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Blending PEG-based polymers and their use in surface micro-patterning by the FIMIC method to obtain topographically smooth patterns of elasticity†

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We have designed and fabricated a library of polyethylene glycol (PEG)-based polymer blends, including blends of two PEG-based polymers that are liquid at room temperature where the optimisation of the blending method allows for the incorporation of higher molecular-weight PEG-based polymers which are solid at room temperature. The absence of a solvent in these blends makes them perfect candidates for use in our recently developed Fill-Molding in Capillaries (FIMIC) patterning method. As our FIMIC samples have shown to be not completely smooth (a small topography up to several nanometers has been seen previously), and this is likely to affect the cellular behaviour, we have improved our technique in order to obtain virtually smooth samples that exhibit a pattern of elasticity only. It is demonstrated that, by taking advantage of the differential swelling of the pattern components, we can level out the undesired topographic difference. In particular, by employing blends of materials, (1) the swelling degree of each component can be fine-tuned to even out any topography and (2) the use of the same blends in the sample, yet with varying cross-linker amounts, ensures the swelling degree and elasticity change without changing the surface chemistry significantly. Genuine, binary patterns of elasticity can thus be fabricated, which are a great asset to study cell migration phenomena in systematic detail.

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Introduction

Surface patterning has proved to have successful applications in the fields of engineering, physics and biology with efforts including the recent development of microfluidic devices, flexible electronics and microarrays for use in cell culture. Micro- and nanosized surface patterns of chemistry, topography and elasticity have been shown to control cell adhesion, migration and proliferation on a large variety of substrates.[1–5] In addition, surface patterns have been shown to influence cell viability, protein production and even stem cell differentiation.[2,6–9] In order to alter the surface of any substrate with great precision, techniques from the disciplines of chemistry, physics and engineering must be employed. Techniques like soft lithography, dip-pen lithography and electron-beam lithography have become part of the toolbox to change the properties of the surface of a substrate.

There is a large amount of interest in using these patterning methods to mold soft materials (*e.g.* hydrogels from polyethylene glycol (PEG) and poly(lactic-*co*-glycolic acid) (PLGA)) in order to fabricate a natural tissue mimic for use in tissue engineering. Soft materials have to be able to be (1) molded if they are to be used in many of the soft lithography techniques and (2) solidified, after the molding step, in order to maintain their shape and integrity. Polymer gels that are UV-curable, *e.g.* polymers with acrylate end-groups, are ideal for this purpose. UV-crosslinking of acrylate groups is fast, efficient, and by using a cytocompatible photoinitiator (PI) the presence of heavy metals or toxic residues is avoided.[10–14] These types of chemically cross-linked gels often show better mechanical strength and gelation behaviour compared to their physically cross-linked counterparts.[15,16]

Polyethylene glycol (PEG) is well known to the scientific community where it has been patterned by UV-curing methods and used in cell studies numerous times. However, because PEG is known to be intrinsically cell-repellent, the polymer was usually functionalised with cell-adhesive proteins to encourage cell attachment and spreading. Interestingly, our group has discovered that (physical and mechanical) surface

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patterns alone are enough to induce cells to adhere to the surface of pure PEG, without any protein functionalisation. We have recently discovered for instance that cells do adhere to and spread on micro- and nano-topographically patterned PEG surfaces as well as adhering to patterns of elasticity, with cells adhering preferentially to stiffer lines of PEG over softer lines.[17–19] This phenomenon of cells "feeling" elasticity has been shown to be crucial in directing cell migration; cells have in fact shown to migrate in the direction of increasing stiffness, a phenomenon known as durotaxis.[5,9,20,21] We believe that PEG can be utilised as an excellent substrate for the study of cell behaviour, as the modification of the surface alone influences cell adhesion and migration, be it chemical, topographical or elastic modification, with non-patterned PEG acting as the inert reference material on which the biointeraction is minimal.

Many of the UV-curing techniques for forming patterns rely on the use of photomasks, a process that can result in unreacted acrylate groups being exposed on the surface and may leave ill-defined borders between patterned areas. The FIMIC (Fill-Molding In Capillaries) (Fig. 1) process has been developed by our group as a method capable of producing patterns of bulk gels with patterns of elasticity and/or chemistry with defined borders, creating a wide range of pattern dimensions, while employing fully cross-linked gels. Our method uses capillary action to fill the micro-channels formed by the PEG mold when in contact with a flat surface. As we have recently demonstrated, patterns of elasticity made by the FIMIC method are shown to control and direct cell behaviour.[17]

However, more recently still we observed that this control of the cell behaviour may in fact have been biased by a topographical effect, rather than attributed solely to the designed stiffness contrast we first believed. We found namely that the processing method resulted in a slight topography between the filler and the mold (shown schematically in Fig. 2A). The topographic landscapes were investigated in detail by atomic force microscopy (AFM) in the dry and swollen state which revealed that the topography present on the samples under ambient conditions could be even further amplified when placed in aqueous medium such as cell culture, due to the water uptake by both the mold and the filler material.[22] Nevertheless, this problem could be rectified by using the differential swelling of materials to our advantage; we have demonstrated to be able to compensate for this unwanted topography and "level out" the FIMIC samples to some extent. By using a filler material that swells appreciably more than the mold, we were able to significantly reduce the topography in the swollen state between the filler and the mold.[22]

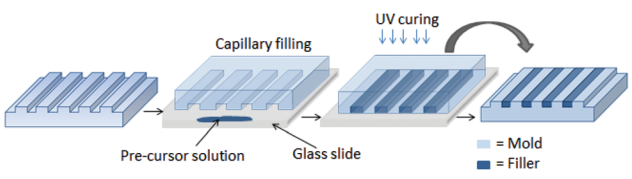


Fig. 1 Schematic diagram of the FIMIC method.

