Enhanced pattern resolution, swelling-behaviour and biocompatibility of bioimprinted casein microdevices

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This work introduces casein microstructures with surface features as a biodegradable biomedical platform technology for enhancing tissue-engineering applications. An optimized fabrication process is presented to reduce the hydrophobicity of intermediate polydimethylsiloxane (PDMS) molds and to transfer high-resolution regular and biomimetic features onto the surface of casein devices. Four different cross-linking reagents, glutaraldehyde, formaldehyde, citric acid and transglutaminase (TG) were investigated to increase the degradation time of casein and their influence on swelling and biocompatibility of the films was studied. TG was found to be the only cross-linker to effectively increase the degradation time and show reduced film swelling after immersion into media, while remaining compatible with cell-culture. The maximum expansion of the films cross-linked via TG was 33% after 24 hours of immersion in cell-culture media. C2C12 cells were successfully cultured on the patterned films for up to 72 hours. The patterned biodegradable casein substrates presented here have promising applications in stem-cell engineering, regenerative medicine, and implantable devices. https://doi.org/10.1063/1.4991783

I. INTRODUCTION

Surface topography is one the parameters which is known to influence cell phenotype. Biological cells cultured on engineered surfaces, with either regular[1–4] or bioimprinted cellular[5–10] micro- and nano-scale features, have shown a distinctive behaviour regarding their proliferation, regulation and response to drugs, as compared to when cultured on flat surfaces. Previously, such patterns have typically been replicated onto non-biodegradable, but cell-compatible materials such as Polystyrene and polydimethylsiloxane (PDMS), which are commonly used for engineering purposes, as well as some metals used in implants.

Recently, plastics based on casein, the main protein in cow's and other mammalian's milk, have started to gain increasing interest in biological applications and as implants.[11] We have previously incorporated the fabrication of regular and biomimetic features onto casein films as biodegradable materials.[12] While not limited to this form, the films in the shape of disks are the simplest of casein microstructures. The previously developed process to replicate the features onto casein films, even though successful, was not capable of maintaining the sub-micrometer details of the original masters. We have thus recently introduced an optimized process to achieve a much higher resolution in the replicated features on casein films.[13]

Casein is water-soluble and devices made of casein, if not cross-linked, will dissolve in water or cell culture media in a short period of time. Hence, casein films need to be cross-linked to increase their stability and degradation time. Cross-linking films will not only influence the degradation time,

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but depending on the type of cross-linker, can influence the amount of water absorption and swelling of the films, once immersed in cell culture media. In this paper, we report cross-linking of casein films using four different cross-linking reagents and we demonstrate the change in the size of films and resolution-improved features on the films for each cross-linker. We finally show, for the first time, the successful use of patterned cross-linked casein films as cell-culture substrates.

II. EXPERIMENTAL

A. Fabrication process

In order to optimize the fabrication process and to increase the resolution of features on the casein films, one step was added to the previously developed fabrication process.[12] In brief, the existing process included designing the patterns using computer program (L-Edit V16, Tanner) and transferring them onto Chrome coated masks using a laser mask writer (μPG101, Heidelberg Microsystems). A silicon wafer was spin-coated with AZ1518 photoresist and it was exposed to UV light through the mask. The photoresist was then baked and developed in AZ326 MIF developer. PDMS pre-polymer (Sylgard 184, Dow Corning) was mixed at a 10:1 w/w ratio, degassed, poured on the silicon wafer and baked. Once solid, PDMS was peeled-off to liquid cast casein onto it.

The process for bioimprinting on the other hand, started by culturing C2C12 cells, a mouse myoblast cell-line, on microscope glass slides. In order to find the same cells at different stages of replication, glass slides were etched with 2.5μm deep grid patterns. This was done by designing the grid patterns using L-Edit, coating the slides with AZ1518 and transferring the patterns onto photoresist using photolithography. The glass slides were then etched by immersion into Hydrofluoric acid for four minutes. The photoresist layer was removed using acetone, and glass slides were sterilized using UV light prior to seeding of the cells. The cells were fixed after 24 hours and premixed liquid PDMS was dispensed on top of the cells and baked. This resulted in a negative PDMS mold, which was peeled off and a second replication could be made off this negative PDMS mold to create a positive PDMS mold. Either of these PDMS molds could be used for liquid casting casein.

