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Self-folding nano- and micropatterned hydrogel tissue engineering scaffolds by single step photolithographic process ☆

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ABSTRACT

Current progress in tissue engineering is focused on the creation of environments in which cultures of relevant cells can adhere, grow and form functional tissue. We propose a method for controlled chemical and topographical cues through surface patterning of self-folding hydrogel films. This provides a conversion of 2D patterning techniques into a viable method of manufacturing a 3D scaffold. While similar bilayers have previously been demonstrated, here we present a faster and high throughput process for fabricating self-folding hydrogel devices incorporating controllable surface nanotopographies by serial hot embossing of sacrificial layers and photolithography.

1. Introduction

Current progress in tissue engineering is focused on the creation of environments in which the relevant cells can adhere, grow and form functional tissue [1]. This proliferation can be controlled by chemical [2] or topographical [3] cues through surface patterning and functionalization. While there has been significant progress in 2D surface-cell interaction, most fall short of presenting the cells with the cues which exist in the native 3D cellular environment in a device combining surface patterning and self-folding methods [4–6]. While many 3D cellular tissue scaffold constructs have been demonstrated in the past [7], these do not offer much control over the geometry, and would not serve in making a purpose designed surface topography and structure. There is therefore a need to extend the applications of defined 2D micro- and nanopatterned methods to the third dimension. Self-folding is one technique of extending the existing and readily available 2D patterning techniques into a viable method of manufacturing more complex 3D cellular environments, and to create reconfigurable structures which can fold or unfold in response to specific environmental cues. An array of self-folding 3D structures [6], employing stimuli responsive materials for cell capture [8] and drug delivery have been made in the past. These have often incorporated a differential bilayer structure [9] with varying environmental sensitivity [10]; from heat-shrink type hinges [11], shape memory polymers [12] to hydrogel films [13]. However these devices lack combined surface patterning on a material which will remain permeable to oxygen and nutrients. The challenge lies in the creation of permeable and patterned 2D templates composed of polymers and hydrogels to provide a basis for self-folding structures constructed using a wider range of biocompatible and biodegradable materials. One possible solution is optical patterning using photolithography [14,15] or soft lithographic methods [16] such as UV-NIL [13,16–18]. Some of these methods have previously been utilized to fabricate hingeless polymeric structures that roll up or fold spontaneously [14,15,17]. Hydrogels are of particular interest in this application because of their high water content, permittivity and mechanical properties which resemble those of nonosseous living tissues. Previous methods of manufacturing thin hydrogel scaffolds have used a two stage photolithographic process or manufacture by two-photon stereo-lithography. These processes are time consuming and require several stages of mask alignment or expensive equipment, as the micro-patterning of biocompatible hydrogel films is generally recognized as a complex task [9]. The key features for a self-folding tissue engineering scaffold are then: pattern-ability for cell contact guidance, nutrient permeability to avoid tissue necrosis, and ease and speed of manufacture.

We propose a novel method for producing environmentally triggered, self-folding, non-fouling and permeable hydrogel scaffolds, combined with nano- and micropatterned on both surfaces in a one-step photolithographic system. The manufacturing

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process uses to the authors' knowledge a new method of sacrificial layer embossing and simultaneous double sided patterning and surface activation. The method allows for high throughput manufacture of "smart" hydrogel scaffolds with the added advantages of employing well developed 2D micro and nanopatterning techniques. In this approach, the previously demonstrated use of poly(acrylic acid) (PAA) as a uniform thin film sacrificial layer [19] is taken a step further with sacrificial layer embossing using an ordered micro- and nanopatterned stamp. This pre-patterning of the sacrificial film allows for the simultaneous patterning and activation of the hydrogel film applied on top of it, either by UV-NIL or UV photolithography. During the subsequent photolithographic step pendant groups of PAA are cross-linked to the poly(ethyleneglycol)dimethacrylate (PEGDMA) hydrogel matrix, forming an environmentally sensitive bilayer, where the grafted side of the thin film swells on exposure to a neutral pH 7 and collapses again in a more acidic pH 4 allowing for repeated spontaneous folding and unfolding with changes in the surrounding aqueous environment. Inter-sheet spacing is affected by exposure dose and ionic concentration of the surrounding media, or fixed in its folded state by additional UV exposure to further crosslink the hydrogel scaffold while it is in its folded state, preserving the roll like structure on further exposure to aqueous media. The finished device is a bifacially nanopatterned self-folding hydrogel scaffold, achieved by fast throughput methods.

