Direct Laser Writing to Generate Molds for Polymer Nanopillar Replication

Colm Delaney,^{1} Niamh Geoghegan,^{1,2} Hossam Ibrahim,^{3,4} Mark O'Loughlin,¹*

Brian J. Rodriguez,^{3,4} Larisa Florea,⁵ Susan M. Kelleher^{1,2}

¹School of Chemistry, Science Centre – South, University College Dublin, Belfield, Dublin 4, Ireland

²CURAM, Science Foundation Ireland Centre for Research in Medical Devices (CURAM), National University of Ireland Galway, Ireland

³Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland

⁴School of Physics, University College Dublin, Belfield, Dublin 4, Ireland

⁵School of Chemistry and AMBER, the SFI Research Centre for Advanced Materials and BioEngineering Research, Trinity College Dublin, the University of Dublin, College Green, Dublin 2, Ireland

E-mail: cdelane5@tcd.ie

Keywords: 2-Photon Polymerization, Direct Laser Writing, Replica Molding, Nanopillars, Nanostructured Polymers.

Abstract

Herein we exploit direct laser writing as a means to fabricate negative masters for the generation of polymeric nanopillars, via replica molding. Through optimization of design parameters, fabrication configuration, and polymerization protocols, we demonstrate for the first time, the fabrication of large-area negative templates which can be used to repeatedly produce some of the tallest ($>1.5\ \mu\text{m}$) and thinnest ($<300\ \text{nm}$) pillars ever achieved in biocompatible photocurable polymers, such as polyethylene glycol diacrylate, polypropylene glycol diacrylate, and polycaprolactone dimethacrylate. This combination serves to add value to two photon polymerization, and to extend its potential to the facile production of millimeter-sized arrays of submicron pillars, of infinite combinations of height, diameter and pitch in a wide variety of compliant, biocompatible, and biodegradable materials.

Introduction

The ubiquity of nanostructures in nature, their ability to alter interactions with light and to control hydrophobicity, adhesion, and even cell-interaction is a justification for the breadth of biomimetic nanostructured materials which have been generated over the past twenty years.¹⁻³ Inspiration taken from lotus leaves, cicadae, and morpho butterflies has been used to generate significant advances in surface coatings, medicine, and photonics.^{4,5} Synthetic analogues of periodic nanostructured materials have been achieved through disparate approaches, serving as relatively crude approximations for the multifaceted and hierarchical assemblies which are characteristic of natural nanostructures.⁶ In particular, hard lithographic techniques, adapted from the semiconductor industry, such as photolithography, electron beam lithography, and etching have been used to produce high-fidelity nanostructures.^{7,8} To date, these techniques have been hampered

by high cost, timescale, and the limited nature of the materials used (silicon, titania, glass, polystyrene).⁹ Particular focus on micro/nanofabrication in soft materials has been driven in recent years by advances in photonics, tissue engineering, and medical device research.^{8–10} These advances have focused on primary (hard lithography) techniques, such as electrospinning, e-beam lithography, and direct laser writing, or secondary (soft lithography) techniques such as nanoimprint lithography and capillary micromolding from high-fidelity hard masters, in materials such as silica, anodic alumina, or common negative tone resists.⁸

Of particular interest are direct write techniques, which operate without the need for mask exposure, subsequent photoresist curing, or etching. Laser-based direct write technology using multiple photon absorption can allow spatial confinement of polymerization with minimal topological constraints. Direct Laser Writing (DLW) *via* 2 photon-polymerization (2PP), in which a laser beam is focused into a tiny volume which serves as a building block within UV-curable photoresists, can yield feature sizes below the diffraction limit of the excitation light.¹¹ Since the advent of the technology, research groups have strived to push the boundaries of achievable resolution and line width through innovative physical and chemical approaches. Kawata *et al.* demonstrated the dramatic effects which could be achieved through the use of additional radical quenchers in the photoresist, which serve to minimize the extent to which created radicals can survive, thereby reducing the effective voxel volume.¹² Other groups have relied on more traditional methods borrowed from photolithographic process chains, such as post-fabrication baking and shrinkage, to achieve sub 100 nm line widths.¹³ In recent years, several groups have studied more adaptable acrylate-based photoresists to achieve impressive resolution and accurate predictions of voxel volumes, with respect to laser power and writing speed.^{14,15}

The flexibility of the 2PP technique, the advancement of throughput in commercially available systems, and the need for arrays of submicron sized structures within optics, photonics, microfluidics, tissue engineering, and cell biology fields, has driven many researchers to make libraries of rapidly prototyped nanostructures in a range of different polymeric materials. Purto *et al.* make use of controllable voxel size in proprietary IP-Dip resin to form nanopillars which were constructed from several vertically joined voxels.¹⁶ Through the use of additional post-fabrication crosslinking they have achieved impressive control of shape, pitch, and periodicity, in addition to controllable diameters (from 120 - 430 nm) and heights (from 330 - 1315 nm).¹⁷ The work of the Martinez Group in Copenhagen is also worthy of significant mention for the creation of 250 μm x 250 μm arrays of polymeric nanopillars with submicron diameters, and heights up to 6 μm , *via* 2PP, from a pentaerythritol triacrylate monomer.¹⁷ Their work, which probes the effect of pitch and surface coverage on the ability of fibroblasts to accept cues from the nano- and micro-topographies represents a small, but ever-developing, body of research focused on the use of 2PP for biological applications. They demonstrate that without a structural pattern, cells are seen to align randomly, while in the presence of nanopillar geometries, the alignment of cells is observed to be reliant on both pillar height and spacing. To date, much other work surrounding the application in the biological realm has focused on photoresists based on sol-gel chemistry of inorganic-organic hybrid photopolymers, which result in hydrophobic structures that do not swell readily in aqueous media. Koroleva *et al.* have shown an example of such materials applied within stem cell studies, showing abilities to control seeding efficiency, proliferation, and even differentiation.¹⁷ Recently, Accardo *et al.* have shown the use of poly(ethylene glycol) diacrylate with phenylbis(2,4,6-trimethylbenzoyl)phosphine oxide photoinitiator to fabricate 3D architectures on the 10 μm feature scale, and have demonstrated their compatibility with cells for

