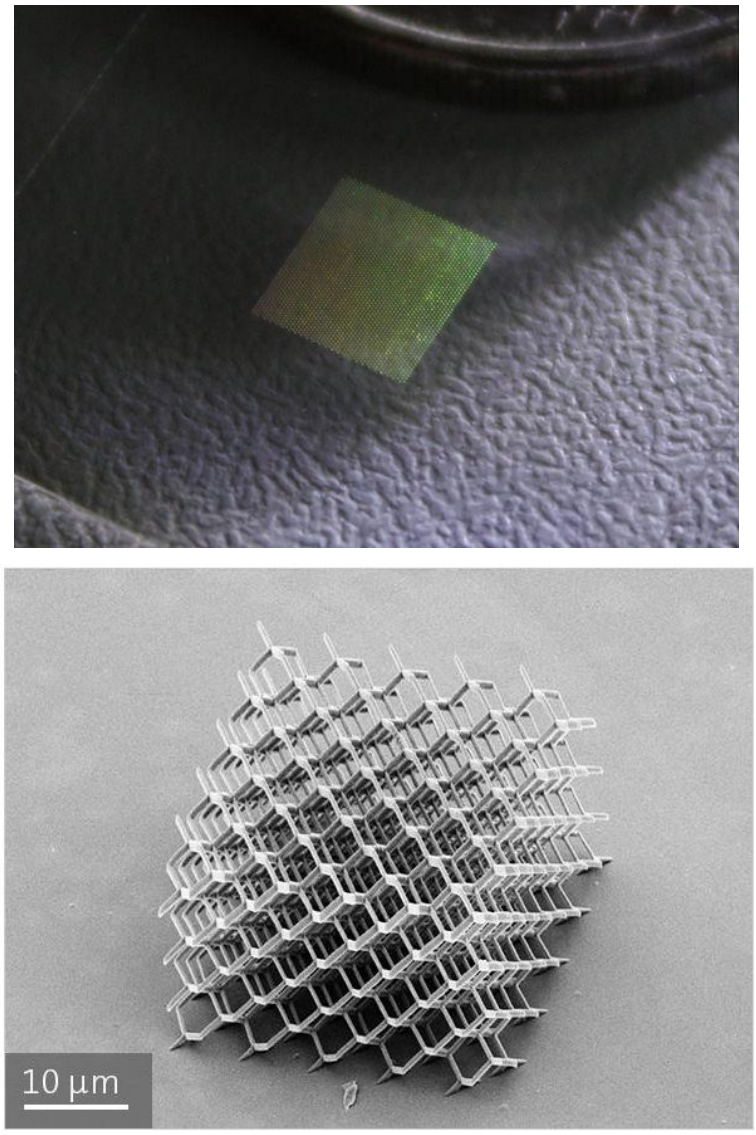

Figure 1.7 (up) A structure comprises of 2500 woodpiles and (down) a 3D photonic crystalline diamond in IP-L 780 [2]

1.3.3 Tissue Engineering

As will be better described in the following chapters, nowadays, an increasing interest in recreating tissue-like structures to better understand cellular processes is developing.

Two-photon polymerization can be used as a powerful tool, not only for its ability to recreate complex structures that can mimic the extracellular matrix but even due to the low probability of damaging bioactive materials due to its polymerization mechanism.

Moreover, using innovative material like hydrogels, two-photon laser lithography can open a wide range of new possibilities in biological applications.

Some examples of the synergic use of hydrogels and two-photon laser polymerization will be presented in chapter 3 and chapter 4.

Chapter 2
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Materials and methods

As seen in the previous chapter, photopolymerization is a chemical reaction that needs the right source of energy to be activated.
But energy is just one of the factors required for the reaction to begin: two-photon active monomers and initiators have to be tailored to obtain a high yield and manageable polymerizations.
This chapter will present a short overview of the resists commercially available as well as some at the cutting-edge alternatives.
Finally, the 3D laser lithography system used in the experimental part of this elaborate will be presented.

2.1 Two-photon active initiators

Energy given by the light beam is generally not absorbed by the monomers which will then form the long polymeric chain. For this reason, a photopolymerizable resist needs some photoactive molecules called photoinitiators which will make the process possible.
Obviously, these molecules need to absorb efficiently the energy coming from the incident radiation to become an active specie which, subsequently, can start the chemical reaction. But this is not the only prerequisite needed: they ought to be transparent to the wavelength of the radiation but active to the half of it. This characteristic gives the possibility for the beam to be focused correctly in every position of the liquid resist without being absorbed by the material it is passed through.
Moreover, to be an efficient initiator, this kind of molecule should have a high two-photon cross-section and, if possible, a high quantum efficiency.
The TPA cross-section is used as a parameter to compare the absorption behaviour of different molecules and can be estimated with equation 2.1 It is a measure for the probability of the absorption process and is related to the intensity of the applied light.

$$\sigma_2 = \frac{8\pi^2 h\nu^2}{n^2 c^2} * Im\left(\chi^{(3)}\right) \qquad (2.1)$$

TPA cross-section unit of measure is Goeppert-Mayer (GM) which corresponds to 10^{-50} cm^4 s $photon^{-1}$.

There are two groups of photoinitiators depending on whether the polymeric reaction is cationic or radical.

Cationic photoinitiators are electronically excitable compounds that can produce cationic initiating species via bond cleavage and, consequently, initiate the cationic polymerization.

They are generally mixed with coinitiators as, for example, photosensitizers to increase initiation efficiency. Onium salts (On^+X) are commonly used to initiate cationic photopolymerization because of their efficiency. [2]

Radical photoinitiators are the ones used in direct writing lithography which relies mainly on acrylate monomers polymerizations.

This group of photoinitiators can be once again divided in three different type of photoactive compounds: Type I, Type II and two-photon active photoinitiators [3] [4]

- Type I: Cleavable compounds which, once the energy is absorbed, are excited to a triplet state and split to produce two free radicals able to initiate the photopolymerization. Since the reactions involves just one molecule, this type of initiators exhibits a high efficiency (e.g. Irgacure I2959)
- Type II: the process of initiation involves two molecules and multiple steps. Firstly, one of the molecule absorbs the energy coming from the radiation and, once excited initiate a fast electron transfer to the second molecule (generally a tertiary amine) followed by a slow proton transfer process. The photoactive molecule undergoes a termination coupling with another radical while the amine reacts with a monomer to start the radical reaction.
- Two-photon active initiators: this group on initiators consist of electron donating and electron acceptors moieties linked together via a π conjugated bridge whose length can be tailored to achieve better performances. These molecules are chemically optimized to exhibit a much larger TPA cross-section in order to enhance the two-photon absorption. (e.g P2CK)

Figure 2.1 describes the different reaction mechanism followed by the radical initiators.

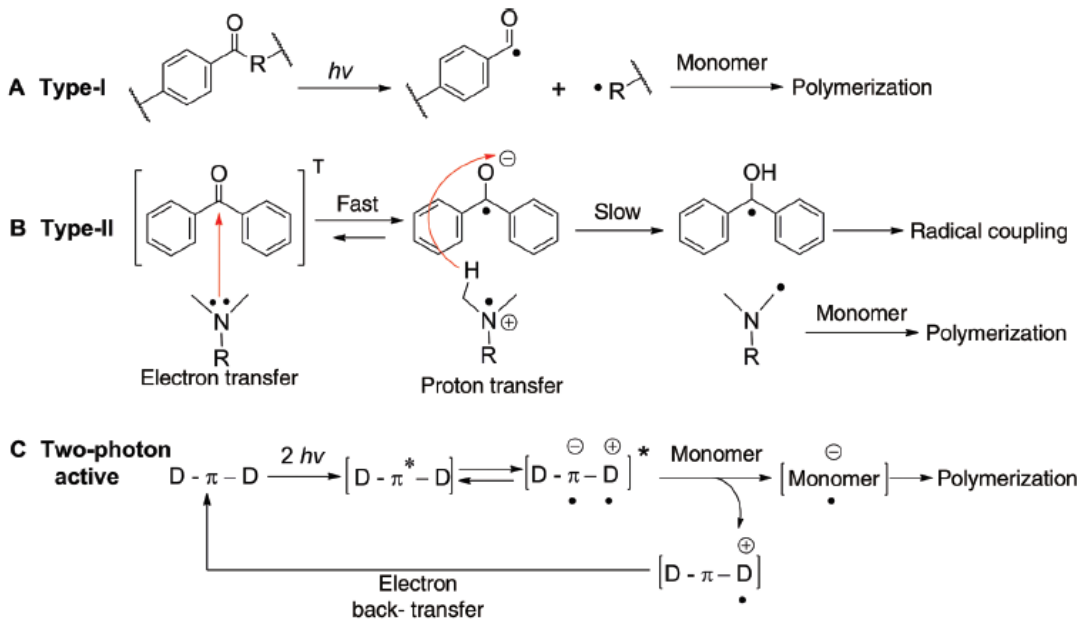


Figure 2.1 Reaction mechanism of Type I photoinitiators (A), Type II photoinitiators (B) and two-photon active initiators (C)

2.2 Resist

Photoactive resist used in lithography can be mostly divided in 2 categories: negative-tone resists and positive-tone resists.

The former group generally starts as a liquid mixture in two-photon lithography consisting of small molecules, monomers or prepolymers, photoinitiator and solvent. Its viscosity and reactivity can be tailored modifying the concentration of the solutes.

During polymerization, monomers, activated by the initiators, undergoes to a chain reaction which induces a phase-changing from liquid to solid due to the formations of long polymeric chains which can be chemically or physically linked one to the other forming a 3D network which forms the desired structure.

Positive-tone resist, on the contrary, are photosensitive solids whose chemical structure can be modified becoming a soluble material that can be removed with a solvent revealing the desired structure made by non-exposed resist.

Design and fabrication of 3D structures will be different in the light of which of these resists will be used. Positive-tone photoresists allow to attain bigger structures faster thanks to the smaller amount of resist that has to be exposed but there should be a continuity between every feature of the structure to let the solvent wash away the modified material.

Moreover, the washing solvent needs a period of time to complete the removing of the exposed resist that depends on the dimensions, number and shape of the structure's features. When fabricating with negative-tone resist, on the contrary, the dimension of the structure is the main restricting parameter. Big or extremely complicated structures can take many hours to be completed

2.2.1 Acrilate resists

Considering commercially available two-photon active resists, most of them are made of acrylate or methacrylate monomers. This kind of molecules, indeed, are generally very reactive especially the former. A high reactivity of the resist allows the polymerization to happen with lower laser's intensity and this is the reason why acrylate resists are favorite over other kind of resist when possible.

2.2.1.1 Nanoscribe resists

In 3D micro and nanolithography, Nanoscribe GmbH is, nowadays, one of the most innovative company.

They developed a Photonic Professional GT printer that can provide submicrometer features as well as the software to control the fabrication process and 4 different high-performance negative-tone resists.

This system is the one used in the experimental part of the elaborate and many applications which will be described in the forthcoming chapters take advantages of the high performance and the resists provided.

IP-L 780 is an acrylate prepolymer which is used to achieve the highest resolution. It is composed of 2-(Hydroxymethyl)-2-[[(1-oxoallyl)oxy]methyl]-1,3propanediyl diacrylate monomers for the 95% of the composition and 7-(dietilammino)-3-(2-tienilcarbonil)-2H-1-benzopiran 2-one for the remaining 5% which is a D-π-D two-photon active initiator.

It is drop deposited on a 170μm glass substrate and then polymerized. Once the fabrication process is completed the sample is immersed in Propylene glycol monomethyl ether acetate (PGMEA) for 20-25 minutes and, successively, in isopropanol for 3 minutes to complete the development.

IP-Dip is a negative-tone acrylate resist which major component is the same diacrylate monomer used for IP-L. while the photoinitiator is 9H-fluorene-9,9-diylbis(4,1-phenyleneoxyethane-2,1-diyl)-bisacrylate. IP-Dip is optimized for DiLL writing mode which is one of the available configuration of Nanoscribe photonic GT printer. This process,

which will be better describe later, requires the resist to directly touch the objective lens and this means that the resist has to be optimized not to damage it.

IP-S is third photoactive prepolymer which is used in mesoscale fabrication. The voxel size that can be obtained with this resist is bigger than those of IP-L and IP-Dip thanks to a higher proximity effect which let the polymerization continue all around the focused spot. A bigger voxel means a limited spatial resolution but, nevertheless, a faster fabrication of big structures.

It is generally used with a 25x objective to further increase the voxel size and, thus, decrease writing time.

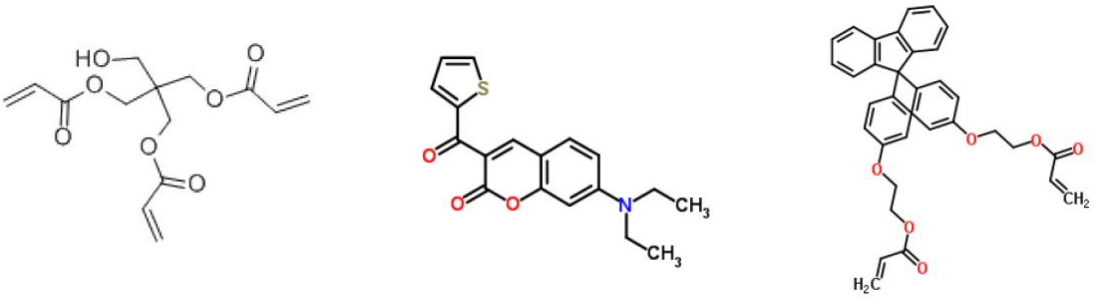


Figure 2.2 From left, 2-(Hydroxymethyl)-2-[[(1-oxoallyl)oxy]methyl]-1,3-propanediyl diacrilate, 7-(Diethylamino)-3-(2-thienylcarbonyl)-2H-1-benzopyran 2-one, 9H-fluorene-9,9-diylbis(4,1-phenyleneoxyethane-2,1-thenediyl)-bisacrylate

2.2.2 Innovative resist

Nanoscribe resists are the best solution when a rapid prototype of polymeric structures with good mechanical properties are required. However, many new fields of research are now willing to take advantage of the great opportunities that two-photon lithography can offer.

An inert polymeric matrix, as the one achievable with Nanoscribe resins, is not functional in these novel applications and new classes of photoactive materials have lately been developed.

Hydrogels, glass-like materials and positive-tone resist are now available and a quick overview of their properties and applications will be now presented.

2.2.2.1 Hydrogels

Hydrogels are polymeric materials whose peculiar hydrophilicity (which relies on the hydrophilic functional groups attached to the backbone) let them absorb a high amount of water without being dissolved.

Their structure is formed by a 3D network consisting in long polymeric chains entangled chemically or physically.

Thanks to their high hydrophilicity and their ability to react to external stimuli such as, pH [5], Ionic strength, solvent composition, light and electrical field (that makes them smart materials), they can be used in several applications. [6]

Hydrogel can be used in hygienic uses (diapers), agricultural uses [7], pharmaceutical uses (drug delivery, contact lens) but, the main breakthrough field of application is tissue engineering. [8]

Hydrogel are soft materials which can easily deform while maintaining their shape and their mechanical properties can be tailored increasing/decreasing physical or chemical links between polymeric chains.

Moreover, thanks to the high quantity of water absorbed they can allow cells diffusion and support assuring, simultaneously, exchange of chemical stimuli.

These unique properties, make hydrogel a perfect material to be used in mimicking extracellular matrix [9].

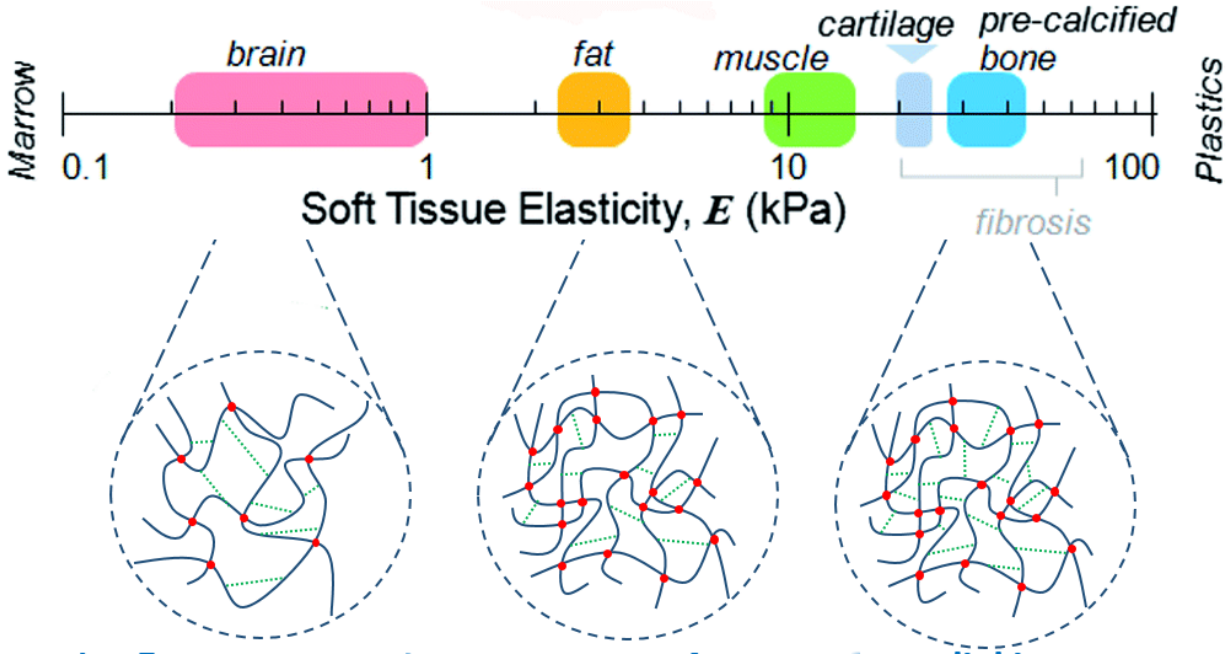


Figure 2.3 Tuneable mechanical properties of hydrogel due to degree of crosslinking and possible applications in tissue engineering

Hydrogel, furthermore, can be thermally or photo polymerized and radical reaction is the main used polymerization mechanism.

Generally, polymerizable hydrogel are liquid solutions containing monomers or prepolymers, crosslinkers and initiators (sometime with coinitiators or photosensitizers) that can solidify due to radical or cationic reactions forming a continuous network of interconnected long polymeric chains.

Hydrogel can be naturals or synthetic. Some noteworthy examples of natural hydrogels are proteins (collagen, gelatin, fibrin, Matrigel), polysaccharides (hyaluronic acid) or hybrid polymers (collagen/HA).

Synthetic polymers often used to generate hydrogels can be, once again, divided in biodegradable (PLA, PGA, PCL) and non-biodegradable (HEMA, PEGDA, Polyacrylamide) polymers.

Some of both natural and synthetic hydrogels will be presented in the following chapters.

Polymeric chains into the network are linked one to the other with chemical or physical crosslinks.

If chains are chemically bonded via covalent bonds the hydrogel is defined chemical hydrogel, however, if chains interact thanks to entanglements they will form a physical hydrogel.

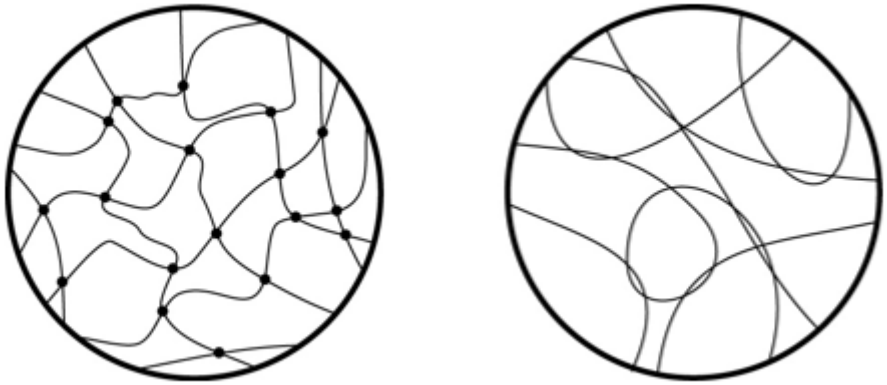


Figure 2.4 cross-links in a chemical hydrogel (on the left) and entanglements of a physical hydrogel (on the right)

To undergo to radical polymerization, monomers or prepolymers containing acrylate or methacrylated functionalities are generally used; natural hydrogels have to be chemically modified, to add these functionalities, as described later.

.

Figure 2.5 Hydrogel samples

Few examples have been recently reported in literature on TPP of hydrogels mainly for biomedical applications. Main challenge is the use of water as solvent and, as a consequence, water soluble and, if necessary, also biocompatible photo initiators. Moreover, high resolution structures and fabrication in reasonable times is still in its infancy.

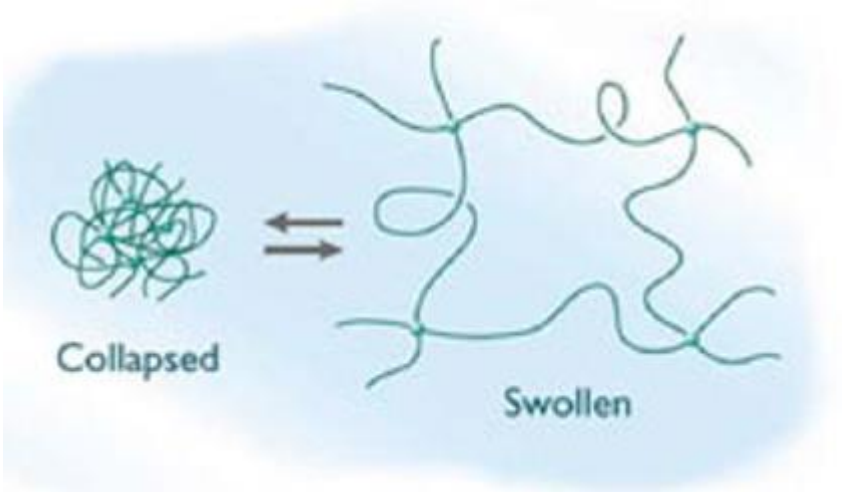
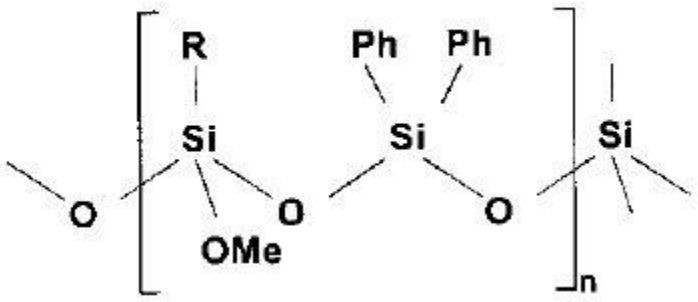


Figure 2.6 Swell of the hydrogel due to absorption of water

2.2.2.2 Ormocers

Ormocore and ormocomp are inorganic-organic hybrid polymers synthesized by sol-gel processing. Their structure is formed by a inorganic backbone linked to organic moieties



Ormocers exhibit peculiar features as:

- UV and two-photon patterning
- Fast curing
- Solvent free
- High mechanical properties
- Low shrinkage during curing
- High thermal properties (stability up to 350°C)

Their density, Young's modulus and thermal expansion coefficient are intermediate values between ceramic materials and polymers.