2. Materials and methods

2.1. Materials

PEGDMA (550 Mn) and Phenylbis(2,4,6-trimethylbenzoyl)phosphine oxide (IR 819) were obtained from Sigma Aldrich, three different molecular weights of PAA (50,000 M_W 25% in H_2O, 100,000 M_W 25% in H_2O and 1800 M_W 67% in H_2O) were obtained from Polysciences, the low molecular weight PAA was diluted to 25% aqueous concentration by the addition of RO Water, while the others were used as received. Sylgard 184 Polydimethylsiloxane (PDMS) and curing agent were obtained from Farnell Electronics and used as received according to manufacturer guidelines.

The micro- and nanopatterned hydrogel device were created through a series of stages to create the foundation for a high throughput one-step lithographic method. For each pattern, a mastering process was required in which quartz (Qz), silicon (Si) or PDMS stamps were prepared, to be used in subsequent hot embossing and photolithography steps of the sacrificial PAA base layer to facilitate lift-off and functionalization of the PEGDMA hydrogel layer.

2.2. Quartz stamp

The nanopatterned stamp utilised for both the pre-patterning of the sacrificial film and subsequent top-side patterning of the hydrogel film (as shown in Fig. 1) was fabricated from a 25 × 25 mm and 1 mm thick, quartz sample. Electron beam lithography, metal lift-off and reactive ion etching (RIE) were used to create the stamp. Electron sensitive poly(methylmethacrylate) (PMMA) 2010 4% was spun at 5000 rpm and baked at 180 °C for 15 min before repeating the process with a second layer of 2041 2.5% PMMA. The purpose of the bi-layer was to provide an undercut in the resist in order to aid a future stage of processing, metal lift-off. Prior to defining the desired nanopattern, with an electron beam lithography tool, a discharge surface layer is required as the quartz/PMMA stack has insufficient electrical conductivity and a build-up of surface charge from the electron beam exposure is known to occur on non-conductive substrates [20]. Such a charge build-up would cause uncontrollable pattern deformity [21]. In this instance 10 nm of Al was evaporated onto the sample to act as the discharge layer.

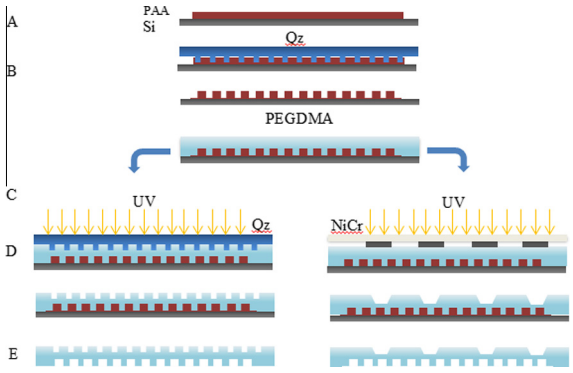

Fig. 1. Fabrication process for manufacture of nano/nanopatterned and nano/micropatterned PEGDMA hydrogel films by PAA sacrificial layer embossing. (A) PAA is spun onto Si wafer. (B) PAA film is embossed with master stamp. (C) Hydrogel is applied to PAA surface. (D) Master stamp or mask is applied and assembly is exposed to UV and developed in IPA. (E) Wafer placed in RO water allowing for dissolution of PAA layer and subsequent lift-off of hydrogel patterned film.

The nanopattern design for this stamp was an ordered array of 250 nm diameter circles with a pitch of 500 nm covering a square area of 5 × 5 mm. This design was defined in the resist using a Vistec Gaussian Vector Beam 6 (100 kV) electron beam lithography tool.