In its untreated state PDMS is very hydrophobic, with a measured contact angle of around 110°. While the initial process involved treating the surface of PDMS with oxygen plasma to increase the surface energy prior to liquid casting of the casein solution, this did not enable the casein solution to reliably penetrate into all the fine details on the PDMS master. As a result, a lack of resolution for the features on casein films was observed. In the improved process, PDMS is made hydrophilic more permanently by first, plasma-treatment for 180 seconds in 30 W oxygen plasma (K1050X Plasma Etcher, Quorum Emitech), and secondly, immersion in a 22.2% (w/v) solution of Polyvinylpyrrolidone (PVP) in DI-water for 1 minute. PVP is a hydrophilic chemical, which binds to the surface of PDMS once it has been plasma treated, and makes PDMS hydrophilic for a much longer period of time.[14] The molds were then taken out of the solution, washed thoroughly with DI-water and dried using Nitrogen gun before being placed in the desiccator under vacuum for 30 minutes. The latter removes air from the bulk of the PDMS and helps to fill features with casein solution. After this time, PDMS molds were placed on a flat surface and casein solution was carefully poured on the molds, and left to dry at room temperature overnight. A schematic of the complete optimized process can be seen in Fig. 1, with the left column showing the process for regular features and the right column for biomprinted features.

B. Cross-linking casein films

Given their intended use, fabrication of casein microstructures with high-resolution surface features alone is not satisfactory if the devices cannot be used as cell-culture substrates. This is the case with non-crosslinked casein devices, as they dissolve in media too quickly or, when used with certain crosslinkers, are no longer biocompatible.

As mentioned earlier, casein films were made by liquid casting casein solution on PDMS molds. A 15% (w/v) casein solution was prepared in 0.2% (w/v) NaOH solution in DI-water. Casein powder