neuronal tissue engineering.¹⁴ While some further work has gone on to demonstrate 2PP fabrication in functionalized gelatin, collagen, and chitosan, with minimal cytotoxicity, adoption of the technology within this field has been rather slow.¹⁸ Reservations in this regard can arguably be attributed either to a relatively limited library of biocompatible monomers, a paucity of water-soluble photoinitiators with a substantial 2 photon absorption (2PA) cross-section, or indeed, inherent limitations caused by chemical reactivity (susceptibility to Michael reactions), and relatively low conversion rates of common acrylate monomers. Of course, these hurdles have not come without impressive solutions, such as photoinitiators based on coumarin scaffolds with improved water solubility and 2PA cross-sections,¹⁹ traditional dye-amine combinations which rely on intermolecular electron transfer followed by hydrogen transfer,²⁰ and even through the addition of biocompatible surfactants to disperse hydrophobic chromophores.²¹ Nonetheless, a tradeoff between reactivity, biocompatibility, fabrication resolution, and processing throughput may continue to be a sticking-point in extending the application of 2PP. Undeniably, the ability of the technology to produce high-resolution prototypes down to the nanoscale may well find its greatest contribution to biological studies through the combination of complementary techniques.

In this regard, several groups have combined the generation of 2PP-generated templates with more traditional soft lithography techniques on the micron scale, usually through a multistep process which proceeds *via* a soft, elastomeric replica. Some of the most impressive work in this field has been performed by the group of Helmut Schift at the Paul Scherrer Institute, Villigen, Switzerland, who have drawn on their knowledge of replication to extend it to 2PP generated masters and draw comparisons with silicon templates achieved *via* dry etch processes. By combining 2PP template generation with supercritical drying, they achieved high aspect ratio photonic nanofences which, *via* an elastomeric polydimethylsiloxane (PDMS) replica molding step, were then used to generate

high fidelity replicas of the master in photocurable proprietary-blend polymers. Through intelligent matches of master and replica materials, their results highlight the potential for using such a technique in high-volume manufacturing protocols.²² Fourkas *et al.* have also made significant inroads in this regard over the past fifteen years. Their original work generated structures using 2PP (tens of microns in height and width) in a monomeric cocktail based on 48 wt% ethoxylated trimethylolpropane triacrylate, 49 wt% tris(2-hydroxyethyl)isocyanurate triacrylate, and 3 wt% Lucirin TPO-L.²³ These structures, with high aspect ratios and truly 3D character, were subsequently used to generate PDMS masters, which could be then used to replicate the original structures in the same cocktail mixtures, without any discernible loss of resolution. The same group has developed on this work, to replicate 3D microstructures with closed loops, and more recently, to replicate submicron size ridges and sawteeth, with widths and heights of several hundred nanometers, *via* the same two-step process which uses a soft elastomeric primary replica and solvent assisted nanotransfer molding.^{24,25} Despite these significant advances, this approach has yet to be extended to soft, biocompatible polymers, on the nanoscale. Using this method to fabricate large arrays of nanostructures, without the use of any intermediary steps, would allow recipes and formulations currently optimized for bulk polymerization and used across tissue engineering and cell biology, to be accurately and reproducibly nanostructured.

Herein we present a selection of 2.5D and 3D structures generated from cocktails of biocompatible monomers achieved through the direct replication of 2PP-produced masters. The ability to generate millimeter-sized arrays in soft biocompatible polymers, with the retention of nanostructure, represents a marriage of high-resolution master development with the flexibility of a well-established fabrication technology. This approach holds significant potential, as control of material properties such as mechanical strength, degradation, and surface chemistry can be optimized in a

relatively facile manner. The ability to use 2PP as a means of generating master templates for the replication of nanoscale grooves, pits, or protrusions, finds great synergy with several decades of work on replication of biocompatible nanotopographies. Moreover, it gives access to high-resolution structuring in materials that have not been accessible with 2PP hitherto.

Results and Discussion

Arrays of negative masters were fabricated using 2 photon polymerization in photocurable cocktails. The fabrication was performed in the dip-in laser lithography configuration using IP-Dip photoresist, a proprietary blend containing 60–80% of 2-(hydroxymethyl)-2-[[[(1-oxoallyl)oxy]methyl]-1,3-propanediyl diacrylate and supplied by Nanoscribe GmbH. Covalent attachment of the polymeric structures was achieved by prior silanization of the fused silica substrates. This enabled repeatable replica molding from the same master. Scheme 1 presents a sample fabrication workflow showing replica molding from a 2PP-generated array.

Scheme 1: A combination of hard and soft lithography used to generate a library of submicron structured polymers. The process starts with the generation of a negative master using 2PP, followed by replication in photocurable polymers.

Since the advent of 2PP, the effect of writing parameters on feature size and resolution has been widely studied in the context of submicron structures, usually through analysis of adjacent lateral lines or *via* suspended line structures. Effective voxel size, line width, and polymer growth all play significant roles in the generation of high-resolution submicron features which are constructed by stacking voxels in three dimensions. Much less is known about the effect these parameters have on the generation of high aspect ratio pores, and the resulting size, shape and morphology of the internal channel. Moreover, in the context of replica molding templates for soft photocurable polymers, it is imperative that a systematic study such as this be performed to act as a blueprint for how these two techniques can be brought together to yield structures of predictable and controllable morphology, shape, and size.

