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The Micropatterns of Glutaraldehyde-crosslinked Gelatin as ECM for Attachment of Tumor Cells

Yu-Cheng Ou[1], Chih-Wen Hsu[1], Lung-Jieh Yang[1,*],
Hsieh-Cheng Han[2], Yi-Wen Liu[2], Chien-Yuan Chen[2]

[1]*Department of Mechanical & Electro-Mechanical Engineering, Tamkang University, Tamsui, Taiwan*
[2]*Institute of Microbiology and Biochemistry, National Taiwan University, Taiwan*

Abstract — **Use This work proposes a novel technique to induce the attachment of tumor cells to the micropatterns of glutaraldehyde (GA)-crosslinked gelatin. It provides another method to crosslink gelatin other than using the photo-sensitizing agents or selective GA-crosslinked technique. The micropatterns of GA-crosslinked gelatin are fabricated by O_2 plasma etching process. This novel technique can ensure the degree of crosslink, prevent the over-crosslink from pattern deformation and enhance the adhesion between the gelatin and glass slide. The best spatial resolution of the micro gelatin bases can be reached to 2 μm. The micropatterns of GA-crosslinked gelatin can still be made successfully by the conventional photolithography. The much less toxic and more biocompatible approaches of strengthening the gelatin microstructures can be developed according to the idea herein.**

Keywords —***Gelatin, Glutaraldehyde, Tumor cells, Attachment***

I. INTRODUCTION

A. Research motivation

In recent years, gelatin is a popular material and broadly introduced into cell and tissue engineering due to its biocompatibility and easy gelation by changing the temperature of its solution [1]. The gelatin is a derivative of collagen, one of the most common extracellular matrix (ECM) proteins. The cell growth and its viability that drive morphogenesis in complex tissues are controlled through modulation of cell binding to the extracellular matrix [2]. Based on the facts that hyaluronan is a ubiquitous component of the extracellular matrix of all connective tissues, the higher weight percentage of gelatin is good for cell growth in a hyaluronan-gelatin hydrogel film [3]. The gelatin immobilized on poly (acrylic acid) can also significantly enhance the cell adhesion [4]. Meanwhile, the different shapes of protein can affect the extension and the migration of tumor cells [5] and gelatin is a suitable biomaterial for drug release [6]. In this work, we have tried to explore the novel potential approach of using a substrate with gelatin micro patterns to attract tumor cells for the application of drug delivery in the future. The gelatin micro patterns fabricated firmly on the substrate have a small size which is purposely comparable to cells, and they 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 [7] and therefore gelatin intrinsically fits the hydrophilic requirement.

The gelatin is first used as a protection layer for low-temperature surface micromachining in 2002 [8-9]. Natural gelatin has the drawback of dissolving in an aqueous environment and therefore requires a crosslinking procedure using appropriate agents. After being added with 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 glutaraldehyde (GA), carbodiimide, dextran dialdehyde and genipin. Gelatin strengthened by these functional group agents has more superior characteristics such as biocompatibility, mechanical strength, anti-water transmission and anti-swelling. These characteristics are very suitable for the aqueous environment of the cell culture base. In 2005, the selective GA-crosslinked gelatin patterns are successfully introduced into cell culture [10]. 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.

B. Advantage compared to other fabrication of gelatin patterns

Gelatin negative resist can be accessed to fabricate micropatterns directly and the line width can be precisely controlled. 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. Moreover, a fabrication of selective GA-crosslinked gelatin patterns was successfully introduced into cell culture. The toxicity of selective GA-crosslinked gelatin had tested by Mesenchymal stem cells. It provided another means to crosslink gelatin other than using the photo-sensitizing agents. However, the lateral dimension error will occur in the crosslinking procedure of the selective GA-crosslinked gelatin patterns. Therefore, the selective GA-crosslinking fabrication needed the proper controlled time to reduce the over-crosslinking distance and to generate fine line width. By the reason, the gelatin patterns without good crosslinking or accurate size encounter a difficulty during being used in the environment of cell culture for several days. For example, the selective GA-crosslinked gelatin patterns may detach from the

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**Contact author: Tel.: +886-2-26215656×2768; fax: +886-2-26209745. E-mail address: Ljyang@mail.tku.edu.tw*

substrate, and the best spatial resolution of the micro gelatin bases can be only reached to 10 μm.

In this work, a new fabrication of the GA-crosslinked gelatin patterns is developed. It provides another method to crosslink gelatin neither by using the photo-sensitizing agents nor by selective GA-crosslinked technique. This novel technique can ensure the degree of crosslink, reduce the lateral dimension error, easily control the line width and enhance the adhesion between the gelatin and glass slide. The GA-crosslinked gelatin patterns can be compatible with the environment of cell culture. The best spatial resolution of the micro gelatin bases can be reached to 2 μm.