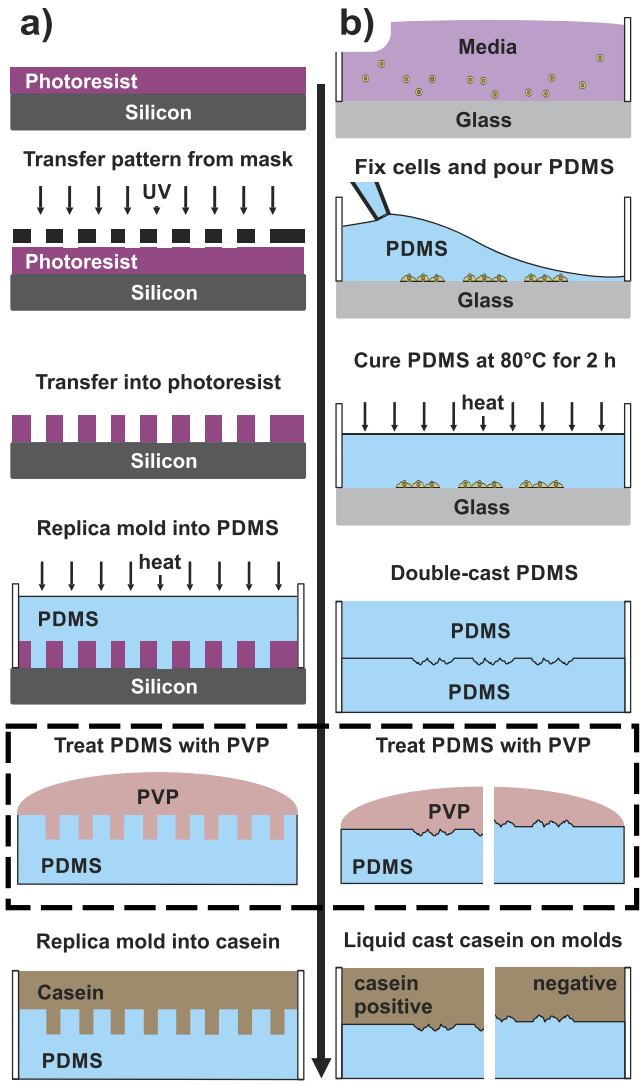

FIG. 1. Schematic of the optimized process to replicate the initial patterns onto casein films using photolithography (a) and bioimprinting (b). For (a), photoresist is spin-coated on a silicon wafer, exposed to UV light through a prefabricated mask, and then developed. Liquid PDMS is dispensed on the photoresist and thermally cured. Surface of PDMS mold is plasma-treated and covered with PVP solution. Patterns are transferred by liquid-casting casein onto the PDMS mold. In case of (b), cells are cultured and then fixed on microscope slides, and PDMS is dispensed onto the cells. PDMS is thermally cured and peeled off. A second replica is made of the negative PDMS mold to form a positive mold. Both molds are plasma- and PVP-treated. Positive and negative casein patterns are made by liquid-casting of the negative and positive PDMS molds, respectively.

(*C7078-500G*, Sigma-Aldrich) was dissolved in the NaOH solution by stirring on a hot plate at 80 °C for 3 hours in a bottle. After this time the bottle was placed in an ultrasonic bath at 60 °C for 30 minutes to dissolve as much of the casein powder as possible. The solution was then filtered through a 200 μm filter to remove undissolved particles from the solution. It was then mixed with 15% glycerol (w/w), to increase the flexibility of the films. As a final step before liquid casting it on PDMS, the solution was placed in the fridge at 4 °C to cool down and remove the air bubbles from the solution.

Unpatterned, non-crosslinked casein films made of this solution, were immersed in DI-water and, as it can be seen in Fig. 2, they repeatedly dissolved in less than 2 hours. This period of time is not long enough for cells to adhere to the films. In order to increase the degradation time of the films and hence be able to use them as cell culture substrates, the films need to be crosslinked.

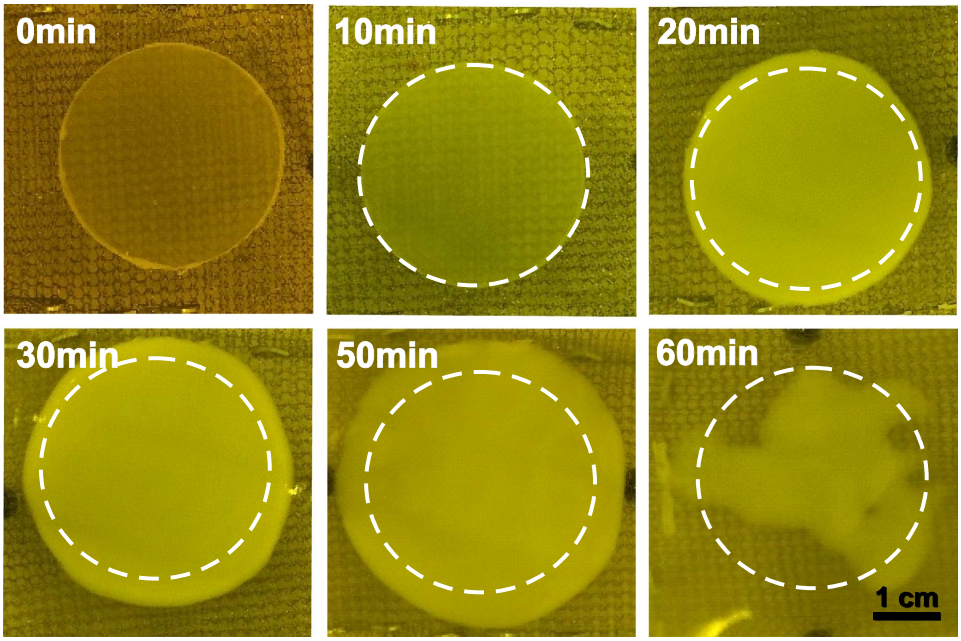

FIG. 2. Swelling and degradation of a non-crosslinked casein film, within 60 minutes after being immersed in DI-water. Dashed circle shows the size of the original film before being immersed in water.

As explained in the following, four different crosslinking reagents were used to crosslink casein films.