Following exposure, the Al discharge layer was removed with tetramethylammonium hydroxide (TMAH) and the PMMA developed in MIBK:IPA solution. The sample was then descummed in oxygen plasma. NiCr has been shown to be an effective hard mask for RIE quartz nanofeatures [22]. Therefore 50 nm of NiCr was evaporated onto the PMMA coated quartz face so as to create the actual nanofeature etch mask. Thereafter the PMMA resist was 'lifted-off' in acetone and a further oxygen plasma descum was performed. The penultimate step in the quartz stamp fabrication was the transfer of the defined hard mask features into the quartz. In order to achieve this, an Oxford Instruments Plasmalab 80 plus RIE machine was used with a mixture of CHF_3 and Ar gases. The quartz was etched to a depth of ~250 nm producing robust 1:1 aspect ratio nanopillars. Finally any remaining NiCr on the top of the pillars was removed by giving the sample an agitated emersion in a solution of ceric ammonium nitrate and nitric acid (chrome etch).

2.3. Si stamp

The Silicon stamp was first fabricated for thermal NIL. A clean 15 × 15 mm Si substrate with a thickness of 500 μm was spin coated with HSQ in which the pattern was written. The design was further transferred to the Si substrate by inductively coupled plasma (ICP) dry-etching [23] The gas used for dry-etching was SF_6/C_4F_8. The dry-etching depth was found to be 260 nm as measured under SEM.

2.4. Release coating

Prior to use in UV-NIL or manufacture of PDMS stamps the Si and Qz masters were washed in acetone, methanol and isopropanol for 5 min each before being cleaned in an oxygen asher for 2 min. Silane deposition was carried out from a 0.0001% v/v solution of silane in heptane for 20 min. After the treatment was completed the master stamps were rinsed in heptane, acetone, and isopropanol.

2.5. PDMS stamps

Sylgard 184 PDMS was mixed at a ratio of 10:1 to the curing agent, and poured onto the master pattern in a salinized glass dish.