II. Materials and Methods

A. Fabrication of glutaraldehyde (GA)-crosslinked gelatin micropatterns

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 2 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 cell on the gelatin micro patterns both individually and in a 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 50°C. Dry at room temperature for 3–4 h.) Second, we dipped the sample in 50 % GA solution to undertake the whole-wafer crosslinked with sufficient reaction time. Third, a masking layer of positively-toned photoresist (e.g. AZP-4620) is spun on the gelatin surface, and the correlated ultra-violet (UV) exposure (365 nm, 16.5 mW, for a minimum of 15 s) for the portion of photoresist defines the crosslinked gelatin etched by the O_2 plasma. Finally, the glass substrate with a positive-toned photoresist mask on the GA-crosslinked gelatin film is etched by O_2 plasma (recipe: the etching rate of the GA-crosslinked film is shown in Table 1 and the etching rate of the positive photoresist, AZP-4620, is shown in Table 2). A reactive ion etching system (MODEL RIE-1C, SAMCO INTERNATIONAL INC.) is employed here. The flow rate is 30 sccm and the power is 80 W. The average etching rate of the GA-crosslinked film is 0.2664 μm/min and the average etching rate of positive photoresist, AZP-4620, is 1.0068 μm/min. In this O_2 plasma etching process, the thickness of GA-crosslinked film is controlled to 0.8 μm and the thickness of positive photoresist, is controlled to 4 μm. Although the average etching rate of positive photoresist is larger than the rate of GA-crosslinked film, the GA-crosslinked micro patterns still can be protected from O_2 plasma etching by the thickness control. Finally the GA-crosslinked micro patterns show up after stripping the masking layer of photoresist by acetone.

The fabricated gelatin patterns are shown in Fig. 2. The Fig. 2(a) shows the photoresist mask on the gelatin micropattern. The Fig. 2(b) shows that the photoresist mask is stripped by acetone and the GA-crosslinked gelatin micro patterns show up. It is found that no crack is produced on the GA-crosslinked gelatin micro patterns. It is proved that the process of the O_2 plasma has no effect on the gelatin patterns. To measure the finest line width and the corresponding thickness of the fabricated gelatin micropattern, an AFM (atomic force microscopy) in non-contact mode is introduced to scan the surface profile of the gelatin micropattern shown in Fig. 3.

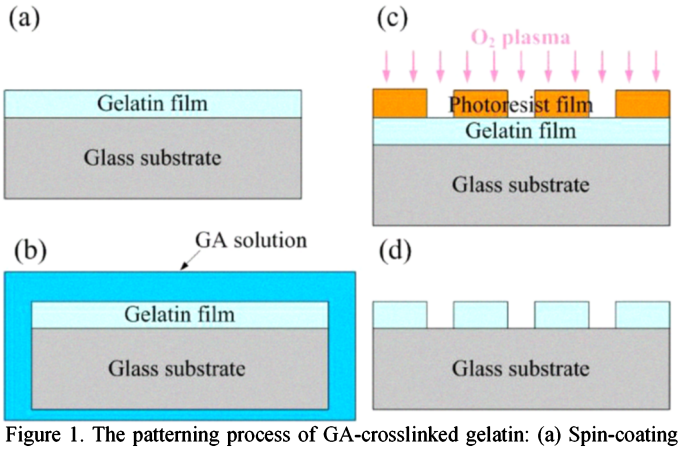

Figure 1. The patterning process of GA-crosslinked gelatin: (a) Spin-coating pure gelatin film at 50 °C, drying for 1 day; (b) Crosslinking gelatin film in 50% GA solution; (c) Patterning a positive-toned photoresist mask on the gelatin film and etching by O_2 plasma; (d) Stripping photoresist by acetone.