1. Glutaraldehyde

Glutaraldehyde is from the aldehyde family and has been used as a crosslinker for casein in medical applications.[11] Glutaraldehyde (*G5882-50ML*, Sigma-Aldrich) with a concentration of 25%, was diluted in DI-water with a ratio of 1:1 to achieve 12.5% of concentration. This was mixed with casein solution to have 0.5 % glutaraldehyde (w/w) in casein solution prior to liquid casting.

2. Formaldehyde

Formaldehyde, similar to glutaraldehyde, is from the aldehyde family and another cross-linker used for cross-linking proteins such as casein.[15] Formaldehyde (252549-*1L*, Sigma-Aldrich) was added to the casein solution to achieve 0.5% formaldehyde (w/w) in casein solution prior to liquid casting.

3. Citric acid

Citric acid is a poly(carboxylic) acid, which is inexpensive and non-toxic, and has been used in textile applications to improve the performance properties of proteins.[16] Food-grade citric acid was purchased locally and dissolved in DI-water with desired concentration. Dried, patterned casein films were immersed in 0.5% citric acid solution (w/v) in DI-water for 10 minutes, without being peeled off PDMS molds. The molds and films were then taken out of citric acid solution and left at room temperature to dry again, before being baked at 150 °C for 1.5 hours on a hotplate.

4. Transglutaminase (TG)

Transglutaminase (TG) is an enzyme that cross-links amino acid residues of glutamine and lysine in proteins and forms inter- and intra-molecular isopeptide bonds in protein molecules or between them.[17] TG was kindly provided by Aginomoto Food Co. Japan. TG powder with 100 U/g activity was dissolved in Phosphate Buffer Saline (PBS) and then mixed with casein solution to a concentration of 10 U per gram of casein prior to liquid casting.

III. RESULTS AND DISCUSSION

A. Resolution enhancement

It was found that treating the surface of PDMS with oxygen plasma and PVP, prior to liquid casting casein on the molds, significantly improved the resolution of the features. Using the grid patterns, individual cells could be tracked and imaged across the different steps of the process, once fixed on glass, on the negative imprint in PDMS and the positive imprint on casein with and without PVP treatment. Fig. 3 shows optical images of the cells and their imprints, as well as corresponding Atomic Force Microscopy (AFM) images of an example cell at each stage. As can be seen from these images, patterns are transferred with extremely high fidelity over large areas and at single cell level. To qualify the transfer process, the cross-section of the same cell at these four stages is compared in Fig. 4, with each scan following the white line depicted in Fig. 3.

