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Realization of Photo-curing Gelatin Hydrogel using a Commercial Projector for Culturing Mesenchymal Cells

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***Abstract*—This paper investigates the realization of synthetic extracellular matrix with visible light photo-patterning gelatin in a simpler manner. A synthetic extracellular matrix provides an initial attachment for the seeded cells on the experimental substrate such as glass plate or multi-well until they realized their the natural extracellular matrix. Here, a commercial Digital Light Projector (DLP) was used to induce gelatin with Rose Bengal as a crosslinking agent to form the thin layer on the experimental substrate. Various gelatin concentrations from 2%-10% were exposed at different times in order to optimize the patterning process. A geometrical characterization on the patterned gelatin, such as contour measurement and resolution, were taken place. Results showed that the thickness of patterned gelatin was in the range of 10 µm – 60 µm depends on the exposure time of the DLP projector. Moreover, a visual method aided by the Fiji toolbox from NIH ImageJ image processing was used to observe the density and spatial arrangement of the cultured cells on the substrate. Ultimately, biocompatibility using MTT assay was also employed to confirm the viability of the cells on the gelatin substrate. The results show that we are able to control the physical and spatial arrangement of the gelatin substrate, and they with cell viability depend on 6 days of observation. It was found that the gelatin substrate provides faster growth on cultured cells compared to the control study. This finding leads to the possibility to realize the automation system in cell culture technology with an affordable investment.**

***Keywords*—extracellular matrix; visible light; photo-patterning gelatin; digital light projector; Rose Bengal; Fiji Sofware.**

I. INTRODUCTION

In tissue engineering, extracellular matrix fabrication has become a concern since it provides the initial mechanical structure for the seeded cells until they fabricate their own natural extracellular matrix. An ideal extracellular matrix (ECM) acts as a biomimetic environment that hosts cells in order to fabricate desired tissues. ECM is required to have a surface geometry that can enhance the proliferation and differentiation of human cells [1]. This condition is fulfilled by the realization of a textured microenvironment that allows the appropriate diffusion of biochemical cues and the removal of cellular waste.

Gelatin is a biodegradable, biocompatible material; it was first used as a protection layer for implantable material [2]. Natural gelatin, however, has the drawback of dissolving in aqueous environments; therefore, it requires a crosslinking procedure that uses appropriate agents [3]. For tissue culture, the 3D networks of a crosslinked polymer facilitate nutrient transport and cellular waste removal in the hosted cells [4]. Ultimately, the crosslinked 3D network promotes the attachment of cells by providing space and mechanical stability for new tissue formation [5]. These properties make hydrogels great potential biomaterial for tissue engineering [6].

The development of many ECM fabrication techniques over the past decade has resulted in significant leaps in creating constructs at the micro-level [7,8]. For example, fabricated porous and degradable hydrogel ECM by using photolithography technology to host skeletal myoblast cells

[9]. A 3D hydrogel ECM with micrometer-sized features was fabricated by using the direct-write assembly technique with acrylamide-precursor as printing ink [10]. In addition, Collins reported work on patterning scaffolds that confined the cells [11].

Photo-induced cross-linking is a fast and convenient way to produce a pattern of crosslinked polymer material that occurs only in areas exposed to light. This patterning process does not require a high temperature or high pressure. Therefore, the process will not influence the characteristics of the material. Materials that have been investigated for the patterning process are poly-ethylene glycol diacrylate, derivatized gelatin, and photo-induced poly-ethylene glycol diacrylate [7,8]. They have been reported as biomedical materials for tissue rehabilitation, molecular drug delivery, and cell culture [7-11]. Several types of polymers that are cross-linkable by ultraviolet and visible light have also been investigated [7-11]. Furthermore, the study of a gelatin micropatterning-applied glutaraldehyde (GA) agent has also been successfully carried out [12,13]. GA solution acted as a developer for gelatin, though it is known to be slightly toxic [14,15]. Therefore, using non-toxic materials as a crosslinking agent such as rose Bengal [16] became the focal point of this research. Rose Bengal is reported to have successfully formed a biosealant with gelatin that was crosslinked by visible light [17].