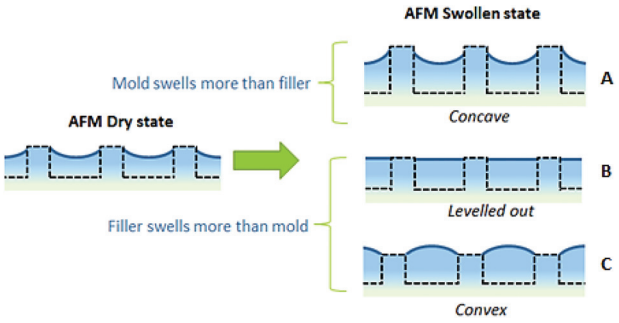


Fig. 2 Schematic diagram outlining the topography that forms in the swollen state on FIMIC samples and the levelling out method we have employed.

These first results demonstrating the efficacy of this strategy were obtained using a block copolymer of PEG and PPG (polypropylene glycol) as a mold and a pure PEG-gel as a filler material that swells more. Apparently, these two polymers differ slightly in their chemical makeup, resulting in local chemical contrasts, which may further affect the cellular behaviour. In the present study, we have aimed at levelling out any chemical differences as well by using blends of polymers.[23]

In this paper, we describe the levelling out of the FIMIC materials by the production of a series of new gel materials that can be used in the process, blending these materials to give a range of highly tuneable gels with different physical but similar chemical properties and successful use of these gels to create FIMIC samples that are topographically smooth, and have no chemistry difference, while exhibiting sharply defined patterns of elasticity. By tuning the swelling properties of these gels we are able to create not only previously unseen "*convex*" FIMIC samples (Fig. 2C) but by then gradually reducing the mold/filler swelling ratio of these samples to reach the desired equilibrium that resulted in the formation of a levelled out pattern of elasticity where the components have similar chemistry (Fig. 2B).

Materials and methods

All chemicals were purchased from Aldrich and used as received unless stated otherwise except for the 8-arm star-shaped PEG (**8PEG**) which was ordered from Jenkem Technology USA. Solvents were of at least analytical grade quality. The silicon masters were purchased from Amo GmbH (Aachen). ^{1}H NMR spectra were recorded on a Bruker Advance DRX-400 spectrometer with trimethylsilane (TMS) as the internal standard and deuterochloroform ($CDCl_3$) as the solvent.

Acrylation

Hydroxy-PEG (shown below for different compounds) and K_2CO_3 were dried in a vacuum oven at 100 °C for 4 h. Hydroxy-PEG (5 g) and K_2CO_3 (20 eq.) were dissolved in dry CH_2Cl_2 (50 mL) under N_2. The solution was then cooled to 0 °C and

acryloyl chloride (15 eq.) was added dropwise. The mixture was stirred at 50 °C for 2 days for the linear PEGs and 60 °C for **8PEG**. The solution was filtered and poured into petroleum ether cooled to −196 °C. The solution was stirred for 10 min and the ether decanted, leaving behind a crude product. This crude product was dissolved in 50 mL of CH_2Cl_2 and then washed 3 times with a saturated NaCl solution. The organic layer was collected, dried with $MgSO_4$, filtered and the solvent was removed under reduced pressure resulting in a colourless liquid. **3BC1**: yield = 64%; ^{1}H NMR ($CDCl_3$): OCH_2CHCH_3O 1.12 ppm, OCH_2CHCH_3O 3.38 ppm, OCH_2CHCH_3O 3.52 ppm, OCH_2CH_2O 3.63 ppm, (C═O)OCH_2 4.30 ppm, ═C–**H** *trans* 5.83 ppm, C**H**═C 6.15 ppm, ═C–**H** *cis* 6.42 ppm. **3BC2**: yield = 75%; ^{1}H NMR ($CDCl_3$): OCH_2CHCH_3O 1.13 ppm, OCH_2CHCH_3O 3.39 ppm, OCH_2CHCH_3O 3.53 ppm, OCH_2CH_2O 3.64 ppm, (C═O)OCH_2 4.31 ppm, ═C–**H** *trans* 5.83 ppm, C**H**═C 6.15 ppm, ═C–**H** *cis* 6.42 ppm. **PEG2**: yield = 70%; ^{1}H NMR ($CDCl_3$) of **PEG**: OCH_2CH_2O 3.64 ppm; (C═O)OCH_2 4.31 ppm; ═C–**H** *trans* 5.83 ppm; C**H**═C 6.15 ppm; ═C–**H** *cis* 6.42 ppm. **8PEG**: yield = 72%; ^{1}H NMR ($CDCl_3$): OCH_2CH_2O 3.64 ppm, (C═O)OCH_2 4.31 ppm, ═C–**H** *trans* 5.83 ppm, C**H**═C 6.15 ppm, ═C–**H** *cis* 6.42 ppm.

Gel preparation and characterization

The gel networks were formed by UV photocrosslinking of an acrylated polymer in the presence of a photoinitiator (PI) (Irgacure 2959) and pentaerythritol triacrylate (PETA) as a crosslinker (CL). In order to fabricate varying stiffness, pre-curing mixtures were prepared by mixing acrylate terminated PEG or 3BC in a small amount of acetone with varying amounts of CL (0–10 wt%) and 1% PI (with respect to the amount of polymer).

Fabrication of micropatterned PEG replicas

Micropatterned silicon wafers were rinsed with acetone, water and isopropanol and dried under a mild stream of nitrogen before use. Prior to the replication the cleaned silicon masters were fluorinated with trichloro(1*H*,1*H*,2*H*,2*H*-perfluorooctyl)-silane 97% (Sigma-Aldrich, Munich, Germany). The selected pre-curing mixture was dispensed on the silicon master, covered with a thin glass coverslip and exposed to UV light (366 nm, 8 cm from the light source) for 5 min. If annealing was required to ensure that the polymer was in liquid form, the polymer was mixed with the PI and then heated in an oven (~60 °C) until the polymer had reached its T_g (~10 min), after which the polymer began to liquefy. The polymer was then carefully but rapidly poured onto a warm silicon master, covered with a warmed glass coverslip and cured straightaway under the UV lamp. Following curing, the transparent polymeric film, with an inverse relief to that on the silicon master, was peeled off mechanically. The stand-alone film (250–300 μm in thickness) could be handled with tweezers.