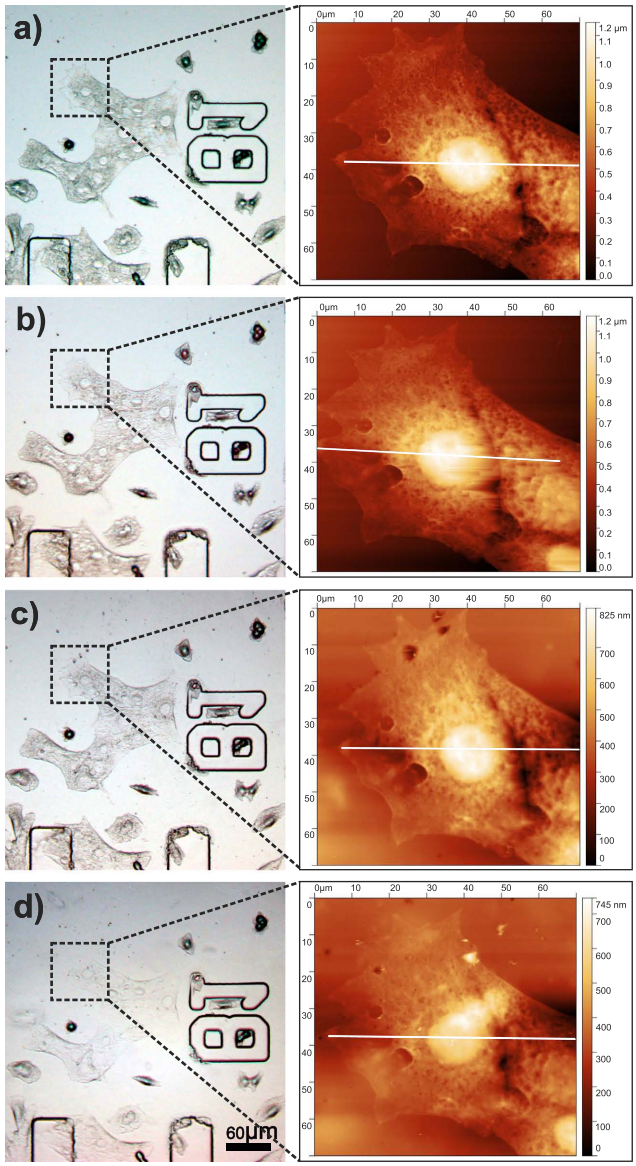

FIG. 3. On the left, optical micrographs of (a) fixed C2C12 cells (mouse myoblast cell line) on glass slide, (b) negative imprint of the cells on PDMS, (c) positive imprint of the cells on casein films, after treating the PDMS mold with PVP, and (d) positive imprint of the same cells on casein films without PVP treating PDMS mold. On the right, AFM images of a single cell at different stages are shown.

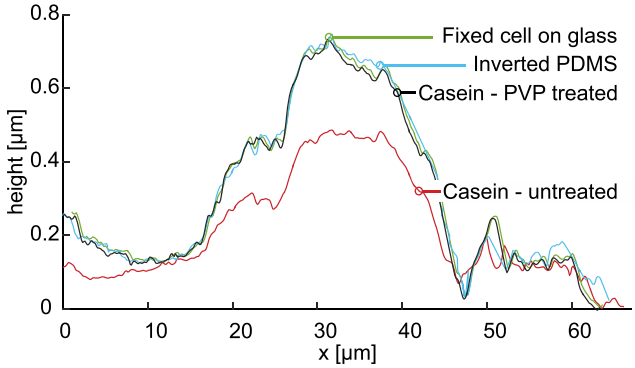

FIG. 4. Profiles of the cross-section of the cell, shown in Fig. 3, at different stages of the replication process. Feature details during the imprint stage can be compared to the original fixed cell.

As can be seen from Figs. 3 and 4, the amount of replication detail of the bioimprints on casein films increases significantly with PVP treatment of PDMS as compared to no treatment and results obtained using our previous process.[12] In addition, it can be observed that, by making PDMS hydrophilic using PVP, the resolution is greatly enhanced such that the features on the final imprint can be directly compared to the original cells on the glass.

B. Cross-linking casein films

Casein films cross-linked with different cross-linking reagents (i. e. glutaraldehyde, formaldehyde, citric acid and transglutaminase) exhibit different behaviors once immersed into media and not all of the cross-linkers are suitable for the proposed application of this work. In the case of the three reagents which were mixed with casein solution prior to liquid casting, patterning the films is a simple process and the resolution of the patterns are high once films are dried and peeled off PDMS molds. On the other hand, the resolution of the patterns is not very high on citric acid cross-linked films, the fourth cross-linker. Since dried patterned films are immersed in citric acid solution for cross-linking, films start to absorb water and swell at the same time as they are being crosslinked. This leads to the films detaching from the PDMS master, thus lowering the resolution of the features on these films.

Even after crosslinking, the films are still biodegradable, which means films absorb water once they are immersed in media. This can also lead to swelling of the films and thus a change in the size of the films and any features on the surface. To investigate this, we characterized how the films crosslinked with each cross-linker behaved when immersed into either Dulbecco's Modified Eagle Medium (DMEM, #11995073, Thermo Fisher) or DI water. Fig. 5 shows the expansion of the diameter of the crosslinked casein devices as a function of crosslinking reagents, immersion liquid and time.

The expansion is given in percentage and is calculated by measuring the diameter of the films, or width of the features, at each time and using the equation below

$$E = \frac{D_t - D_0}{D_0} \times 100$$

where D_t is diameter of the film at any given time and D_0 is diameter of the film before immersion into water or media.

The maximum expansion of film diameter after 24 hours in water or media, ranges from 12% of the original diameter, for formaldehyde or citric acid cross-linked films in water or media, to almost 100% for TG cross-linked film in water. Other studies, investigating water absorption of casein films based on the change in the weight of the films have shown casein films cross-linked via some of aldehyde cross-linkers (i. e. glyceraldehyde and glutaraldehyde) can absorb water up to four or five times their original weight once immersed in water,[18] which consequently influences the size of the films as observed in this work.