To truly understand the effect of fabrication parameters on the generation of high-fidelity replica-molding masters, a single design file of arbitrary dimensions was generated. To create a 3D object from a standard stereolithography (.stl) file using DLW, a solid scaffold is achieved with a series of outline (contour) and filling (hatching) lines. The designs presented herein incorporated a series of 100 nm slices fabricated on top of each other, with lateral hatching performed perpendicular to the previous layer. This served to negate the effect of fabrication direction on the morphology of the fabricated pore features, which becomes of significant importance at submicron feature sizes. As discussed, fidelity of structures achieved using 2PP, especially in the submicron regime, are a complex product of material, fabrication parameters, and design. To initially probe fabrication parameters for a negative template, when isolated from variables of design, an .stl file

presented in Figure 1A containing pores of 800 nm diameter and 800 nm pitch and of 1.5 μm depth was used. In the absence of contouring, which can be used to define the outline of the cylinder, variation of fabrication parameters can have a dramatic effect on the generated pores. The reader is directed to Figure S1 and Table S1 for analysis of the arrays across a range of 20 parameter combinations, varying laser power from 7.5 – 15 mW and writing speeds from 1000 – 8000 μms^{-1} .¹ All of these parameters yielded pristine free-standing arrays which were fully attached to the fused silica substrate, as demonstrated by the SEM image shown in Figure 1B. Figure 1C presents a representative sample of these arrays, demonstrating, as expected, the profound effect of variation of laser power (increased from 12.5 – 15 mW) on the dimensions of the resulting pores, and the minor effect of varying writing speed through 1000 – 3000 μms^{-1} . The effect of laser power is maximized at slower writing speeds. At laser powers below 10 mW, a significant deviation from circularity was observed. This resulted in pores which tended towards a square geometry with top-down diameters nearing 1 μm (such as those fabricated at 7.5 mW and 1000 μms^{-1} ; seen in Figure S1). At higher powers (and lower writing speeds) the resulting geometry had an oval shape, as shown in Figure 1, which can be supported by previous studies documenting the effect of power and writing speed on the effective voxel shape and aspect ratio.¹⁵ Increasing laser power above 17.5 mW resulted in additional polymerization within the pores, thereby confirming a suitable parameter window for the presented design (Figure S1 and Table S1).

Figure 1: Generation of primary templates; A) .stl design file and B) SEM image of the fabricated master consisting of a 15x15 pore array. C) SEM images showing the effect of laser power and writing speed on the resulting pores (5x3 pore array shown). Top row fabricated at 12.5 mW laser power and writing speeds of 1000, 2000, and 3000 μms^{-1} , respectively. Bottom row fabricated at 15 mW laser power and writing speeds of 1000, 2000, and 3000 μms^{-1} , respectively. Scale bar represents 2 μm . All samples were coated with a 10 nm gold layer to enable imaging and reduce charging.

While top-down analysis of pore diameter proves a useful tool in providing an indication of cylindrical pore size, the true impact of this work is in the generation of 2.5D and even 3D objects. Success in template fabrication can only be measured in the ability of these arrays to generate

replica molded submicron structures, and the height, shape and size thereof. Using a replica molding procedure, outlined in detail in the experimental section, a monomeric cocktail of poly(ethylene glycol) diacrylate (M_n 575) (PEGDA) with 1 wt% phenylbis(2,4,6-trimethylbenzoyl)phosphine oxide (PBPO) was introduced to the gold-coated (10 nm) masters *via* capillarity between the substrate slide and a non-silanized top slide, with a channel height of 0.6 mm. Polymerization was then achieved using a white light source, for 120 mins, with irradiation from above and below the sample. A large proportion of the presented arrays successfully replica molded structures in the PEGDA polymer, as outlined in Table S1. For pores created using lower powers/higher writing speeds, the replicated pillars exhibit a notable ellipsoidal geometry, with a bulbous base and tip. This can be observed, in completion, in Figure S2 or a pertinent example (at 12.5 mW laser power) is observed for pillars replicated from templates fabricated using different writing speeds in Figure 2A, shown in the tilt SEM images. For templates fabricated using writing speeds $< 2000 \mu\text{ms}^{-1}$, replicated PEGDA pillars exhibit a traditional cylindrical geometry with uniform diameters from top to bottom, while above speeds of $5000 \mu\text{ms}^{-1}$, the replicated pillars exhibit a hyperboloid geometry with a noticeable overhung plateau at the top. This is particularly pronounced at laser powers of 10 mW and below, as seen in Figure S2. Conversely, exploiting lower writing speeds, to generate uniform pores in the master template, results in arrays of cylindrical pillars whose diameter can be easily controlled through the laser power used in master fabrication. Two such examples are shown in Figure 2. Tilt SEM images show pillars replicated from pores fabricated at 12.5 mW power (Figure 2B) with an average diameter of 520 ± 13 nm and 15 mW power (Figure 2C) with an average diameter of 300 ± 12 nm, respectively, as measured at 60° tilt. Top down analysis of the primary arrays had diameters of 779 ± 21 nm and 726 ± 22 nm, respectively. For a designed array with 800 nm pores, this serves to highlight the profound

impact writing parameters can have within submicron regimes. Moreover, it further reinforces the assertion that a combination of 2PP and replica molding can serve to generate potential that is greater than the sum of its constituent parts.

Figure 2: SEM images of PEGDA pillars replicated from 2PP templates. A) shows the effect of writing speed used to form the template on the morphology of resulting pillars (12.5 mW laser power, writing speeds from 1000 – 6000 μms^{-1}) imaged at 45° tilt ; B) shows pillars replicated

from pores fabricated at 12.5 mW and 2000 μms^{-1} C) shows pillars replicated from pores fabricated at 15 mW and 1000 μms^{-1} . B and C were imaged at 60° tilt. Scale bar represents 1 μm for all images. All samples were coated with a 10 nm gold layer prior to imaging.

