The micro patterning of glutaraldehyde (GA)-crosslinked gelatin and its application to cell-culture†

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This paper proposes a novel technique for fabricating micro patterns of glutaraldehyde (GA)-crosslinked gelatin. It provides another means to crosslink gelatin other than using photo-sensitizing agents, and the micro patterns of GA-crosslinked gelatin can still be made successfully by accessing conventional photolithography. A much less toxic and increased biocompatible approach to strengthening the gelatin microstructures can be developed according to this idea. This paper also describes a potential methodology for using GA-crosslinked gelatin patterns as single-cell culture bases. The best spatial resolution of the micro gelatin bases can reach 10 μm, and the selective growing density of human Mesenchymal stem cells on the gelatin patterns surpasses the density on the glass substrate by 2–3 orders of magnitude.

Introduction

Research motivation

The development of tools for the manipulation of single cells in parallel way is a very attractive issue for the rapidly growing areas of genomics and proteomics. Thin film electrode arrays have been demonstrated in order to monitor the electrical signals generated from cells *in vitro*.[1] However, the adhesion of the monitored cells on the desired electrode is randomly distributed, and difficulties are encountered in monitoring many cells in parallel. A micro-robot based on a polypyrrole–gold bilayer actuation has provided a potential tool for handling a single cell by moving arms gently and precisely.[2] The integration complexity of the micro-robot system is high, but the minimum size of cells which the micro-robot can grasp depends on the spatial resolution of the bio-compatible microfabrication technology.

Based on the fact that the higher weight percentage of gelatin is good for cell growth in a hyaluronan–gelatin hydrogel film,[3] in this paper, we have tried to explore the novel potential approach of using a substrate with gelatin micro patterns to attract single cells without micro-robots or moving parts. The gelatin micro patterns fabricated firmly on the substrate have a small size purposely comparable to cells, and are supposed to produce a selective attraction effect during the stages of falling and attachment of cell culture. Another reason to use gelatin as the culture bases of the cells is its surface property. It was empirically reported that the more hydrophilic substratum stimulates cell growth,[4] and therefore gelatin intrinsically fits the hydrophilic requirement.

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Gelatin

Gelatin, a polymer made from natural sources, is a biodegradable, biocompatible material, first used as a protection layer for low-temperature surface micromachining in 2002.[5,6] Natural gelatin has the drawback of dissolving in an aqueous environment and therefore requires a crosslinking procedure using appropriate agents. After being added to a photo-sensitizer, *e.g.*, potassium dichromate ($K_2Cr_2O_7$), the gelatin gel acts like a negatively-toned photoresist, and can be used to fabricate many micro patterns with good mechanical strength and good resistance to chemicals. However, the photo-sensitizers are always highly toxic therefore forbidding the practical application in a biomedical environment, *e.g.*, the cell culture incubator in this work. This pushed many biomaterial researchers to use other, less toxic, types of crosslinking agents, such as formaldehyde, glutaraldehyde (GA),[7] carbodiimide,[8] dextran dialdehyde[9] and genipin.[10] Gelatin strengthened by these functional group agents has more superior characteristics such as biocompatibility, mechanical strength, anti-water transmission and anti-swelling.

Review of other patterning techniques of proteins or collagens

In 2004 a biolithographic technique used gelatin film as the masking for chitosan culture bases.[11] The pattern transfer is accomplished by applying a heated stamp to shape the gelatin and selectively crosslink the underlying chitosan. The spatial resolution of the bio-patterns was limited to the mm range only. Further work[12] on the patterning of gelatin was done by the lift-off technique using immobilized *N*-isoprophylacrylamide. However, the resolution of gelatin patterns is still solely confined to a size larger than 100 μm.