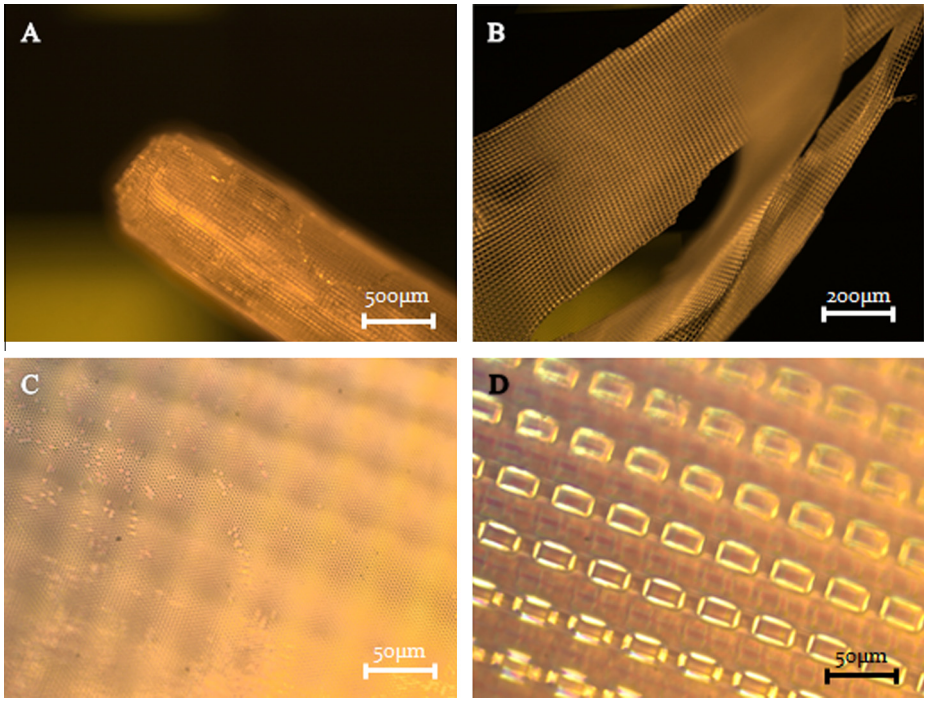

Fig. 2. (A) Rolled micropatterned PEGDMA hydrogel film after exposure to pH 7 buffer and air drying. Scale bar, 500 μm. (B) Unrolled micropatterned PEGDMA hydrogel film after unrolling in pH 4 buffer and air drying. Scale bar, 200 μm. (C) Bottom surface after lift-off from embossed PAA layer. Scale bar, 50 μm. (D) Top surface of film showing pattern from UV exposure. Scale bar, 50 μm.

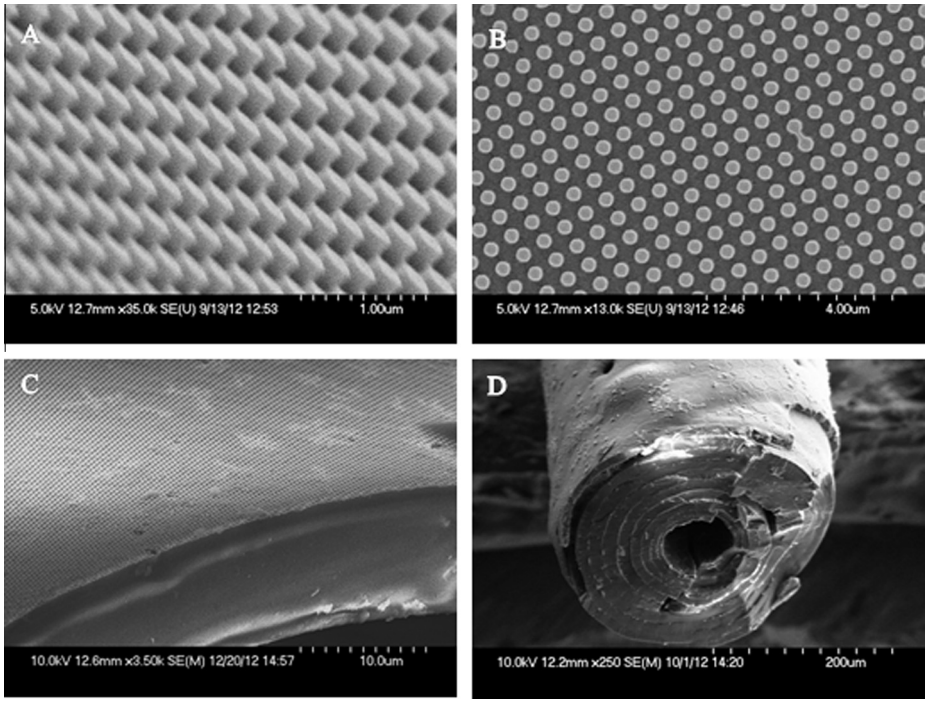

Fig. 3. (A and B) SEM images of 250 nm wide and 270 nm tall pillars remaining on PEGDMA film after pattern transfer from PAA sacrificial layer. Scale bars, 1 μm and 4 μm, respectively. (C) PEGDMA film nanopatterned edge. Scale bar, 10 μm. (D) Cross-section view of rolled PEGDMA scaffold. Scale bar, 200 μm.

It was then sonicated for 2 min and de-aerated in a vacuum for 30 min prior to curing in an oven for 3 h at 70 °C. The cured PDMS slab was cut into individual patterned 15 × 15 mm squares.