The effect of fabrication parameters on pore array templates, and their subsequent impact on replica molded pillars has been documented hitherto. However, it is worthy of mention that the design of the fabrication file can also have a considerable impact on the fidelity of the negative structure, and the subsequent replica molded features. Figure 3 offers a direct comparison between design files when an additional outline is written at the perimeter of the cylindrical array. This outline, which is written prior to subsequent hatching, serves to greatly reduce the pore size achievable from the 800 nm features in the design file and limits the drastic effect of fabrication parameters on the resulting features. Figure 3A shows a set of pores fabricated, in the absence of an additional contouring line, at 10 mW laser power and writing speeds from 1000 –3000 μms^{-1} with top-down diameters measured from 1119 ± 31 nm to 903 ± 12 . The addition of a contouring line reduces this diameter, and further limits the effect of writing speed on the subsequent pores, with measured diameters of 528 ± 21 nm and 557 ± 21 nm for writing speeds of 1000 and 3000 μms^{-1} , respectively (Figure 3B). This had a profound effect on achieved diameters of replica molded pillars, which were measured from tilt SEM images to be 276 ± 2 nm (1000 μms^{-1}) and 285 ± 2 nm (3000 μms^{-1}), respectively. Most apparent, from Table S2 and Figure S4 is the narrow window of fabrication parameters which can be used to generate arrays that result in replica molding, when contour lines are employed. Further investigation is warranted to understand if this phenomenon is purely limited by pore dimensions, or if the variation of template modulus, caused by increasing levels of crosslinking along the contour line, is a contributing factor for replication of polymer structures on the nanoscale.

Figure 3: SEM images showing the effect of design on pore template and resulting replicated PEGDA pillars, when 10 mW laser power and writing speeds of 1000 – 3000 μms^{-1} are employed for master fabrication. A) in the absence of a contour line at the pore perimeter; B) with the inclusion of contour line for the same fabrication parameters (top); replica molded pillars in PEGDA at 45° tilt (bottom). Scale bar represents 1 μm for all images. All samples were coated with a 10 nm gold layer prior to imaging.

To truly exploit the potential of the methodologies presented herein, it is necessary to demonstrate the extension of this approach to larger-scale footprints, with retention of submicron resolution, homogeneity in heights, and applicability to a wider variety of materials. In this regard,

the results presented in Figure 4 serve to address the extension of this approach to larger arrays. Accurate replication of a 900-pore array with the same feature dimension and pitch but a much larger footprint ($50\text{ }\mu\text{m} \times 50\text{ }\mu\text{m}$; fabricated at 12.5 mW power and $2000\text{ }\mu\text{m s}^{-1}$) is demonstrated in the SEM image in Figure 4A. Adaptation of the original .stl design allows for fast and facile redesign of the template array. With the ability to seamlessly stitch $100\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$ blocks, each with a fabrication time of a few minutes, the generation of a millimeter sized submicron structured array is easily achievable within a reasonable timeframe.

Figure 4: Production and characterization of larger scale replica molded arrays in PEGDA. A) SEM image of 30x30 replica molded PEGDA pillar array imaged at 55° tilt. Scale bar represents $10\text{ }\mu\text{m}$. Sample was coated with a 10 nm gold layer prior to imaging; B) 3D reconstruction of AFM

height analysis performed in tapping mode; C) and D) AFM height analysis and example of corresponding height profiles across the three traces shown.

Moreover, as demonstrated in Figure 4A, the significantly increased size of the array, and separation forces from >600 additional pillars posed no problem in replication of the master templated in PEGDA. Most importantly, AFM imaging of the PEGDA shows the homogeneity in height profile across a 15 μm x 15 μm section, as seen in Figure 4B. Analysis of height traces, some examples of which are shown in in Figure 4C and Figure 4D, show an average height of 1440 ± 43 nm ($N = 80$ pillars). Not only does this confirm such uniformity across the array, but it also serves to verify that infiltration of the prepolymer cocktail occurs to the very bottom of the 1.5 μm cylindrical pores. After polymerization, complete removal of the newly formed pillars, with retention of the as-formed surface morphology, and minimal adherence or covalent attachment to the functionalized slide, offer much hope for the possibility of migrating to more compliant polymers.

Developments in 2PP for biological applications continue to make inroads in achieving 2D and 3D structures in biocompatible polymers. While some effective solutions to biocompatibility have been achieved through variation of photoinitiators, solvents, and suitable monomers, the nature of fabrication using DLW does necessitate a high degree of crosslinking, to enable small, self-standing features. This can be achieved, as in the case of proprietary cocktails, through the use of multiarm acrylate monomers, or through the inclusion of additional crosslinking agents in bespoke prepolymer blends. The ability to accurately and predictably program the modulus of polymeric matrices is critical in biological applications, from gene therapy, to stem-cell differentiation. If successful, replica molding from high-resolution 2PP masters using traditional biocompatible prepolymer cocktails may serve to generate arrays of polymeric nanostructures with greater

adaptability of physical and chemical properties, than can currently be achieved using 2PP. To demonstrate the applicability and flexibility of this approach, a single 30x30 master array was fabricated without contour lines at 10 mW power and 1000 μms^{-1} . From this same master, three cocktails were subsequently replica molded. The resulting pillar arrays are shown in Figure 5, and demonstrate complete replication across short-chain hydrophilic monomers, such as PEGDA (M_n 575) and PPGDA (M_n 880), and the longer chain hydrophobic PCLDMA (M_n 1350) monomer. Impressively, the retention of structure height (1.5 μm) and width (< 500 nm) in an elastomer of this nature, confirms the potential that this approach may hold in the generation of soft, submicron structured biocompatible and biodegradable polymers.