Compared to other guided micro-cell-culturing methods using polydimethylsiloxane (PDMS) substrates with contact-printed lamini,[13] or using robotic protein printing onto amino-silane-modified glass slides,[14] or using a fibronectin self-assembled monolayer (SAM) on a patterned metal surface,[15] the gelatin micro patterns in this work attach directly

onto the glass slides without linker agent immobilized on the substrate in advance. Therefore it has the advantage of a more concise concept and simpler process while achieving a comparable protein pattern-size smaller than 100 μm for the cell culture.

One reason for choosing gelatin as the working protein for cell culture is down to economic factors. The processing cost of the GA-crosslinked gelatin micro patterns depicted in this paper is much lower than other biological methods. Hence, the application potential of extending this technique to large-area, wafer-level processing is highly promising. Second, the chip size of gelatin micro patterns after substrate dicing can be adjusted as small as possible to reduce the required amount of cells needed for seeding. Finally, the gelatin micro patterns, after proper crosslinking by GA solution, have the advantage of a longer shelf life than other patterned proteins before coming in contact with the cell culture.

Experimental methods

Fabrication of glutaraldehyde (GA)-crosslinked gelatin micro patterns

In this paper we have combined the concepts of GA-crosslinking and conventional photolithography to fabricate the gelatin microstructures with a bio-patterning resolution as fine as 10 μm, and applied this technique to the cell culture. Such a small size of gelatin pattern can not only be assigned as the bio-compatible platform for cell culture, but also has a size comparable to cells so as to possibly attract a very confined amount of cells or even single cells on the gelatin micro patterns both individually and in parallel.

The fabrication process for GA-crosslinked gelatin micro patterns is depicted in Fig. 1. First, we spin-coated a gelatin film on a glass substrate. (Preparation of pure gelatin: Dissolve gelatin powder in water at 40–50 °C. Filter to remove bubbles. Apply to glass plates by spin-coating at 40 °C. Dry at room temperature for 3–4 h.)[16] Second, a masking layer of positively-toned photoresist (*e.g.* AZ-4620) is spun on the gelatin surface, and the correlated ultra-violet (UV) exposure (365 nm, 5 mW, for a minimum of 45 s) for the portion of photoresist which defines the crosslinked gelatin is undertaken. The processing temperature of the photolithography must be well controlled near ambient temperature to prevent melting of

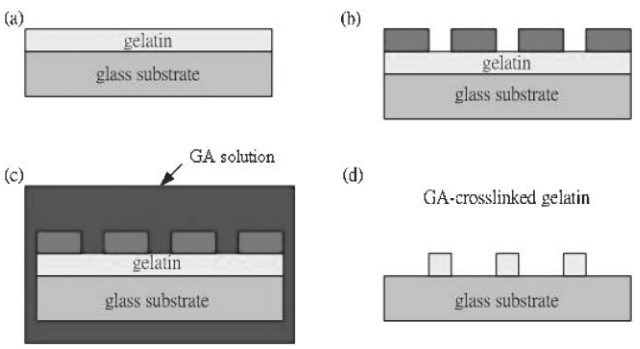

Fig. 1 Fabrication process of GA-crosslinked gelatin: (a) spin-coating gelatin film; (b) photolithography; (c) dipping in GA; (d) removing photoresist and uncrosslinked gelatin.

the uncrosslinked gelatin underneath the photoresist. Third, we dipped the sample in GA solution, *e.g.*, 45 wt%, to undertake the crosslinking with proper control of reaction time. Finally the GA-crosslinked micro patterns show up after both stripping the masking layer of photoresist by acetone and removing the uncrosslinked gelatin in hot water, sequentially. The thickness of the GA-crosslinked gelatin, depending ultimately on the spin-coating characteristic of the gelatin gel, can be made up to 10 μm.