Ormocers are used in many applications as, for example, moulded gratings, micro lenses, micro lens arrays and prism.

Moreover, ormocers are non-toxic and biologically inert and ormocer-based cellular matrix are already commercially available as Definite or Admira.

If mixed with a radical initiator, ormocomp can be photopolymerized with two-photon laser lithography [10] as shown in figure 2.7

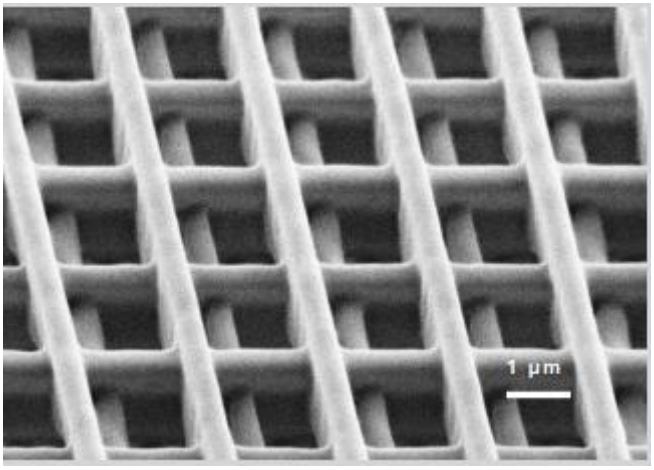


Figure 2.7 SEM image of a ormocomp 3D photonic crystal structure

2.2.2.3 AZ

AZ resists are based on the phenolic resins chemistry and are synthetized and commercialized by Microchemicals GmbH.

There are many different resists belonging to the AZ group so they are classified using a 4 digits number which follows the "AZ" in the name. First two digits represent the class of the resist while the last two digits are the thickness of the layer obtained if spin coated at 4000 rpm.

AZ resist are exposed to light and then developed in alkaline solutions able to dissolve all the previously exposed regions. Phenols are transformed in phenolates that are, successively, solvated by the acid solvent.

AZ resist can be used with Nanoscribe system mainly as positive-tone photoresist using air configuration and 20x, NA 0.5 objective.

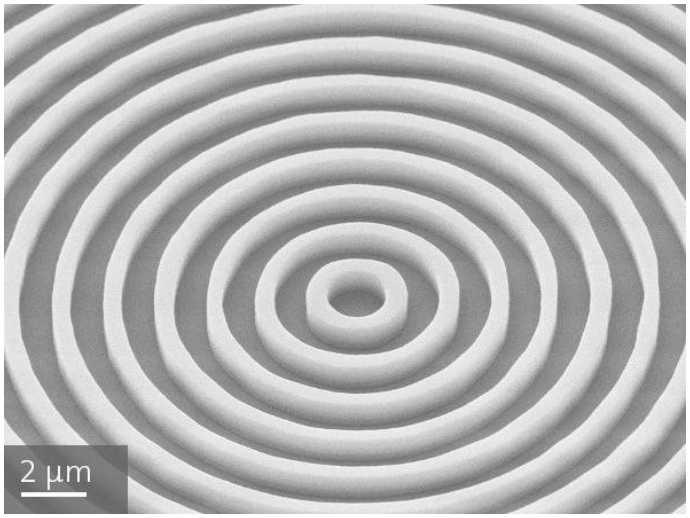


Figure 2.8 Circle test pattern using AZ701and Nanoscribe system

2.2.2.4 Foturan

Foturan is a photosensitive glass consisting of a lithium alumino silicate glass doped with Silver, Cerium and Antimony and it's commercialized by Schott Corporation.

Cerium, originally present as Ce3+ ions can release an electron through exposition to light radiation becoming Ce4+. Free electrons can, therefore, reduce Silver ions to metallic Silver Once the exposition is complete, during a heat treatment Silver starts to diffuse forming nanometric clusters when temperature is about 500°C.

Increasing temperature up to 600 °C the silver clusters are used as nucleus by a crystalline phase of lithium metasilicate that starts to grow into the amorphous glass. Thanks to the higher solubility of this new crystalline phase compared to the amorphous glass, the exposed regions can easily be etched by a diluted solution of Hydrofluoric acid.

Foturan is generally photoactivated by a UV light source and then etched following the work flow in figure 2.9. However, many papers nowadays reported 3D microstructuring in Foturan by femtosecond lasers. [11] [12]

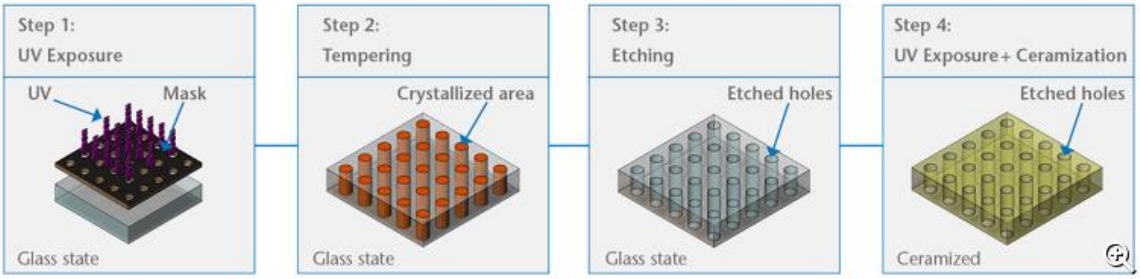
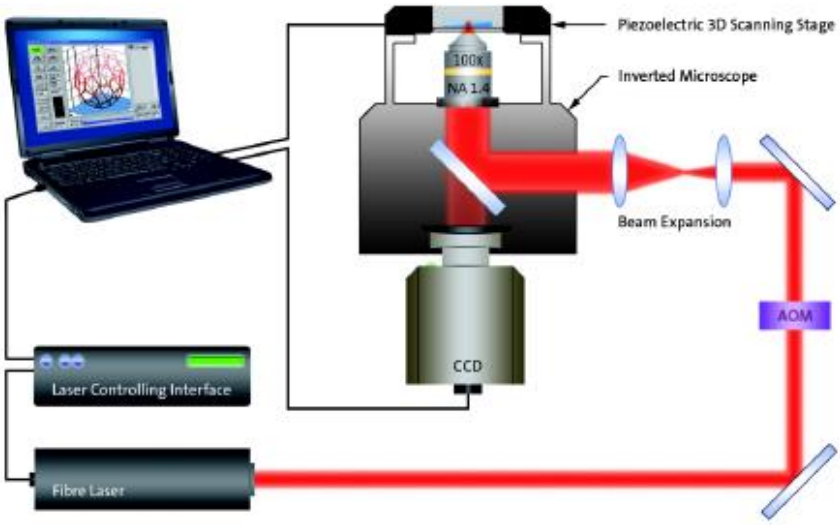


Figure 2.9 Work flow of 3D structures embedded into Foturan glass by UV photoactivation

2.3 Nanoscribe



Nanoscribe photonic GT professional is a 3D laser lithography system equipped with a pulsed Erbium doped femto second laser source at a center wavelength of 780nm.
This lithographic system takes advantage of an acousto-optic modulator and of an objective to focus an intensity modulated laser beam in a photoreactive resist.
In order to define the structure, the focused spot has to move along x, y and z direction. As for x-y movements two different scan modes can be used:

- Piezoscanmode: the sample moves thanks to the movement of the sample holder over a fixed laser beam
- Galvoscanmode: the beam is moved via a galvo scanner.

Moreover, Nanoscibe's system can be used in three different printing configurations:

- Oil immersion configuration: the objective is immersed in a matching index oil and the laser beam is focused through the substrate into the resist. The maximum structure hight is limited by the objective's working distance and by the substrate thickness
- Dip-in laser lithography (DiLL): the objective is directly immersed into the resist. In this configuration, the spherical aberration is minimized and he height of the structure is limited only by the sample holder used. DiLL configuration can be used only with DiLL compatible resist of nanoscribe.

- Air configuration: objective is immersed in air, the focus quality depends on the z position of the focus in the resist and the maximum height of the structure is defined by the working distance of the objective

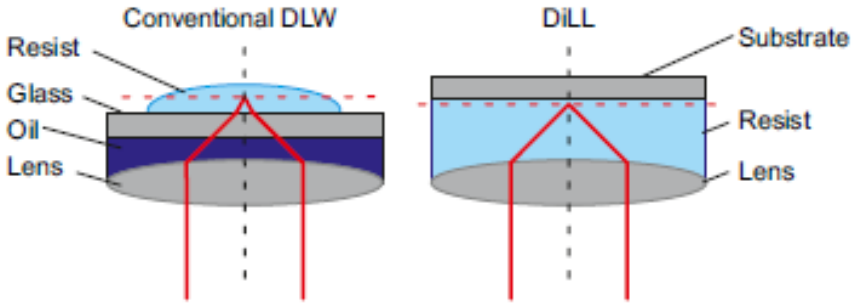


Figure 2.10 Comparison between oil configuration and DiLL configuration

2.3.1 Fabrication Procedure:

First step is the sample preparation: depending on which kind of resist and objective the fabrication will required the substrate can be:

- fused silica: for IP-Dip with 63x and 100x objectives in DiLL configuration
- a 170µm glass coverslip: IP-L or IP-G with 63x and 100x with oil configuration
- Silicon: AZ resist with 20x in air configuration
- ITO coated glass: IP-S with 25X objective in DiLL configuration.

All the substrates should be cleaned with acetone or IPA, blow dried and then resist can be drop casted on the surface of the suitable substrate. IP-G as opposed to IP-L and IP-Dip need a prebake for 1 hour at 100 °C.

Once the sample is ready, it can be loaded into Nanoscribe's system using the supplied sample holder and writing process can be started.

Using Nanoscribe's editor DeScribe a STL file (StereoLithography file) can be opened and the model of the structure can be sliced defining two parameters: Hatching distance (lateral distance between two adjacent lines within a layer) and slicing distance (height of a single layer). If the structure is too big it has to be divided in different sections that will be fabricated one after the other.

When the writing process is complete, the sample can be unloaded and developed.

Development consists of a first step in PGMEA for 20 or 30minutes, followed by a second step in IPA for about 2 minutes. The sample can finally be blow dried.

Chapter 3
-
TPP of GelMA for biomedical applications

3.1 GelMA

Gelatin methacrylate is a natural polymerizable material derived from a hydrolytic degradation of collagen and proper chemical modification.

Due to its natural derivation, it has many active sites for biological interaction as for example integrin binding motifs which allow cellular adhesion and metalloprotein degradation sites for enzymatic degradation.

GelMA is highly hydrophilic and, once the polymeric networks are formed it can absorb a high quantity of water becoming a perfect substrate for cellular culture, giving variable stiffness around 0.1-10 kPa that mimic different natural tissues. Swelling and mechanical properties can be tuned modifying the methacrylation degree and the gel concentration.

If mixed with a photoinitiator, GelMA can be photopolymerized via radical polymerization involving lateral methacrylate groups.

GelMA synthesis was performed in our lab following J.W. Nichol et al. procedure. [13]

Porcine skin gelatin (G2500, Sigma Aldrich) is dissolved into Dulbecco's phosphate buffered saline (DPBS) at 60°C to obtain a 10%(w/v) concentration.

Solution is mixed for 1 hour at 50 °C and 200 rpm. Methacrylic anhydride (8% (v/v)) is, then, added and the mixture is, one again, mixed for 3 hours at 60°C.

DPBS is finally added to terminate the reaction and a dialysis against distilled water is carried on for 7 days at 40°C and 500 rpm.

The solution is then filtered and lyophilized for 1 week to obtain a white porous foam.

Once the synthesis is completed, GelMA can be stored and solubilized in water according to the monomeric concentration desired.

Figure 3.1 shows schematically how GelMA is obtained from gelatine and how lateral methacrylate groups reacts due to the initiation to form a 3D continuous network.

As, said before, mechanical properties of GelMA can be tailored modifying the degree of methacrylation and the concentration of this monomer into the prepolymeric solution. The

more methacrylate groups are bonded to the backbone or, the higher the concentration of GelMA, the more the chains are linked to each other inside the polymer.

This results in an increase of the mechanical properties of the polymerized gelMA and in a simultaneous decrease of water absorption because of the tighter network.

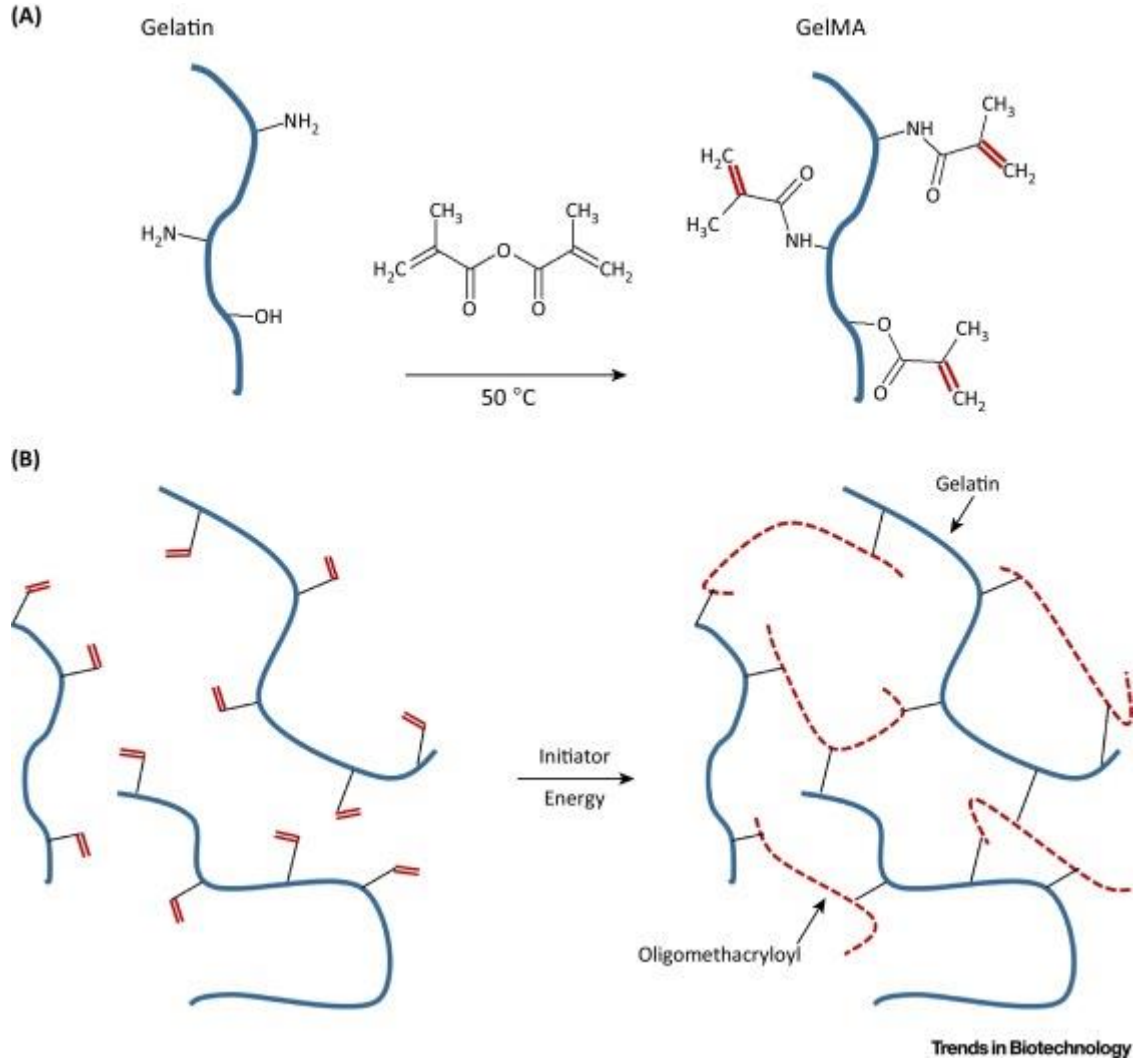
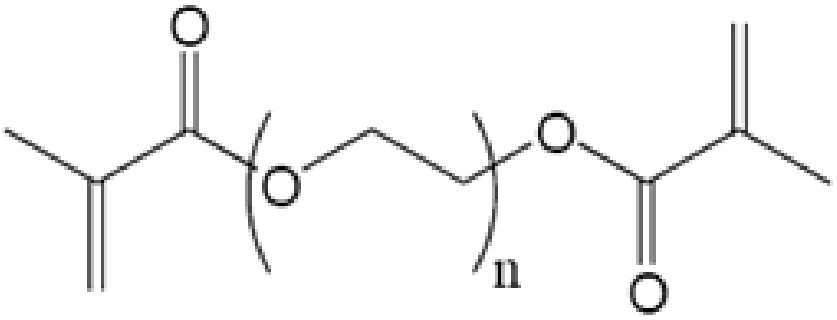


Figure 3.1 (A) GelMA schematic synthesis and (B) photolymerization of gelMA oligomers to continuos network

Possibility of properties tuning and its cell-responsive nature make this material a perfect alternative for mimicking extracellular matrix in cell-cell and cell-ECM interaction studies.

3.2 PEGDA

PEGDA is a PEG derived synthetic hydrogel formed by a PEG backbone with two acrylate lateral groups.



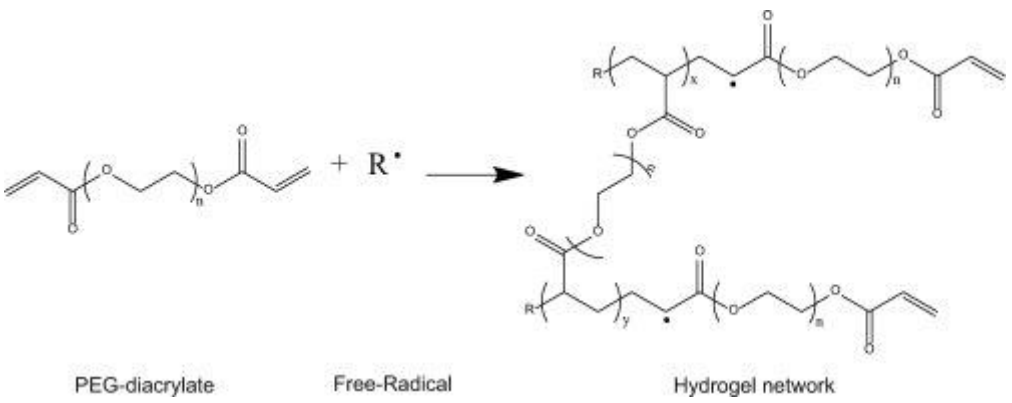
PEGDA is considered a biologically inert material ("blank slate") and this is why is largely used in cellular biology.

Its highly reactive acrylate groups permit a polymerization via a radical mechanism.

This polymerization has a low threshold and is easy to be performed so PEGDA can be easily polymerized through thermal or photo-induced initiation.

PEGDA can be polymerized to reach mechanical properties that can be tuned over a wide range of different moduli.

Due to its possibility to be polymerized as a soft matrix and thanks to the high quantity of water that can absorb becoming a hydrogel, PEGDA is becoming a viable alternative for tissue engineering and regenerative medicine. In fact, it is considered a biologically inert material ("blank slate") and this is why is largely used in cellular biology after proper functionalization. For this reason, 3D printing and also 2Photon polymerization studies have been recently developed.



3.3 Biocompatible initiators

Biological research is continuously increasing its interest toward two-photon laser lithography. Having the possibility to mimic extracellular matrix both for the material (using hydrogel) and shapes in micro and nanoscale, being able to study one single cell and how it behaves according to different mechanical stimuli, materials composition as well as spatial context is an amazing opportunity to better understand and control biological processes.

Human and animal cells, however, are very sensitive to cytotoxic monomers, initiators or polymerization by-products even though in very small concentration.

This is a major constrain for two-photon polymerization and has to be taken into account when prepolymer mixtures are prepared.

Since cellular studies is becoming one of the most important field of research in two-photon lithography, and due to the lack of any possibility to change monomers cytotoxicity, many researchers are now concentrating in developing new two-photon active initiators which can be used without damaging or even modifying cellular behaviour.

This new class of photoinitiators has to fulfil many different requirements:

- TPA cross-section as large as possible in order to decrease laser power needed
- Lack of cytotoxicity
- High quantum yield for a proper radical formation so that minimum amounts of initiator are involved
- solubility in water instead of aromatic solvents

Satisfy these conditions is possible only if the chemical structure of photoinitiator's molecule is custom-made.

Photopolymerization were used in biological applications before two-photon lithography was possible. Some photoinitiators were already available and one of them is noteworthy.

Irgacure 2959 is a type I photoinitiator, is the state-of-art in UV encapsulation of cells due to its low cytotoxicity and its hydrophilicity, and it is used as a benchmark for every photoinitiator. Its chemical structure is represented in figure 3.2. However, Irgacure 2959, has a small TPA cross-section and needs high laser power and low writing speed to initiate the two-photon polymerization.

Many other water-soluble photoinitiators are available (e.g Xanthene dyes) but generally their TPA cross-section is not so high and their biocompatibility is generally low.

Thanks to chemical synthesis, however, nowadays different well-known water-soluble initiators which lack of cytotoxicity has been proven and that were used in hydrogel fabrication in presence of a living organism are accessible.

One of them is a benzylidene cyclopentanone dye which is a two-photon active photinitiator with π conjugation synthetized in 2008 by Xiaojun Wan [14]. This initiator has a TPA cross-section of 287 GM at 800 nm and a low energy threshold of 0.51 mW at 400 nm.

Moreover, Torgensen et al. in 2012 synthetized a water-soluble initiator called WSPI whose biocompatibility was verified with a living test organism. Its TPA cross-section is 120 GM at 800 nm. [15]

Professor Robert Liska, at Technische Universitat Wien in 2013 published a paper in which a series of cyclic benzylidene ketone-based two-photon initiators are described. P2CK, G2CK and E2CK are characterized by a TPA cross-section at 800 nm of 176 GM, 163 GM and 201 GM respectively. Their cytotoxicity was tested with two different cell types and P2CK exhibits a cytotoxicity as low as Irgacure 2959. [16]

Lately, in 2016 Liska's group announced a new Hyaluronan based photoinitiator called HAPIs which was used in the fabrication of a methacrylated gelatin photopolymerization.