Figure 5: Multiple replications from a single 2PP master to produce submicron pillars in poly(ethylene glycol) diacrylate (M_n 575) (PEGDA), poly(propylene glycol) diacrylate (M_n 880) (PPGDA), and poly(caprolactone) dimethacrylate (M_n 1350) (PCLDMA). Replica molded arrays are imaged at 45° tilt. Scale bar for all samples represents 10 μm . All samples were coated with a 10 nm gold layer prior to imaging.

The mechanical properties of the nanostructured polymer surfaces were elucidated using AFM (see ESI) and compared to their 2PP fabricated analogues. Force volume mapping of the replica-molded samples demonstrated notably different stiffness regimes across the same sample. Applying masks allowed for analysis of three distinct regions on the nanostructured polymer surfaces; 1) bulk polymer; 2) pillar; and 3) flat regions at base of pillars. Images of modulus maps, masks used, and distribution of measurements can be found in Figures S5-S7. The bulk planar region of the replica molded PEGDA polymer exhibited a Young's modulus of 765 ± 73 MPa, showing a considerable difference from the analogous PEGDA 2PP-fabricated sample, with a measured Young's modulus of 45 ± 4 MPa. Such reduction in Young's modulus for 2PP-fabricated structures compared to bulk has been previously reported and is proposed to be a result of the different approaches used to induce polymerization.^{26,27} In the case of 2PP, microstructures are constructed by building voxels on voxels, while bulk photoinitiated polymerization is achieved by light-flooding illumination. The higher molecular weight PPGDA gives a bulk polymer with a smaller Young's modulus of 374 ± 41 MPa, as the flexibility of the chain increases.

The flat areas at the base of replica-molded pillars showed a noticeably lower Young's modulus for both polymers (405 ± 88 MPa for PEGDA; 262 ± 93 MPa for PPGDA) than in the bulk regions. Of significant importance is the noticeable difference in Young's modulus measured for the replica molded pillars ($111 \text{ MPa} \pm 44$ PEGDA, 76 ± 22 MPa for PPGDA) in comparison to the bulk regions of the respective polymers. This is best explained by the effect of polymerization in confined, submicron spaces. Although much of the previous studies on free radical polymerization in confined spaces focuses on linear polymer chains,^{28,29} the lower moduli of the submicron pillars could be a consequence of a lowered polymer glass transition temperature, caused by restriction of the polymeric network in a confined space.

Conclusions

We have successfully created a highly adaptable and accessible means for low-cost generation of structured polymers, through the combination of 2PP and replica molding, which yields large arrays of nanostructures in soft, biocompatible polymers. Through optimization of 2PP design and fabrication parameters, we have demonstrated achievable control of feature size and shape of the negative 2PP master. Subsequent generation of highly reproducible pillared surfaces in photocurable polymers, such as PEGDA, PPGDA, and PCLDMA was then carried out, with size and morphology controlled by the master fabrication parameters. This strategy allows for the fabrication of desired 3D shapes in a variety of well-known, biocompatible materials with pre-defined and tailored modulus. The marriage of sub-micron resolution 3D fabrication with a wide library of materials of controlled mechanical properties, holds enormous potential for next-generation 3D cell assays. These large area, highly reproducible, structured polymers can be used to study invasion processes of cancer cells, or to probe the effect of the microenvironment on the differentiation of stem cells.

Experimental

Fabrication of Cylindrical Pore Arrays

Two photon polymerization of cylindrical pore arrays was carried out using a commercial direct laser writing apparatus, Photonic Professional (Nanoscribe GmbH). The Dip-In configuration was used, employing a 63x objective. DiLL substrates and DiLL monomer photoresists were purchased from Nanoscribe. Prior to laser writing, the fused silica substrates (25 x 25 mm²; thickness 0.7 mm) were sonicated in ethanol for 30 min, followed by UV Ozone treatment for 15 min to activate the substrate surface (Digital UV Ozone System, PSD Pro Series). The surface of the DiLL

substrates was modified by immersion in a solution containing 3% v/v 3-(trimethoxysilyl)propyl methacrylate (Sigma Aldrich) and 0.1% v/v acetic acid in ethanol for 1 hr. After rinsing, and drying under nitrogen, the substrates were cured for 10 mins at 40 °C. A single drop of the monomeric photoresist, IP-Dip (Nanoscribe GmbH) was deposited onto the center of the substrate before the holder was placed into the system. The design (.stl) file of the desired array of cylindrical pores was loaded into the software with distances of 100 nm used for both slicing and hatching. For pore array fabrication, the laser power and scan speed were varied between 10 – 17.5 mW and 1000 – 10000 $\mu\text{m s}^{-1}$. After laser writing, the structures were cleaned by immersing in propylene glycol methyl ether acetate for 20 min followed by 2-propanol for 2 min. The structures were then rinsed dried under a stream of nitrogen and coated with a 10 nm layer of gold.

Fabrication of Nanopillars

Replica molding from 2PP cylindrical pore arrays was carried out using a 1% w/w solution of phenylbis(2,4,6-trimethylbenzoyl)phosphine oxide in poly(ethylene glycol) diacrylate (M_n 575) (Sigma Aldrich), a 1% w/w solution of phenylbis(2,4,6-trimethylbenzoyl)phosphine oxide in poly(propylene glycol) diacrylate (M_n 880) (Sigma Aldrich), or a 2% w/w solution of phenylbis(2,4,6-trimethylbenzoyl)phosphine oxide in poly(caprolactone) dimethacrylate (M_n 1350) (Sigma Aldrich). Two PDMS spacers (thickness 600 μm) were placed firmly onto the Au-coated DiLL substrates and positioned either side of the 2PP structures. A cover glass (22 x 22 mm) was placed firmly onto both PDMS spacers. The monomeric cocktail was introduced to the space beneath the sealed cover glass until the 2PP structures were fully covered. The cocktail was cured under white light (Edmund Optics, MI 150 Illuminator) for 120 min.