Bad morphology of gelatin patterns with improper protection

Some corrugated surface profiles of the crosslinked gelatin show poor surface morphology after photolithography of the masking resist and the GA crosslinking process. This result can be explained due to weak resistance of pure gelatin which is vulnerable to the high pH-value alkaline developer (AZ400K) used at the end of the resist developing. In other words, no sooner had the resist-developing process ended, than the pure gelatin exposed to the alkaline developer and was immediately etched away.[6]

The best way to protect against the attack of alkaline developer is therefore to under-develop the masking resist. We decreased the developing time and recess thin layer of the UV-exposed photoresist on purpose. The residual layer of the UV-exposed photoresist, several tens of nanometers thick, can prevent the pure gelatin underneath from attack of the developer, and can be removed rather easily by O_2 plasma (the so-called "descum" process) before the GA crosslinking process.

The abnormal gelatin profile with longer time of GA-crosslinking

After both stripping the masking layer of photoresist with acetone and removing the uncrosslinked gelatin in hot water (50 °C), the GA-crosslinked micro patterns should appear. However, some residual of the gelatin near the edge of micro patterns can still be left on the substrate. Once again, the O_2 plasma "descum" process (100 W for just 1 min—too much time induces cracks on the gelatin) is mandatory and required after the hot water developing. High-contrast micro patterns without a fringe hue show up accordingly.

Due to the intrinsic diffusion mechanism of GA molecules inside the gelatin chain-like protein, or due to the finite adhesion strength between the gelatin surface and the masking photoresist, the over-crosslinking at the edge of gelatin occurs regularly. The abnormal and imperfect gelatin patterns extrude from the edges as can be observed by the "cross" and the "number" patterns in Fig. 2(a) and 2(b). We thereafter measured the surface profile across a gelatin strip, which has the over-crosslinking edges. As shown in Fig. 3, the original line pattern is confined by the trail with the twin-peaks, B and C.

Fig. 4 describes the quantitative data of the lateral over-crosslinked distance *vs.* crosslinking time of gelatin subject to different weight percentage concentrations of GA solution. The square-root behavior of the gelatin edge front with respect to the crosslinking time in Fig. 4 demonstrates that the respective plateau areas in A–B and C–D in Fig. 3, extruding

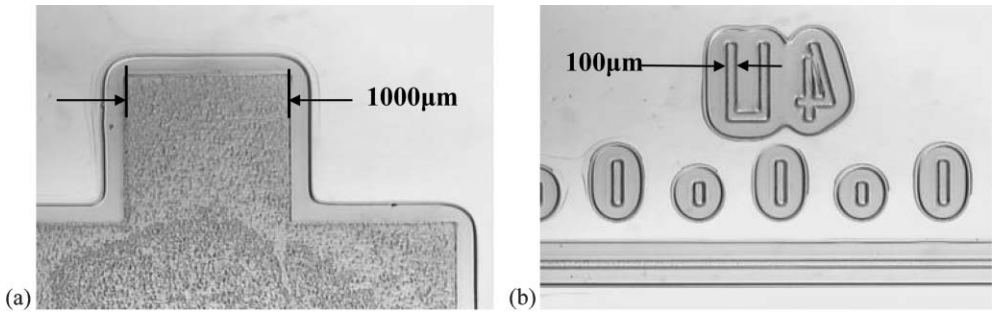

Fig. 2 GA-crosslinked gelatin patterns with longer time control of crosslinking: (a) partial view of a "cross" pattern with the arm width of 1000 μm; (b) the straight line and "4U" patterns with a line-width of 100 μm.