Cytocompatibility was also evaluated finding a reduction of its value if compared with P2CK and E2CK. [17]

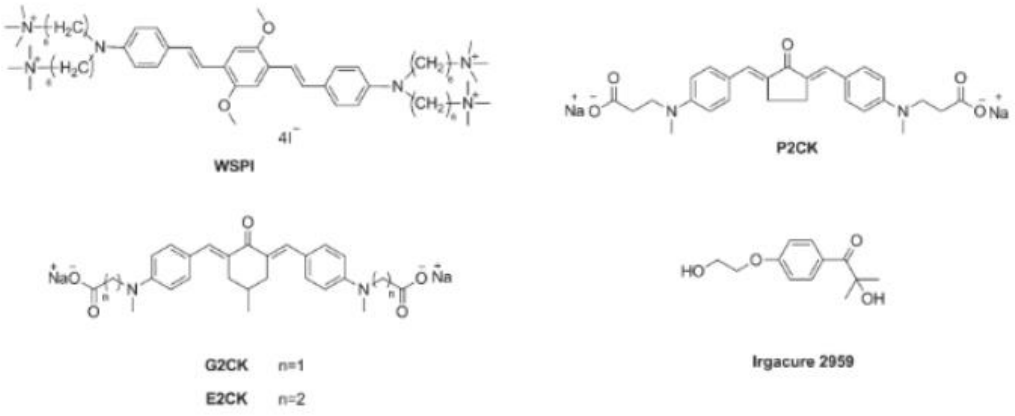


Figure 3.2 Liska's group photoinitiators and I2959 as a reference

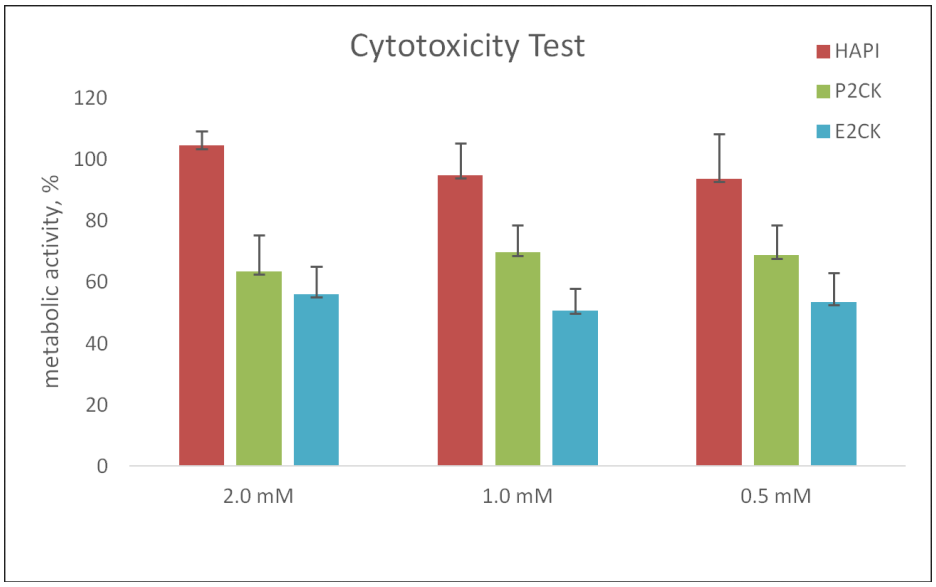


Figure 3.3 Comparison between cytotoxicity tests of P2CK, E2CK and HAPI

Thanks to the collaboration with Liska's group, P2CK was successfully used in our TPP experiments of GelMA.

3.4 3D microfabrication of perfusable channels

Tissue engineering is the application of engineering and life science principles to restore, maintain or improve tissue function. This relatively new field of research combines many different knowledges from material science, biology, genetic and engineering and its focus is recreating a natural-like tissue where to study cellular behaviour and morphogenesis.

Also, more innovative is 3D organoid technology, that can be used to model human organ development and various human pathologies "in a dish".

Recently advances in 3D culture technology have shown that embryonic and adult mammalian stem cells can exhibit their remarkable self-organizing properties, and the resulting organoids reflect key structural and functional properties of part of organs such as kidney, lung, gut, brain etc.

To mimic most of these epithelia, porous 3D scaffold made by biocompatible materials that can recreate a suitable environment for cells should be used; in this respect, hydrogel materials are able to mimic at least in part the complexity of ECM. Their stiffness and texture con imitate the one typical of most of the tissues due to proper modification of the mechanical properties (stiffness tuneable between 0.1 and 10 kPa) combined to a high porosity in order to allow cell penetration, adhesion and remodelling.

Lack of vascularization, however, is one of the most important limitation of these artificial tissues and many researches are now focusing on how to overcome this problem.

The goal now is to simplify as much as possible a vascular system and analyse, one-by-one all the possible facets of the problem to better understand how to deal with it.

The purpose of this project is to fabricate a hydrogel hollow structure as 3D scaffold for micro vessel formation, which can allow a liquid perfusion and whose mechanical properties are high enough to bear the pressure gained. To this purpose, we used GelMA based hydrogel as scaffold which is adequate for cell culture and vessel epithelia generation. On top of it organoid culture could be then designed.

3D two-photon lithography can become a crucial tool thanks to its intrinsic tridimensionality and high resolution.

This study is carried on a microfluidic chip consisting of a glass substrate 170µm thick, a PDMS hollow structure and a hydrogel igloo-like structure.

The two structures are placed on the opposite sides of the glass coverslip and are interconnected thanks to a micrometric hole passing through the glass thickness (figure 3.4).

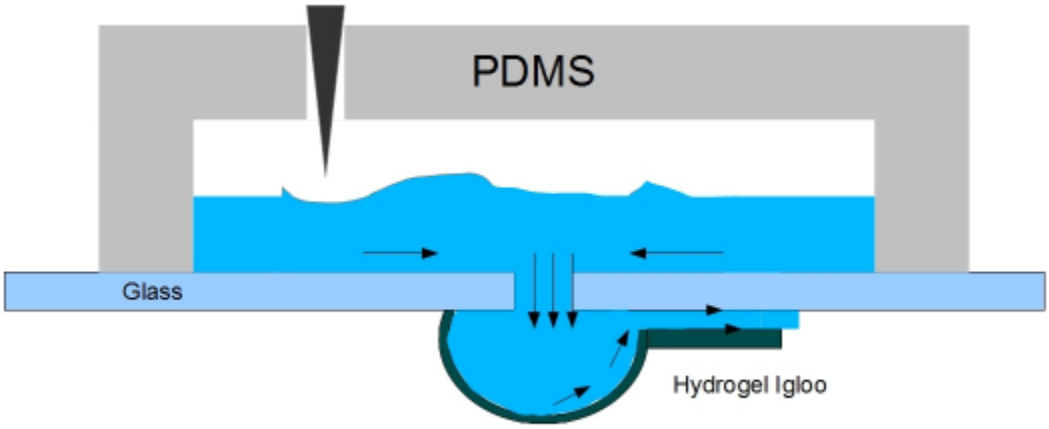


Figure 3.4 microfluidic chip configuration

Long-term objective is to add a second hole in the glass, fabricate another PDMS chamber and a second igloo structure which will be coupled to the first one through a small capillary. A liquid would be injected from the first PDMS chamber, should pass through the hydrogels structures and be collected in the second PDMS chamber as can be seen in figure 3.5.

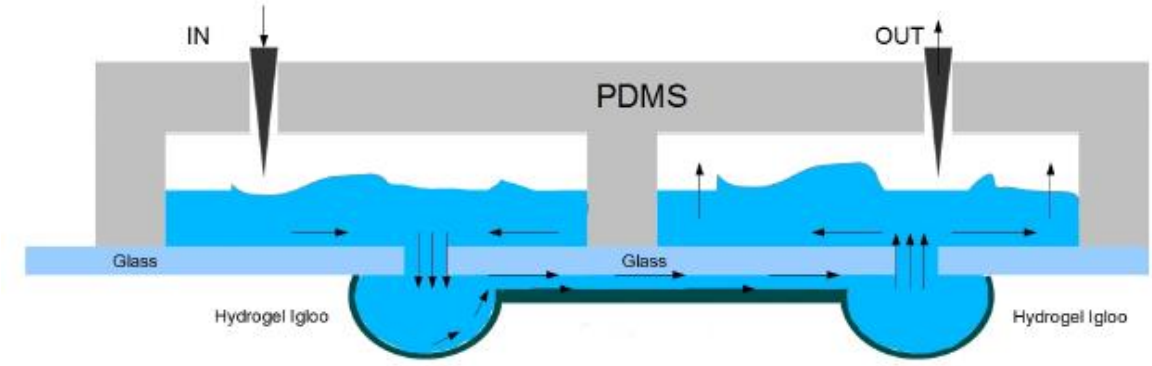


Figure 3.5 microluidic chip with a continuos perfusable system

3.4.1 Design and fabrication

As said before, thinking about future biological application, a aqueous gelatin-based prepolymer (GelMA) was chosen. The photopolymerizable solution should not need in principle any further cross-linker thanks to the multiple functional groups that can already develop a 3D network.

Radical photoinitiated reaction of GelMA, however, is not so efficient with two-photon laser lithography because of the low reactivity of methacrylate groups for radical polymerization. This means that completing a single igloo structure with only this monomer would take too long and a better mixture has to be prepared to increase fabrication feasibility.

This is why GelMA was mixed with a second crosslinker, PEGDA, which is a diacrylate polyethylene glycol that, thanks to its acylate groups (instead of methacrylate ones) can rapidly reacts with lateral groups of GelMA and form the 3D network.

Moreover, a two-photon active compound has to be added to allow the photoinitiation.

P2CK from Liska's group of TU Wien was chosen for its low cytotoxicity and its high TPA cross-section. [16]

Considering that opposed to GelMA that is a natural polymer, PEGDA is synthetic which tends to impede cell adhesion, a trade-off for these monomers ratio is needed to allow fast writing and good cell compatibility.

First mixture's composition is described in table 3.1. This first solution has a small amount of PEGDA trying to enhance cell interactions.

Compounds	Function	Concentration
GelMA	Main monomer	10% w/v
PEGDA 700	Crosslinker	1% v/v (GelMA)
P2CK	Initiator	1% v/v (GelMA+PEGDA)
Water	Solvent	

Table 3.1 mixture's compostition of first fabrication test

Two-photon polymerization of this mixture gave good results and, the structure obtained can be seen in figure 3.6.

A second test was performed trying to increase fabrication speed. To achieve this purpose monomers concentration in water was increased as well as the ratio PEGDA:GelMA. The resulting mixture composition is in table 3.2.

Compound	Function	Concentration
GelMA	Main monomer	35% w/v
PEGDA	Crosslinker	20% v/v (GelMA)
P2CK	Initiator	1% v/v (GelMA+PEGDA)
Water	Solvent	

Table 3.2 mixture's composition for second polymerization test

Fabrication starts with a 3D CAD model of the structure desired. For this application the model used can be seen in figure 3.6: an igloo-like hollow structure whose radius is circa 120 μm with a lateral branch needed for the liquid to flow out of the structure.

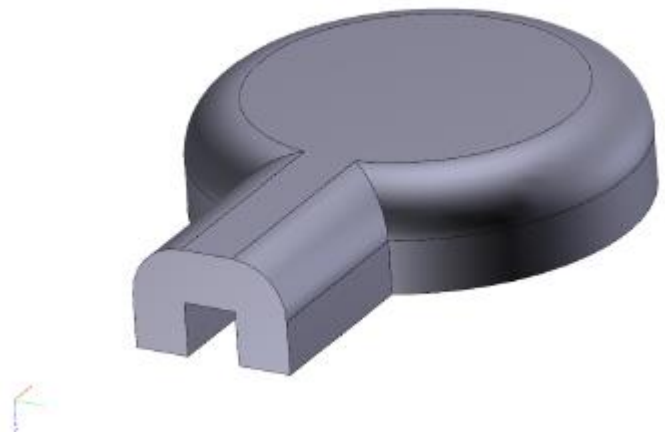


Figure 3.6 CAD model of igloo hollow structure

Once the model is ready, it is converted in STL file with can be read by Nanoscribe's software DeScribe 2.0. Operator now, decide how DeScribe should divide the structure choosing the monolayer fabrication height (slicing distance), the distance between every single line drew by the laser (hatching distance).

Moreover, operator decide three major fabrication parameters which are laser power, power scaling and writing time as already described in Chapter 2.

DeScribe can then convert STL 3D model into a GWL file that which is a command file that can be read by Nanowrite.

Furthermore, DeScribe can compute and display a simulation of the writing process giving an estimated writing time.

The GWL file is then loaded in Nanowrite software which can control the writing process thanks to the commands provided by GWL file.

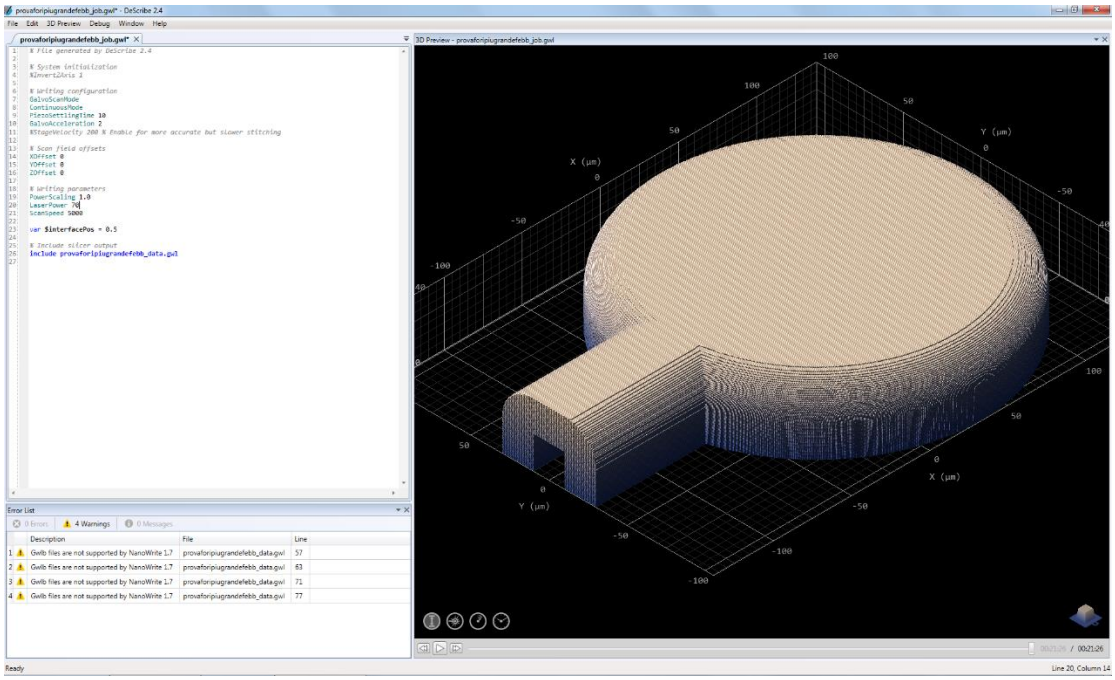


Figure 3.7 Describe's window presenting the division of the structure, the estimated time and the writing commands.

Before starting the writing, is important to prepare and load the sample where the 3D structure will be fabricated.

Hydrogel are highly hydrophilic materials but, when a single drop of them is placed over the substrate to start the polymerization, a certain amount of the absorbed water can evaporate modifying the solution concentration, its properties and the writing conditions.

Therefore, the sample has to be prepared in a sandwich-like configuration before being loaded into Nanoscribe system.

This configuration consists of one functionalized coverslip (to enhance the adhesion between glass and hydrogel), a drop of the solution to polymerize and another plain glass coverslip right above the resist. This upper coverslip spreads the resist all over the substrate and reduces the rate evaporation of the solvent. The prepared sample can now be loaded in Nanoscribe sample holder and fabrication can start.

Both the fabrication of the first and second prepolymer mixtures provided good results with structures matching the 3D CAD model, but, as we can see from table 3.3, scan speed achievable with second mixture is higher. This result proves that, modifying the solution composition, increasing the content of PEGDA, there is actually a change the writing parameters required for a correct fabrication.

Solution	LaserPower	PowerScaling	ScanSpeed
Solution 1	70	1	3000
Solution 2	70	1	5000

Table 3.3 Writing parameter for fabrication of GelMA 3D structures

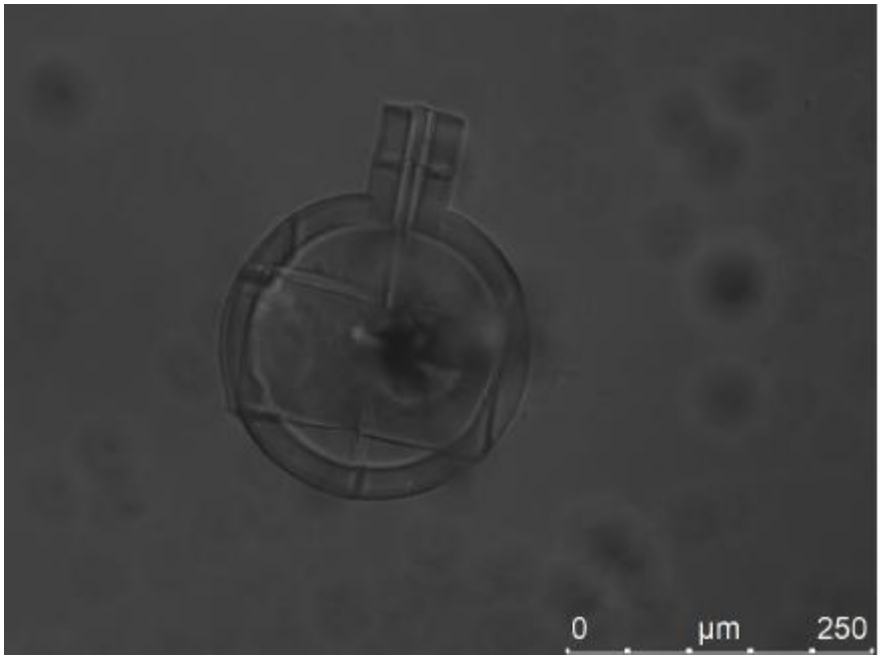


Figure 3.8 GelMA polymerized structure as can be seen from the Nanoscribe internal camera

GelMA igloo were fabricated Nanoscribe oil configuration (See Chapter 2 for more details). Trying to avoid oil to flow through the hole in the substrate, fabrication was, at first, carried on placing the drilled coverslip on top of the resist and fabricating the structure in a downward direction from the upper resist-glass interface down toward the lower glass substrate.

Once the sample was placed inside Nanoscribe system we succeeded in finding the interface between resist and the lower glass substrate but, moving upward, no other interface could be found. This is probably due to the fact that the resist layer between the two coverslips was too high and the upper interface was out of the objective working distance (190µm).

This configuration was therefore immediately abandoned due to the high viscosity of the resist and limitation of the system.

Preparation of the sample was therefore modified placing the functionalized substrate on the bottom and the plain coverslip on the top. This configuration allowed to the fabrication to be completed but the obtained structures exhibited a lack of polymerization in the region over the hole of the substrate.

The index matching oil, perhaps, flowing through the hole, removed the photoactive resist preventing the polymerization to happen in that region.

Achieving results as good as the one in figure 3.7 means trying many combinations between the three mains writing parameter: Laser Power, Power Scaling and scan speed.

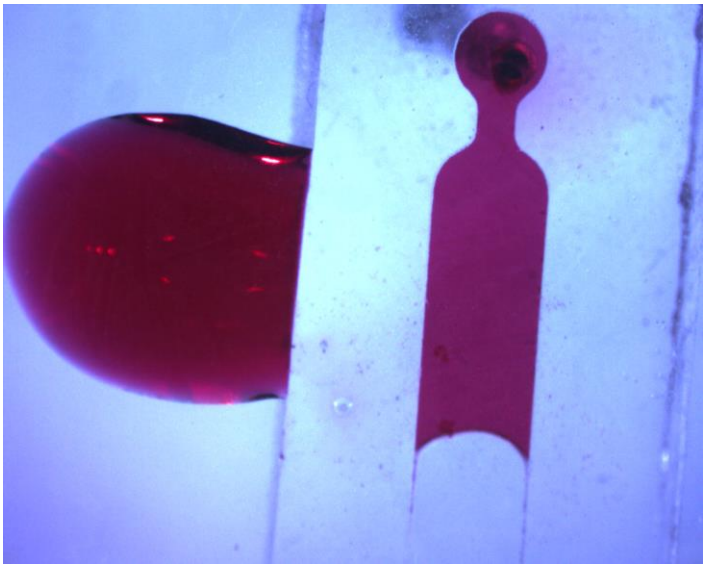
This is long trials and errors procedure that won't be described here. However, is crucial to underline how optimizing a two-photon lithography process generally means hours or even days to fully understand how writing parameters and material composition modify the fabrication.

Even though a complete polymerization was not achieved, a perfusion test was performed to evaluate the adhesion of the structure to the substrate.

A microfluidic channel made by PDMS was attached to the opposite side of the glass substrate and red fluid was injected with a syringe with a constant flow of 100nL/min.

However, during perfusion, a loss of liquid happened due to an insufficient adhesion between PDMS and glass and this precluded the perfusion of the scaffold.

Lack of adhesion between PDMS and glass in wet condition is a major problem for this kind of devices.



3.5 3D microfabrication of pierced septum

This second project involving 3D microfabrication of GelMA hydrogel for biological purposes is born from a collaboration between industrial engineering department and neuroscience department of University of Padua on human hearing apparatus regeneration study. This project was possible due to positive results achieved in the previous example,

where fabrication results highlighted how modifying mixture's composition can decrease writing time of 3D GelMA structures.

Here, even if this project has just preliminary results on 2D hydrogel films, it will be possible to understand how achieving a higher fabrication speed can simultaneously decrease cell viability on the material.

Human hearing system consists of many different tissues and types of cells. However, many pathologies that involve this system relies on the lack of conversion of the mechanical impulses in electronic stimuli that then can reach the brain via the nervous system.

This problem can cause deafness or disabling hearing impairment and approximately 5% of the world population suffer from this disease. [18]

Two different types of cells are involved in conversion and transmission of mechanical stimuli which takes place inside the cochlea: cochlear hair cells and auditory nerves fibers.

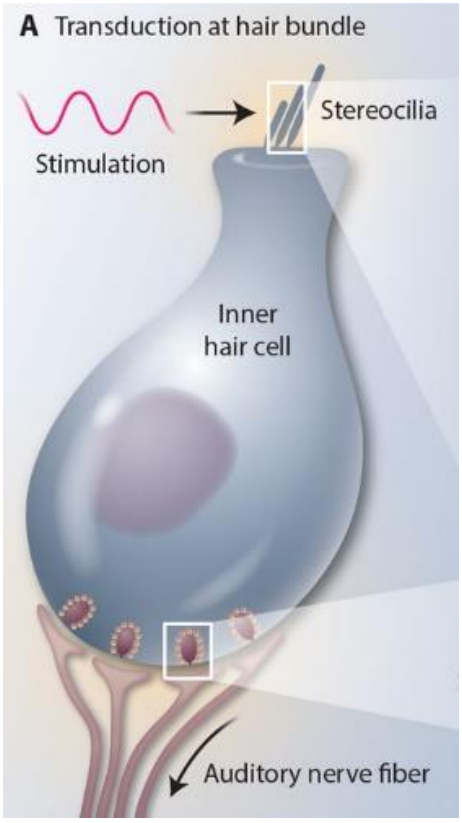


Figure 3.9 hair cell and auditory nerve fiber connection in choclea

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The aim of this study is fabricating a 3D hydrogel structure where these different kinds of cells can live, develop and interact one with the other.