2.6. Sacrificial layer

PAA solutions of various molecular weight were spun at 4000 rpm for 30 s onto a 15 mm by 15 mm silicon wafer of 500 μm thickness and allowed to settle at room temperature for 2 min, the spin speed vs thickness for varying molecular weights of PAA are shown in Fig. 4. The spun film of PAA was then heated at 90 °C for 30 min to remove residual solvent [24]. The dehydrated samples were placed into an Obducat NIL 2.5 nanoimprinter, a patterned Qz master was placed on top, and pattern transfer was then carried out as follows. The master and wafer stack was set to preheat to 105 °C under a pressure of 3 bar for 5 min. The temperature

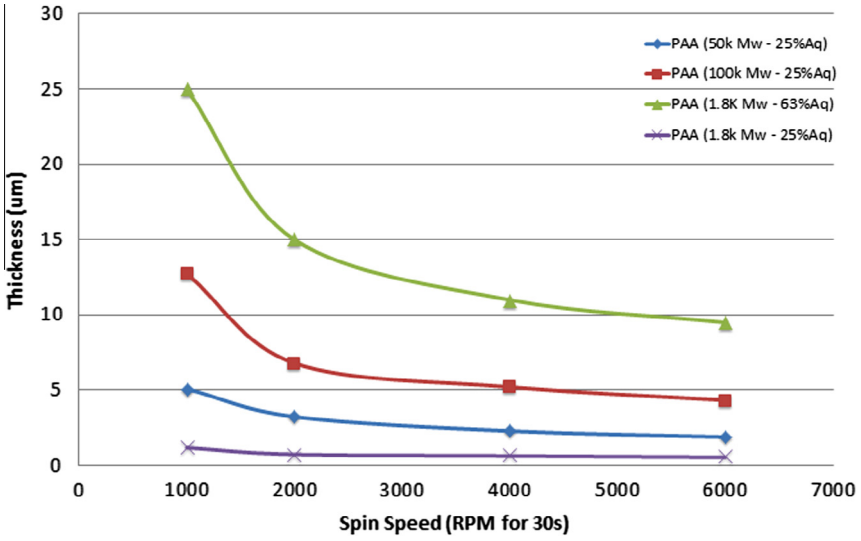

Fig. 4. Layer thickness plotted against spin speed for different molecular weights and concentrations of commercial PAA spun for 30 s.

was then raised to 115 °C and a pressure of 15 bar for 6 min before dropping to 10 bar at which point the sample was air-cooled to 60 °C under a constant pressure. De-moulding of the master was carried out at room temperature.

2.7. Hydrogel solution

To create the photopolymerizable PEGDMA solutions, IR819 photo initiator was dissolved in ethanol (EtOH) or isopropanol (IPA) at a ratio of 1:10 w/v and vortexed for 1 h followed by 5 min sonication. PEGDMA was then combined with the prepared initiator solution at a ratio of 10:1 v/v resulting in an initiator concentration of 1:100 w/v. The whole solution was then agitated with nitrogen for 10 min, at which point the glass container was sealed and left for 18 h before use.

2.8. Photolithography

Silicon wafers with a pre-patterned PAA sacrificial layer were placed in a Suss MA6 mask aligner with a patterned Ni-chrome mask. A drop of PEGDMA solution was applied immediately followed by hard contact with the mask. The sample was exposed to a dose varying from 59.2 mJ/cm^2 to 118.4 mJ/cm^2 to produce sheets of varying flexibility as outlined in Fig. 5. The photo cross-linked sheets were developed in IPA followed by rehydration overnight in RO water, which facilitated the dissolution of the sacrificial layer and subsequent lift-off of the nanopatterned films.

2.9. UV-NIL

The fabrication of nanofeatures on the hydrogel top layer was carried out using a transparent nanopatterned master stamp and a procedure similar to that in photolithography. For this application either a transparent Qz or PDMS stamp was applied to the PEGDMA solution in a Suss MA6 mask aligner and exposed as before. Following exposure the sample was developed in IPA and sliced into individual sheets of 5 × 5 mm prior to rehydration overnight in RO water.

2.10. Triggering

After development in IPA and rehydration in RO water the samples of free floating gel were then triggered to fold and unfold by applying aqueous pH 7 and pH 4 buffer, respectively.