This paper investigates the possibility of fabricating an ECM with visible light photo-patterning gelatin in a simpler manner. A commercial Digital Light Projector (DLP), which was casually used in a classroom, was used to induce the gelatin with Rose Bengal as a crosslinking agent. Various gelatin concentrations and exposure times were tested in order to realize patterning gelatin. A geometrical characterization took place, and a biocompatibility test was also employed by culturing mesenchymal cells.

II. Material and Method

A. Preparation of the gelatin solution

First, porcine gelatin (G2500, Sigma) was dissolved in 25 mL of distillate water and kept at 40°C. Second, Rose Bengal (Aldrich) was added to the solution at a ratio of 1:3 w/w to the gelatin. The concentrations of the gelatin in the water were 2%, 5%, and 10% w/w.

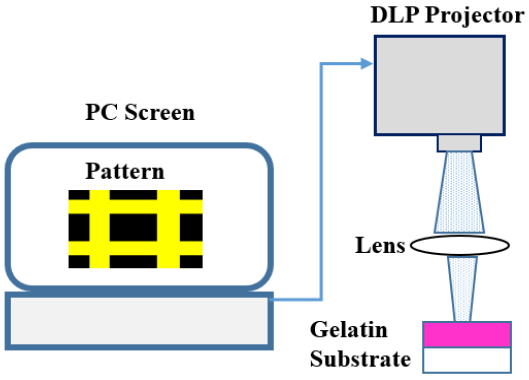

Fig. 1 The experimental setup of photo-patterning of gelatin solution coated onto a glass plate surface with a predetermined pattern using commercial DLP projector

B. Photo-patterning process

A gelatin-Rose Bengal mixture was coated on the surface of the glass plate coverslip by using a spin coater to obtain uniform thickness. Next, the mixture was exposed to a commercial DLP Projector Infocus™ at 500–550 nm (approximately yellow-green at the visible light level), as depicted in figure 1 [18]. This study applied a yellow color (in the Red Green Blue (RGB) color model: 255,255,0) as the light resource. Ultimately, the projector formed a pattern on the surface of the coverslip (as predetermined on a personal computer).

C. Development of the gelatin solution

Exposed gelatin was then developed in distillate water until it showed a model (as stated above. Meanwhile, the temperature was kept at 40°C in order to wash away undeveloped gelatin.

D. Gelatin patterning characterization

Patterned gelatin was photographed using a stereomicroscope AxioCam™ and digital-microscope Dynolite™. The resolution of the patterned gelatin was quantified by image processing. Then, the contour was observed by an Accretech 2900SD3 Surfcom™.

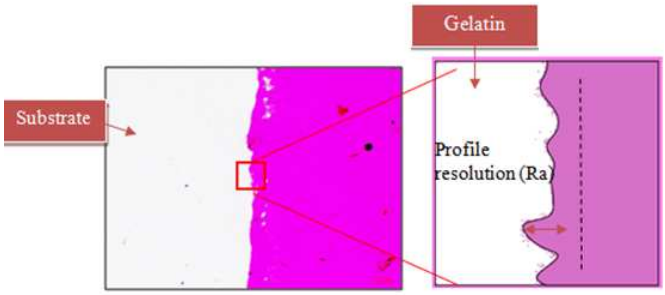

Fig. 2 Method for determining resolution by measuring average straightness on the edge of the patterned gelatin on a glass plate

Fig. 2 shows how a resolution is measured in this study. Briefly, it can be observed that the photopatterning result will explain a discrepancy with respect to the borderline as predetermined. This discrepancy is then measured to indicate the accuracy of the photo process.

E. Cell Image Analysis

Images of the cells were captured with an inverted microscope (Olympus IX81, Olympus Corporation, Tokyo, Japan) at 10-fold magnification in four random fields after day 2 and day 6 observation. Fiji was used for all image analyses and manipulations [19]. The individual fluorescence images from a time series were corrected by normalizing the intensity of a region containing mostly cells to the same mean concentration. Image contrast was set to show the localization of cells clearly and is set to the same level when the direct comparison between the figure is presented.

F. Biocompatibility tests

Biocompatibility tests were conducted using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. Cell proliferation and the

correlation between the toxic effects of the ECM were observed. The mesenchymal stem cells (MSCs) were provided by the Stem Cell Medical Technology Integrated Service Unit, Cipto Mangunkusumo Central Hospital, Jakarta [20].