2D pattern fabrication by the FIMIC process

The polymer replica was placed upside down on a glass slide and a small amount of a second liquid polymer was carefully dispensed at the edge of the open channels. The viscous mixture (either neat or melted depending on the polymer) was allowed to fill the capillaries for 1 min after which the assembly was exposed to UV light for 20 min. After the exposure time was complete, the hybrid construct was easily detached from the glass substrate mechanically and turned upside down to proceed with surface characterization and cell culture. The resulting polymer composite was a robust, free-standing, transparent film.

Swelling tests

Circular discs (~1 cm in diameter and 1 mm thick) of the as prepared, "dry" gels were weighed and immersed in deionized water at 37 °C. After an appropriate time, the gels were taken out from the water, blotted dry with tissue paper and weighed again immediately. The water uptake was determined according to the equation for calculating the swelling degree (SD):

$$\mathrm{SD} = (M_s - M_d)/M_d \times 100 \quad (1)$$

where M_s is the gel mass after swelling and M_d is the dry gel mass. Reported results were averages of measurements on three samples.

Rheological measurements

Rheology measurements were conducted using a Gemini 200 HR (Malvern Instruments) by determining the appropriate frequency and vertical force on the sample (strain-controlled mode). An 8 mm plate was used and measurements were taken at room temperature. Samples were kept under ambient conditions for measuring in the dry state, while samples were immersed in deionized water for at least 12 h for measurements in the swollen state before recording data. During the measurement of swollen samples a solvent trap was utilized to avoid loss of water during the experimental run. First, the linear elastic range of the samples was determined with the help of the amplitude sweep. This is observed when the Storage Modulus (G') (indicating the elastic properties of the network) and the Loss Modulus (G'') (indicating the viscous properties of the fluid) are yielding a constant plateau. The value was transferred to the frequency sweep, where suitable values were ascertained within a range of 0.01–10 Hz. 1 Hz as the applied frequency and 0.0001–0.01 as the deformation value (γ) were chosen as appropriate parameters for all measured samples. The value of the observed plateau was recorded and the bulk elasticity was calculated by the following equation as described by Flory,[24]

$$E = 3G' \quad (2)$$

where E is Young's modulus and G' is the storage modulus. Each distinct material composition was measured 5 times.

Atomic force microscopy (AFM)

An atomic force microscope (JPK Instruments, Nanowizard II) was used in order to measure the topography and surface elasticity of samples in the dry and swollen state.

Topographical imaging

Imaging was done in contact mode (dry samples) and intermittent contact (swollen samples) using silicon nitride cantilevers (PNP TR tips) ($k \approx 0.08$ N m^{-1}, $f_0 \approx 17$ kHz; Nanoworld Innovative Technologies) with a chromium–gold coating. Images were edited with NanoWizard IP Version 3.3a (JPK Instruments). Samples measured in the swollen state were immersed for at least 12 h in deionized water prior to measuring.

Surface elasticity

Surface elasticity was calculated using force–distance curves measured on the same scanning probe microscope (JPK Instruments, Nanowizard II). In order to obtain quantitative values for surface elasticities of hydrogel samples, 64 single force–distance curves in the range of 100 μm × 100 μm were recorded and repeated on at least three different areas on the surface. After every set of measurements, the cantilever was newly calibrated (by applying the thermal noise method) before starting with the next set of force–distance measurements. Out of those 64 different values, a mean value with standard deviation was calculated, and from the three values obtained again, a mean value along with the according standard deviation was obtained. This value was then taken as surface-elasticity. Silicon nitride cantilevers (PNP TR tips) with a chromium–gold coating ($k \approx 0.08$ N m^{-1}, $f_0 \approx 17$ kHz; Nanoworld Innovative Technologies) were used. Images were edited with NanoWizard IP Version 3.3a (JPK Instruments). PNP TR tips (Nanoworld) exhibiting a pyramidal tip-shape (face angle 35°) were used and the tip-geometry has been taken into account by applying the Bilodeau formula[25] in order to fit force–distance curves. This is a modification of Sneddon's model. The fitting was implemented in the Nanowizard IP software and the resulting values for the E-modulus were accordingly obtained.

Optical microscopy

Light microscopy images were taken using an inverted Axiovert 100A Imaging microscope (Carl Zeiss, Goettingen, Germany) using an AxioCam MRm digital camera and analysed using the AxioVisionV4.8.1 software package (Carl Zeiss, Goettingen, Germany).

Cell culture

Mouse connective tissue fibroblasts (L-929) were kindly provided by Dr J. Lehmann (Fraunhofer Institute for Cell Therapy and Immunology IZI, Leipzig). L-929 cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS, PAA Laboratories GmbH) and 1% Penicillin/Streptomycin (PS, 100×, PAA Laboratories GmbH) at 37 °C, a 5% CO_2 atmosphere and 100% humidity. The cells were grown in 75 cm^2 cell culture flasks (Greiner Bio-One) until confluence, washed with Dulbecco's phosphate buffered saline solution (Dulbecco's PBS, PAA Laboratories GmbH) and treated with trypsin–EDTA (PAA Laboratories GmbH). After incubation for 2 min at 37 °C, detached cells were suspended in cell culture medium. The cell suspension was transferred into a falcon tube (VWR International GmbH) and centrifuged for 5 min at 1300 rpm and 4 °C. Finally, the cell pellet was re-suspended in fresh medium and cells were counted using a haemocytometer (Paul Marienfeld GmbH & Co. KG). In this work, L-929 cells were used between passages 9 and 40; cell culture medium was refreshed every second day.

Cytocompatibility

L-929 cells were used to investigate the cytocompatibility of smooth samples of PEG-based polymer gels. Cytotoxicity was determined as colony forming ability using varying amounts of a crosslinker (1% PI; 0% CL and 10% CL).