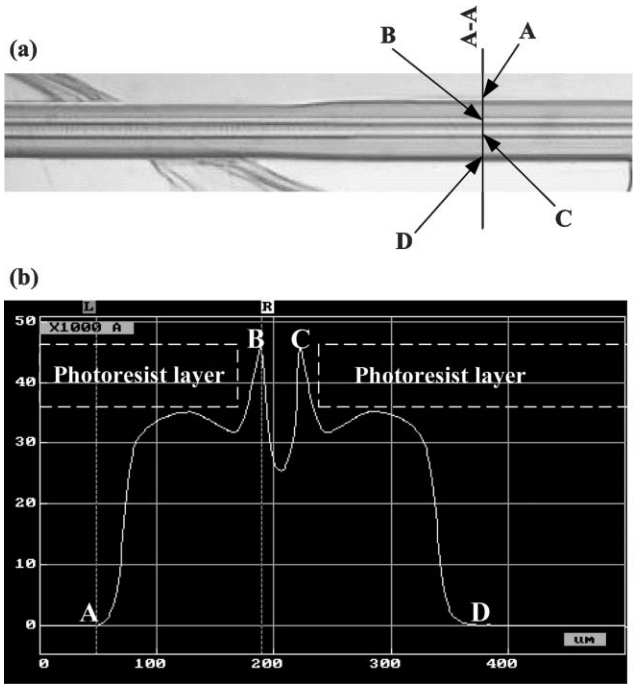

Fig. 3 Surface profile of a GA-crosslinked gelatin pattern with longer time control of crosslinking: (a) the location definition of the measuring points; (b) the profile of the A–A cross section measured by Alpha-Step-500.

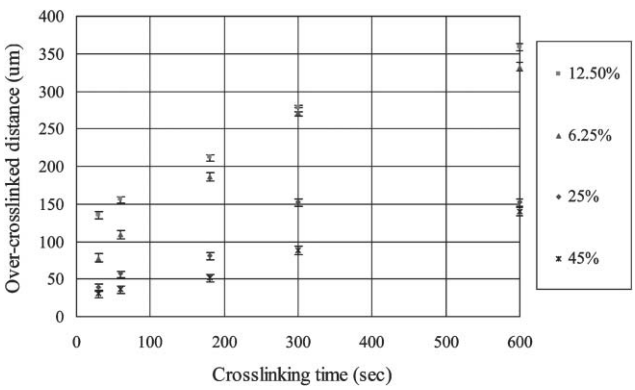

Fig. 4 Lateral over-crosslinked distance *versus* crosslinking time of gelatin subject to different weight percentage concentrations of GA solution.

laterally from both sides of the original line-pattern, are mostly controlled by the diffusion mechanism of GA agent in pure gelatin.

From Fig. 4, we observe that the crosslinking time of GA solution for gelatin should be less than 1 min for avoiding a large dimension error at the edge of the micro patterns. Moreover, the high weight percentage concentration of GA solution, *e.g.*, 45%, is better recommended for using because the smaller amount of dissolved water in GA solution induces a less serious problem of undesirable gelatin swelling. Additionally, the bonding strength of GA-crosslinked gelatin films with hydrophilic substrate (*e.g.*, glass substrate) increases with increasing aldehyde content in the film.[17] Finally, the high concentration of GA solution is also beneficial to the fast crosslinking of gelatin for preventing an overgrowth issue at the gelatin edge as mentioned previously.

The modified processing of GA crosslinking for gelatin

Combining the processing remedies dealing with undesirable problems described in previous sections, we can summarize a modified process of GA crosslinking for gelatin micro patterns:

(1) Spin-coating pure gelatin film at 40 °C.

(2) Photolithography of a positively-toned resist with under-development at room temperature.

(3) O_2 plasma descum (100 W, 1–2 min) for the masking photoresist.

(4) Crosslinking in 45% GA solution with proper time control, *e.g.*, 8 s.

(5) Stripping photoresist by acetone and removing pure (uncrosslinked) gelatin by hot water (50 °C).

(6) O_2 plasma descum (100 W, 1 min) for the residual pure gelatin.

By the modified process mentioned above, micro patterns with better morphology and contrast are fabricated and shown in Fig. 5. The thickness of the gelatin patterns is 1.5 μm. The reliable minimum line-width is as fine as 5 μm, and the minimum gap between two patterns is thus far 10 μm.