Preliminary tests to evaluated how different types of hydrogels change cell viability have already been performed in 2D films of Polyacrylamide hydrogel and 2 different mixtures of GelMA and PEGDA.

These films are produced via UV photopolymerization using Irgacure 2959 thanks to its low cytotoxicity.

Hydrogel solutions were dropped on a functionalized substrate and a second glass coverslip was then placed on the top of the resist. To initiate the photopolymerization these sandwich-like samples were placed under a UV light and exposition was carried on for 2 minutes.

Once the hydrogel was polymerized, the top glass coverslip was removed and the sample was placed in water.

Composition of the three different mixtures are reported in table 3.4.

Two different ratios of GelMA and PEGDA were tested in order to see how this composition parameter (which can modify two-photon laser lithography writing speed) can change cellular behaviour

solution	composition	concentration	solvent
PAA	Acrylamide Bisacrylamide I2959 (1% v/v (AA+BAA))		water
GelMA/ PEGDA 80/20	GelMA 80% wt/wt (GelMA+PEGDA) PEGDA 20% wt/wt (GelMA+PEGDA) I2959 1% wt/wt (GelMA+PEGDA)	35%	water
GelMA/PEGDA 50/50	GelMA 50% wt/wt (GelMA+PEGDA) PEGDA 50% wt/wt (GelMA+PEGDA) I2959 1% wt/wt (GelMA+PEGDA)	35%	water

Table 3.4 compositions of solutions used for hydrogels films

Viability test was performed in neuroscience department of University of Padua using OC-k3 cells.

Adhesion and vitality controls were performed 24h, 48h, 72h after cell seeding using MTS method.Results of these can be seen in figure 3.10 and 3.11.

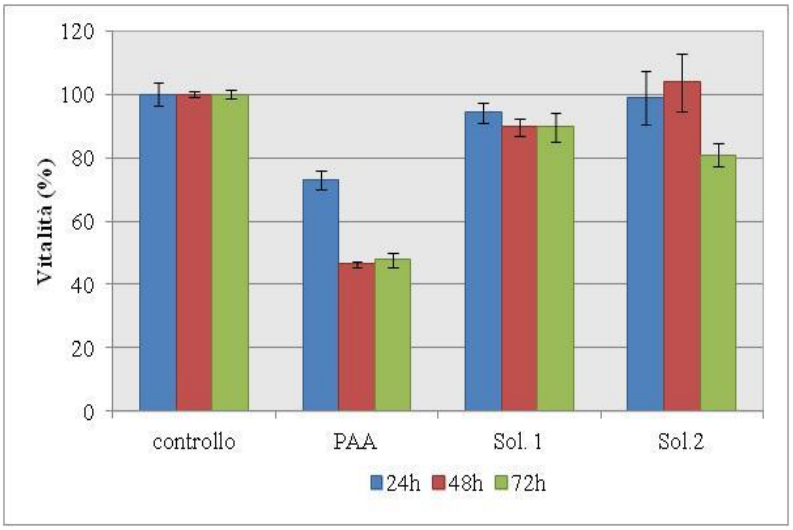

Figure 3.10 percentage of OC-k3 alive after 24,48,72 h

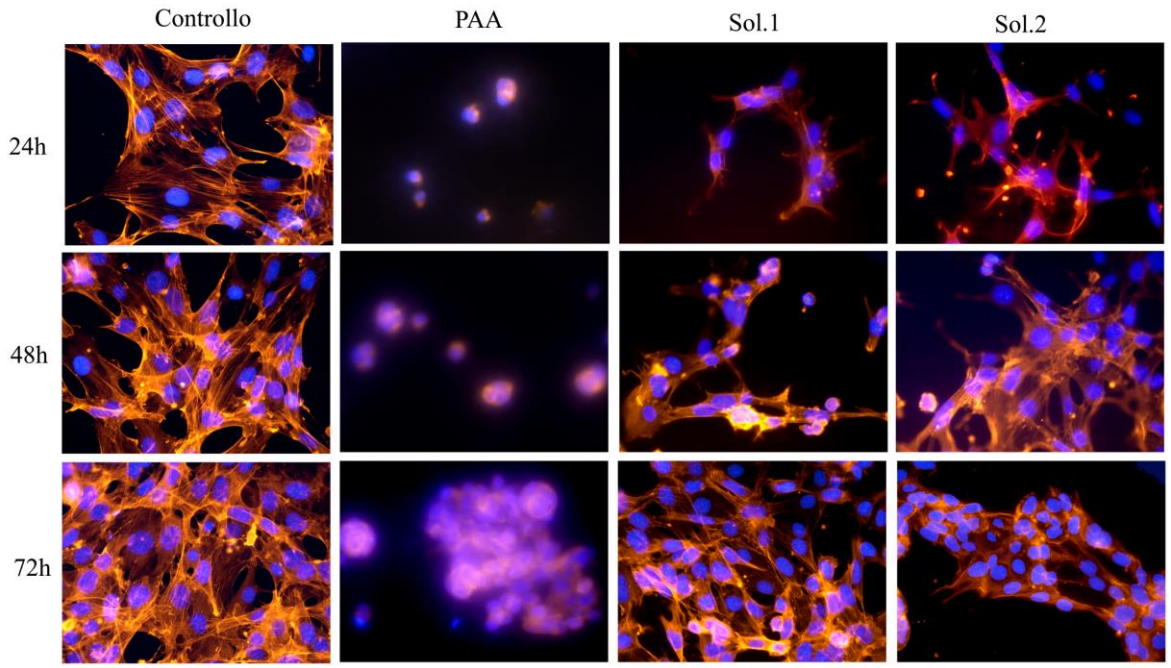

Figure 3.11 Morphologic coloration of OC-k3 in order to highlight cytoskeleton and nuclei

Conclusions derived from these preliminary tests are that:

- PAA is not toxic but on this substrate cell cannot adhere and grow,
- Sol 1 and Sol 2 hydrogels allow cell adhesion and development of cells
- Vitality on PAA hydrogel decrease of 30% after 24h and 55% after 48-72h
- Vitality on Sol 1 hydrogel doesn’t show any decrease
- Vitality on Sol 2 hydrogel decrease of 20% after 72h

These are just preliminary results but show how GelMA is a good alternative to the extracellular matrix and, on this hydrogel, cells can adhere and develop. Maybe, PEGDA can modify slightly cell vitality and growing.

Many more tests are mandatory to have a complete overview on how these substrates interfere with cellular development.

Once adhesion and viability tests will be concluded, a 3D structure will be fabricated using two-photon laser lithography.

The 3D CAD model of this structure is presented in figures 3.12-3.13

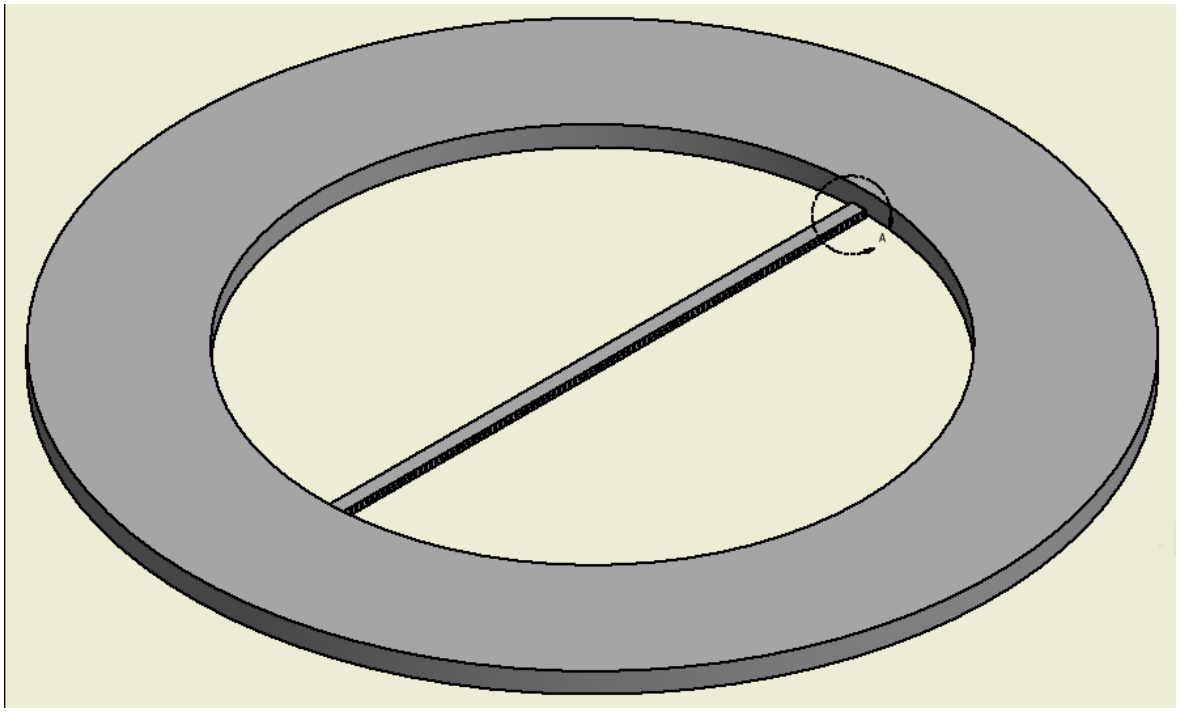

Figure 3.12 3D structure for choclear cells study

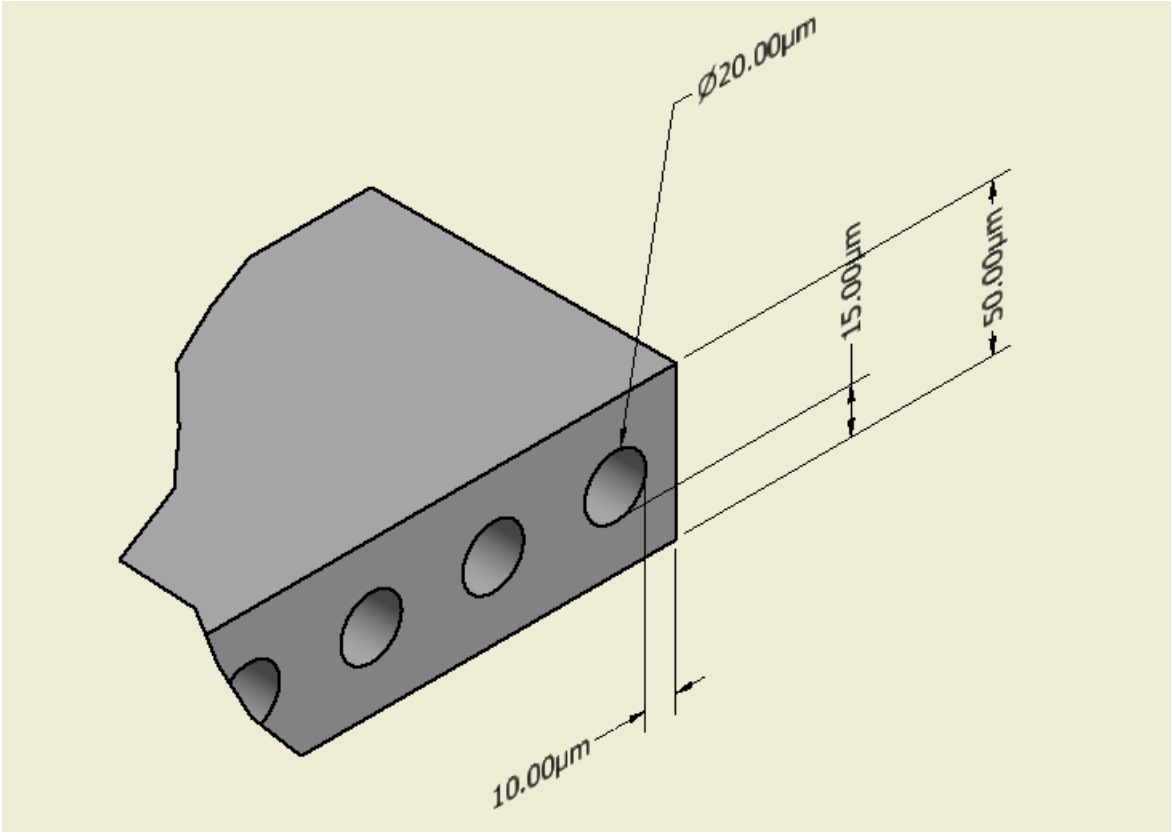

Figure 3.13 Detail of hydrogel pierced septum

This structure will consist of a pierced septum of hydrogel fabricated via two-photon polymerization and a PDMS gasket all around the septum in order form two different chambers for the two different cochlear cells.

The Hydrogel septum will be a meso-scale structure and a deep optimization of process, design and material will be required to complete the writing process in an appropriate writing time.

Chapter 4

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TPP of acrylate polymers for mimicking intestinal crypts

This project is a collaboration between Industrial engineering department and molecular medicine department (Dr Stefano Piccolo) of the University of Padua. Two-photon lithography was performed at TE.SI laboratory in Rovigo while cellular analysis were performed by Stefano Piccolo's group in Padua.

4.1 3D shapes for cellular branching studies

Many human and animal organs as for example the kidney, the lung, the mammary gland exhibit a treelike structure. Their peculiar structure is naturally developed through a reiterative cellular branching process which can transform a pre-existing epithelium in a treelike tissue.

Being able to understand how this morphogenic mechanism develop in humans and animal tissue and which cues need to be taken into consideration would be a major step forward in understanding and controlling how such organs grow and mature.

Nelson et al. [19] published in 2006 a study proposing a simple tubular shape mimicking the mammary rudiment to study how positional context can modify cells behaviour and branching mechanism.

This same simple shape can imitate morphologically many other human tissues as for example the crypt structure of intestinal gland (figure 4.1).

This structure is, therefore, a perfect starting point to study how spatial context can alter cells behaviour.

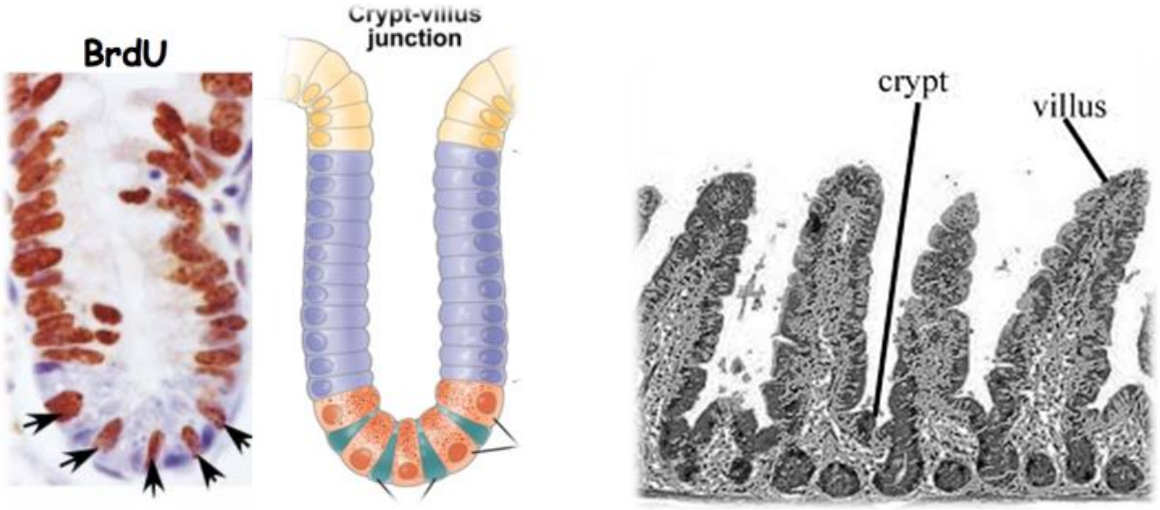

Figure 4.1 crypt-vilius structure in intestinal gland

Fabrication of the tubules in a gelled collagen was made via a micropatterning technique using an elastomeric mould (replicated from silicon mould made by lithographic techniques) and a matrix of collagen physically gelifed at high concentration (figure 4.1)

This fabrication methods allowed to obtain simple 2.5D structures whose shape modified cellular behaviour as for differentiation and development.

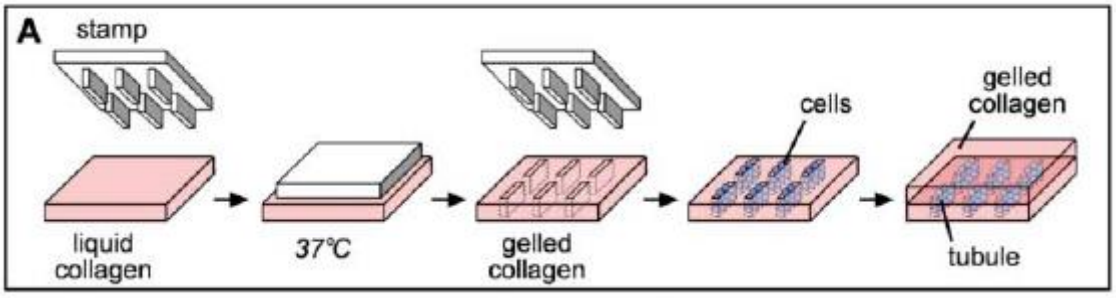

Figure 4.2 schematic micropatterning fabrication method used by Nelson et al. [19]

Once mouse mammary cells were embedded and epidermal growth factor added, multicellular branches were observed from the two ends of the tubular cavities proving that spatial context can indeed alter cellular morphogenic process (figure 4.2).

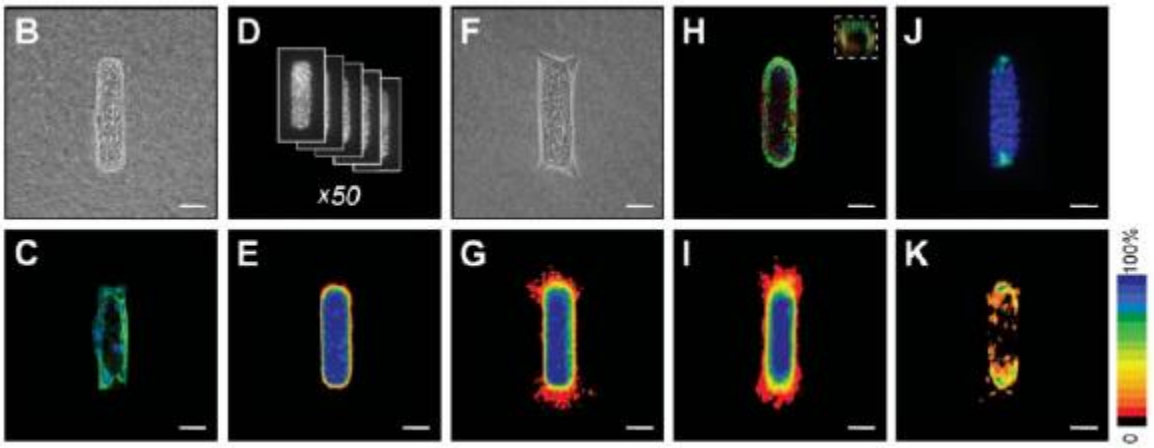

Figure 4.3 [19]

This study provides remarkable results but has a major limitation from the fabrication point of view: changing the shape of the elastomeric moulds means to fabricate each time a different silicon negative mould of the desired shape and this limits the versatility of the method.

A development of this study needs to overcome this shortcoming and expand the achievable shapes decreasing, simultaneously, the fabrication time needed to obtain a new mould.

Two-photon lithography can, therefore, become a perfect tool to attain these goals.

4.2 Moulds fabrication

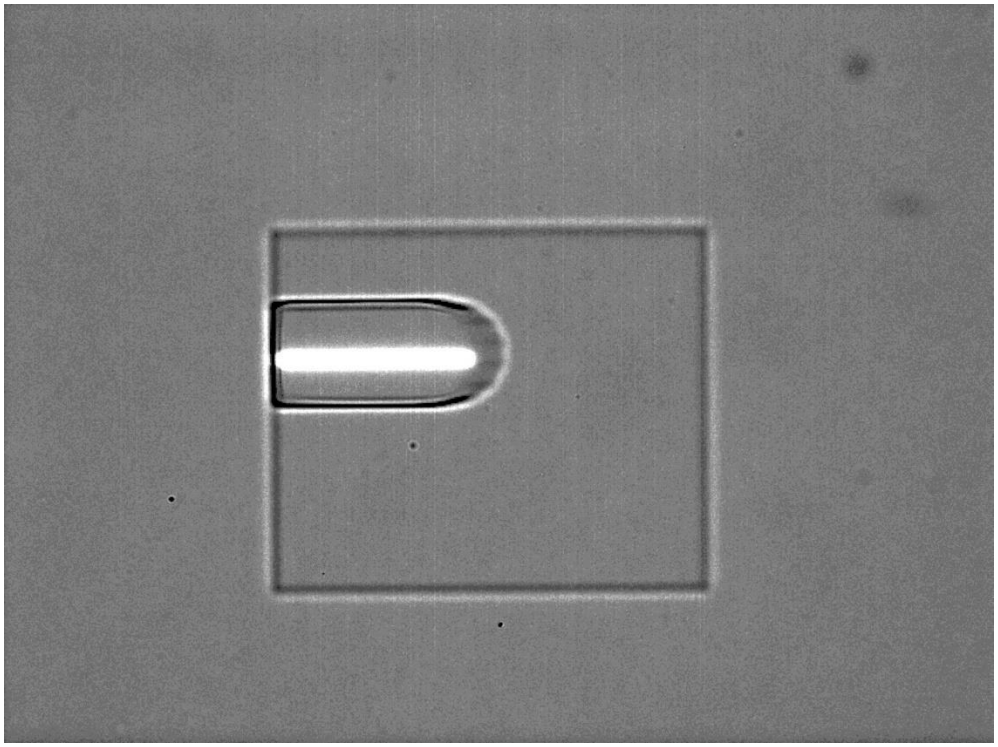

Figure 4.4 structure's fabrication as can be seen from nanoscribe's internal camera. White line is the laser beam scanning the resist.

First step of this project was to obtain similar results described by Nelson's paper via micromolding coupled with two-photon fabrication of the tubules' moulds. Then, once results were confirmed, the aim is working on different and more complex shapes to further study cell behaviour.

First, a 3D CAD model of the mould was drew using Autodesk Inventor (figure 4.3). The mould consists of 6 pillars with a 200μm x 50μm base. As for the first test, a 100μm height was chosen in order to obtain good microprinting results keeping writing time acceptable.