Colony forming ability (CFA)

L-929 cells were covered with the polymer gels (approximately 16 mm in diameter and 0.05 mm thick) for 24 h. After the incubation period, the polymer gels were removed, cells were washed with Dulbecco's PBS and detached from the bottom of the dish using Trypsin–EDTA. After adding the medium, the cell suspension was centrifuged for 5 min at 1300 rpm and 4 °C. The cell pellet was resuspended with fresh medium and the cells were counted with a haemocytometer. For the determination of the colony forming ability, 300 cells were seeded onto 6 cm petri dishes (Greiner Bio-One). After eight days, the colonies were washed with Dulbecco's PBS, fixed with ethanol (99.5%, Carl Roth GmbH & Co. KG), and stained with Giemsa solution (1 : 20 in deionised water, Carl Roth GmbH & Co. KG). The number of colonies was counted and expressed as the relative percentage of the negative control; values were determined in triplicate and averaged.

Cell morphology

Samples were cut out into circles (1.4 cm in diameter), washed with ethanol (70%), rinsed in Dulbecco's PBS and placed in 24 well plates (Becton Dickinson). 50 000 cells mL^{-1} were seeded on top of the samples and incubated for 24 h at 37 °C, 5% CO_2 and 100% humidity. After 24 h, the cells were washed with Dulbecco's PBS to remove unattached cells as well as the remaining medium components and fixed for 30 min with 4% formaldehyde, pH 7 (Carl Roth, Karlsruhe). Cell adhesion was observed with light microscopy (Axio Observer.Z1, Carl Zeiss) and scanning electron microscopy (SEM, Hitachi S-2700). SEM samples were subsequently dehydrated in a graded acetone or ethanol series after fixation. Finally, the samples were dried with critical point drying (CPD 030, Baltec), sputtered with gold using a sputter coater (SCD 030, Balzers) and observed with a SEM using 20 kV. Light microscopy and SEM pictures were analysed using the Axio Vision software (V4.8.1, Carl Zeiss) and a Digital Image Processing System (2.6.20.1, Point Electronics), respectively.

Results and discussion

One of the main reasons for using **PEG1** (PEG, M_W = 575 g mol^{-1}) and **3BC1**, a tri-block copolymer (3BC) of polyethylene glycol (PEG) and polypropylene glycol (PPG, M_W = 4400 Da), in the fabrication of our previous samples was because they are both liquid at room temperature. Despite finding these polymers useful in levelling out our samples, we wanted to investigate polymers that were (1) non-linear in nature *e.g.* multi-arm PEG and (2) contained more PEG than the high level of PPG in **3BC1** (to ensure that we maintained the excellent cell-repellent behaviour of the PEG). The polymers we now include in our library are outlined in Fig. 3. Alongside the state of the polymer at room temperature and the molecular weight, we have listed in the table in Fig. 3 the calculated theoretical percentage of PEG in the polymer, with pure PEG containing 100% PEG and the block co-polymer containing either 30% PEG in the case of **3BC1** or 80% in the case of **3BC2**. It is useful to be aware of the PEG content in these polymers as it can be described as one of the factors at play when understanding the physicochemical properties of the gels.

The addition of both polymers with longer chains **PEG2** (M_W = 3400 Da) and **3BC2** (M_W = 8400 Da) and multiple arms, **8PEG** (M_W = 15 000 Da), will allow us to fabricate polymer networks with very different characteristics than those made before, with more possible crosslinking points in the case of the multi-arm PEG or longer distances between them (*i.e.* higher molecular weights between crosslinks). In addition to using the favourable **3BC1**, the **3BC2**, another tri-block copolymer, has a lower ratio of PEG : PPG present, allowing us to precisely control the amount of "non-PEG" we add to the system.

Important to remember is that one of the main requirements for the FIMIC patterning method is the need to use liquid precursors, both to make replicas from silica masters *via* soft lithography and to fill the capillaries formed by these stamps and the glass surface. The combination of specific chemistry and low molecular weight of these three new polymers (**PEG2**, **3BC2** and **8PEG**) means that they are, in contrast to **PEG1** and **3BC1**, solids at room temperature and need to be processed into liquid form for blending and use in FIMIC. Here the first successful attempts to meet this challenge are presented.

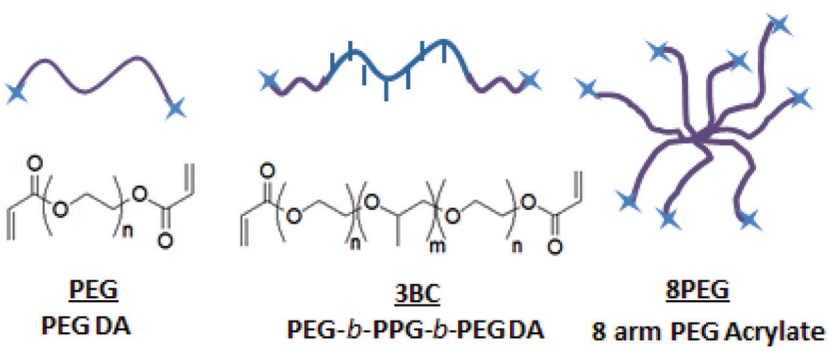
Processing of pure polymers

To process the polymers into moldable gels, we must ensure that they are (1) in a liquid state and (2) homogeneously mixed with the photoinitiator (PI) (1% PI). In the case of the two liquid polymers (**PEG1** and **3BC1**), we disperse the PI evenly throughout the mix using a small amount of acetone, which we remove after mixing. Solid polymers, on the other hand, require additional treatments for them to become processable.