In the MTT assay, ECM and cultured cells were tested with Vybrant®, and the absorbance value was determined by a UV-Vis Spectrophotometer at a 570 nm wavelength. ECMs were incubated in alpha-MEM (Gibco, USA) on the first and sixth days with MSCs and cell culture media. The complete media contained penicillin/ streptomycin (final concentration 100U/mL), amphotericin B (final concentration 2500ng/mL), 1% L-Glutamine (Lonza 17-605C), and 10% TC (Indonesian Red Cross). The cultures were also supplemented by 10% human AB serum (Gibco 34005-100).

A10 µl of MTT reagent was added to all the wells, which were incubated for 4 hours at 37°C. Right after formazan crystals were clearly identified, 100 µl of sodium dodecyl sulfate (SDS) 10% in 0.1 N hydrochloric acid (HCL) (stopper) was added. Cells and medium without the gelatin were observed as the control.

III. Result and Discussion

A. *Patterning characterization results*

Figure 3a shows the pattern predetermined by a personal computer and applied on a coverslip substrate. The width of the patterned gelatin was predicted to be dependent on the concentration of gelatin and exposure time during patterning. Figure 3b shows the outcomes of the patterned gelatin as measured by the digital-microscope.

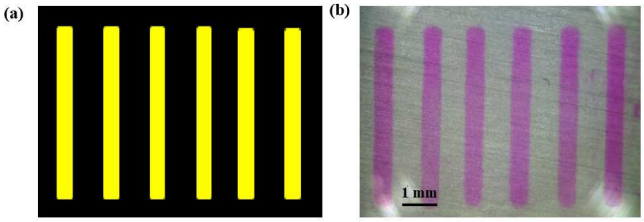

Fig. 3 The pattern of gelatin substrate in a computer screen; and (b) realized patterned gelatin on a glass plate

The measurement using image analysis results that the width was found to be 0.534±0.32 mm, 0.659±0.64 mm, and 0.834±0.44 mm at gelatin concentrations of 2%, 5%, and 10%, respectively. It can be concluded that the width of the patterned gelatin is dependent on the concentration of the Rose Bengal crosslinker. The pattern of the gelatin gets wider when solidification begins finishing. The solidification occurred when the temperature was lower than 40°C (which is the melting point of gelatin). A higher concentration of gelatin provided a wider patterned line; this is because a higher concentration of Rose Bengal brings more degrees of crosslinking to a solution. The exposure duration during the photo-patterning process was varied at 3, 6, and 9 minutes. However, the statistical calculation shows that the exposure duration does not give significantly different results. This result indicates that an exposure of 3 minutes has completely solidified the gelatin.

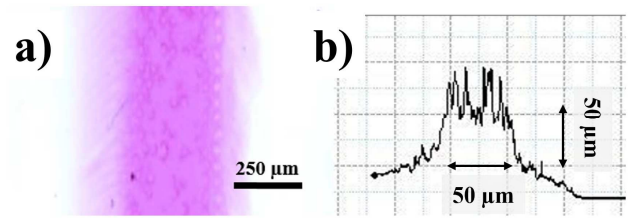

Fig. 4 a) The realized patterned gelatin in 10X magnification, and (b) the result of the contour measurement.

Figure 4a shows the patterned gelatin at a 10% gelatin concentration with a predetermined pattern at 500 µm full. Figure 4b indicates a gelatin thickness of around 50 µm on the glass substrate after the photo-patterning process. The contour shows a rough surface and a bell-shaped distribution, which was caused by a higher decomposing rate of gelatin on the edge area (figure 4b). This rough surface indicates a higher surface-to-volume ratio of the patterned gelatin compares to the surface without a pattern. This top surface might benefit to the seeded cells. In order to host the cells, the gelatin substrate required a 3D structured profile. Furthermore, figure 5a summaries the result of the thickness measurement of gelatin with various concentrations of gelatin at different exposure times. The statistical analysis shows that the gelatin thickness has dependent on its concentration. On the other hand, the exposure duration does not give significantly different results.