Moreover, a 10μm height rectangular base was added so as to improve structure's adhesion to the substrate.

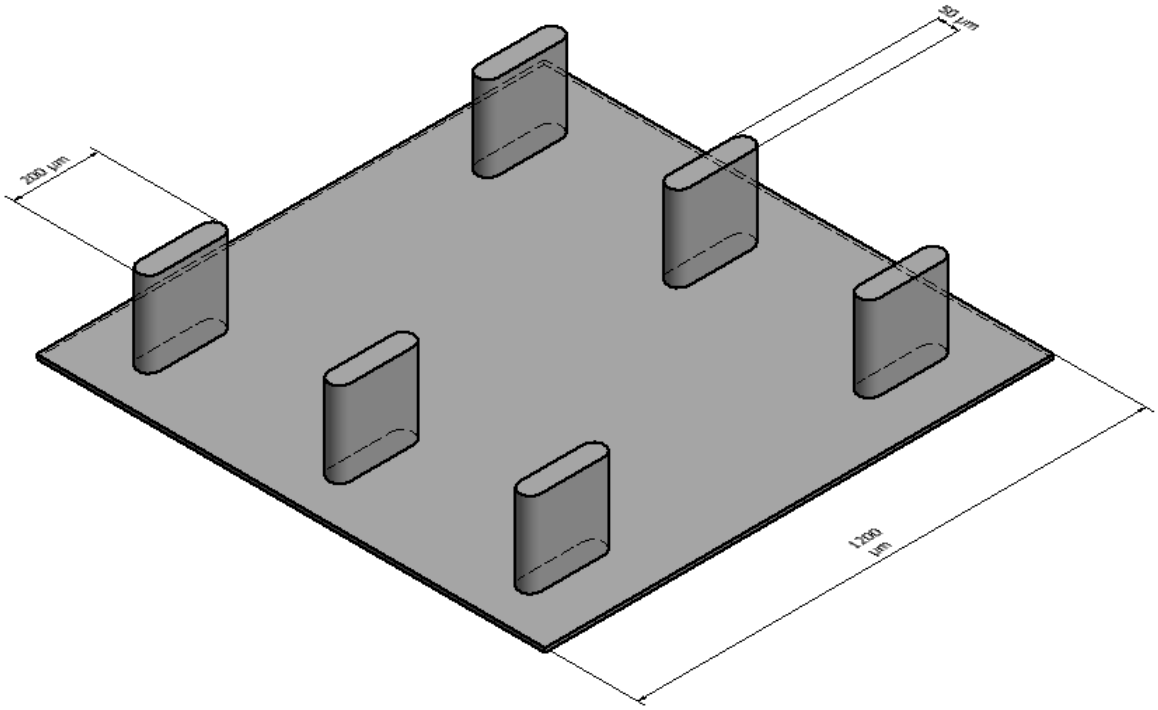

Figure 4.5 CAD model of the mould

Limiting as much as possible writing time is achievable using a commercially available resist already optimized per two-photon laser lithography. Choosing a polymeric resist and not a hydrogel for this application is a good choice considering that this structure will be used just as a mould and will not ever come into contact with cells, even though its biocompatibility has been demonstrated. However, the rigidity and composition of this polymer would not allow to mimic ECM characteristics. Moreover, the idea is to use this stamp several times to replicate the desired features in a suitable hydrogel.

Among Nanoscribe available resists, IP-S seemed to be the right choice due to the high writing speed achievable and since the fabrication with this resist is optimized in DiLL configuration (high heights possible) with 25x objective. While increasing writing speed, this configuration has the major drawback of a limited obtainable resolution. This is not a problem for this application due to the lack of small features in the 3D model but is something to keep in mind for futures perspectives.

Writing parameters were set following Nanoscribe's user manual and are presented in table 4.1. No difficulties were found during fabrication and the structure obtained was used to micropattern a polyacrylamide and GelMA hydrogel.

This step was achieved placing a PDMS gasket around the pillars and pouring a thermally polymerizable solution of polyacrylamide inside the gasket.
Once the hydrogel matrix was formed, trying to divide polyacrylamide from structures caused the pillars to be ripped off from the substrate.
This step pointed out the problem of this kind of moulds: many of the acrylate lateral groups on the surface of the IP-S structure are probably not saturated and they can react with polyacrylamide chains during radical polymerization.
Thinking about how to solve this problem two different alternatives were found:

- Modify the mould's fabrication choosing a different photoactive resist
- Modify the micropatterning procedure

As for the first possibility, this kind of resist should be photopolymerizable, highly reactive in order to minimize the writing time and, above all, should possess non-adhesive properties. For all these reasons, a hybrid organic-inorganic silicone material synthetized by Evonik Industries was chosen: Evonik TEGO RC 711.

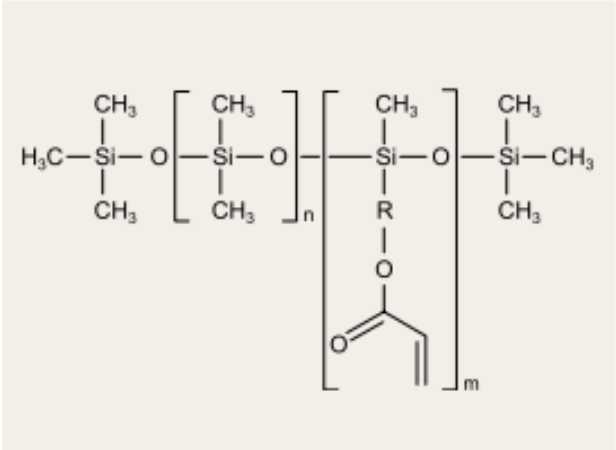

Figure 4.6 Silicone acrylate chemical structure

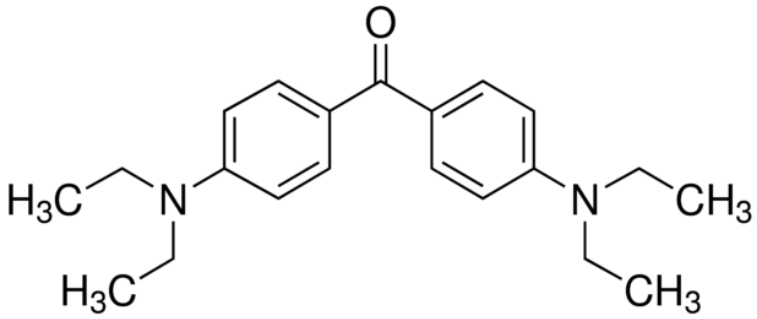

Figure 4.7 4,4'-Bis(diethylamino)benzophenone chemical structure

Due to the organic lateral chains bonded to the inorganic backbone, this silicone is polymerizable via radical polymerization. However, in order to activate this polymerization mechanism using Nanoscribe system's laser beam, RC 711 was mixed with 1% wt of 4,4′-Bis(diethylamino)benzophenone, a two-photon active photoinitiator.

Solution was prepared pouring silicone resin into a vial, adding the photoinitiator in powder and then mixed together the two compounds using an anchor and a magnetic stirrer for 4 hours. Once the initiator was dissolved the solution was degassed through a sonication process.

Because of lack of information on how this material can interact with the glass lens of the Nanoscribe's objective, a DiLL configuration fabrication was too risky so the oil writing mode was the best option.

Changing prepolymer solution and Nanoscribe's writing mode means that many tests should be done to find the best fabrication's parameters. But is not just a matter of Laser power, power scaling and scan speed. Design of the structure and slicing method should be modified as well.

Moreover, in oil configuration, the objective is immersed in a matching index oil on the lower side of the glass substrate while silicone resist in poured inside a PDMS gasket on the upper side. (figure 4.4).

Figure 4.8 Upper side of the sample for evonik sillicone fabrication. Liquid silicone is contained by a PDMS gasket placed on the glass substrate

So, there are, at least, two different limitations in maximum achievable height for the mould's pillars:

- Objective nominal working distance
- Initial distance between objective and glass substrate

Objective working distance reported on Nanoscribe's user manual for the 25X objective is 380µm but, based on previous experience, the structure's height attainable is generally lower than this nominal distance. Therefore, testing the parameters to achieve a good fabrication was made with a 100µm height structure.

Finding the best writing parameter is a trials and errors procedure that mainly relies on laser power, power scaling and scan speed. Generally, increasing laser power and power scaling a higher energy dose is provided to the resin. On the contrary, increasing scan speed will decrease it. The aim of changing these three parameters is to find the right energy dose that stands between polymerization energy threshold and laser induced breakdown of the material.

Best parameters inside these two boundaries are chosen looking for the trade-off between good quality fabrication and high writing speed.

This procedure, applied to Evonik TEGO RC 711 gives, as best writing parameters, those reported in table 4.1.

Resist	Laser Power (%)	Power scaling	Scan Speed (µm/s)
IP-S	50	1	50000
TEGO RC 711	38	1	41000

Table 4.1 Comparison between IP-S and RC 711 writing parameters

As can be seen from the comparison on table 4.1, both the two resists can be written with high scan speed. Using Evonik resist, structure was completed in about 45 minutes.

Unfortunately, trying to use the silicone mould obtained, the same results already seen for IP-S was observed: the hydrogel matrix polymerized bonding covalently with the mould's surface. Trying to divide hydrogel from silicone cause the ripping off of the pillars damaging the mould.

Since the change in resist didn't provide any improvements neither for writing time nor for micropatterning results, Evonik silicone was abandoned.

Moreover, since the result is the same, IP-S has the advantage that can be written in DiLL mode limiting the restrictions described above.

A second way to deal with the problem was to change the micropatterning process in order to use the IP-S moulds.

Instead of a single step micromoulding, a two-step process was developed and consist of:

- First stage using two replicas in PDMS and then Ormocomp
- Second stage using the final hydrogel (polyacrylamide, gelatin or collagen)

As for the mould that was used with this improved procedure, it is a IP-S 300µm height fabricated in DiLL writing mode that can be seen in figures 4.9-4.10.

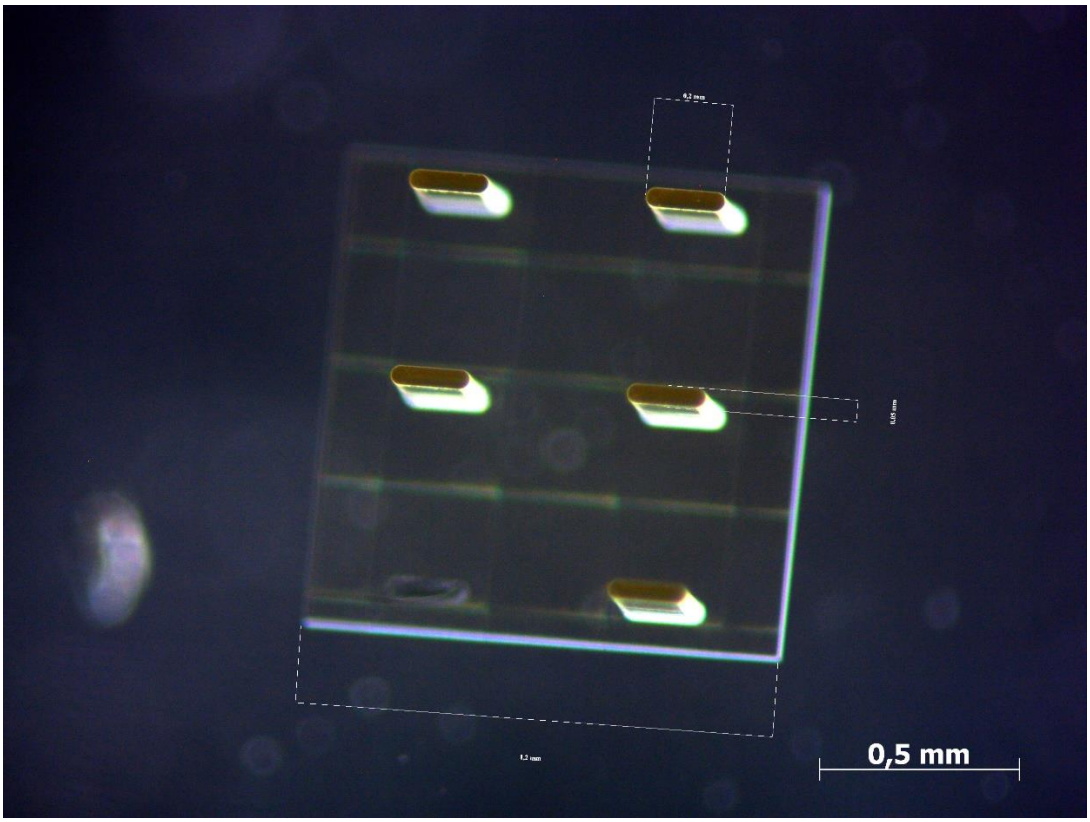

Figure 4.9 IP-S final mould from the top

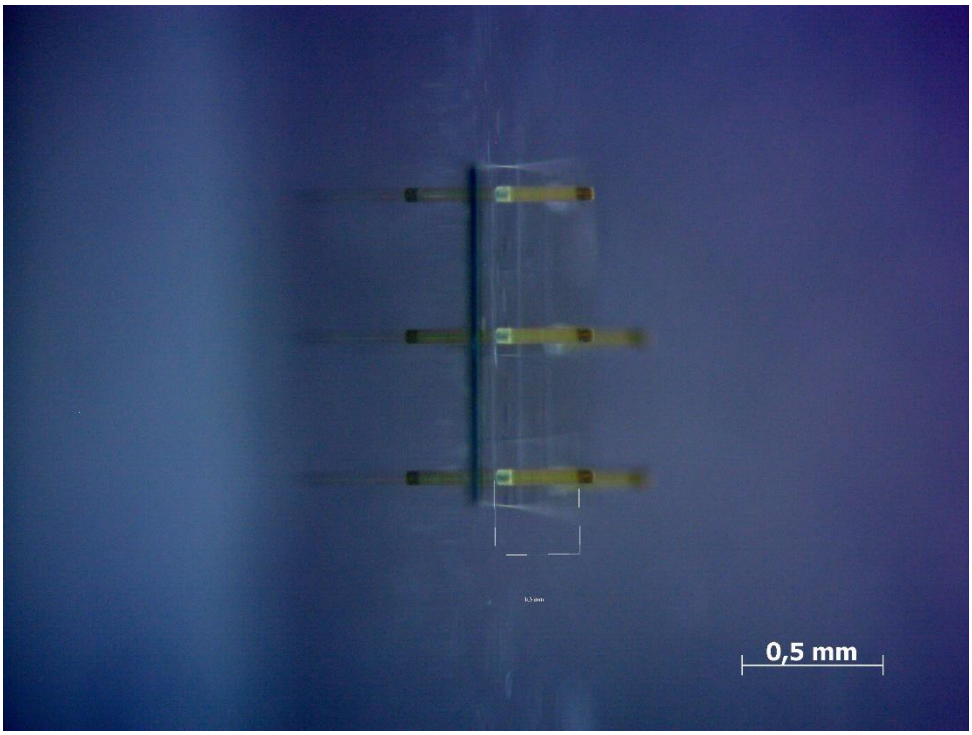

Figure 4.10 IP-S mould seen from the side

4.3 Replica moulding

As already said, an improved process of nanoimprinting was developed and will be now described highlighting how and why are multiple step needed.

4.3.1 First stage replica moulding using PDMS (Sylgard) and ormocomp

First step of replica moulding is preparing, using polydimethylsiloxane, a negative-mould of the IP-S master obtained via photolithography.

In contrast with hydrogel seeing before, Sylgard doesn't rely on radical polymerization of acrylate groups. The different polymerization mechanism avoids any possible reaction between IP-S and PDMS preventing adhesion.

Moreover, Sylgard provide a good quality replica of the mould succeeding in reproduce small features too.

This silicone matrix is formed starting from an elastomer precursor that polymerize when mixed to a curing agent which is added in a 1:10 ratio (wt).

The solution is then mixed and degassed in order to preclude any bubble formation inside the mould.

Once degassing is complete, a gasket is placed on the glass substrate of IP-S mould comprising all the pillars and PDMS is poured inside. The sample is then heated at 80 °C for 1.5 hours inside an oven. PDMS can now be easily peeled off from the mould

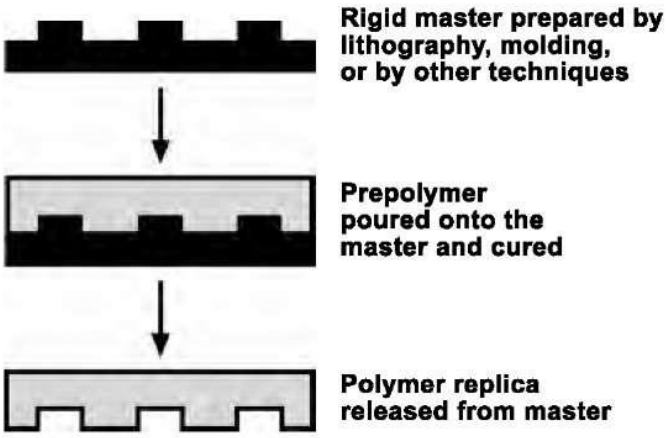

Figure 4.11 PDMS replica process

A further step is necessary to reobtain a positive mould and is performed using ormocomp.

The process is similar the one just described: a gasket is now placed on the PDMS negative mould and Ormocomp is then poured inside.

To better achieve a good quality replica, the sample is heated on a hot place at 60°C for 10 minutes. Heating Ormocomp up decrease its viscosity allowing the resist to completely fill the negative mould. The sample is then placed under a UV lamp and exposed for 2 minutes. Ormocomp, however, is a hybrid organic-inorganic material and contains acrylate groups that can react with hydrogel chains during radical polymerization.

Thanks to the inorganic backbone, Ormocomp can be functionalized with octadecyltrichlorosilane (OTS). This compound can form a continuous self-assembled monolayer on the Ormocomp surface shielding unsaturated double bonds preventing their reaction.

Functionalization is performed activating mould surface with a 10 minutes UV-ozone cleaning. Then, the activated samples are placed into a desiccator over a becher containing OTS, the desiccator is connected to a vacuum pump and placed over a hot plate. OTS evaporates due to the high temperature (140 °C) and forced to pass against the mould due to the vacuum. Functionalization is complete after 1 hour.

4.3.2 Second stage replica moulding with GelMA and Polyacrylamide

Second step of the replica moulding is using Ormocomp sample to recreate tubular shapes in a hydrogel matrix.

Two different hydrogels were used to compare how they can replicate simple structures and how will interact with cells. These two hydrogels are gelMA and Polyacrylamide.

GelMA and polyacrylamide were both tested with thermal and photo-induced polymerization.

As for thermal polymerization, Ammonium persulfate (APS) and TEMED are used as initiator and polymerization takes 17 minutes to complete. Photo activated polymerization was performed under UV light using I2959 as photoinitiator and takes 2 minutes.

GelMA and polyacrylamide have both advantages and disadvantages: while polyacrylamide exhibit a better replica quality, it has to be functionalized after molding with adhesive proteins (i.e. fibronectin) due to its non-cell-adhesive properties. On the contrary, GelMA is a natural hydrogel and is fully cell-compatible but its ability to reproduce the mould is limited: no sharp angle can be obtained with GelMA replica.

4.4 Preliminary cell results

Hydrogel matrix were sent to molecular medicine department where Stefano Piccolo's group performed cellular studies.

Preliminary cellular seeding results are encouraging but many more tests are necessary to understand clearly how the shape induce behaviour modification in cells.

As can be seen from figure 4.13 cells can feel the curvature of the tubular shape allowing a translocation of a transcription factor inside the nucleus (nucleus becomes green instead of black), indicating that shape has strong effect on cell behaviour. In particular this translocation has the immediate effect to give to the cell a stemness-like behaviour, as occurs in real natural intestine crypts.

Thanks to these results and to the possibility of varying easily and in a broad range stamp dimensions and geometries, further experiments will be made to better investigate this phenomenon from a biological point of view.

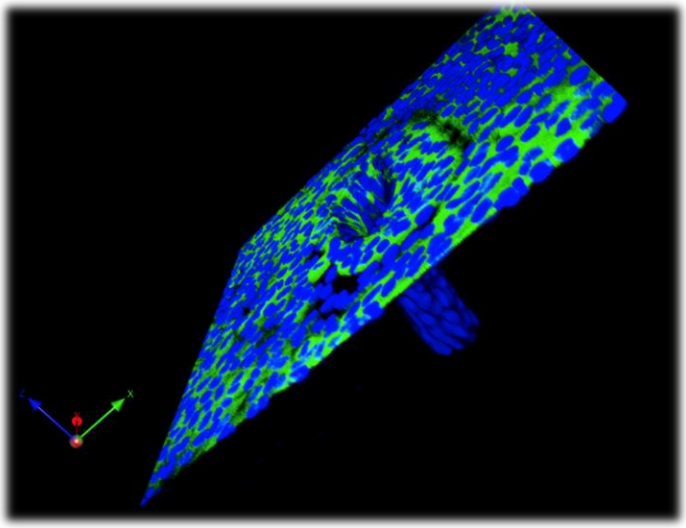

Figure 4.12

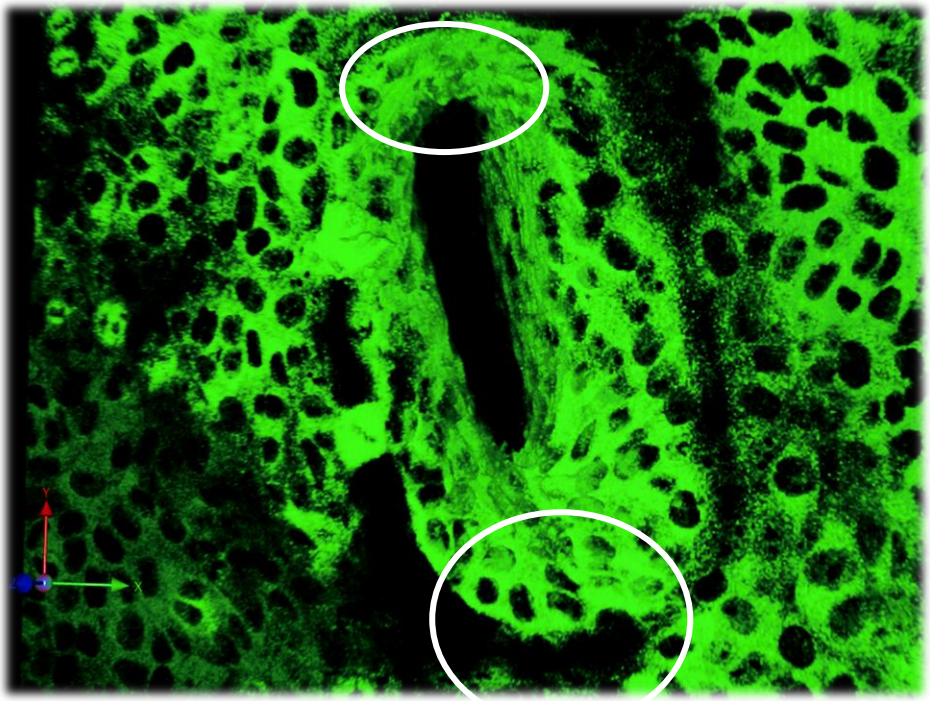

Figure 4.13 cells with black nucleus along the straight sides of the tubular shape and green nucleus along the curved regions

Chapter 5

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TPP fabrication of structures for solid state nanolasers

5.1 Nanolaser

Lasers are intense, coherent and ultrafast sources of light based on optical amplification due to stimulated emission. This kind of light source found many applications in scientific, military and medical fields but many more applications are hindered by physical dimensions and lack of tunability in wavelength emission.