Results and discussion

The application of GA-crosslinked gelatin to cell-culture

Before using gelatin micro patterns as the micro culture bases, we must consider how "toxic" is the organic stain, which

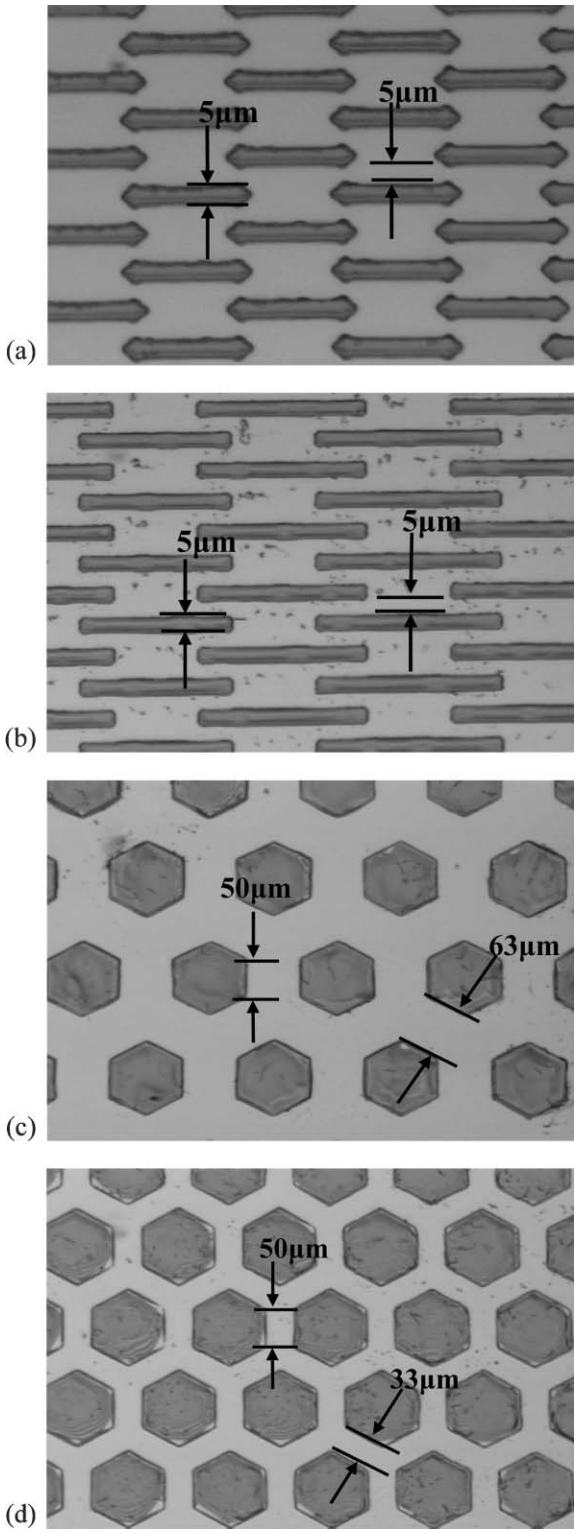

Fig. 5 1.5 μm thick gelatin patterns prepared by the modified GA-crosslinking process: (a) microstrip lines of 30 μm long, 5 μm wide and 5 μm gap; (b) microstrip lines of 60 μm long, 5 μm wide and 5 μm gap; (c) micro honeycomb patterns of 50 μm wide, 63 μm gap; (d) micro honeycomb patterns of 50 μm wide, 33 μm gap.

comes from the photoresist in the modified MEMS process of GA-crosslinked gelatin. Therefore, this work used two sample chips covered with GA-crosslinked gelatin film (without micro patterns) to verify the toxicity of photolithography (the same procedure (2)–(6) in the previous section): one was run with photolithography and the other was not. These two sample chips were incubated with Mesenchymal stem cells for 72 h, as shown in Fig. 6. As shown by the experiment results in Table 1, the density of cells decreases from 1.8×10^4 to 1.5×10^4 cells cm^{-2} with the pre-treatment of photolithography. In other words, only 16.7% attenuation of cell growth was observed due to photolithography.