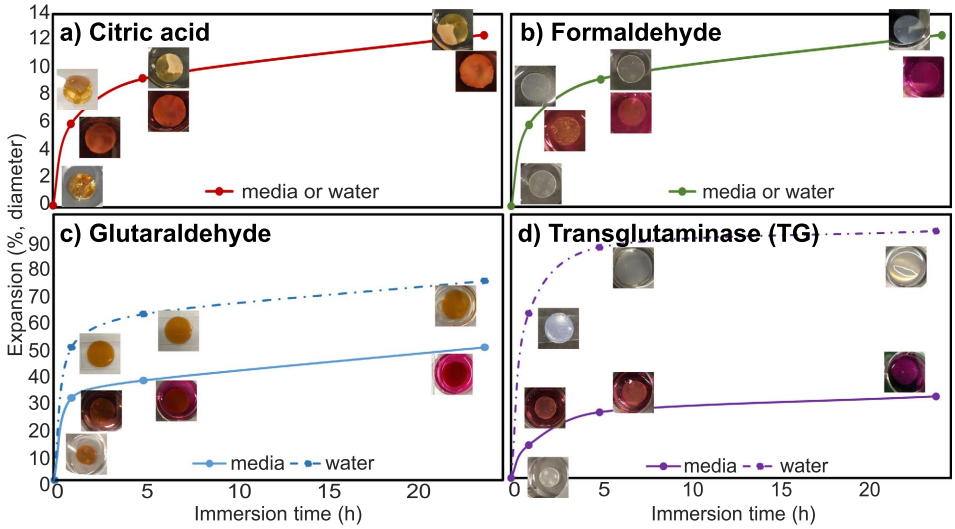

FIG. 5. Expansion of diameter of casein films crosslinked with (a) citric acid, (b) formaldehyde, (c) glutaraldehyde, and (d) TG in water and DMEM over 24 hours. Photos show the films in water and media at each measurement point. A single line means films behaved the same in media and DI water.

C. Swelling of patterned, TG cross-linked casein films

Cross-linking of casein films using the four different cross-linkers increased the degradation time from a few hours to in excess of several weeks. While this makes the casein devices suitable for tissue-engineering applications requiring extended cell-culture, crosslinking can also influence the biocompatibility of the films. Glutaraldehyde and formaldehyde, which are also used as cell fixatives, may leach out of the films into cell-culture media and lead to cell death. When using glutaraldehyde-crosslinked casein conduits as implants in rats, Wang et al. observed indications of increased immune response,[11] but there was no indication whether this was due to possible leaching of the crosslinker. Furthermore, in-vivo implantation of a casein devices into healthy tissue does not entirely mirror the conditions encountered in in-vitro cell-culture, where individual suspended cells need to establish themselves on a potentially hostile surface. Citric acid on the other hand influences the surface chemistry of the films and stops cells from adhering to the film surface. TG, through the process of bond formation, does not appear to affect the biocompatibility of the films and patterned, TG cross-linked casein films can be used as cell-culture substrates. In order to study the change in the size of the features on TG cross-linked films, the size of a test pattern with shape of a cross was measured within 24 hours of immersing the film into water and media. Fig. 6 shows the expansion of the example feature by microscopy and dimensional change, determined using the same equation as for the expansion of the films. The features were observed to expand homogenously, which means the difference between the expansion in x and y direction is negligible.

As it can be seen in Fig. 6(a), the TG cross-linked casein films immersed in media absorb the most amount of water in the first hour, and expand to almost 20% of their original size. Within this time very few cells would attach to the films. In the next 4 hours, the expansion of the features is less than 10% and this can be accounted for during initial design of the pattern prior to lithography. A duration of five hours is enough time for the cells to adhere to the films and spread. While this amount of swelling results in a change in the size of the features, it is yet to be seen how this change would influence the cultured cells. To understand this better we are currently in the process of investigating cells cultured on casein substrates with pattern sizes adjusted during fabrication, as well as on different substrates, one made of biodegradable casein, which will expand and the other, made of polystyrene, which will retain its original size. The measured change in the size of the cross feature on TG cross-linked films immersed in media and water is plotted in Fig. 6(b). Interestingly, films immersed in water expand almost three times more than films immersed in media. Previous work by others on the water holding