Since 1980s many efforts have been made to miniaturize lasers devices and big improvements have been made during recent years especially due to semiconductors advances. [20]

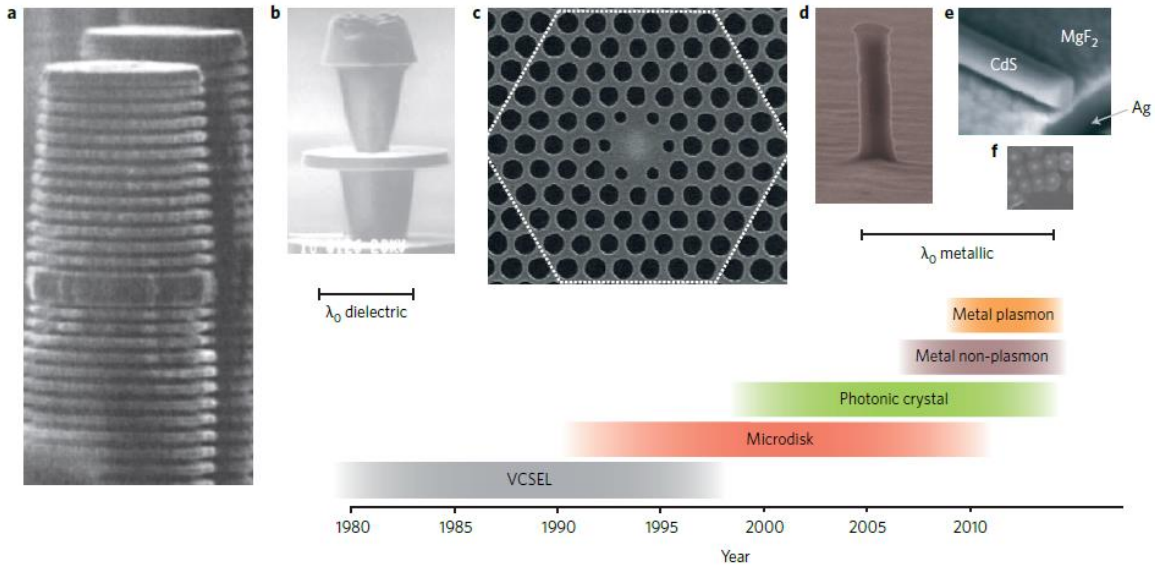


Figure 5.1 Development timeline of small lasers: (a) VCSEL, (b) Microdisk laser, (c) Photonic crystal laser, (d) Metallic non-plasmon mode laser, (e) Metallic propagating plasmon mode laser , (f) Localized plasmon mode laser [20]

These devices, however, are based on differences of refractive index to confine light so the size of the laser is larger than the emitted radiation's wavelength.

Latest improvements in nanolaser fabrication as, for example, Plasmonic nanostructures [21] and photonic crystal nanocavities arrays give now the possibility to modulate emission properties modifying structural parameters while decreasing devices dimensions.

Miniaturization, nowadays, is not the only concern about nanolaser, many studies are now focusing on how to produce a tuneable multiwavelength lasers through FEM assisted design and fabrication engineering.

Thanks to the 3D microfabrication, it could be possible develop an efficient nanolaser consisting of nanocavities based on 2D plasmonic nanoarrays and 3D plasmonic crystals in optically active polymeric matrix.

One key aspect of this nanocavities is to obtain a dye doped polymeric matrix with a refractive index as low as possible in order to simulate a homogenous dispersion of fluorescent molecules in air. While conventional polymeric materials have a refractive index oscillating around a value of 1.4, a swollen hydrogel has a refracting index similar to the one of the water (1.33) due to the high hydrophilicity owned by this material (they can contain up to 99% of water).

Taking advantage of the pioneering experience on 2Photon fabrication of hydrogel and in general of innovative materials in this work of thesis we wanted to give a contribution to this project studying how hydrogel optimization and two-photon laser lithography can be used as powerful tools for developing tuneable solid state nanolasers.

A further improvement will be implement this technology on a flexible/stretchable substrate in order to obtain a dynamic control of the lasing emission.

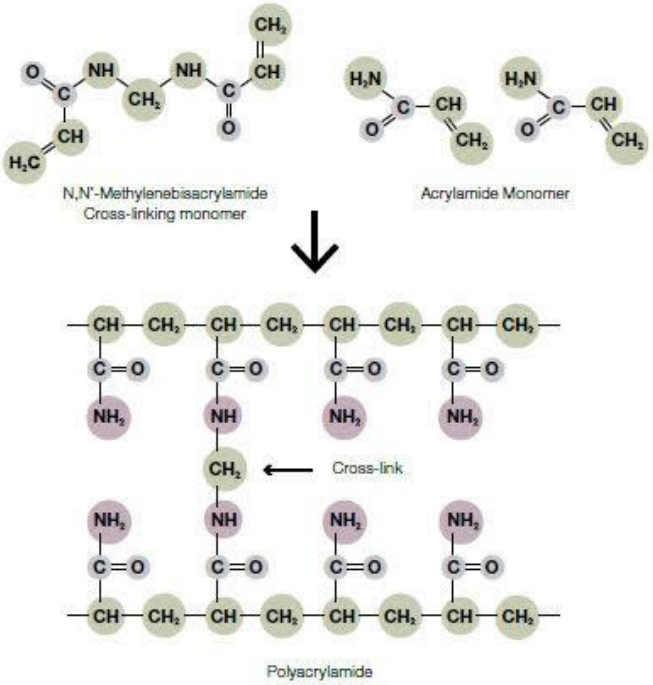
The work consisted of testing how PAA and PEGDA hydrogel as well as nanoscribe resist can be modified using fluorescent dyes and then fabricated via UV photopolymerization and two-photon laser lithography.

As for the resist, nanoscribe's IP-L, polyacrylamide hydrogel and PEGDA hydrogel have been used.

Most of the testing work performed trying to obtain good results on two-photon laser lithography of polyacrylamide has been done during nanoscribe's training at Karlsruhe Institute of Technology.

This was possible thanks to the training, tutoring and support of Dr Klaus Bade, Dipl.-Ing. Stefan Hengsbach and Florian Rupp.

5.2 Polyacrylamide



Polyacrylamide is the polymerization product of acrylamide monomers usually mixed with a crosslinker called bisacrylamide and a thermal of photo-induced initiator.

Polyacrylamide gels are largely used in many applications like electrophoresis [22], as a soil conditioning [23] or as a flocculator in water treatments [24].

Usually, acrylamide polymerization is performed thermally using Potassium persulfate [25] or APS mixed with TEMED [26] as initiators. Otherwise, it can be performed via UV photopolymerization using Igacure 2959. These kinds of polymerization are fast, high yield and very reliable and this is why many papers use these techniques in their experimental section.

However, just few papers describe two-photon polymerization of acrylamide hydrogel.

Only Campagnola et al. reported good results with two-photon polymerization in 2000 [27] and 2010 [28].

In the first fabrication, he succeeded in polymerize a 3D pyramid and some nanorods with width of 500µm (figure 5.2), while in 2010 a freeform nanostructure with gold nanorods was reported. (figure 5.3)

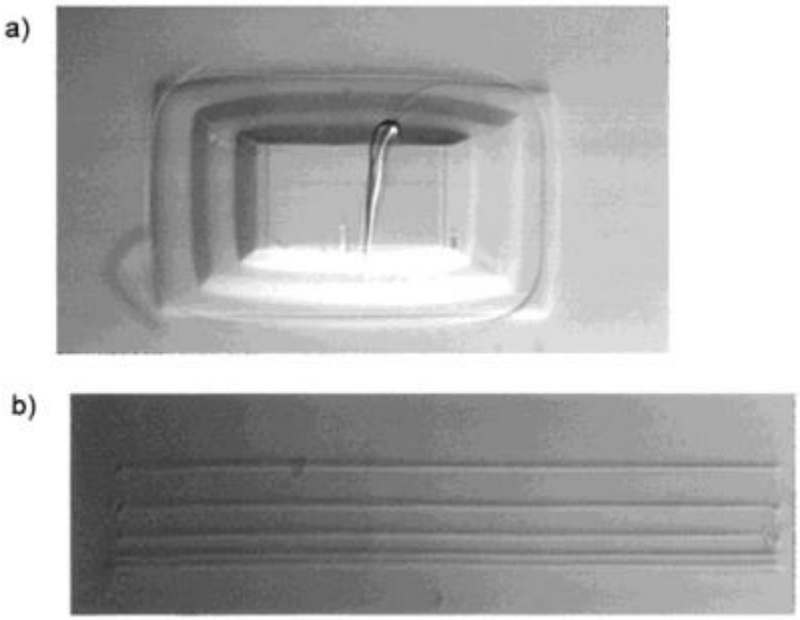


Figure 5.2 A three level pyramid with step height of 1 µm and (b) five rods with widths of approximately 500 nm [27]

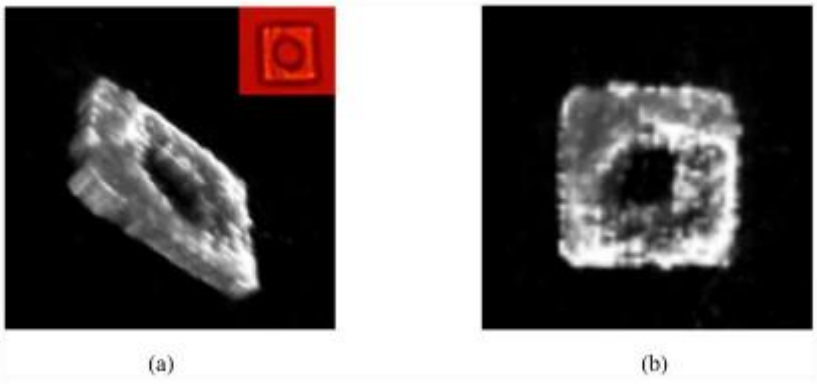


Figure 5.3 3D TPP microstructure imaged by 3D TPL and 2D TPL [28]

Due to the good results reported, first step in this study was to reproduce these fabrications using the same solutions described in the two papers that are reported in table 5.1. In both cases the solution consists of acrylamide as the main monomer, biacrylamide as a crosslinker and an initiator-coinitiator system of a xanthene dye (Rose Bengal) and triethanolamine.

Name	Concentration	Composition	Quantities
C2010	30% in water	• AA • BAA 1:74 (AA) • RB 2mM • TEA 0.1 M • DMSO 10% v/v	• 0.0020 g BAA • 0.1483 g AA • 10 µL TEA • 50 µL DMSO • Water to reach 500 µL
C2000	40% in PBS (pH=8)	• AA • BAA 1:29 w:w (AA) • TEA 0.1M • RB 0.2mM	500 µL • 0.1937 g AA • 0.0066 g BAA • 7 µL TEA • 3.37 µL RB sol

Table 5.1 solution's composition of Campagnola's tests

Even though polymerization seems to happen during laser scanning, scan speed required for polymerization was too slow to think about a possible application. Indeed, with 100% of laser power, polymerization happened with scan speed of 20µm/s (Nanoscribe's resists can be polymerized with a scan speed of 50000 µm/s) and the shape of the structures was very different from the one designed.

Moreover, once the samples were developed, it was possible to observe that polymerization continued beyond exposed areas.

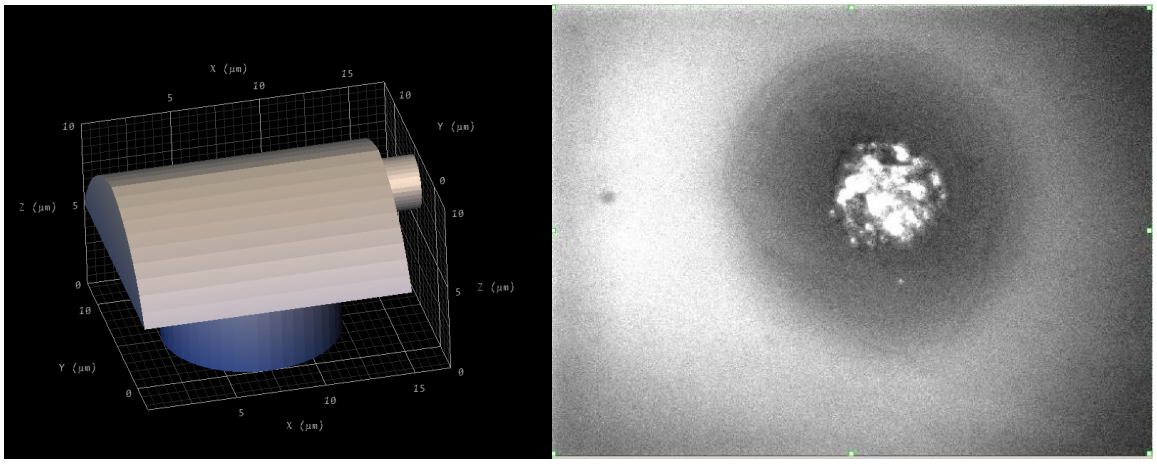


Figure 5.4 CAD model of the structure (left) and structure obtained by two-photon polymerization (right) with solution C2010. Is possible to observe a darker ring all around the structure due to the polymerization propagation

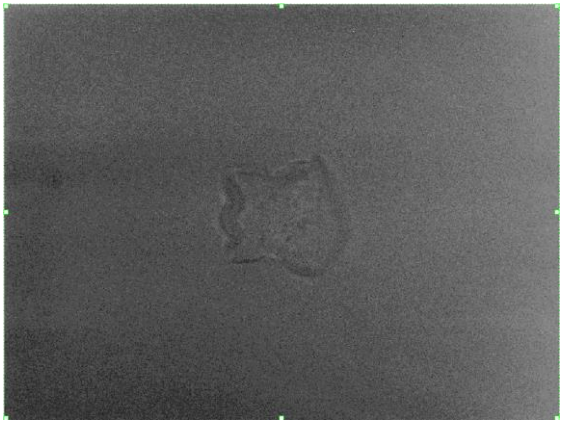


*Figure 5.5 7 levels pyramid (base 20*20µm) fabricated with C2000*

Fabrication testing with Campagnola's solutions shows two main problems of these mixtures:

- If scan speed is too low, no polymerization happens
- Decreasing scan speed in order to increase the energy dose provided to the solution is causing an uncontrollable polymerization

For these reasons, an optimization of the solution was done first through a bibliographic research and then with several experimental tests to better understand which aspects are involved in the polymerization of acrylamide and how to modify them to achieve better results.

As for the solvent, in 2010 De Sterk and Roel Vaneerdeweg studied how water molecules interact with acrylamide through different simulation models. [29]

This study shows that the most important phenomenon between acrylamide and water is solvation. Water and acrylamide can arrange in different configurations and, if more than one molecule is involved, water can act as bridging or non-bridging molecule between acrylamides. Water as a bridging molecule is favourite and decreases the free energy of solvation. An improvement of the solvation is then followed by a higher reactivity of acrylamide towards radical polymerization.

According to the different simulation methods used in this paper, the presence of water can decrease the energy threshold for the radical polymerization by 4 to 10 kJ/mol if compared to lack of solvent.

Moreover, Khomikovskii in 1979 already studied how rate of polymerization, average molecular weight and the ratio between propagation constant and termination constant (k_p/k_t) can change depending on the solvent choice. [30]

All the three parameters decrease when organic solvents as DMSO or THF are used instead of water.

Solvent	$k_p/k_t^{1/2}$, litre$^{1/2}$ mole$^{-1/2}$ s$^{-1/2}$ (40°C)	E_p, kcal mole^{-1}	$10^{-7}A_p$, litre mole^{-1} s^{-1}	E_t, kcal mole^{-1}	$10^{-8}A_t$, litre mole^{-1} s^{-1}
Water (pH 7)	4.1	2.7	1.0	2.7	780
Water (pH 13)	3.6	3.6	2.2	1.0	9.7
Formamide	1.0	5.0	4.4	0.8	7.2
DMSO	0.32	5.4	3.0	0	2.5
Tetrahydrofuran	0.05				

Figure 5.6 polymerization of acrylamide in various solvents

Solvent can, therefore, modify interactions between acrylamide molecules: in water, acrylamide is in a solvated configuration and there are no hydrogen bonds between different molecules. This situation increases acrylamide reactivity and promotes radical polymerization.

In DMSO and THF, on the contrary, acrylamide is in an associated form and a higher amount of energy is required in order to allow molecules' dissociation.

A second aspect that can change the polymerization of acrylamide is the ratio between acrylamide and bisacrylamide.

Let's consider PDB as pendant double bonds and

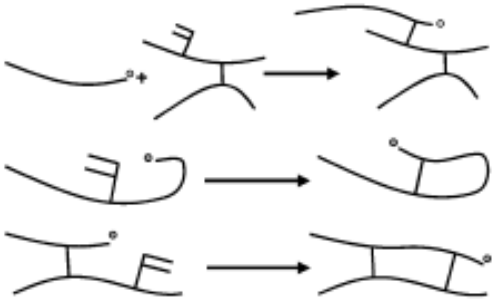
$$w_c = \frac{m_{\text{crosslinker}}}{m_{\text{crosslinker}} + m_{\text{monovinyl}}}$$

$$w_t = \frac{m_{\text{crosslinker}} + m_{\text{monovinyl}}}{m_{\text{crosslinker}} + m_{\text{monovinyl}} + m_{\text{water}}}$$

Different studies show how there are three possible reactions between acrylamide and bisacylamide: Cross-linking, primary cyclization and secondary cyclization. [31] [32] While cross-linking use PDB in order to form a continuous network and is the mechanism on which polymerization is based on, cyclizations are unwanted reactions that consume PDB.

Tobita and Hamielec [31] show how, if solution has a w_t=5.6%, about 80% of total PDB available are used by primary cyclization. This phenomenon is found for a wide range of w_c (from 0.02% to 28%).

Other studies, as for example, Naghash and Okay [33] found some slightly different data but shares the same main result: primary cyclization consumes almost all the available PDB.



CROSSLINKING

PRIMARY CYCLIZATION

SECONDARY CYCLIZATION

Secondary cyclization is more effective than crosslinking as well and the combination of primary and secondary cyclization is more effective than propagation.

Primary and secondary cyclization depends on monomers concentration thus, modifying the composition of the solution, a better polymerization should be attainable.

While secondary cyclization is proportional to the crosslinking concentration, primary cyclization is inversely proportional to the total amount of monomers w_t.

Authors of the paper proposed an empiric equation to link wt to a primary cyclization coefficient which seems to follow experimental data:

$$\eta_{cp} = \exp(-3.356\ \omega_t^2)$$

Considering results reported in these papers, some general rules should be followed to improve radical crosslinking:

- Increase viscosity of the solution to prevent termination of the reaction
- Avoid excessively diluted solution to reduce primary cyclization's effect
- Decrease concentration of bisacrylamide so as to decrease the effect of the secondary cyclization
- Add small amount of isopropanol in order to limit the average length of polymeric chains and, therefore, the secondary cyclization

One last improvement can be done to the solution: a different photoinitiator.

Solution reactivity can be increased replacing Rose Bengal (which has a TPA cross-section of 10 GM and is a type II photoinitiator) with a more efficient two-photon active initiator as, for example 7-Diethylamino-3-thenoylcoumarin or P2CK.
In view of the above, improving the radical reaction should be done changing:

- Type of solvent
- Type and amount of photoinitiator
- Concentration of monomers
- Ratio between acrylamide and bisacrylamide

Different solutions were prepared and tested. For each solution, many tests were performed trying to achieve a good fabrication result of a small structure chosen as a sample. The CAD model is presented in figure 5.7.

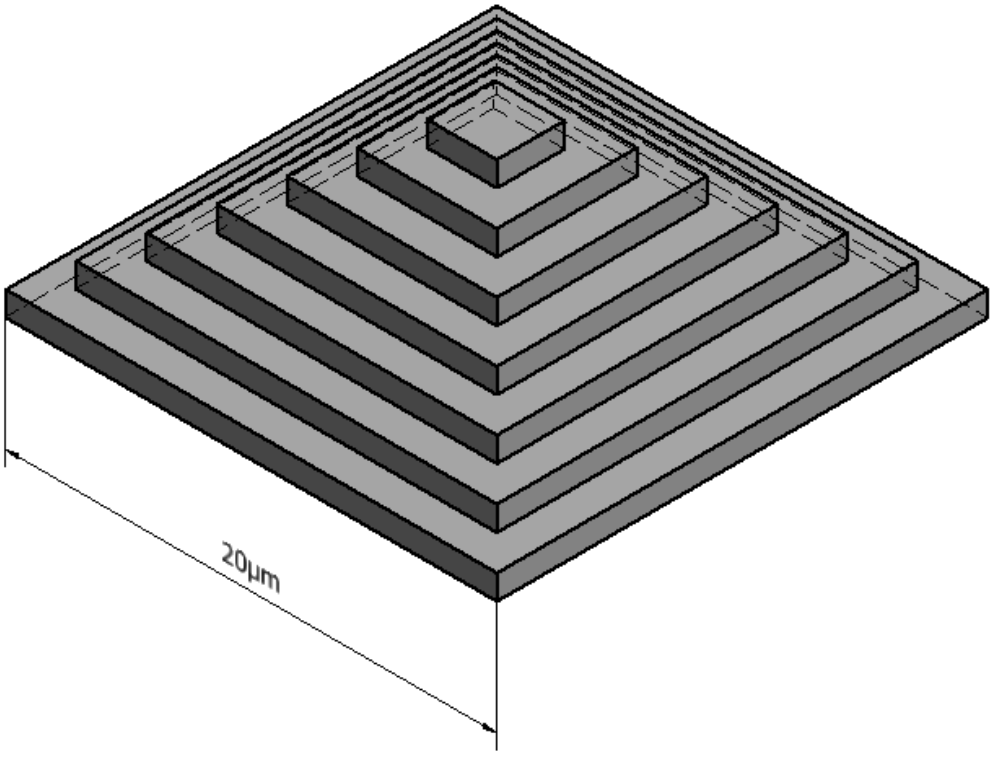


Figure 5.7 Test structure used to perform the optimization of the writing parameters

All the fabrications were performed in oil configuration with an 100X NA 1.4 objective. Preparation of the sample was done using sandwich-like configuration using a functionalized substrate and a plain coverslip. The plain glass coverslip is attached to Nanoscribe's sample holder and a drop of the matching index oil Immersol 518 is placed on the lower glass surface.