The sequence for cell-culture was performed as follows. First, the glass slides with the square size of 25 mm were fabricated with GA-crosslinked gelatin micro patterns. Second, the sterilization of glass slides was subject to 1 h UV exposure (365 nm, 15 μW) and then treated with phosphate buffered saline (PBS). Third, glass slides were seeded with human Mesenchymal stem cells and culture in α-minimum essential medium (α-MEM) with 10% fetal bovine serum (FBS) for 3 days at 37 °C in 5% CO_2 humidified atmosphere. Finally, after 3 day culture, the cells were counted quantitatively by phase-contrast microscopy. The stem cells on the same GA-crosslinked gelatin pattern for different culturing times are shown in Fig. 7.

Selectivity of culturing cells on the gelatin micro patterns

From Fig. 7 and Table 1, the stem cells attach to the gelatin micro patterns successfully and selectively after 3 days of incubation. The cell density is estimated as 6.48×10^4 cells cm^{-2},

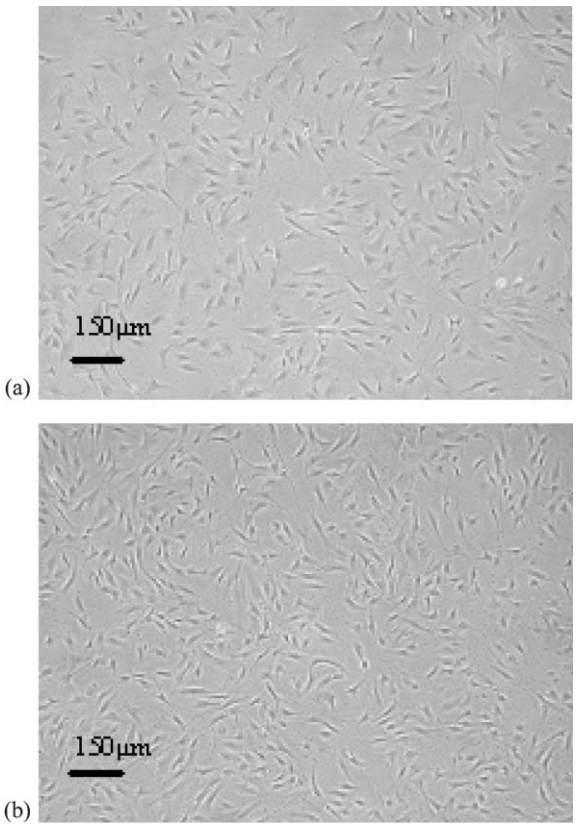

Fig. 6 Human culture Mesenchymal stem cells on un-patterned GA-crosslinked gelatin on glass substrates after 72 h: (a) with photolithography treatment; (b) without photolithography treatment.

Table 1 Densities of human Mesenchymal stem cells on 3 samples after 72 h culturing

Sample	Condition	Density of Mesenchymal stem cells
1	On the un-patterned GA-crosslinked gelatin without photolithography treatment	1.8×10^4 cells cm^{-2}
2	On the un-patterned GA-crosslinked gelatin with photolithography treatment	1.5×10^4 cells cm^{-2}
3	On the GA-crosslinked gelatin micro patterns above the glass substrate	6.48×10^4 cells cm^{-2}
	On the bare glass substrate instead of the GA-crosslinked gelatin micro patterns	400 cells cm^{-2}