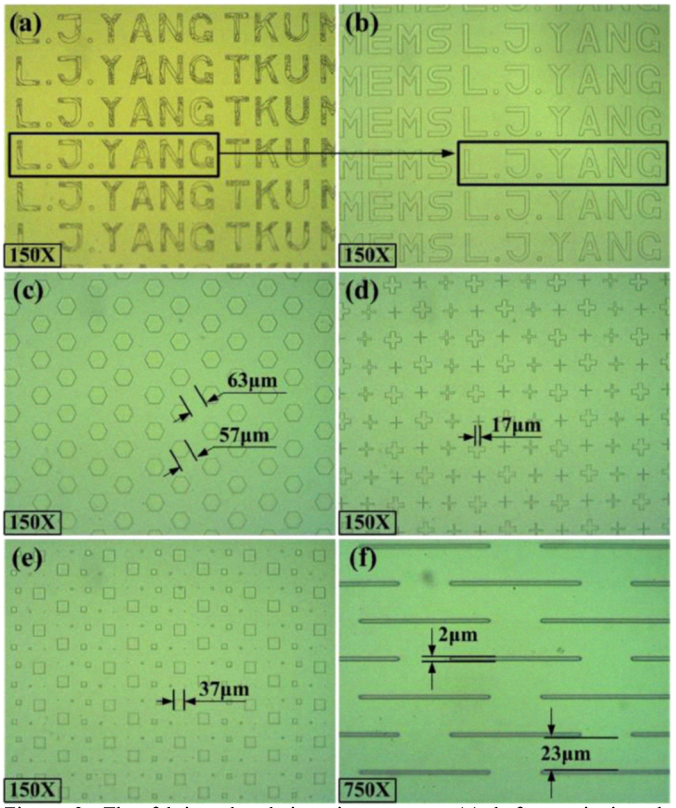

Figure 2. The fabricated gelatin micropatterns: (a) before stripping the photoresist mask; (b) stripping the photoresist, micro letter pattern of 17 μm width; (c) micro honeycomb patterns; (d) micro "cross" patterns, the largest width is 17 μm; (e) micro square patterns, the largest width is 37 μm; (f) microstrips lines with 2 μm width, 90 μm length and the gap between two lines is 23 μm.

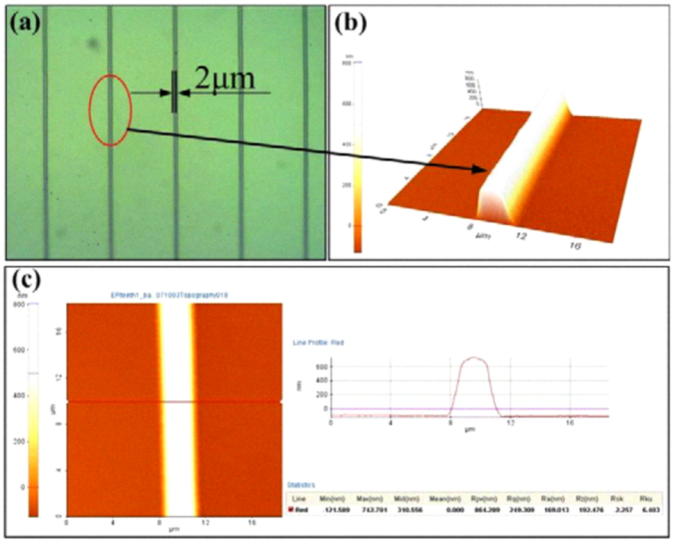

Figure 3. Thickness measurement of gelatin micropattern scanned by AFM: (a) the scanned sample of microstrips lines with 2 μm width; (b) AFM scanning picture of microstrip gelatin; (c) the cross-section line profile of microstrips lines with 0.8 μm thickness.

TABLE I. THE ETCHING RATE OF THE GA-CROSSLINKED FILM(μm/MIN)

Item	etching rate
GA-crosslinked gelatin film 1	0.2908
GA-crosslinked gelatin film 2	0.2869
GA-crosslinked gelatin film 3	0.2474
GA-crosslinked gelatin film 4	0.2600
GA-crosslinked gelatin film 5	0.2664
GA-crosslinked gelatin film 6	0.2469

TABLE II. THE ETCHING RATE OF THE POSITIVE PHOTORESIST(μm /MIN)

Item	etching rate
sample 1	0.9935
sample 2	1.0000
sample 3	1.0005
sample 4	1.0075
sample 5	0.9910
sample 6	1.0480

B. Contact angle of glutaraldehyde (GA)-crosslinked gelatin

Another reason to use gelatin as the culture bases of the cells is its surface property. It is reported that the more hydrophilic substratum can stimulates cell growth and the hydrophilic surface of gelatin can significantly enhanced the cell adhesion. In this work, the contact angle of surface on glutaraldehyde (GA)-crosslinked gelatin film is also measured. The measured contact angles of the GA-crosslinked gelatin film and the glass substrate are shown in Fig. 4. The contact angle of the GA-crosslinked gelatin film is measured about 57°. The contact angle of the glass substrate is measured about 41°. The contact angles of the different samples are shown in Table 3. The measurement shows that the average contact angle of GA-crosslinked gelatin film is higher than the average contact angle of the glass substrate.