Then, a drop of the solution is deposited on the upper side of the coverslip and a functionalized glass substrate is placed over the solution to spread it and to prevent evaporation of the solvent (figure 5.8)

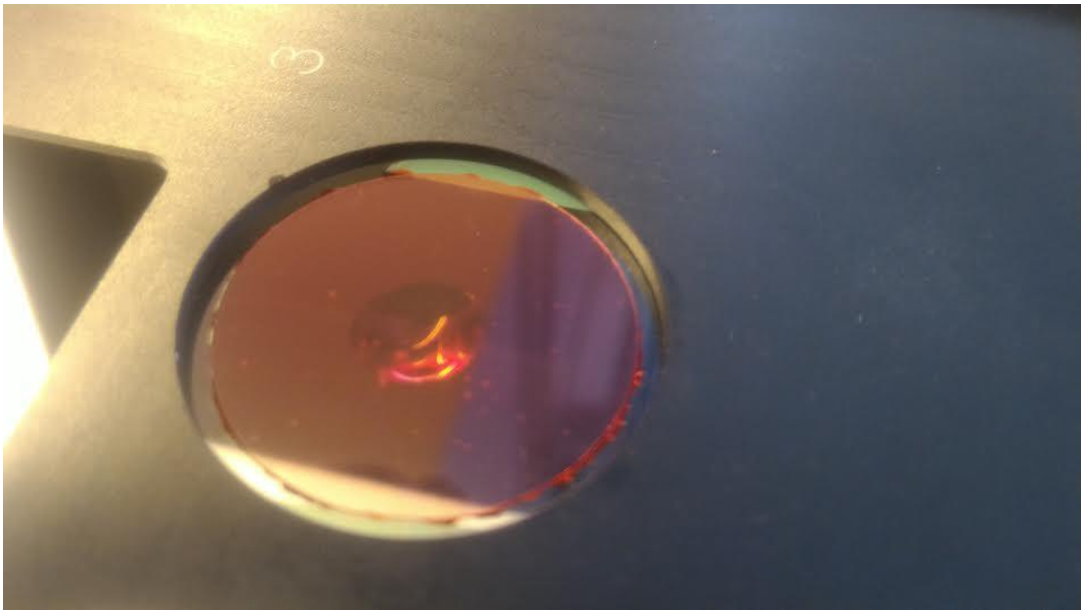


Figure 5.8 Sample preparation for polyacrylamide's solutions tests .In this picture the red colour is due to P2CK initiator. It is possible to see the oil drop on the lower side of the sample

First tests were performed using water instead of DMSO maintaining the same initiators and the same monomer ratio (solution A1RB and A1RB30 on table 5.2). Two solutions were prepared varying monomers total concentration between 30% and 40% but no consistent results were found. No suitable writing parameters were found to achieve a good structure

Keeping the water as the solvent, one more solution was prepared using 7-Diethylamino-3-thenoylcoumarin instead of Rose Bengal and Triethylamine.

7-Diethylamino-3-thenoylcoumarin is a two-photon active initiator, with a high TPA cross-section which should promote two-photon polymerization.

Unfortunately, this initiator has the major drawback that it is not soluble in water. For this reason, a solution of DMSO and 7-Diethylamino-3-thenoylcoumarin was prepared and then mixed with acrylamide, bisacrylamide and water.

Moreover, 7-Diethylamino-3-thenoylcoumarin has a limit of solubility in DMSO so in order to add a higher amount of photoinitiator in the solution, a higher quantity of DMSO is necessary.

Considering everything said before, increasing DMSO should prevent the radical polymerization so, instead of trying to add more 7-Diethylamino-3-thenoylcoumarin, a different photoinitiator was chosen.

This solution (K13 in table 5.2) was tested anyway but no polymerization happened even with 100% of laser power and scan speed down to 10µm/s.

Finally, 4 different solutions were prepared using P2CK as photoinitiator and a modified ratio between acrylamide/bisacrylamide.

P2CK is a two-photon active photoinitiator which has a TPA cross-section of 176 GM and is water-soluble. These four solutions (P10, P20, P30, P40) were prepared varying the total monomer concentration from 10% to 40%.

These four solutions gave the best results between all the solutions tested; varying the writing parameters, pyramids structures with a good shape and sharp edges was fabricated with P20 and P30.

Even though polymerization was possible with P10 and P40 no writing parameters were found to succeed in fabricate good structures.

Figures 5.9 shows some of the best results obtained with P20 solutions including the writing parameter used for each structure while figure 5.10 shows a comparison between results achieved with the four solutions using the same writing parameters.

With P20 a highest scan speed up to 75µm/s was achieved.

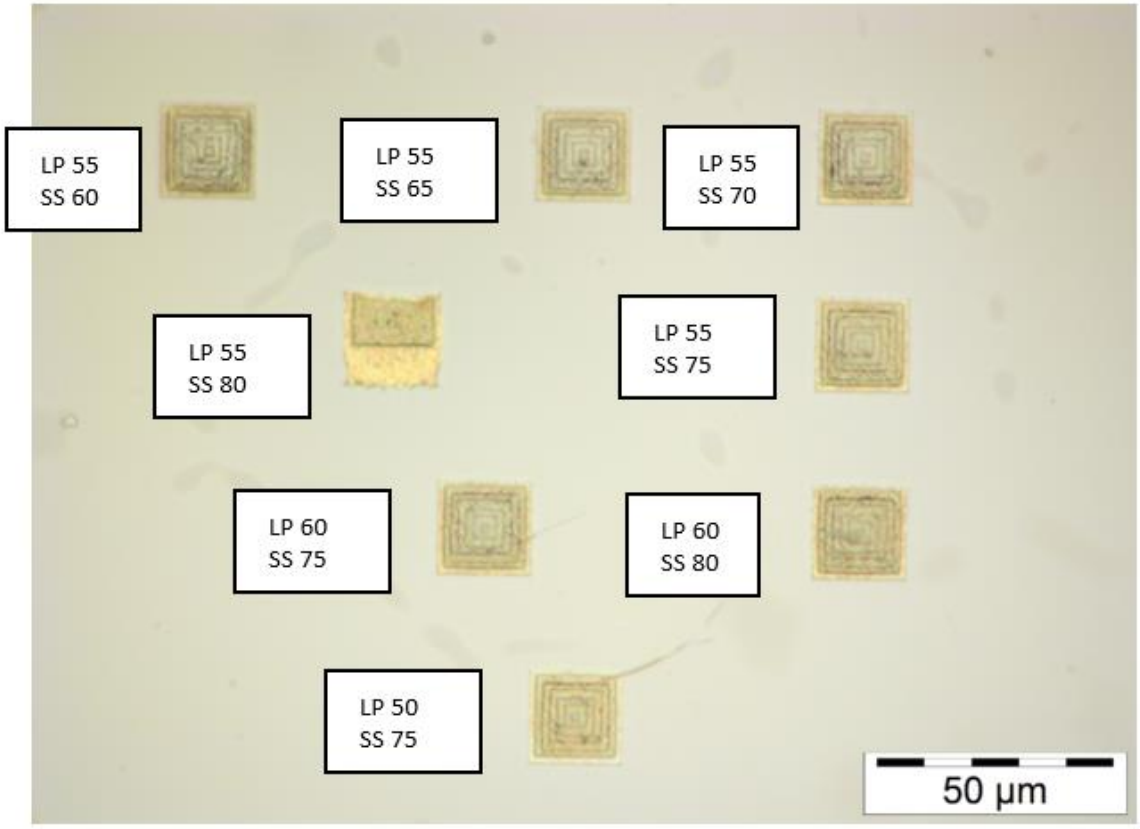


Figure 5.9 Fabrication results using solution P20

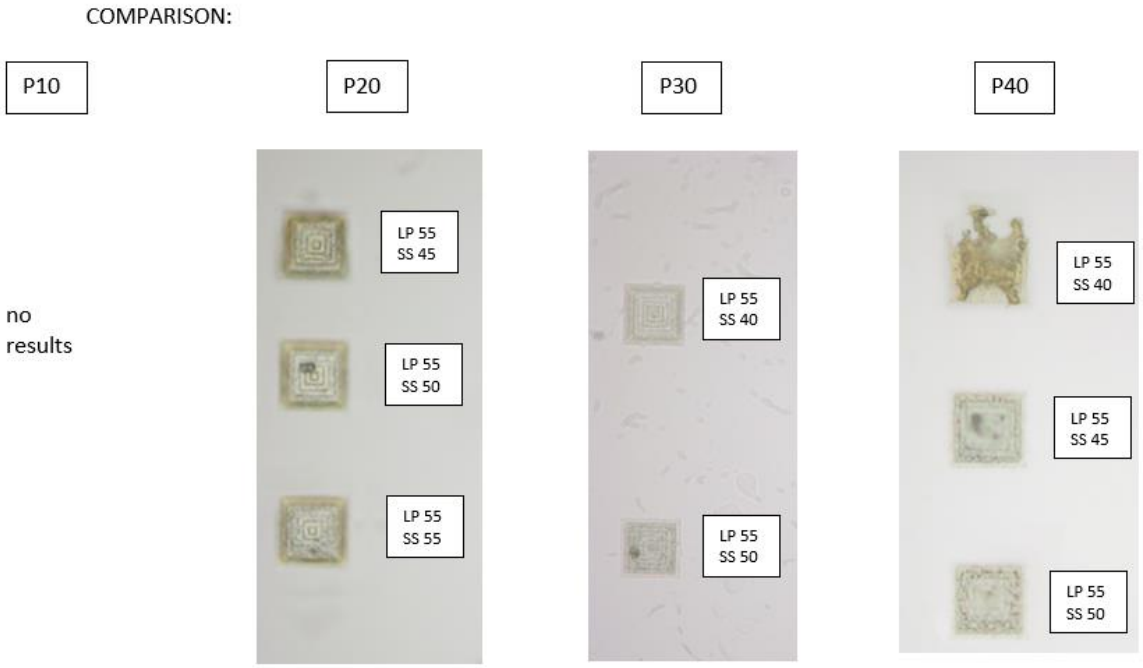


Figure 5.10 Comparison between structures obtained with P10, P20, P30 and P40

Name	Concentration	Composition
A1RB	40% in water	AA/BAA 29:1 (40% in water) Rose Bengal 0.2 mM Triethylamine 0.1M
A1RB30	30% in water	AA/BAA 29:1 (40% in water) Rose Bengal 0.2 mM Triethylamine 0.1M
K13	40% in water + DMSO	AA/BAA 29:1 (40% in water) 7-Diethylamino-3-thenoylcoumarin 2mM
P10	10% in water	AA BAA 7% wt/wt (AA) P2CK 1% wt/wt (AA+BAA)
P20	20% in water	AA BAA 7% wt/wt (AA) P2CK 1% wt/wt (AA+BAA)
P30	30% in water	AA BAA 7% wt/wt (AA) P2CK 1% wt/wt (AA+BAA)
P40	40% in water	AA BAA 7% wt/wt (AA) P2CK 1% wt/wt (AA+BAA)

Table 5.2 composition of the solutions tested with Nanoscribe

The last improvement of the composition was substitute bisacrylamide with an 8-ARM peg based prepolymer with acrylamide groups at the end of each arm in order to enhance the tridimensionality of the network obtained and to increase the writing speed thanks to its higher reactivity compared to bisacrylamide monomers.

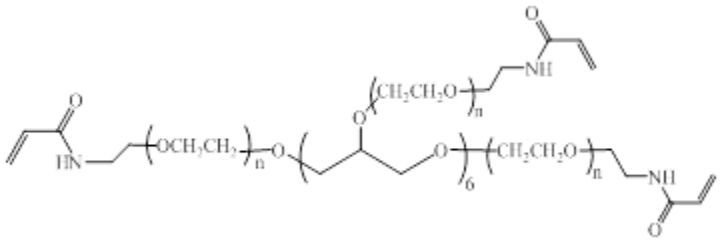


Figure 5.11 Chemical structure of the 8-ARM PEG prepolymer with acrylamide lateral groups

The structure represented in figure 5.12 was successfully fabricated with scan speed up to 2600µm/s (about 35 times faster the previous tests)

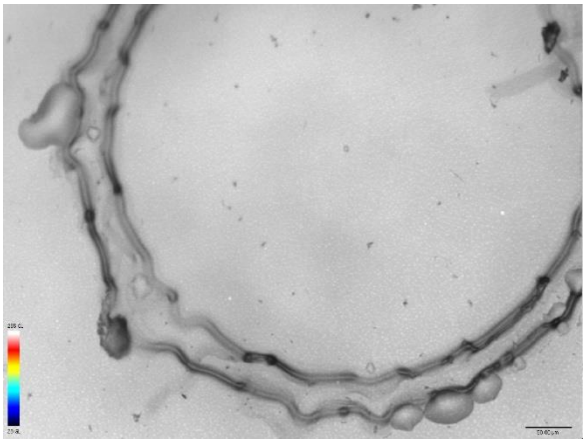


Figure 5.12 test structure for acrylamide-4ARM PEG solution

Although 3D structures were successfully fabricated by improving the fabrication process and modifying the composition of the solution, writing speed and quality of the structures are far from being usable in high resolution two-photon lithography. More tests will be made to chase a high quality and reliable two-photon polymerization of polyacrylamide

Considering the results obtained with 3D fabrication of polyacrylamide, we decided to develop the further experimental tests following two different directions:

- Perform preliminary 2D studies with PAA to be used once the 3D fabrication process will be optimized
- Use a dye doped Nanoscribe's IP-L resist in order to obtain some reliable results regarding how addition of a fluorescent dye can modify the fabrication of a surely fabricable resist.

5.3 Preliminary 2D test

Different films were produced via UV polymerization using solutions of PAA or Nanoscribe's IP-L mixed with Rhodamine 6G.

This fluorescent dye was chosen due to the wide gap between the peak of absorption and the peak of emission in order to obtain more consistent results.

As for PAA, solutions were prepared mixing the monomers (acrylamide and bisacrylamide) with 1% wt/wt of I2959 as UV photoinitiator and with different concentrations of Rhodamine 6G (0.2% wt/wt, 0.5% wt/wt, 1% wt/wt based on monomers' weight).

For each solution two films were fabricated with two different thickness of about 20µm and 40µm.

Samples were prepared using a functionalized glass substrate with a drop of the solution on the top and a second plain glass coverslip covering the solution in order to spread the prepolymer and prevent solvent evaporation.

This sandwich-like samples were exposed to UV radiation until polymerization was completed then, the plain coverslip was removed and the lower substrate covered by the hydrogel films is stored in a petri dish submerged in water.

IP-L was mixed with 0.2% wt/wt of Rhodamine 6G using an anchor and a magnetic stirrer for four hours and then, a film of the solution was produced using a 400nm wavelength UV lamp.

5.3.1 Spectrofluorimetric analysis

An emission spectrum was obtained for an aqueous solution of the dye (as a reference) and for each hydrogel film through a spectrofluorimetric analysis performed with a Jasco FP-6300. The excitation wavelength was set to 527nm (as specified on the box of the fluorescent dye) and the emission was analysed between 500nm and 900nm.

The aim of this analysis is to observe how embedding the fluorescent dye in a polymeric matrix can modify its emission.

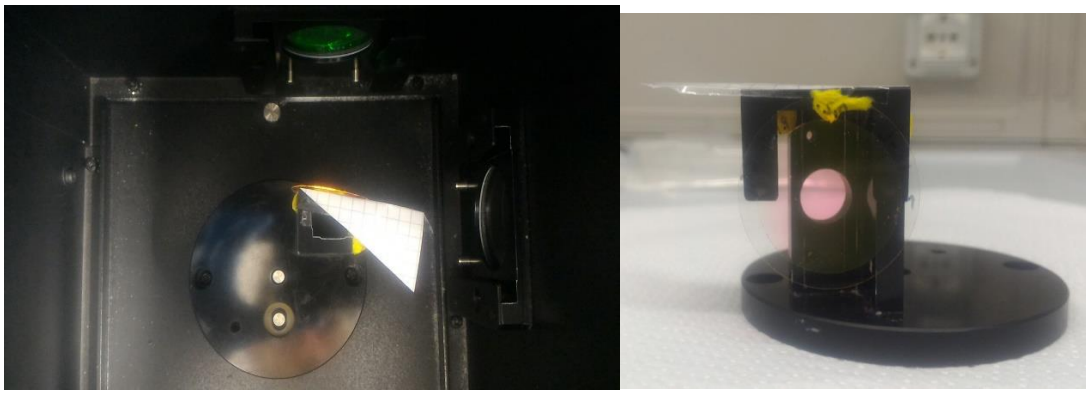


Figure 5.13 Set-up used for emission's analysis performed on polymeric films

The set-up used during analysis is represented in figure 5.11, the sample is tilted in order to avoid the reflection of the excitation radiation toward absorption window.

5.3.1.1 Results

Comparing the spectra obtained from the aqueous solution (figure 5.12) and from the hydrogel films it's possible to observe a blue shift of the main peak for all the polymeric matrix. This blue shift is even more accentuated for IP-L.

Moreover, the measured emissions depend on the thickness of the film and this phenomenon is probably due to the higher number of excited molecules that are present in thicker films.

In spectra obtained for hydrogel films containing 0.2% and 0.5% and for IP-L there is a shoulder peak that according to F.M. Zehentbauer et al [34]is due to the formation of dimers or complex agglomerates as a result of an incomplete solvation of the solute.

Considering that the shoulder is present even in spectrum of diluted solutions, better results can be probably achieved improving the mixing process.

To better understand if the fluorescence behaviour is linked to the matrix or to the composition of the solution, all the samples were sent to the physics and astronomy department of the University of Padua in order to obtain a better characterization.

These results will be available on July and will be used to optimize solution preparation and fabrication process.

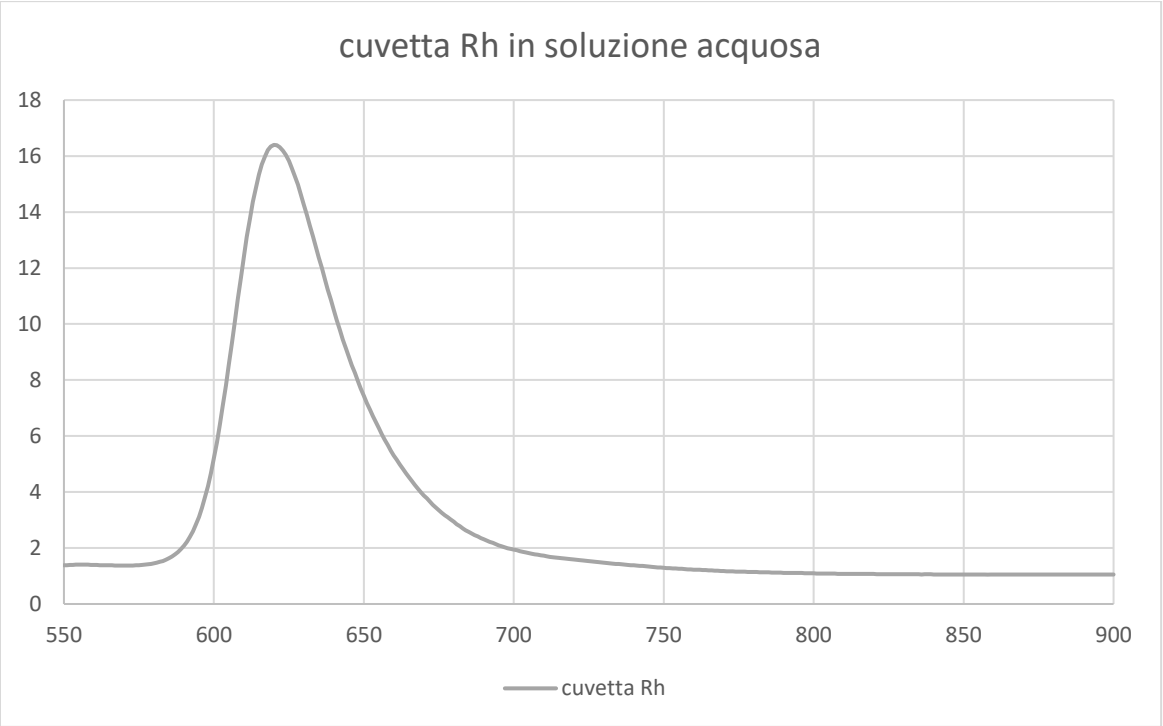


Figure 5.14 emission spectrum of aqueous solution of Rhodamine 6G

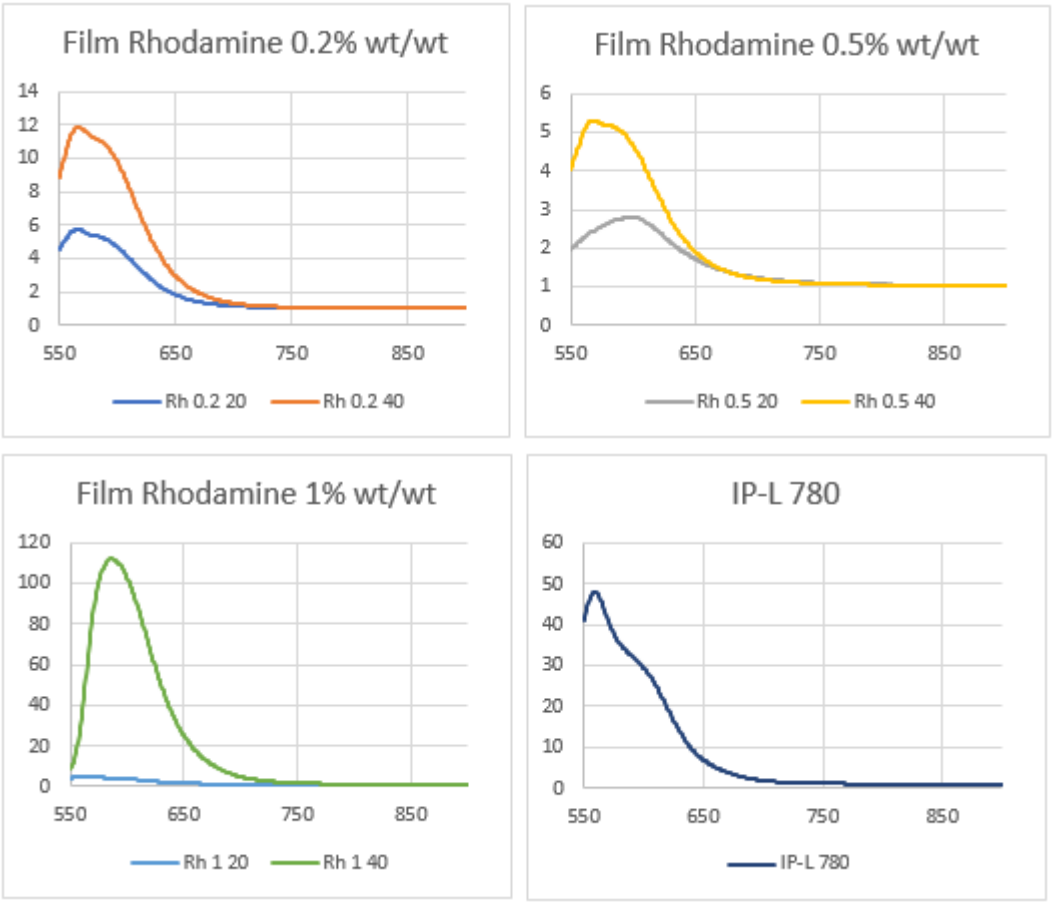


Figure 5.15 Emission spectra of dye doped polymeric films

5.4 Design and fabrication of 3D patterns

To observe how adding a fluorescent dye can change the results achievable with two-photon laser lithography, IP-L and dye doped IP-L were used to fabricate some 3D periodic arrays with a periodicity of 0.9μm.