Dissolving the solid polymers (**PEG2**, **3BC2** and **8PEG**) and the PI in water allows for the formation of very soft but strong hydrogels, depending on the amount of water used in the process. However, our FIMIC process is not compatible with water-based gels at present, due to either rapid dehydration of highly hydrated molds during the filling step or indeed hydration of unhydrated molds being filled with water-based polymer solution, both phenomena that are followed by curling off of the molds from the surface, so we have to avoid using water in our system. For this reason we have used the method of heating the solids above their glass transition temperature (T_g) to give us melted polymers to allow us to handle them in a liquid form and therefore enabled the molding of these materials without any solvent.

The window of processing time for this liquid processing is less than five minutes, after which time precursors begin to solidify. Polymers that were heated to the correct temperature (above their T_g) and were rapidly cast into molds produced transparent, homogeneous samples. Analysis using optical microscopy and atomic force microscopy confirmed the homogeneity of the samples (results not shown). Highly crystalline polymers, *e.g.* **PEG2**, were the most difficult to make into a transparent, homogeneous gel, sometimes showing the formation of spherulites[26] upon molding (and concurrent crystallisation). This can be avoided by working quickly with the melt and warming the molds and glassware used in the casting.

The gels we make are easy to handle, flexible and transparent and thus ideal for using in the eventual cell studies.



	Molecular weight	% PEG	State at r.t.
PEG1	575 gmol^{-1}	(100)	**Liquid**
PEG2	3400 Da	(100)	Solid
3BC1	4400 Da	30	**Liquid**
3BC2	8400 Da	80	Solid
8PEG	15,000 Da	(100)	Solid

Fig. 3 The general structure of the three types of polymers used in this work, *i.e.* **PEG**, **3BC** and **8PEG**, and the properties of five selected examples that were investigated.

Table 1 The characteristics of the pure polymers and their ability to form transparent, homogeneous gels (+++ = very good, + = not very good)

Polymer	T_g (or Mp) °C	SD (%)	Chain length (kDa)	Gel formation
PEG1	12–17	28	0.6	+++
PEG2	58–65	179	3.4	+++
8PEG	~50	157	~2	+++
3BC1	5	6	4.4	+
3BC2	~52	204	8.4	++

After fabricating samples from our five pure polymers, we calculated the swelling degree (SD) of each sample after 24 h in deionised water at 37 °C (Table 1).

Of the pure PEG-based gels, **PEG2** swells the most after 24 hours. This can be explained by the longer chain length compared to the other two derivatives (**PEG1** and **8PEG**) and therefore the cross-linking points may be further apart. The molecular weight (*e.g.* chain length) between crosslinks has been shown to be related to the pore size and water uptake of gels like these.[12] The swelling ability of the block co-polymer **3BC1** is significantly poorer than the pure PEG counterparts, due to the chemistry of the block copolymer, with a large section of the polymer consisting of the hydrophobic PPG. In addition, having relatively short chains means that **3BC1** hardly swells at all in water. Interestingly, on the other hand, the block co-polymer **3BC2** has the highest swelling degree of all the gels, showing that despite the presence of the PPG moieties, the longer chain length produces, upon crosslinking, a "loose" network that is capable of taking up more water.

The swelling data are vital for us to understand as we use the swelling degree of the gels to our advantage to manipulate the topography produced in the FIMICs and subsequently level out our samples. From Table 1 it becomes clear that, based on their large swelling ability, **3BC2** and **8PEG** are the most promising candidates to use in our strategy of employing a filler material that swells more than the mold. Nevertheless, these two derivatives are also the most challenging to make processable.

Moreover, the chemistry of these two polymers is quite different; **3BC2** contains 20% PPG whereas **8PEG** contains only PEG. In order to rule out any chemistry differences on our FIMIC samples, we should use one type of material, it being one of the five pure polymers or blends of two (or more) polymers in fixed ratios. Blending building blocks gives us more versatility in tuning the properties of the resulting gels; the physicochemical properties such as swelling degree lie in between those of the gels formed from the pure constituents (see below). By adding different amounts of crosslinker (CL), we can keep the chemistry of two gels the same, while adjusting the stiffness. Samples of homogeneous gels with different levels of cross-linker (0%, 5% and 10%) and therefore tuneable physicochemical properties were fabricated.

Blending

Blending the two liquid pre-polymers (*i.e.* **PEG1** and **3BC1**) was thought to be the least complicated combination to physically

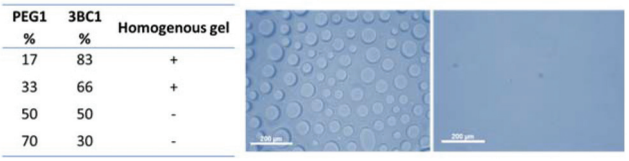


Fig. 4 (a) Successful formation of good gels depends on the ratio of **PEG1 : 3BC1**; (b) phase separation gel formed from a mixture of **PEG1 : 3BC1** (50 : 50); (c) a mixing ratio of **PEG1 : 3BC1** (33 : 66) gives a transparent gel (the limit for the transparency lies at approximately 40% **PEG1**).

mix and so we began with that. Yet, attempting to blend the two polymers in different ratios showed that the homogeneous blending reached a limit (Fig. 4a). Gels with a smaller ratio of **PEG1 : 3BC1** were better able to form a homogeneous gel, with mixes containing a higher percentage of **3BC1** shown to form a phase-separated material. This phase-separated material was recognised by the naked eye by its opaqueness, and optical microscopy confirmed the presence of micrometre-sized droplets (around 50–100 μm in size; Fig. 4b). Nevertheless, when the % **PEG1** was smaller than approximately 40% (**3BC1** was ~60% or more) we saw the formation of a homogeneous, transparent gel (Fig. 4c).

The swelling degrees of the successful, homogeneous blends (*i.e.* **PEG1 : 3BC1** in mixing ratios below 40 : 60) as well as the swelling degree after 24 hours of the pure polymer gels were measured (Fig. 5). The more **PEG1** and/or less crosslinker (CL) present in the gel, the more it swells. This graph shows that by altering these two factors we can fabricate gels within a wide range of swelling degrees. The liquid PEG-based gels we use have a maximum swelling ratio of about 30%.