C. Swelling test for the aqueous environment of the cell culture

Before using gelatin patterns as the culture bases, the swelling effect of the medium to cell culture must be tested for practicability. The sequence for cell-culture was then performed as follows. First, substratum with GA-crosslinked gelatin micropatterns were diced into slides. Second, the slides were subject to UV sterilization and treated with PBS (Phosphate buffered saline). Third, the slides were culture in MEM (Minimum Essential Medium) with 10 % FBS (Fetal bovine serum) at 37°C in 5 % CO_2 humidified atmosphere. Finally, after 2-day, 3-day and 6-day culture, the slide with GA-crosslinked gelatin micropatterns is observed by a phase-contrast microscopy. The results of the sequential soak in the medium are shown in Fig. 5. The PH value is also measured about 7.64. There is no obvious swell in the GA-crosslinked gelatin micropatterns. It is ensured the practicability that the GA-crosslinked gelatin micropatterns can be successfully introduced into the culture base.

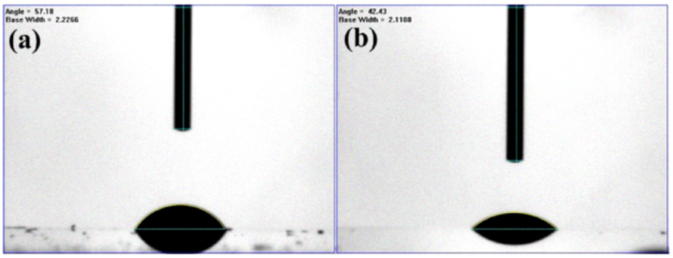

Figure 4. Contact angle measurement: (a) the contact angle of the GA-crosslinked gelatin film; (b) the contact angle on the glass substrate.

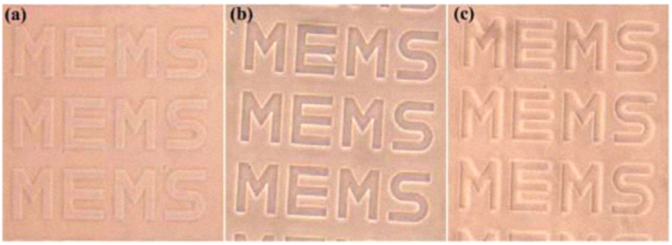

Figure 5. Swell effect of the sequential soak in the medium: (a) soaking for 2 days; (b) soaking for 3 days; (c) soaking for 6 days.

TABLE III. CONTACT ANGLES OF THE DIFFERENT SAMPLES

Item	Contact angle
GA-crosslinked gelatin film 1	57.18°
GA-crosslinked gelatin film 2	57.57°
GA-crosslinked gelatin film 3	57.87°
glass substrate 1	42.43°
glass substrate 2	41.75°
glass substrate 3	40.24°

III. Results and Discussion

The different GA-crosslinked gelatin micropatterns are fabricated for observing the cell extension. The behavior of the tumor cells growing on the gelatin micropaterns preliminarily observed by phase-contrast microscopy is not usually the same. The behavior depends on the kinds and properties, e.g. cell attaching, cell migration, proliferation, cell morphology and cell attachment. In this work, the HepG2, HeLa and NS-1 cells are first introduced into the observation of attachment induced by micropatterns of GA-crosslinked gelatin.

The HepG2 cells attaching on the gelatin micropaterns are shown in Fig. 6. The HeLa cells growing on the gelatin micropaterns are shown in Fig. 7. It is found that the growth of tumor cells base on the side wall of the gelatin micropatterns during the stages of falling and attachment of cell culture. Furthermore, the tumor cells can extend to the shape of the micropatterns along the side wall during the stage of cell division. Due to the scale of micropatterns, it is also found that two sides of tumor cells would anchor the side walls between the different gelatin micropatterns.

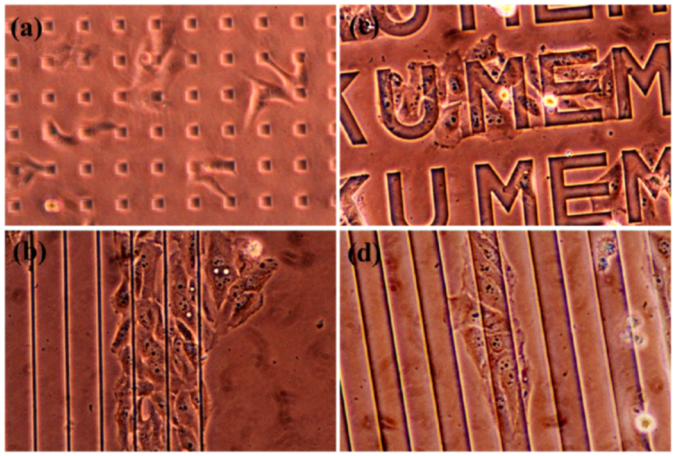

Figure 6. The attachment of the HePG2 cells: (a) the HePG2 cells attaching on the micro square patterns; (b) the HePG2 cells attaching on the gelatin lines with 2 μm width and growing along the gelatin lines; (c) the HePG2 cells attaching on the different micro "words"; (d) the HePG2 cells attaching on the gelatin lines with 37 μm width.