IP-L resist was chosen because, among nanoscribe's resist, it is the one with the higher spatial resolution achievable. This characteristic will be crucial when the final model of the nanostructures will be fabricated.

Different tests were performed varying the dimension of the array and the geometrical parameters of the pillars: table 5.3 reports all the tests done with both the resists.

This parameters variation was done in order to understand if IP-L resolution was precluded by the dye addition and how required writing time changes due to the array's dimension.

	Dimension of the array (μm)	Diameter of the pillars (μm)	Distance between pillars (μm)	Height of the pillars (μm)
Structure 1	100*100	1	9	1
Structure 2	200*200	1	9	1
structure 3	100*100	1	9	2.7
Structure 4	200*200	1	9	2.7
Structure 5	100*100	0.9	2.7	2.7
Structure 6	200*200	0.9	2.7	2.7
Structure 7	100*100	0.9	1.8	1.8
Structure 8	200*200	0.9	1.8	1.8

Table 5.3 geometrical parameters of 3D periodic arrays

All the structures were successfully fabricated both with IP-L and dye doped IP-L, however, some problems in interface finding happened with modified IP-L due to an incomplete solubilization of the fluorescent dye. (figure

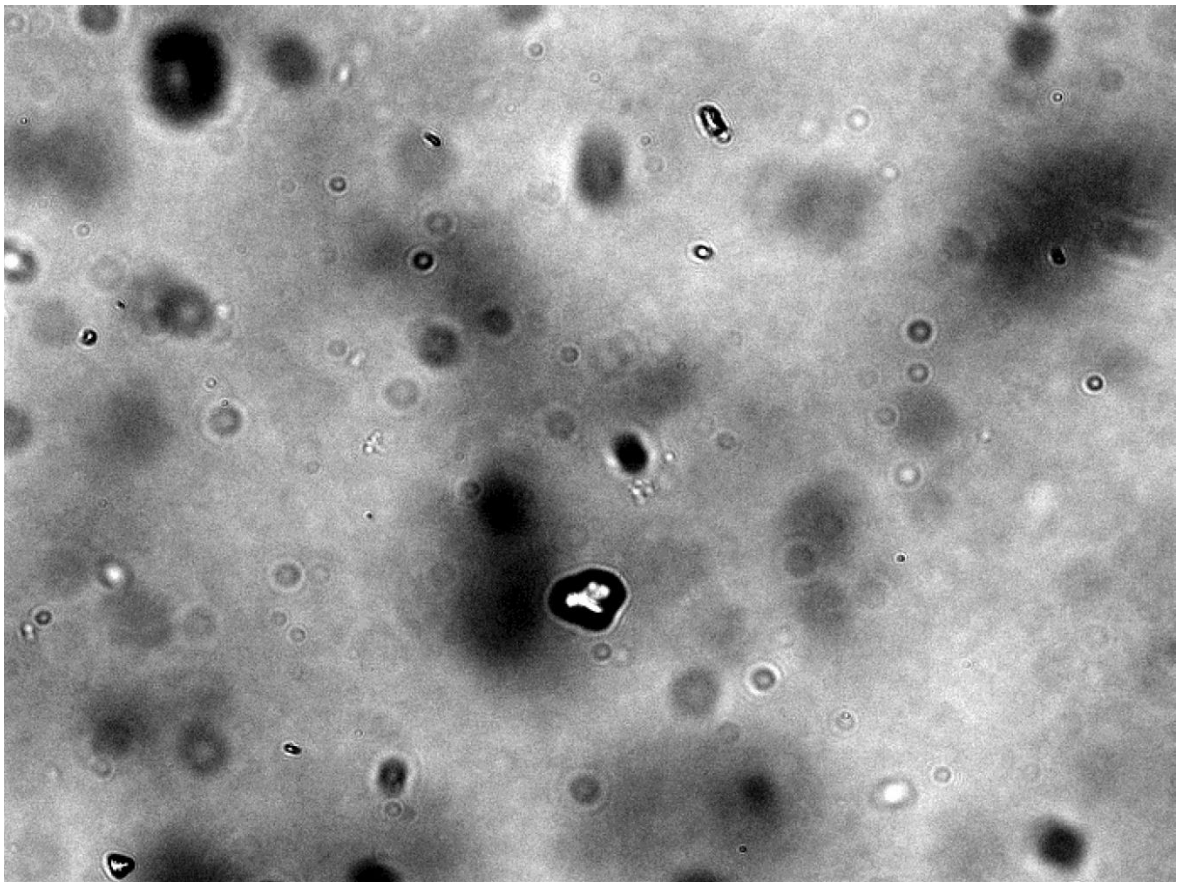


Figure 5.16 dye doped IP-L how can be seen from Nanoscribe's objective. It's possible to see small agglomerates of Rhodamine 6G

Results obtained show that adding Rhodamine 6G to IP-L shouldn't modify the fabrication's parameters but an improvement of the solution should be done to obtain more reliable results.

Moreover, all the arrays were completed in less than one minute so an increase in array's dimensions or in pillars' height should be easily attainable.

As already done for the 2D films, these 3D patterns were sent to the physics and astronomy department for a better characterization which will be the starting point for future developments.

The future prospect is fabricating a 3D periodic structure as the woodpile that can be seen in picture 5.17.

The feasibility test of the fabrication of this kind of structure was already performed with hydrogels materials and figure 5.17 presents the result.

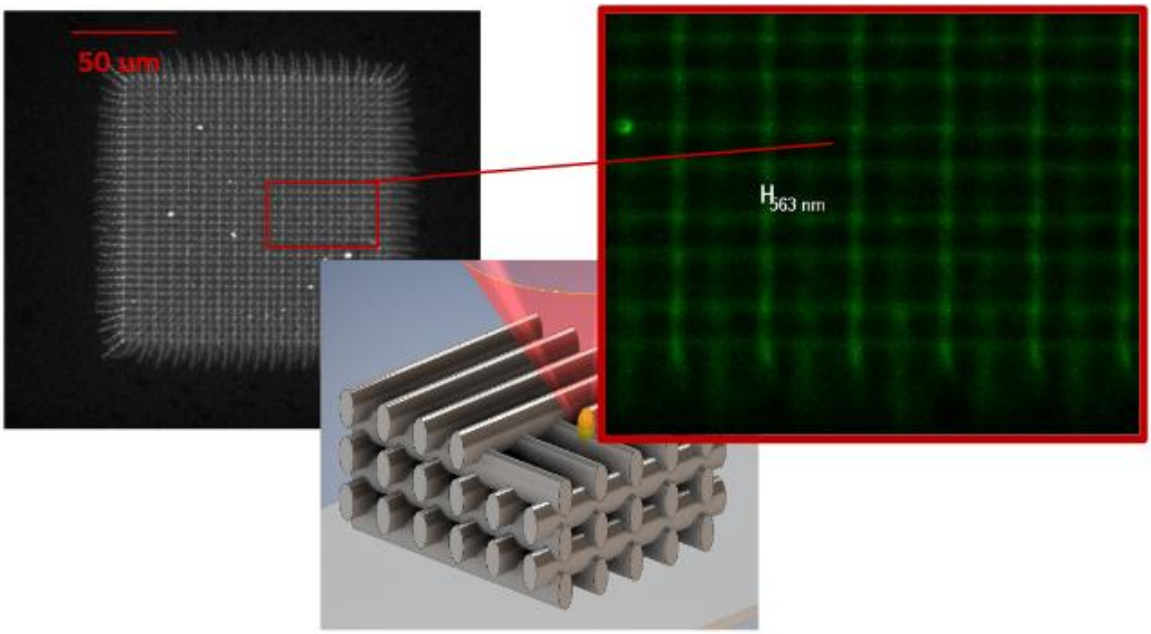


Figure 5.17 Woddpile structure and feasibility test with hydrogel

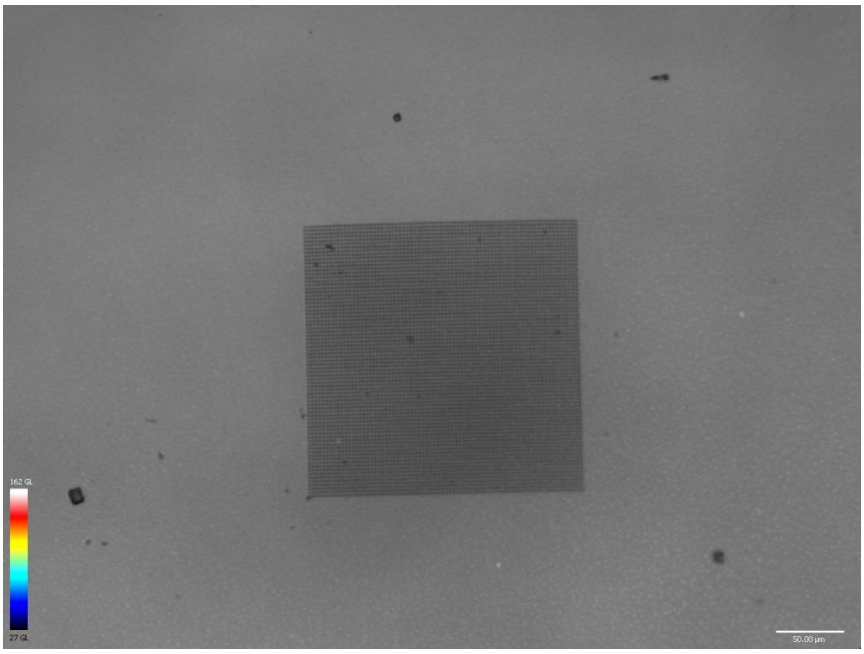


Figure 5.18 dye doped IP-L resist. 200x200µm array, pillars: height 2.7 µm, diameter 0.9µm, distance 2.7µm

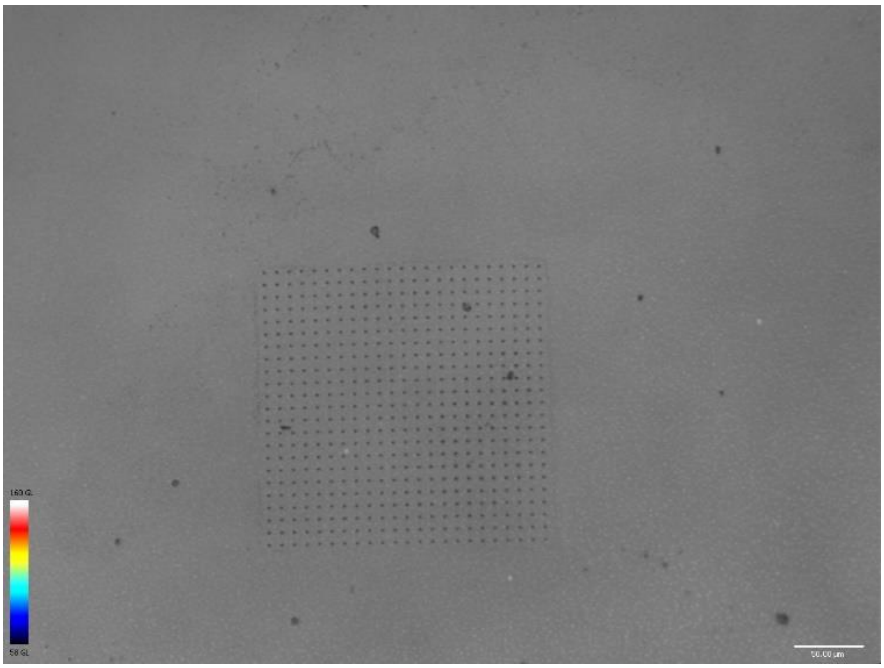


Figure 5.19 dye doped IP-L resist. 200x200µm array, pillars: height 0.9µm, diameter 0.9µm, distance 9µm

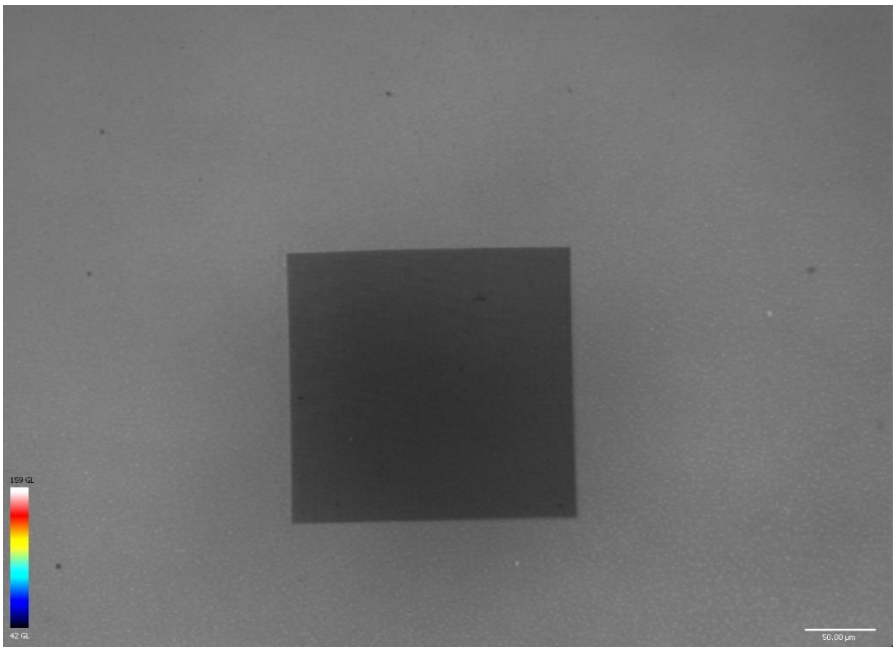


Figure 5.20 dye doped IP-L resist. 200x200µm array, pillars: height 1.8 µm, diameter 0.9µm, distance 1.8µm

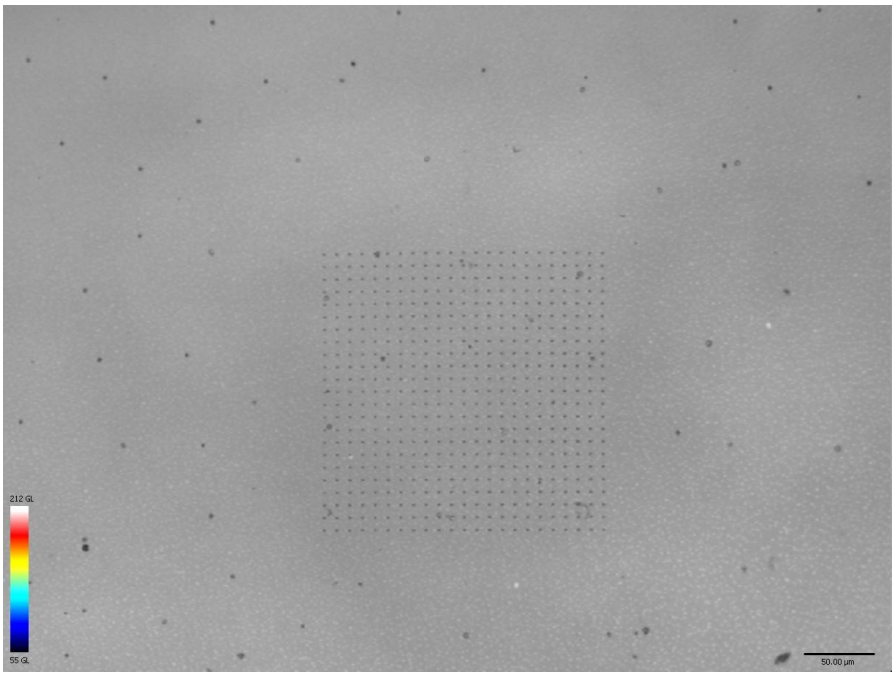


Figure 5.21 IP-L resist. 200x200µm array, pillars: height 0.9µm, diameter 0.9µm, distance 9µm

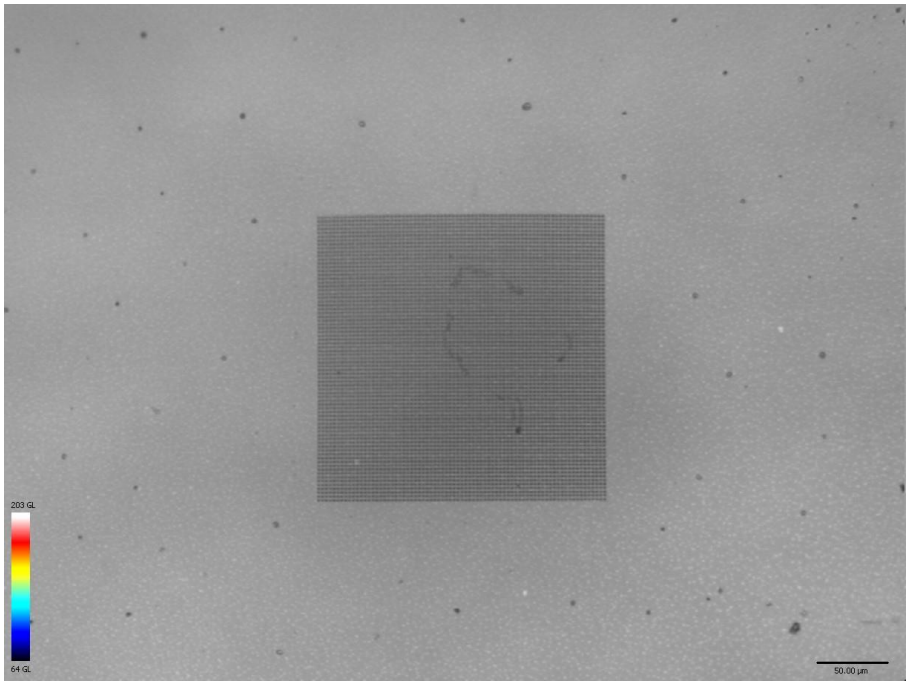


Figure 5.22 IP-L resist. 200x200µm array, pillars: height 2.7 µm, diameter 0.9µm, distance 2.7µm

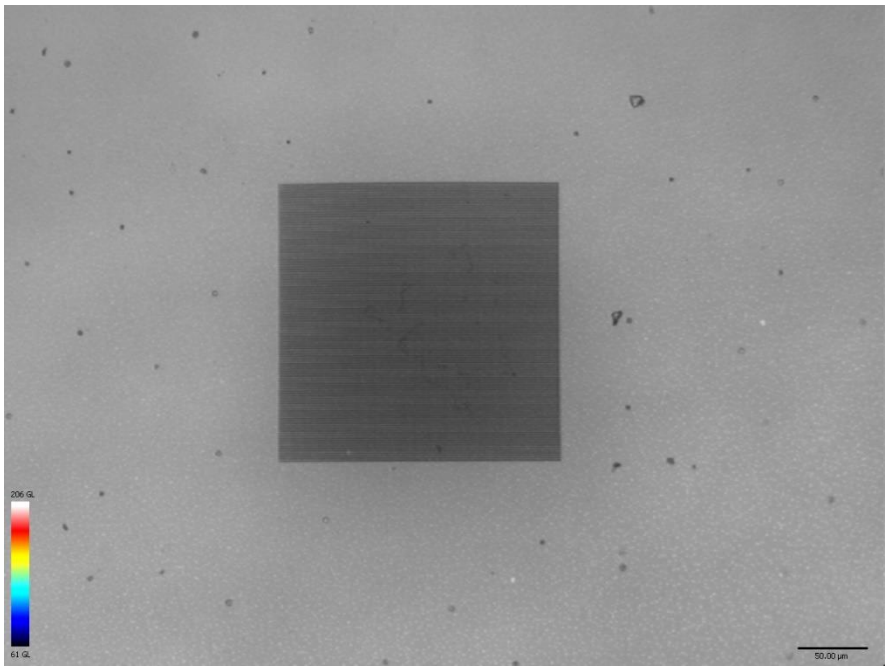


Figure 5.23 IP-L resist. 200x200µm array, pillars: height 1.8 µm, diameter 0.9µm, distance 1.8µm

Conclusion

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This work of thesis was aimed at presenting how an innovative microfabrication system, together with suitably optimized new natural and synthetic materials, can allow to face challenges that were still unimaginable just some years ago.

Let's for example think about the fabrication of the hollow microstructure presented in chapter two. Once considered the chemistry of the monomers involved and how this aspect can change the reactivity of the solution toward photopolymerization, we managed to improve the solution to obtain a reliable and fast fabrication. Moreover, thanks to some preliminary tests, it was possible to observe that this natural material can be 3D shaped and be used in further applications such as in structures that will show how cochlear cells can interact between each other.

Good results were achieved even on the fabrication of micrometric mould for micromoulding. In this case, the main challenge was to study if a custom made photoactive solution can be used instead of a commercial resist. This means understanding how this custom prepolymer should be prepared and how the consequent modify of the fabrication configuration limits the achievable structures.

A second optimization of the micromoulding process was needed in the light of the obtained results.

A fully optimized route was developed choosing the right materials for each step and developing a functionalization process to avoid the adhesion between the mould and the hydrogel matrix.

Thanks to this achievement, it was possible to allow a pioneering study of the dependence of the cell behaviour on the curvature of the substrate, a biological fundamental behaviour of cell in human body. As future development, new geometries and sizes will be explored, thanks to the versatility of the two-photon lithography method for the production of different shapes.

As for the hydrogel used for the final microtubules substrate, both a natural and a synthetic hydrogel were tested. These tests gave us interesting results on how they can replicate a simple shape via micromolding that can be used in future projects.

Another important result was achieved in two-photon polymerization of polyacilamide. As for this kind of hydrogel there are no papers available talking about a multiphoton fabrication except for two made by Campagnola et al. The results obtained till now are not yet reliable but are promising for future developments. Succeeding in fabricating polyacrylamide hydrogels via two-photon laser lithography is an objective that will be pursued in order to obtain a significant result in the microfabrication of this synthetic hydrogel.

As for solid state nanolaser, the preliminary 3D microfabrication results showed the possibility to produce periodic arrays with a dye doped resist without any loss in spatial resolution. This is an important result that can be used in the future development of an efficient nanolaser.

Finally, each case of study in this thesis will be continued as a collaboration with as just many different departments of the University of Padua. The results obtained will be used as a starting point for future developments.

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