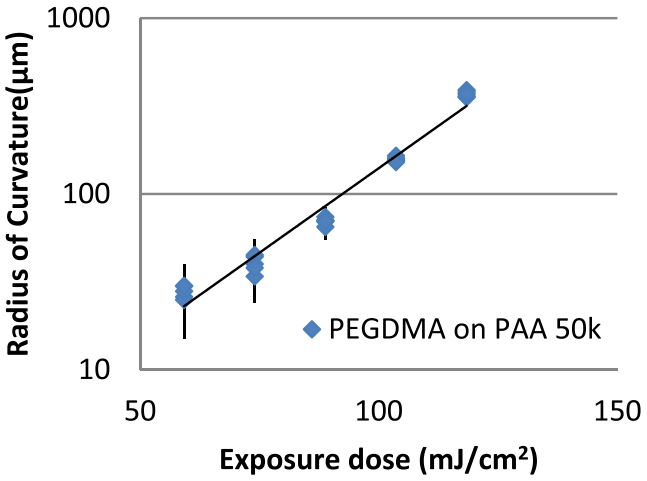

Fig. 5. PEGDMA hydrogel film roll radius of curvature for different UV exposure doses, after scaffold development in IPA, lift-off in RO water, and swelling in pH 7 buffer.

2.11. Analysis

The presence of PAA in the hydrogel films was confirmed by placing hydrated sheets in a 15% toluidine blue EtOH solution where staining of PAA carboxyl groups was confirmed. To compare composition films with and without a PAA sacrificial layer present during manufacture were also dried and examined with FTIR-ATR**.

3. Results and discussion

The gel films resulting from this fabrication method possess sensitivity to environmental pH and will fold when exposed to pH 7 buffer. The folding of these structures as a result of changes in aqueous pH is caused by a differential swelling. The swelling differential is a product of differences in water absorption between the PAA and PEGDMA layers due to protonation or deprotonation of the PAA pendant carboxyl groups when exposed to pH 7 buffer. PAA is an anionic polymer at pH values above the polymers p_{Ka} value of 6.5 and is fully ionized at pH > 9.5 and fully de-ionized at pH 4 [25], thus the side chains of PAA will deprotonate and acquire a

negative charge resulting in rapid swelling and water absorption at which point hydrogen bonding interaction become dominated by polymer interactions with the polar aqueous environment. As PEGDMA is neutral it does not experience a significant change in swelling when exposed to pH 7 buffer. When this bilayer structure is in a sufficiently alkaline environment this causes a swelling through thickness differential in the hydrogel film. This unequal response resulted in a rolled tubular structure as shown in Figs. 2 and 3. The rolled 5 mm square sheet can create spiral tubes of varying thickness with a varied inter-sheet spacing, reducing further when the scaffold was dried. The folded sheet can be unfolded on exposure to pH 4 buffer with some agitation as the layers tend to stick in acidic pH due to the development of polymer to polymer interactions. The sheets were subsequently folded and unfolded over many cycles by switching the aqueous pH from 4 to 7. This behaviour was still observable in sheets which had been stored in RO water for 7 days.

By varying the Mw of the PAA sacrificial layer, the spin thickness and thus possible feature size can be adjusted as shown in Fig. 4. The pH response was observed to be reduced or elevated, with lower Mw PAA providing a quicker response, this could be caused by the increased difficulty for long chains to migrate in the PEGDMA network. Additionally the resistance posed by the PEGDMA to the swelling of the PAA in high pH media was affected by the film level of crosslinking. Highly cross-linked films created by elevated doses of UV exposure lost flexibility, lagged in folding response and had an inhibited radius of curvature, resulting in larger diameter tubes as shown by the correlation in Fig. 5.