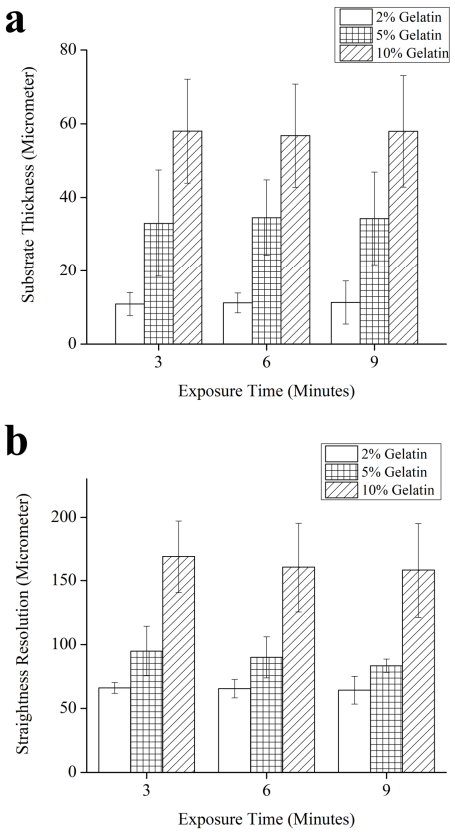

Fig. 5 (a) Thickness of the gelatin substrate at various concentration and exposure time; and (b) resolution of patterned gelatin at different concentration and exposure time

In the fabrication process, the resolution is a measure of precision, and it is determined by comparing the predetermined pattern with the result. This method of measurement was proposed to confirm the straightness of the line formed at the edges of the patterned gelatin. Figure 5 shows the measurements obtained by calculating the average resolution with image processing. Furthermore, the outcomes depicted in figure 5b show resolutions of 66±4 µm and 95±19 µm at concentrations of 2% and 5%, respectively. It was also found that, for the 10% gelatin, the gelatin substrate had a wider resolution. Similarly to previous results, the exposure duration does not have a significantly different effect on the resolution (figure 5b).

B. Cell image analysis

The biocompatibility of the gelatin substrate (with concentrations of 2%, 5%, and 10%) was determined by examining the cell viability and cell proliferation. The gelatin substrate was inserted into adipose-derived MSCs-filled wells and observed until the sixth day. Figure 6 shows the cell proliferation on a control substrate in comparison with the gelatin substrate with various concentrations of gelatin. The control substrate was a glass slipcover without gelatin patterning. The observations were done until the sixth day after the cell seeding on the substrate.

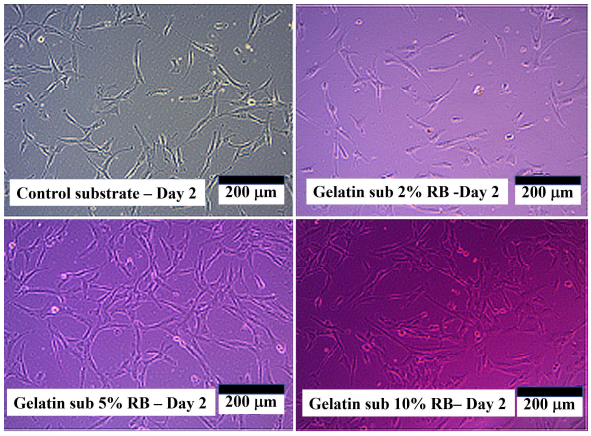

Fig. 6 Cell proliferation on the second-day observation at a 10X magnification for control substrate and gelatin substrate at 2%, 5%, and 10% concentration

It can be indicated that on the second day of observation, the MCs attachment on the control substrate similarly to the 2% gelatin substrate. On the other hand, a denser cell attachment is indicated on the 5% and 10% gelatin. Further observation shows that the MSCs proliferates more after the sixth day for both the control substrate together and the gelatin substrate (fig. 7). However, a closer view shows that the cell proliferates more on the 5% gelatin substrate compare to that control substrate. On the other hand, the 2% gelatin shows a similar result to the control substrate, whereas the 10% gelatin shows a lower density of MSCs (not viewed in the figure).