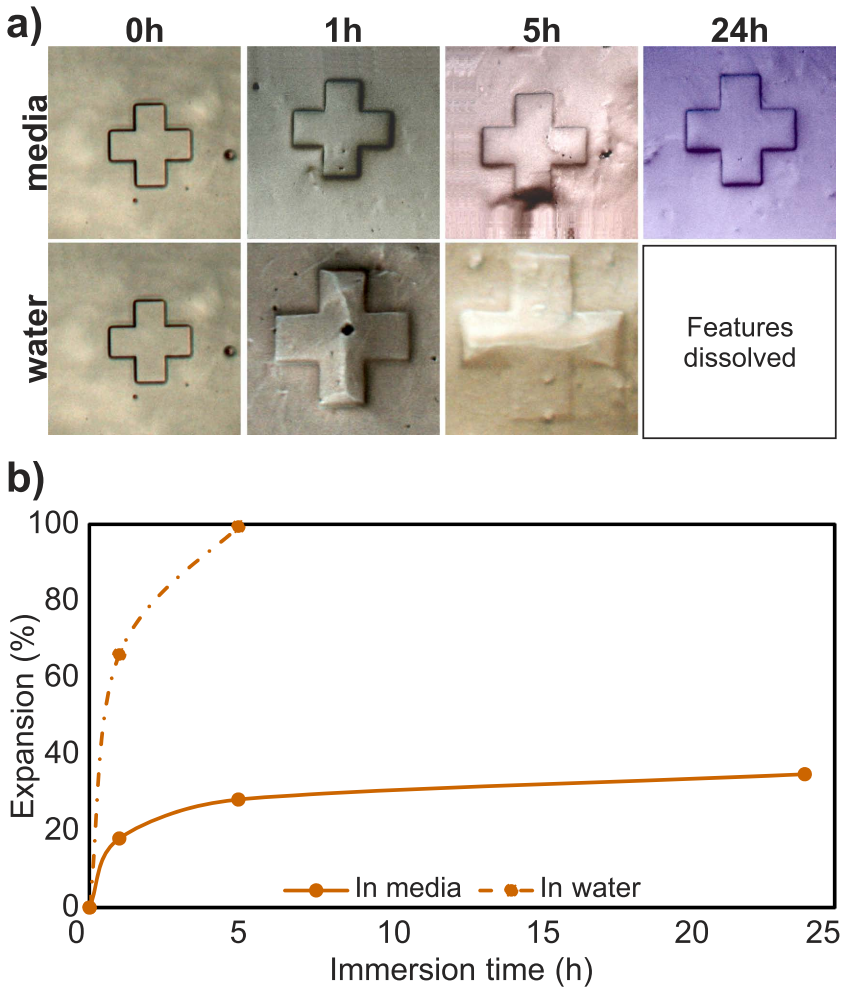

FIG. 6. Effect of water absorption on surface patterns. (a) Optical micrographs showing the change in the size of a cross-shaped test feature replicated on TG cross-linked casein film, immersed in DMEM and DI-water within 24 hours. When immersed in DI water the pattern has disappeared at 24 h, while it is still present in media. (b) Measured change of dimensions of the example feature on TG cross-linked casein film after immersion in water and media within 24 hours.

capacity and swelling of TG-cross-linked sodium caseinate found that the casein gels swell more when immersed into solutions of low salt concentrations, as compared to higher concentrations.[19] Cell-culture media contains significantly higher concentrations of salts than DI-water, which may explain the observed difference in swelling behavior in DMEM. In fact, the same work indicates that the salt concentration of the solution, in which casein samples are immersed into, can be used to adjust the swelling ration and thus expansion of the surface features from below 1 (shrinking) to above 3 (expanding) independent of temperature.[19]