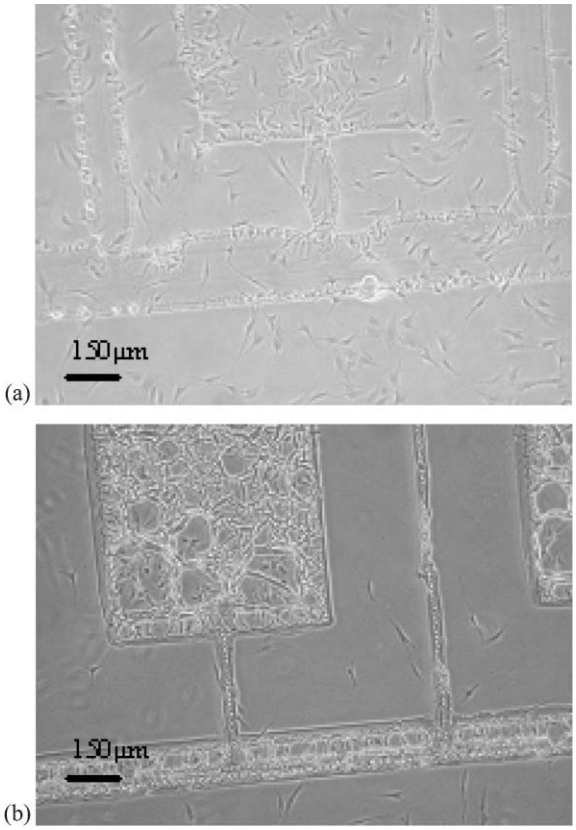

Fig. 7 Situation of human cultured Mesenchymal stem cells on GA-crosslinked gelatin micro patterns on glass substrates: (a) after 48 h; (b) after 72 h.

and surpasses the density on the bare surface of the glass substrate 150 times. Such an interesting phenomenon preliminarily verified the feasibility of culturing cells on the GA-crosslinked gelatin micro patterns selectively as proposed in this paper. More experimental data of other cells or even of the single-cell culture will be collected in the future.

Toxicity of residual GA to cell viability

Although not so detrimental as potassium dichromate, the residual GA on the test samples is still toxic to the seeded cells, and the toxicity of residual GA to cell viability should be additionally addressed. In this study we reduced the residual amount of GA as much as possible by good crosslinking of gelatin. Good crosslinking of gelatin usually enhances the adhesion of gelatin micro patterns to glass slides. Therefore, the gelatin micro patterns with apparent residual GA have poor crosslinking characteristic and are quite easily washed off from the glass slides by rinsing with water for 5–10 min. We washed our samples carefully to remove the "bad" or "poisonous" gelatin patterns, and the "good" or "non-toxic" gelatin patterns that remain are then generated for the culture results of Fig. 7.

The modified effect of UV sterilization on the properties of gelatin

The gelatin micro pattern which is subject to cell culture in this paper involves a step of 1 h exposure to UV for sterilization. The exposure power of the hour-long UV sterilization before the cell culture is actually measured as 15 μW, and the maximum estimated cumulative dosage is 54 mJ. Such an exposure dosage is apparently smaller than the UV dosage of 250 mJ provided by photolithographic mask-aligners. From some empirical investigations, however, the minimum duration of UV exposure required for effective crosslinking of gelatin without photo-sensitizer is no less than 10 h.[18] It's therefore hard to estimate exactly the gelatin crosslinking attributed from the UV sterilization. Anyhow, greater crosslinking time seems to have no negative influence on the properties of crosslinked gelatin. Consequently, the crosslinking of gelatin that results from the UV sterilization is only of secondary concern here.

Summary of experimental results

In summary, the patterning technique and the fabricated sample chips of GA-crosslinked gelatin are initially studied in this paper. We addressed and explained the over-crosslinking and the edge-diffusion phenomena of GA solution in gelatin accordingly. The modified processing procedure for GA-crosslinking gelatin is also proposed and realized. The best spatial resolution of the micro gelatin bases can be fabricated and adjusted to the size of 10 μm. With the micro patterning of gelatin film, a toxicity test was performed on the cell culture of human Mesenchymal stem cells. The cell growth density was not observed to decrease obviously after the treatment of conventional lithography. We also found that the selective growing density of stem cells on the gelatin patterns surpasses the density on the glass substrate by 2–3 orders of magnitude after 72 h incubation. We believe that this biocompatibility, mechanical strength, chemical resistance, anti-water transmission and anti-swelling of the cross-linked gelatin will in future provide useful microstructures for cell-culturing, beneficial to biomedical quantitative studies, *e.g.* single cell incubation and manipulation.

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