Although we are able to process the individual solid PEG polymers (**3BC2**, **PEG2** or **8PEG**) to get transparent, homogeneous gels, we found that blending two solid PEG polymers by heating and mixing was unsuccessful. Therefore, to add fluidity to the mixtures of solid polymers, we decided to mix a solid PEG-derivative with a liquid one, namely with **PEG1** or **3BC1**.

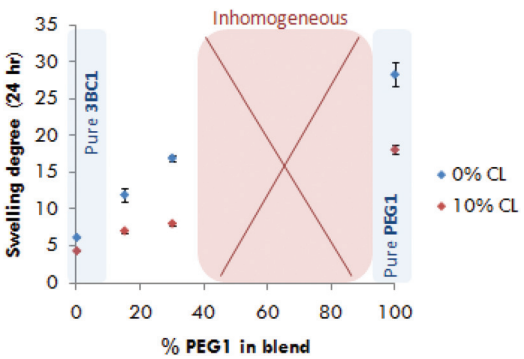


Fig. 5 The swelling degrees of pure **PEG1**, **3BC1** and the homogeneous blend gels (described in terms of their **PEG1** content). Higher amounts of crosslinker (CL) reduce the swelling ability of the gels.

	Liquid	Solid	Good gel formation
1	PEG1	PEG2	-
2	PEG1	3BC2	+
3	PEG1	8PEG	+
4	3BC1	PEG2	-
5	3BC1	8PEG	-

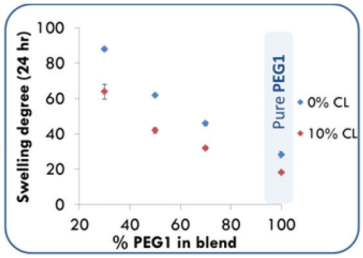


Fig. 6 (a) Table – the outcome of blending liquid–solid pre-polymers to try get a homogeneous blend; (b) chart – the swelling degrees of the **PEG1/8PEG** blends as a function of their composition and amount of crosslinker (CL).

The table in Fig. 6a summarises the combinations of liquid polymers with solid polymers that resulted in the formation of homogeneous, transparent gels. The best (*i.e.* the easiest to form, most homogeneous/transparent and stable within the timeframe of processing) liquid/solid blends that were fabricated involved the **PEG1** liquid polymer. Mixing melts of each **3BC2** and **8PEG** with liquid **PEG1** easily gave gels that were homogeneous and transparent upon curing (*entries 1 and 2*). On the other hand, the blending of **PEG1** with the solid, longer chain **PEG2** was unsuccessful in the formation of a homogeneous gel as the **PEG2** recrystallizes upon mixing with the **PEG1** leading to opaque gels full of crystals (*entry 3*). Finally, the blending of liquid **3BC1** with the two solid, pure PEG-polymers (**PEG2** and **8PEG**) did not result in any homogeneous gel formation as they were immiscible (*entries 4 and 5*).

Thus, the successful blending of the combinations of both the liquid–liquid blend **PEG1/3BC2** (*vide supra*) and the liquid–solid blends of pure PEG, *i.e.* **PEG2/8PEG** or **PEG1/8PEG**, yielded a range of gels with different swelling ratios. In particular, the swelling degree and crosslinking variation of the **PEG1/8PEG** gels show promise in being an excellent candidate for use in the levelling out experiment, since **8PEG** itself swells more than **PEG1**, and the blends therefore combine good processability with a larger swelling range than the pure constituents. The chart in Fig. 6b shows the swelling degree (SD) of blends of three different ratios of **PEG1/8PEG;** obviously, the more **8PEG** in the blend, the more the gel swells.

We confirmed the non-cytotoxic characteristics of the polymer gels prepared from the five gels by investigation using a colony forming ability assay. As expected, no cytotoxic effect was observed.[27,28]

Using blends in FIMIC

The most promising blend materials, namely the liquid–liquid blend of **PEG1/3BC1** in the correct mixing ratio and the liquid–solid blend of **PEG1/8PEG**, were then employed in our patterning technique, FIMIC. To make sure that the polymers are in their liquid state for the FIMIC, the **PEG1/3BC1** blend is used as is and the **PEG1/8PEG** is heated until melted and prepared using pre-warmed glassware. The dimensions of the silicon master from which we make the polymeric mold determine the eventual dimensions of the patterned FIMIC sample. We have selected two pattern dimensions on which to focus, namely "20–10" and "10–50", in which the first number corresponds to the width (in μm) of the silicon groove and the second number to the width of the ridge on the silicon wafer. It is the first of the two numbers which is the eventual width of the polymer mold in the FIMIC and the second of the two that corresponds to the filler dimensions.

Blend 1 – PEG1/3BC1

We began with the liquid blend sample as it was the easiest to process, being liquid at room temperature. We produced two "20–10" FIMIC samples (filler occupies a groove of 10 μm in width) using a blend ratio of **PEG1 : 3BC1** (33 : 66). In the "Filler swells *less*" sample, the filler contained 10% CL and the mold contained 5% CL, for the "Filler swells *more*" sample, the filler contains 5% CL and the mold contains 10% CL. The topography of the surfaces of these samples was measured by AFM in the *dry state* and the *swollen state* (Fig. 7).

For as prepared samples in the dry state, the topography difference between the mold and the filler is typically 0.2–0.4 μm. The use of force maps confirms that there is a definitive pattern of elasticity between the mold and filler elements of the FIMIC samples (ESI Fig. S1†). Using the **PEG1/3BC1** blends, we show conclusively that our strategy to control the height of the filler by altering the swelling ability works alongside the fact that using these blended materials we also now have the advantage of the same chemistry between the filler and the mold. The difference in the topography landscape of the samples between the dry and swollen states has been decreased by using this swelling ability of the filler to our advantage. We next focused our attention on the highly swelling blends of **PEG1/8PEG**, a blend which would open up a much wider range of compositions and swelling abilities.