SEM Imaging and Analysis

Scanning Electron Microscopy of the 2PP cylindrical pore arrays and replica molded nanopillars was carried out using a Zeiss Ultra Plus Scanning Electron Microscope. An accelerating voltage of 5 kV was used under SE2 mode to acquire all images. Prior to SEM imaging, the structures were coated with 10 nm Au-Pd layer using a Cressington Sputter Coater 208HR. A 57 x 0.1 mm Au-Pd target (TED PELLA INC.) was used to coat the structures under an inert atmosphere of Argon for 10 s. Pore diameter was measured from top-down SEM images using the particle analysis function on ImageJ. A minimum of sixty pore diameter measurements were obtained per pore array fabricated to ensure a representative sample of the pores. The Feret diameter obtained for each pore was averaged and standard deviation obtained. The diameter of the nanopillars was obtained manually by measuring the width of the mid-section of the pillars using the measure tool.

AFM Imaging and Analysis

Atomic Force Microscopy of the 2PP cylindrical pore arrays and replica molded nanopillars was carried out using amplitude modulation AFM (Asylum Research, MFP3D) and a silicon probe (Budget Sensors, TAP300AI-G) with an aluminium reflective coating, a nominal resonance frequency of 300 kHz, and a nominal cantilever stiffness of 40 N/m. Prior to imaging, the substrate was secured onto a glass microscope slide. AFM images were post-processed and analyzed using Gwyddion. Line profile data was obtained following image post-processing and plotted in Origin. AFM force volume spectroscopy measurements were performed on the same system. IP-Dip samples were characterized using diamond probes (Adama, NM-RC) with a nominal cantilever stiffness of 350 N/m. For PEGDA and PPGDA samples, measurements were performed using Si probes (BudgetSensors, tip C, All-In-One-AI) with a nominal cantilever stiffness of 7.4 N/m. For

all AFM force spectroscopy measurements, the cantilever spring constants were determined experimentally using the Sader method.³⁰

Force volume mapping was implemented as a 100 x 100 array of force curves across a 2.5 μm x 2.5 μm area for IP-Dip samples (10,000 indentations per force map with a 25 nm spacing) and as a 150 x 150 array of force curves across a 2.5 μm x 2.5 μm area for replica-molded samples (22,500 indentations per force map; 17 nm spacing). For PEGDA fabricated using 2PP, the force volume mapping was implemented as a 150 x 150 array across a 5 μm x 5 μm (22,500 indentations per force map; 33 nm spacing). A relative 5 μN trigger force was used for IP-Dip samples, while a relative 250 nN trigger force was used on PEGDA and PPGDA samples. The trigger forces were selected to ensure that indentations were limited to 10% of the sample thickness.^{31,32} Young's modulus was determined using the Asylum software by fitting the extension part of the force-indentation curves to the Hertz model³³ using Poisson's ratios of 0.49 for IP-Dip samples and 0.35 for PEGDA and PPGDA samples.^{34,35}

ASSOCIATED CONTENT

Supporting Information

The Supporting information is available free of charge at

SEM data, Replica molding results, AFM measurements. (PDF)

AUTHOR INFORMATION

Corresponding Author

E-mail: cdelane5@tcd.ie

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENT

This work was funded by Science Foundation Ireland, through the Starting Investigator Research Grant (SIRG) Scheme (Grant Number 15/SIRG/3429). SK and NG also acknowledge SFI and the European Regional Development Fund (ERDF) (Grant Number 13/RC/2073 and 17/RC-PhD/3480), through CURAM, the SFI Research Centre for Medical Devices. SK and MOL also acknowledge the Irish Research Council (IRC) (Grant number GOIPG/2017/1037). LF acknowledges the European Research Council (ERC) Starting Grant (project number 802929-ChemLife) and SFI-ERDF (Grant Number 12/RC/2278_P2). The 2PP-DLW fabrication and SEM imaging for this project was carried out at the Additive Research Laboratory (AR-Lab) and the Advanced Microscopy Laboratory (AML), Trinity College Dublin, Ireland. The AR-Lab and AML are SFI supported centres, part of the CRANN Institute and affiliated to the AMBER centre.

REFERENCES

- (1) Zhang, S.; Chen, Y. Nanofabrication and Coloration Study of Artificial Morpho Butterfly Wings with Aligned Lamellae Layers. *Sci. Rep.* **2015**, *5* (1), 16637. <https://doi.org/10.1038/srep16637>.
- (2) Narasimhan, V.; Siddique, R. H.; Lee, J. O.; Kumar, S.; Ndjamen, B.; Du, J.; Hong, N.; Sretavan, D.; Choo, H. Multifunctional Biophotonic Nanostructures Inspired by the Longtail Glasswing Butterfly for Medical Devices. *Nat. Nanotechnol.* **2018**, *13* (6), 512–519. <https://doi.org/10.1038/s41565-018-0111-5>.