D. Biocompatibility

To demonstrate the suitability of the casein microdevices as cell-culture substrate for tissue-engineering applications, we transferred a large-scale 15 × 15 μm square grid pattern into TG-crosslinked casein using the optimized process. These substrates were then presented to suspended C2C12 cells and the cells left to adhere in DMEM. Cells were checked at 3, 6, 9, 22, 24, 48 and 72 hours after seeding. Figure 7 shows optical images of the successful culture of C2C12 cells on patterned TG cross-linked casein films and non-patterned PS controls at 6, 9, 24 and 72 hours. We observed cells adhering, spreading and proliferating on the substrate. After initial attachment, cells could be seen interacting with and aligning along the surface patterns. At 72 h after seeding, cells were

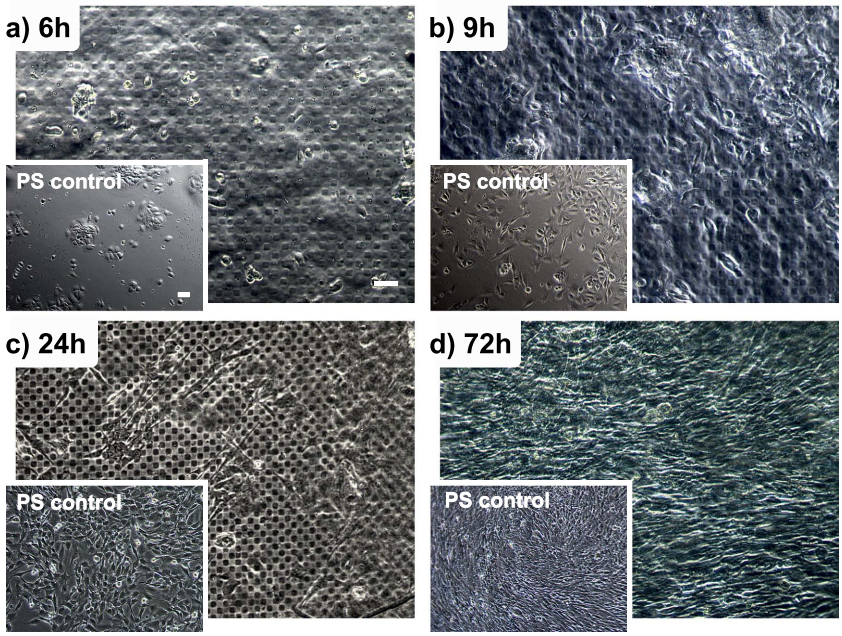

FIG. 7. Optical micrograph of C2C12 cells (mouse myoblast cell line) cultured on TG cross-linked and patterned casein film recorded at a) 6 h, b) 9 h, c) 24 h, and d) 72 h post seeding. Insets show C2C12 cells cultured concurrently on flat PS as control. Morphology of the cells is influenced by the square patterns on the casein substrate, leading to cell alignment and directed spreading. After 72 h cells have reached confluence and are starting to overgrow, at which stage subjacent patterns can no longer be imaged. Scale bars are 50 μm.

confluent and surface patterns could no longer be imaged through the cells. Cells exhibited similar morphologies as previously observed, on the same pattern imprinted onto a polystyrene surface,[4] thus demonstrating that the casein-based surfaces can be used as a direct replacement in tissue-engineering applications. We are currently investigating how the biodegradability of the casein material affects further cell development and muscle cell differentiation. Ultimately, the goal is to tailor the pattern degradability to match C2C12 myoblast differentiation and myotube formation, as well as to study whether the integration of bioimprinted surface features onto casein microdevices may effect cell phenotype and reduce immune response when used as implants.

IV. SUMMARY AND CONCLUSIONS

We have successfully enhanced the resolution of regular and bioimprinted surface features replicated onto casein microdevices. We have also demonstrated the cross-linking of the patterned films using TG and used them as cell-culture substrates. Optical images of features on TG cross-linked films after 24 hours of immersion into media and water, showed an expansion of approximately 35% and 100% respectively. C2C12 cells cultured on the patterned and cross-linked casein showed morphologies common for such micropatterns, demonstrating that the casein-based surfaces are suitable as direct replacement to existing materials used in tissue-engineering applications. We are currently investigating the influence of swelling of the features on cell-culture, as well as how cells would behave on the biodegradable casein bioimprints with different imprinted cell types.

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