Blend 2 – PEG1/8PEG

PEG1/8PEG was selected as our most useful liquid–solid polymer blend as it was (1) the easiest to handle and process, (2) had a high swelling ratio for these types of polymers and (3) consists of pure PEG-constituents, which are anti-adhesive to cells. On such PEG-surfaces, any aided cell adhesion must be attributed to the designed elasticity pattern or to eventually remaining, undesired topographic effects.[22] For these FIMICs, we chose again, for both the filler and mold, blends that contained the same ratio of the two components, **PEG1** and **8PEG** (70 : 30, **8PEG : PEG1**), but different amounts of cross-linkers.

We worked on a series of **PEG1/8PEG** FIMICs using a mold with 50–10 pattern dimensions. This master resulted in a FIMIC where the filler spanned 50 μm and the mold constituted 10 μm lines. As a filled channel of 50 μm in width contains much more polymer material than one that was 10 μm wide, the 50 μm wide filler would then also take up water and swell much more than the 10 μm narrow one. It was believed that this would give the filler a much better chance of either

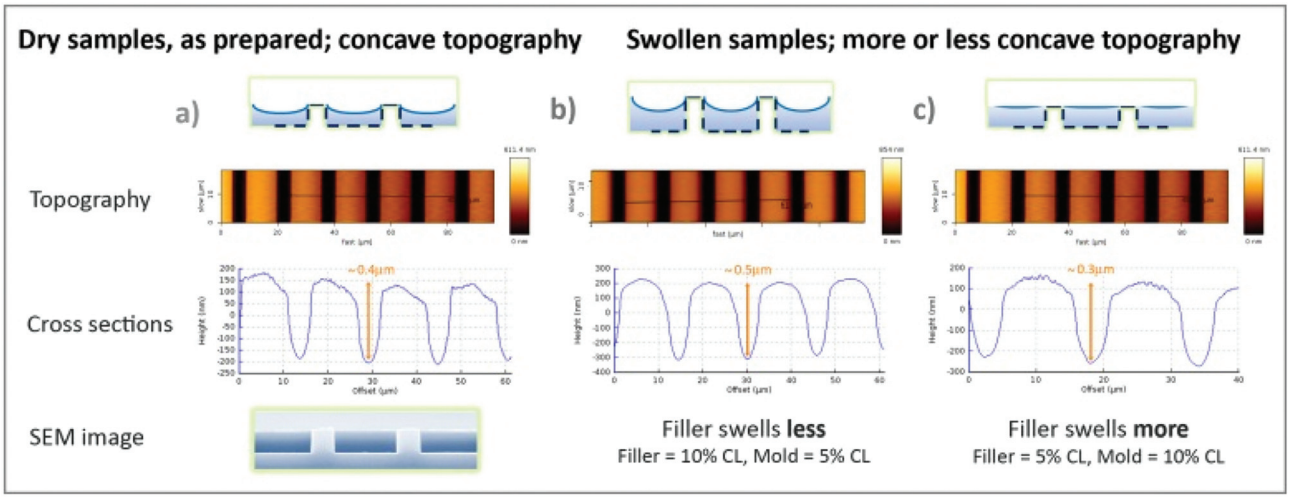


Fig. 7 The atomic force microscopy images of FIMICs made from the **PEG1/3BC1** blend: (a) the topography measurements of as prepared samples in the dry state show a height difference between the mold and the filler of 0.4 µm; a scanning electron micrograph of a real cross-section is shown to reveal the real dimensions; the cartoons show an exaggerated picture; (b) the topography difference between the filler and the mold in a sample where the filler swells less is shown to increase compared to the dry state to a height of 0.5 µm; (c) the topography difference between the filler and the mold in a sample where the filler swells more has decreased compared to the dry state to a value of ~0.3 µm.

reaching the level of the mold or of even "sticking out" from the sample.

We created seven different FIMIC samples using this blend; each sample contained a filler that we thought would be guaranteed to swell a large amount as it contained 0% CL. The molds making up each individual samples then contained a varied amount of CL ranging from 10% right down to 0%. This would give us a range of topographies where we might be able to reach the levelled out stage. The samples were prepared and left to swell in deionised water for 12 hours before the topography was measured with the AFM. Upon measurement in the swollen state, we observed that in fact in all samples, the filler protruded from the mold, *i.e.* we had formed previously unseen *convex* samples (as schematically represented in Fig. 2C). For the samples where there was a high % CL (*e.g.* 10), the filler protruded up to 1 µm from the mold. Consequently, as this % CL was reduced, we were able to greatly reduce the amount that the filler protruded using the differential swelling of the gels we produced.

Fig. 8a plots the AFM topographic cross-section of the filler protrusion of all samples against one another, showing that as you decrease the % CL of the mold (and essentially allow it to swell enough to "meet the filler") we see a great reduction in topography difference between the two. Fig. 8c shows the height of the "sticking out" filler relative to the mold plotted against the % CL in the mold. This work elegantly shows that we can use our blending method to make FIMIC samples that are either *convex*, *concave* or close to *level*.

Although the filler protrudes from the sample where there is 0% CL in the filler and the mold (resulting in a slight topography of 100 nm), relative to the 50 µm of the filler this topography is so minimal that these samples are essentially smooth for cell studies. In cell experiments, we see selective

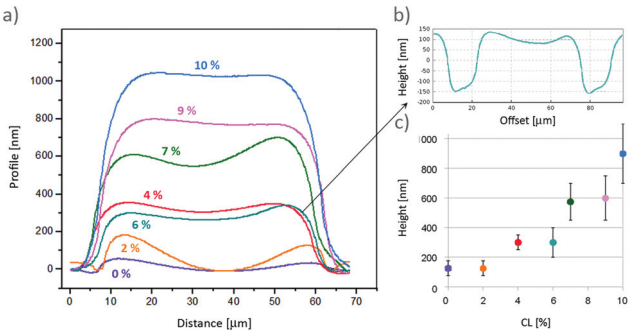


Fig. 8 Graphs showing the relationship between the height difference between the filler and the mold in the FIMIC samples using the **PEG1/8PEG** blend, and the % CL in the mold (while the % CL in the filler remains at 0%). (a) The height of the protrusion of the filler on each sample compared side-by-side; (b) one representative AFM cross-section; (c) graph showing the relationship between the % CL in the mold and the measured height difference.

cell adhesion on FIMIC samples where the chemistry is different but where there is a pattern of elasticity (Fig. S-2A†). When there is no difference in either elasticity or chemistry, we indeed see no cell adhesion on the FIMIC sample (Fig. S-2B†).