The process for lifting off patterned PEGDMA films is shown in Fig. 1. The extent of film folding after exposure to pH 7 buffer is shown in Figs. 2 and 3. In addition to the transition of PAA into the hydrogel network from the underlying sacrificial layer, the features prefabricated into that layer are also evident in the hydrogel film confirmed by AFM** and also by SEM shown in Fig. 3. The nanofeatures present on the PEGDMA surface after pattern transfer from the PAA surface are 270 nm in width and 200 nm deep which is close to that of the original master stamp, although some diffusion should be expected, resulting in slight feature enlargement in the final product. The top surface can be varied as seen in Fig. 1 from large microfeatures obtained by photolithography to nanofeatures obtained by UV-NIL by nanopatterned quartz stamp, the results of which are shown in Figs. 2 and 3 respectively. The presence of PAA in the hydrogel network which was confirmed by staining with a 15% toluidine blue solution in EtOH and further confirmed by analysing dry films with FTIR-ATR**. Both transmittance spectra show the presence of carboxyl groups in the gel network. A characteristic C=O stretching in the acrylic acid carboxy carbonyl COOH group at 1710 cm^{-1}, a slight shift from that of the pure PEGDMA. Additionally C–O stretching was noted at 1292 cm^{-1}, symmetric COO^- vibrational mode at 1380 cm^{-1} and asymmetric COO^- vibrational mode at 1547 cm^{-1} were visible suggesting a not fully ionized state as the gels were rinsed in RO water prior to drying. However, the spectrum of the PEGDMA films obtained by PAA lift-off, is somewhat obscured by pendant carboxyl group interaction with atmospheric water vapour due to its hydrophilic nature, leading to further confirmation as these interactions were not apparent in the PEGDMA hydrogel films where no carboxyl groups were present.

We suggest that the incorporation of PAA onto the PEGDMA gel network arises from diffusion of initiating radicals to the sacrificial PAA layer causing it to tangle with the many methacrylate groups above to form a semi inter penetrating network with the PEGDMA gel even though there was no initiator present in the PAA solution when spun. This method of embossed sacrificial UV-NIL is based on hydrogel technology using simple wet chemical methods, a highly flexible and controllable patterned tissue scaffold has been fabricated that can be switched and controlled by external media. The fabrication technology is inexpensive, scalable, and rapid, reducing fabrication stages and reducing the use of expensive photo initiators to create the bilayer structure.

While some diffusion occurs, which creates the bilayer structure, the PAA remains relatively insoluble in IPA and EtOH in the PEGDMA pre-solution. As a result the predefined pattern in the sacrificial layer remains intact long enough to force the above solution into a replicated shape during crosslinking and does not inhibit subsequent lift-off of the patterned structure.

PEGDMA further offers the benefit of being a non-fouling and stealthing material [26] which does not absorb proteins, allowing inter-sheet chemical cellular interaction, while the grafted PAA layer can immobilize collagen and aid the formation of the ECM [27]. This process provides an effective means of producing self-folding, patterned hydrogel scaffolds. The scaffold incorporates a dual surface micro- and nanopatterned structure with controlled topography for scaffold-cell mechanical interaction and enhanced nutrient permeability.

4. Conclusions

We have demonstrated a new approach to the manufacture of self-folding hydrogel scaffolds by the use of readily available and fast throughput methods. The process shows effective pattern transfer by first embossing a sacrificial layer and using it as a soluble mould in the fabrication process. The use of a sacrificial layer of PAA imparts environmental sensitivity to the hydrogel film on only one surface. The subsequent swelling of the PAA inter-penetrating network (IPN) in elevated pH causes a swelling differential across the film, causing it to roll to accommodate the difference in surface area between the two surfaces. The surface functionalization and patterning stages are thus combined into one photolithographic operation. The net result is a method of producing environmentally triggered self-folding all hydrogel scaffolds by a, to the authors' knowledge, novel use of sacrificial layer embossing. The patterned hydrogel films can be triggered consecutively allowing for successive rolling and unrolling depending on the aqueous pH. The choice of PEGDMA hydrogel provides a versatile platform for creating a variety of hydrogel scaffolds, and while being non-fouling and nontoxic it is permeable to proteins. Furthermore PEGDMA can be modified to produce biodegradable and cell adhesive hydrogels for a variety of biomedical applications.

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**SEI Available: AFM imaging of nanotopographies, additional images of hydrogel rolls, FTIR-ATR analysis of PEGDMA films.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mee.2013.04.003.

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