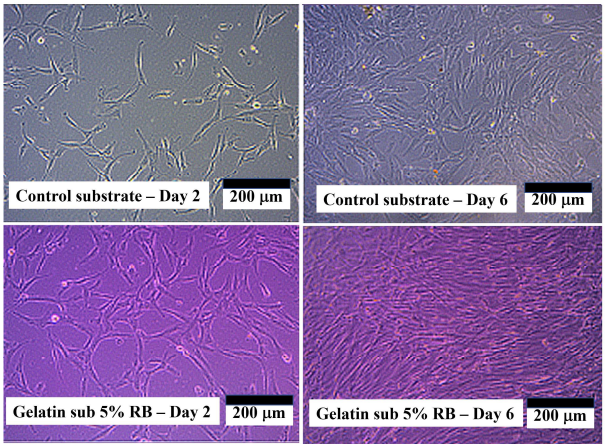

Fig. 7 Cell proliferation on the second and sixth days at a 10X magnification for control substrate and gelatin substrate at 5% concentration

Figuratively, the substrate with a 5% gelatin surface has a rougher surface, as viewed in figure 4b. Hence, it gives a more porous structure as that able to host more cells rather than on a flat glass as a control surface. The 2% gelatin might not have enough volume as the 5% concentration. The thickness measurement in figure 4a confirms that the 5% gelatin has a thicker surface than to the 2% gelatin. Therefore, cell attachment and proliferation of less than 5 % gelatin.

Moreover, figure 8 shows the attachment of cells closely on the substrate during cell proliferation. The software enables us to measure the length of the cell by putting the scale at the specific object (fig.8a). Further processing allows us to detect cells on the substrate using edge detection combine with black and white contrasting processing to indicate the cell density (Fig. 8b-c). The cell density measurement was aimed to confirm the thickness measurement of the gelatin substrate.

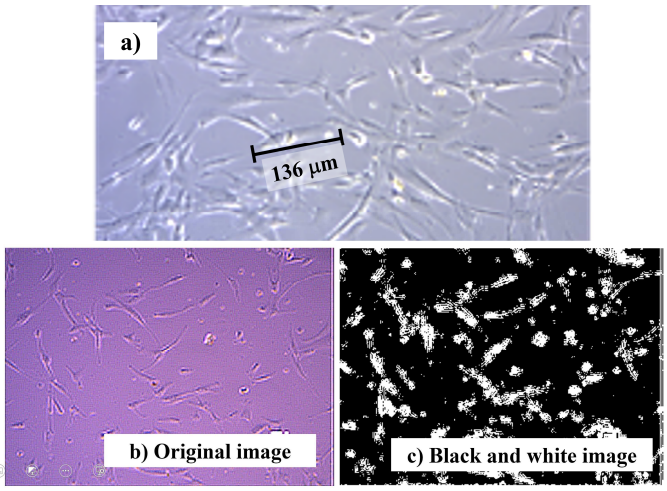

Fig. 8 Image processing using Fiji software from NIH ImageJ that enable us to measure cell length and cell density during cell proliferation

Figure 9a summaries the length measurement of the attached cells on the substrate at various concentrations together during the day of observation. The first notion that can be stipulated is cells tend to have longer dimensions

whenever cultured on the gelatin substrate. The highest growth was achieved by cells attached on 5% and 10% gelatin substrate at 2-days observation. However, the cells attached to the control substrate ultimately matched the cell length of those cells on the gelatin substrate after a 6-days culturing period. Here, it can be reported that cell length achieved is around 160 µm. This result agrees to studies by Riekstina and Witherick that concluded that cells have a length of approximately 120-150 µm [21,22].

Figure 9b summaries cell density measurement that resulted from the processing tools from Fiji software. Shortly, it confirms the previous finding on the measurement of substrate thickness. It is indicated that the cell density depends on the geometrical of the substrate that influenced by gelatin concentration (figure 7). The measurement shows that the higher concentration of gelatin on the substrate would positively host more cells to be attached to the substrate (figure 9b).