- (3) Hsiung, B. K.; Siddique, R. H.; Stavenga, D. G.; Otto, J. C.; Allen, M. C.; Liu, Y.; Lu, Y. F.; Deheyn, D. D.; Shawkey, M. D.; Blackledge, T. A. Rainbow Peacock Spiders Inspire Miniature Super-Iridescent Optics. *Nat. Commun.* **2017**, *8* (1), 1–8. <https://doi.org/10.1038/s41467-017-02451-x>.
- (4) Zyla, G.; Kovalev, A.; Grafen, M.; Gurevich, E. L.; Esen, C.; Ostendorf, A.; Gorb, S. Generation of Bioinspired Structural Colors via Two-Photon Polymerization. *Sci. Rep.* **2017**, *7* (1), 1–9. <https://doi.org/10.1038/s41598-017-17914-w>.
- (5) Kelleher, S. M.; Habimana, O.; Lawler, J.; O’ Reilly, B.; Daniels, S.; Casey, E.; Cowley, A. Cicada Wing Surface Topography: An Investigation into the Bactericidal Properties of Nanostructural Features. *ACS Appl. Mater. Interfaces* **2016**, *8* (24), 14966–14974. <https://doi.org/10.1021/acsami.5b08309>.
- (6) Watson, G. S.; Green, D. W.; Cribb, B. W.; Brown, C. L.; Meritt, C. R.; Tobin, M. J.; Vongsvivut, J.; Sun, M.; Liang, A.-P.; Watson, J. A. Insect Analogue to the Lotus Leaf: A Planthopper Wing Membrane Incorporating a Low-Adhesion, Nonwetting, Superhydrophobic, Bactericidal, and Biocompatible Surface. *ACS Appl. Mater. Interfaces* **2017**, *9* (28), 24381–24392. <https://doi.org/10.1021/acsami.7b08368>.
- (7) Wi, J. S.; Lee, H. S.; Lim, K.; Nam, S. W.; Kim, H. M.; Park, S. Y.; Lee, J. J.; Hong, C. D.; Jin, S.; Kim, K. B. Fabrication of Silicon Nanopillar Teradot Arrays by Electron-Beam Patterning for Nanoimprint Molds. *Small* **2008**, *4* (12), 2118–2122. <https://doi.org/10.1002/sml.200800625>.
- (8) Lipomi, D. J.; Martinez, R. V.; Cademartiri, L.; Whitesides, G. M. Soft Lithographic Approaches to Nanofabrication. *Polym. Sci. A Compr. Ref. 10 Vol. Set* **2012**, *7*, 211–231.

<https://doi.org/10.1016/B978-0-444-53349-4.00180-1>.

- (9) Acikgoz, C.; Hempenius, M. A.; Huskens, J.; Vancso, G. J. Polymers in Conventional and Alternative Lithography for the Fabrication of Nanostructures. *Eur. Polym. J.* **2011**, *47* (11), 2033–2052. <https://doi.org/10.1016/j.eurpolymj.2011.07.025>.
- (10) Mijangos, C.; Hernández, R.; Martín, J. A Review on the Progress of Polymer Nanostructures with Modulated Morphologies and Properties, Using Nanoporous AAO Templates. *Prog. Polym. Sci.* **2016**, *54–55*, 148–182. <https://doi.org/10.1016/j.progpolymsci.2015.10.003>.
- (11) Varapnickas, S.; Malinauskas, M. Processes of Direct Laser Writing 3D Nano-Lithography. *Preprints* **2018**, *2018120119*, 1–31. <https://doi.org/10.20944/preprints201812.0119.v1>.
- (12) Sun, H. B.; Maeda, M.; Takada, K.; Chon, J. W. M.; Gu, M.; Kawata, S. Experimental Investigation of Single Voxels for Laser Nanofabrication via Two-Photon Photopolymerization. *Appl. Phys. Lett.* **2003**, *83* (5), 819–821. <https://doi.org/10.1063/1.1598293>.
- (13) Haske, W.; Chen, V. W.; Hales, J. M.; Dong, W.; Barlow, S.; Marder, S. R.; Perry, J. W. 65 Nm Feature Sizes Using Visible Wavelength 3-D Multiphoton Lithography. *Opt. Express* **2007**, *15* (6), 3426. <https://doi.org/10.1364/oe.15.003426>.
- (14) Accardo, A.; Blatché, M. C.; Courson, R.; Loubinoux, I.; Vieu, C.; Malaquin, L. Two-Photon Lithography and Microscopy of 3D Hydrogel Scaffolds for Neuronal Cell Growth. *Biomed. Phys. Eng. Express* **2018**, *4* (2). <https://doi.org/10.1088/2057-1976/aaab93>.
- (15) LaFratta, C.; Baldacchini, T. Two-Photon Polymerization Metrology: Characterization

- Methods of Mechanisms and Microstructures. *Micromachines* **2017**, *8* (4), 101. <https://doi.org/10.3390/mi8040101>.
- (16) Purto, J.; Verch, A.; Rogin, P.; Hensel, R. Improved Development Procedure to Enhance the Stability of Microstructures Created by Two-Photon Polymerization. *Microelectron. Eng.* **2018**, *194* (December 2017), 45–50. <https://doi.org/10.1016/j.mee.2018.03.009>.
 - (17) Purto, J.; Rogin, P.; Verch, A.; Johansen, V. E.; Hensel, R. Nanopillar Diffraction Gratings by Two-Photon Lithography. *Nanomaterials* **2019**, *9*, 1495.
 - (18) Nguyen, A. K.; Narayan, R. J. Two-Photon Polymerization for Biological Applications. *Mater. Today* **2017**, *20* (6), 314–322. <https://doi.org/10.1016/j.mattod.2017.06.004>.
 - (19) Nazir, R.; Danilevicius, P.; Ciuciu, A. I.; Chatzinikolaidou, M.; Gray, D.; Flamigni, L.; Farsari, M.; Gryko, D. T. Π -Expanded Ketocoumarins As Efficient, Biocompatible Initiators for Two-Photon-Induced Polymerization. *Chem. Mater.* **2014**, *26* (10), 3175–3184. <https://doi.org/10.1021/cm500612w>.
 - (20) Nguyen, A. K.; Gittard, S. D.; Koroleva, A.; Schlie, S.; Gaidukeviciute, A.; Chichkov, B. N.; Narayan, R. J. Two-Photon Polymerization of Polyethylene Glycol Diacrylate Scaffolds with Riboflavin and Triethanolamine Used as a Water-Soluble Photoinitiator. *Regen. Med.* **2013**, *8* (6), 725–738. <https://doi.org/10.2217/rme.13.60>.
 - (21) Jhaveri, S. J.; McMullen, J. D.; Sijbesma, R.; Tan, L.-S.; Zipfel, W.; Ober, C. K. Direct Three-Dimensional Microfabrication of Hydrogels via Two-Photon Lithography in Aqueous Solution. *Chem. Mater.* **2009**, *21* (10), 2003–2006. <https://doi.org/10.1021/cm803174e>.