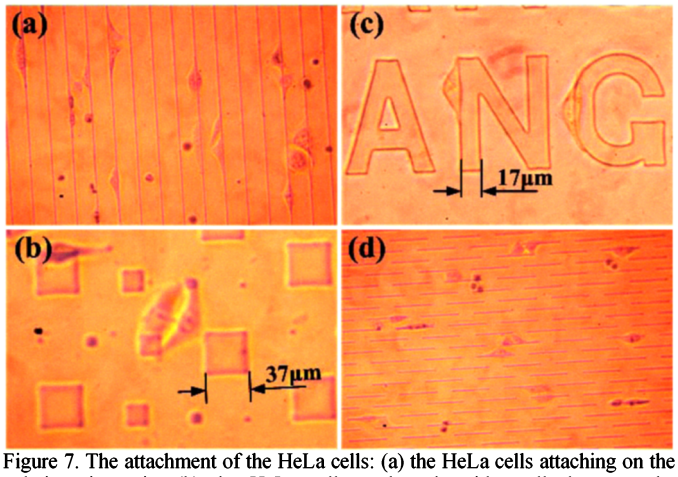

Figure 7. The attachment of the HeLa cells: (a) the HeLa cells attaching on the gelatin microstrip; (b) the HeLa cells anchor the side walls between the different gelatin micropatterns; (c) single HeLa cells attaching on side walls of the micro letter patterns; (d) the HeLa cells attaching on the gelatin and extend to the lines with 2 μm width and 90 μm length.

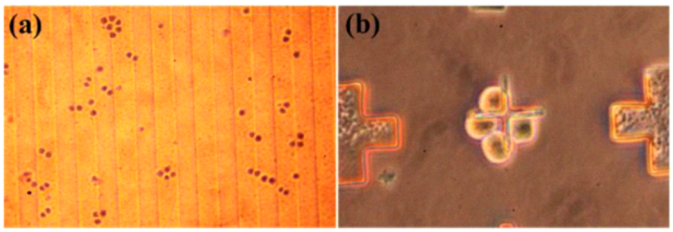

Figure 8. The attachment of the NS-1 cells on the gelatin micropaterns: (a) the NS-1 cells attaching on the microstrips lines (b) the NS-1 cells attaching on the "cross" pattern.

The NS-1 cells attaching on the gelatin micropaterns are shown in Fig. 8. The NS-1 cells attach on the side walls and then directly divide during the stage of cell division. There is no obviously cell migration occurred in the NS-1 cells.

In the O_2 plasma etching process, it is found that the lateral dimension of the all GA-crosslinked gelatin micropatterns will reduce about 3μm compared to the original design of the photo mask. To obtain the correct line width and reduce the lateral dimension error, the line width of the photo mask is modified according to lateral dimension error caused by the O_2 plasma etching process.

IV. Conclusions

The In summary, the patterning technique and the fabricated sample chips of GA-crosslinked gelatin are studied and modified in this paper. The micropatterns of GA-crosslinked gelatin can be made successfully by the conventional photolithography. The best spatial resolution of the micro gelatin bases can be fabricated and adjusted to the size of 2μm. The different GA-crosslinked gelatin micropatterns have fabricated for observing the cell adhesion, extension and migration. The hydrophilic surface property of GA-crosslinked gelatin is preliminarily measured and confirmed by the contact angle of the GA-crosslinked gelatin film to be about 57°. The swelling effect of the medium to cell culture has also tested and there is no obvious swell found in the GA-crosslinked gelatin micropatterns. It is ensured the practicability that the GA-crosslinked gelatin micropatterns can be successfully introduced into the culture base. The behavior of the tumor cells is not usually the same and depends on the kinds and properties, e.g. cell attaching, cell migration, proliferation, cell morphology and cell attachment. In the future, we believe that this biocompatibility, mechanical strength, chemical resistance, anti-water transmission and anti-swelling of the cross-linked gelatin micropatterns will provide useful microstructures for tumor cells. The correlated trapping tumor cells for observing cell extension, exploring the effects of drug delivery to tumor cell, and so on are promising to be under way.

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