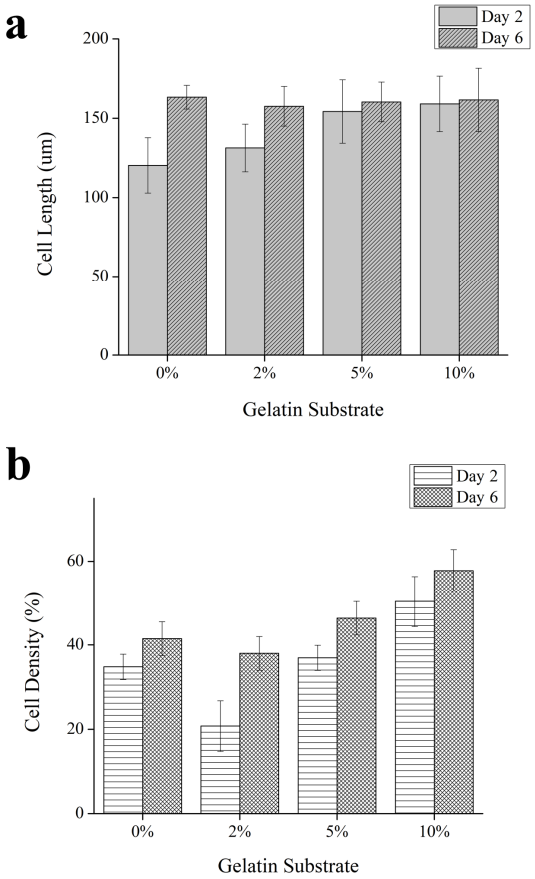

Fig. 9 image processing results of Fiji software to summarize the cell length and cell density at various gelatin concentration for 2-day and 6-day observation

Moreover, figure 10 depicts the result of biocompatibility test by cell counting for all substrates after the sixth-day observation using MTT assay. It can be indicated that 5% of gelatin has the highest cell concentration after the counting process. On the other hand, 10% of gelatin has the lowest cell concentration, among other substrates. Although the previous visual observation suggests the highest cell density (figure 9b), it can be indicated that the cell might have a rejection at a 10% gelatin concentration due to a higher amount of Rose Bengal in the gelatin, although it has a thicker substrate, among others.

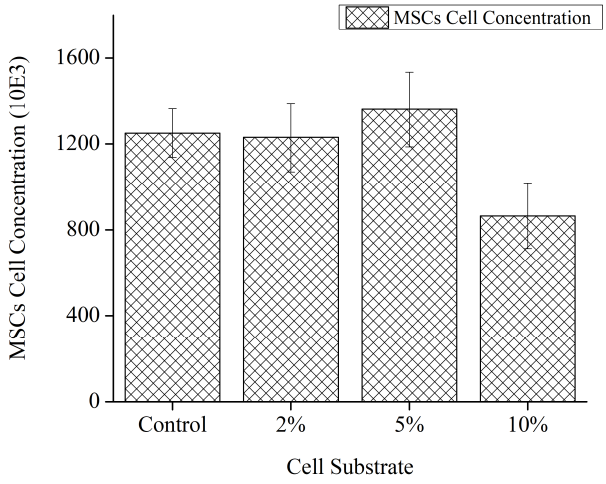

Fig. 10 Cell concentration on the sixth days at various gelatin concentrations using MTT assay.

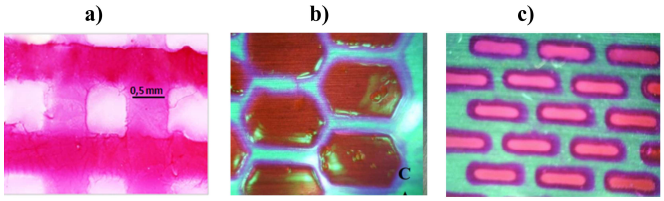

Fig. 11 Cell concentration on the sixth days at the control and gelatin substrate with various concentrations.

Figure 11 shows the versatility of this system to realize various patterns to be applied in several applications. The fig. 11a demonstrated the layering of the substrate to have a thicker substrate. Fig. 9b depicts a thick layer that encapsulates cells onto the gelatin substrate, whereas Fig. 9c shows how a gelatin substrate enables us to do drug screening similar to that multiwell plate.

This gelatin substrate experiment demonstrates the utility of gelatin patterning in controlling concentration and spatial of cultured stem cells; however, there are still many exciting aspects of the cell culturing that have yet to be explored. For instance, we need to investigate the effects of the substrate on stem cell proliferation and differentiation.

IV. Conclusions

A potential method for patterning a gelatin substrate was successfully performed by using a simple procedure at a low investment cost. This study showed that the optimum conditions were obtained at a 5% concentration of gelatin (in terms of the physical geometry). The biocompatibility test also confirmed that the gelatin mixed with rose Bengal at 5% caused no adverse effects to be applied as an extracellular matrix in cultured mesenchymal stem cells. Moreover, this composition of gelatin enhanced cell adhesion and grow at the initial stage of cell culture.

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