- (22) Cadarso, V. J.; Chidambaram, N.; Jacot-Descombes, L.; Schiff, H. High-Aspect-Ratio Nanoimprint Process Chains. *Microsystems Nanoeng.* **2017**, *3* (September 2016), 1–12. <https://doi.org/10.1038/micronano.2017.17>.
- (23) LaFratta, C. N.; Baldacchini, T.; Farrer, R. A.; Fourkas, J. T.; Teich, M. C.; Saleh, B. E. A.; Naughton, M. J. Replication of Two-Photon-Polymerized Structures with Extremely High Aspect Ratios and Large Overhangs. *J. Phys. Chem. B* **2004**, *108* (31), 11256–11258. <https://doi.org/10.1021/jp048525r>.
- (24) Sun, X.; Hourwitz, M. J.; Baker, E. M.; Schmidt, B. U. S.; Losert, W.; Fourkas, J. T. Replication of Biocompatible, Nanotopographic Surfaces. *Sci. Rep.* **2018**, *8* (1), 1–9. <https://doi.org/10.1038/s41598-017-19008-z>.
- (25) LaFratta, C. M.; Li, L.; Fourkas, J. T. Soft-Lithographic Replication of 3D Microstructures with Closed Loops. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103* (23), 8589–8594. <https://doi.org/10.1073/pnas.0603247103>.
- (26) Nakanishi, S.; Shoji, S.; Kawata, S.; Sun, H. B. Giant Elasticity of Photopolymer Nanowires. *Appl. Phys. Lett.* **2007**, *91* (6), 2005–2008. <https://doi.org/10.1063/1.2767995>.
- (27) Cadarso, V. J.; Chidambaram, N.; Jacot-Descombes, L.; Schiff, H. High-Aspect-Ratio Nanoimprint Process Chains. *Microsystems Nanoeng.* **2017**, *3* (September 2016), 1–12. <https://doi.org/10.1038/micronano.2017.17>.
- (28) Sanz, B.; Ballard, N.; M. Asua, J.; Mijangos, C. Effect of Confinement on the Synthesis of PMMA in AAO Templates and Modeling of Free Radical Polymerization. *Macromolecules* **2017**, *50* (3), 811–821. <https://doi.org/10.1021/acs.macromol.6b02282>.

- (29) Tarnacka, M.; Maksym, P.; Zięba, A.; Mielańczyk, A.; Geppert-Rybczyńska, M.; Leon-Boigues, L.; Mijangos, C.; Kamiński, K.; Paluch, M. The Application of Spatially Restricted Geometries as a Unique Route to Produce Well-Defined Poly(Vinyl Pyrrolidones) via Free Radical Polymerisation. *Chem. Commun.* **2019**, 55 (45), 6441–6444. <https://doi.org/10.1039/C9CC02625H>.
- (30) Sader, J. E.; Chon, J. W. M.; Mulvaney, P. Calibration of Rectangular Atomic Force Microscope Cantilevers. *Rev. Sci. Instrum.* **1999**, 70 (10), 3967–3969. <https://doi.org/10.1063/1.1150021>.
- (31) Costa, K. D.; Yin, F. C. P. Analysis of Indentation: Implications for Measuring Mechanical Properties with Atomic Force Microscopy. *J. Biomech. Eng.* **1999**, 121 (5), 462–471. <https://doi.org/10.1115/1.2835074>.
- (32) Wojcikiewicz, E. P.; Zhang, X.; Moy, V. T. Force and Compliance Measurements on Living Cells Using Atomic Force Microscopy (AFM). *Biol. Proced. Online* **2004**, 6 (1), 1–9. <https://doi.org/10.1251/bpo67>.
- (33) Dimitriadis, E. K.; Horkay, F.; Maresca, J.; Kachar, B.; Chadwick, R. S. Determination of Elastic Moduli of Thin Layers of Soft Material Using the Atomic Force Microscope. *Biophys. J.* **2002**, 82 (5), 2798–2810. [https://doi.org/10.1016/S0006-3495\(02\)75620-8](https://doi.org/10.1016/S0006-3495(02)75620-8).
- (34) Greaves, G. N.; Greer, A. L.; Lakes, R. S.; Rouxel, T. Poisson's Ratio and Modern Materials. *Nat. Mater.* **2011**, 10 (11), 823–837. <https://doi.org/10.1038/nmat3134>.
- (35) Lemma, E. D.; Rizzi, F.; Sileo, L.; Spagnolo, B.; Dattoma, T.; Qualtieri, A.; Vittorio, M. De; Pisanello, F. Static and Dynamic Mechanical Characterization of Two-Photon

Lithography Photoresists. **2015**, 2–3.

TOC ENTRY

Direct Laser writing is used as a means to generate easily adapted templates for replica molding polymeric nanopillars. Optimization of fabrication and design parameters show the significant impact on pore arrays and the resulting nanopillars. Variation of monomeric cocktails shows the ability to extend the methodology to nanostructure soft, biocompatible, biodegradable polymers.