Conclusion

We herein provided a detail investigation into the fabrication of a library of novel PEG hydrogels, which were molded by soft lithography techniques. We designed both a method for the heated molding of solid PEG precursors (**8PEG** and **PEG2**) and a method for blending solid and liquid PEG precursors together (**8PEG/PEG1** blend) to produce hydrogels with highly

tuneable physical and chemical properties. We also show the successful blending of two liquid PEGs (**PEG1** and **3BC1**) which enabled us to gain excellent control over the swelling properties of the material. Both the heated PEGs and the PEG blends were successfully used in the soft lithographic approach, FIMIC, to fabricate patterned biomaterial structures. The structures consisted of micro-lines of alternating chemical and/or elastic properties. It was observed by AFM that the FIMIC method could leave a slight topography between the lines on the patterned surface. The differential swelling of the blended materials was used to our advantage to alter this topography and essentially level out the patterned surfaces, leaving us with a pattern of elasticity with no chemical difference between the lines. Cells were found not to bind to a levelled out FIMIC surface when the sample components had the same stiffness and chemistry.

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Notes and references

1 A. Curtis and C. Wilkinson, *Biomaterials*, 1997, **18**, 1573.
2 A. S. G. Curtis and C. D. Wilkinson, *J. Biomater. Sci., Polym. Ed.*, 1998, **9**, 1313.
3 C. M. Lo, H. B. Wang, M. Dembo and Y. L. Wang, *Biophys. J.*, 2000, **79**, 144.
4 N. M. Alves, I. Pashkuleva, R. L. Reis and J. F. Mano, *Small*, 2010, **6**, 2208.
5 D. E. Discher, D. J. Mooney and P. W. Zandstra, *Science*, 2009, **324**, 1673.
6 C. S. Chen, M. Mrksich, S. Huang, G. M. Whitesides and D. E. Ingber, *Science*, 1997, **276**, 1425.
7 C. D. W. Wilkinson, A. S. G. Curtis and J. Crossan, *J. Vac. Sci. Technol., B*, 1998, **16**, 3132.
8 L. S. Chou, J. D. Firth, V. J. Uitto and D. M. Brunette, *J. Cell Sci.*, 1995, **108**, 1573.
9 A. J. Engler, S. Sen, H. L. Sweeney and D. E. Discher, *Cell*, 2006, **126**, 677.
10 P. Kim, D. H. Kim, B. Kim, S. K. Choi, S. H. Lee, A. Khademhosseini, R. Langer and K. Y. Suh, *Nanotechnology*, 2005, **16**, 2420.
11 N. A. Peppas, J. Z. Hilt, A. Khademhosseini and R. Langer, *Adv. Mater.*, 2006, **18**, 1345.
12 K. S. Anseth, C. N. Bowman and L. BrannonPeppas, *Biomaterials*, 1996, **17**, 1647.
13 K. S. Anseth, A. T. Metters, S. J. Bryant, P. J. Martens, J. H. Elisseeff and C. N. Bowman, *J. Controlled Release*, 2002, **78**, 199.
14 P. Kim, D. H. Kim, B. Kim, S. K. Choi, S. H. Lee, A. Khademhosseini, R. Langer and K. Y. Suh, *Nanotechnology*, 2005, **16**, 2420.
15 H.-L. Wei, Z. Yang, L.-M. Zheng and Y.-M. Shen, *Polymer*, 2009, **50**, 2836.
16 W. E. Hennink and C. F. van Nostrum, *Adv. Drug Delivery Rev.*, 2002, **54**, 13.
17 M. Diez, V. A. Schulte, F. Stefanoni, C. F. Natale, F. Mollica, C. M. Cesa, J. Chen, M. Moeller, P. A. Netti, M. Ventre and M. C. Lensen, *Adv. Eng. Mater.*, 2011, **13**, B395.
18 V. A. Schulte, M. Diez, Y. Hu, M. Moeller and M. C. Lensen, *Biomacromolecules*, 2010, **11**, 3375.
19 V. A. Schulte, M. Diez, M. Moeller and M. C. Lensen, *Macromol. Biosci.*, 2011, **11**, 1378.
20 A. Buxboim, I. L. Ivanovska and D. E. Discher, *J. Cell Sci.*, 2010, **123**, 297.
21 A. Buxboim, K. Rajagopal, A. E. X. Brown and D. E. Discher, *J. Phys.: Condens. Matter*, 2010, **22**, 10.
22 S. Kelleher, A. Jongerius, A. Löbus, C. Strehmel, Z. Zhang and M. C. Lensen, *Adv. Eng. Mater.*, 2012, **14**, B56.
23 C. Strehmel, Z. Zhang, N. Strehmel and M. C. Lensen, *Biomater. Sci.*, 2013, **1**, 850.
24 P. J. E. Flory, *Principles of Polymer Chemistry*, Cornell University Press, Ithaca, New York, 1956.
25 G. G. Bilodeau, *J. Biomed. Mater. Res.*, 1992, **59**, 519.
26 G. W. Ehrenstein, *Polymeric materials: structure, properties, applications*, Hanser Publishers, 2001.
27 P. D. Drumheller and J. A. Hubbell, *J. Biomed. Mater. Res.*, 1995, **29**, 207.
28 M. J. Roberts, M. D. Bentley and J. M. Harris, *Adv. Drug Delivery Rev.*, 2002, **54**, 459.

