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Fibroblast and THP-1 Cell Response to Multi-Arm PEGNHS-Modified Decellularized Porcine Pericardium †

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Abstract: The adhesion between an implant and a wound could result in over-bleeding when attempting to separate the two. To address this issue, a cell-repelling implant is preferred. In this study, a cell-repelling membrane was prepared by modifying decellularized porcine pericardium with multi-arm polyethylene glycol. With this modification technology, we switched the surface properties of the decellularized porcine pericardium from cell-adhering to cell-repelling. The result showed that this pericardium was successfully modified without any effect on the original properties of the pericardium and also maintained a low inflammatory response. The level of cell adhesion on the surface of the membrane was significantly reduced.

Keywords: decellularized porcine pericardium (dP); polyethylene glycol (PEG); surface modification; cell repelling; inflammatory response

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1. Introduction

Decellularized tissue is an acellular extracellular matrix (ECM) prepared by decellularization process to remove cellular components from the tissue sources. It has low immunogenicity, good biocompatibility, and strong mechanical properties, especially when prepared using the high-hydrostatic pressure (HHP) method [1]. Among membrane tissues, the pericardium membrane has strong mechanical properties and is easy to handle, large in size, and flat in shape. It has been used for decades in tissue engineering and regenerative medicine applications [1,2]. Due to the enhanced cell growth of decellularized porcine pericardium (dP), it cannot be used in cell-repelling applications as a cell adhesion effect is observed. Therefore, we introduced a new function of dP as a cell-repelling membrane by modifying its surface with polyethylene glycol (PEG) to switch its properties from natural cell-adhering to cell-repelling. PEG is non-toxic, biocompatible, repels proteins and cells [3–14], and is stable in vivo [4]. Commercial PEG products are available in different forms, such as linear and branch, and different effects in terms of cell-repelling ability [15], enzymatic degradation [5,16], and the immune response [17,18] have been reported.

In this study, we fabricated cell-repelling membranes by modifying dP with three different multi-arm PEGs (2-arm PEG, 4-arm PEG, and 8-arm PEG) and we also evaluated the different effects of multi-arms on the modification process and cell-repelling ability.

2. Materials and Methods

2.1. Preparation of Samples

The serous pericardium was pressurized at 1000 MPa at 30 °C for 10 min with a cold isostatic pressure machine (Dr. CHEF; Kobelco, Hyogo, Japan). Then, it was shake-washed with a series of saline solutions to prepare decellularized porcine pericardium (dP) [19,20].

It was then immersed in different multi-arm PEGNHS [α-succinimidyloxyglutaryl-ω-succinimidyloxyglutaryloxy-polyoxyethylene (2-arm PEGNHS), pentaerythritol tetra (succinimidyloxyglutaryl) polyoxyethylene (4-arm PEGNHS), and hexaglycerol octa (succinimidyloxyglutaryl) polyoxyethylene (8-arm PEGNHS)] (NOF, Tokyo, Japan) at 4 °C for 3 h. The modification molar ratios between the NH_2 of the pericardium and the NHS of PEG were 1:1 and 1:2. The samples were abbreviated as [2,4,8: number of multi-arm PEG], [dP: decellularized porcine pericardium], [1,2: modification molar ratio].

2.2. Analysis Procedure

After decellularization, hematoxylin–eosin (H and E) staining and deoxyribonucleic acid (DNA) quantification were used to analyze the tissue structure and the residual DNA, respectively.

A ninhydrin assay evaluated the remaining NH_2 after modification [21]. Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) (NOF, Tokyo, Japan) was used to confirm the chemical bond on the surface of the dP after modification. Immunohistochemistry staining using 10 μg mL^{-1} antibody polyethylene glycol (PEG) polyclonal antibody (CAU30011, Biomatik, Wilmington, DE, USA) was used to track the presence of PEG.

The 4.5×10^4 human leukemia monocytic cells (THP-1 cells) editing procedure with a HiBiT-tagged IL-1β assay was used to detect the inflammatory level of the samples [22]. A preparation of 2×10^3 cells per cm^2 mouse fibroblast (NIH3T3 cells) were seeded on the membrane to detect cell adhesion and proliferation ability.

Data are presented as the mean ± standard deviation. The multiple treatments were compared using one-way ANOVA with a post-hoc Tukey HSD (honestly significant difference) test. A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Decellularization

Figure 1a shows that the structure of dP, shown through H and E staining, was well preserved and showed a wavy, curly formation similar to native porcine pericardium (PP). Figure 1b shows that the DNA residue of the PP significantly reduced from 1446.93 ng mg^{-1} to 6.30 ng mg^{-1} after HHP treatment. This DNA residue follows the recommendation of a DNA residue level below 50 ng mg^{-1} [23]. This dP was used as the base material for modification.

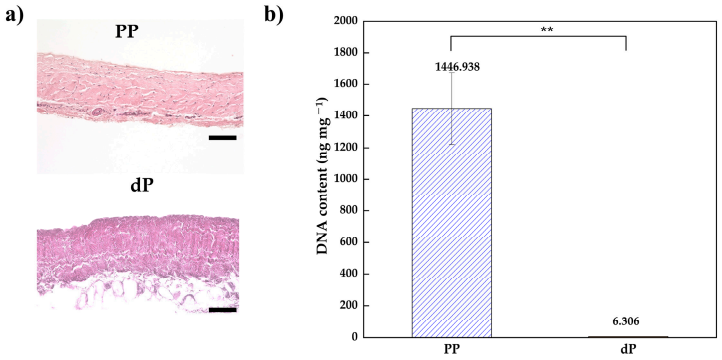

Figure 1. (**a**) H and E staining; (**b**) DNA residue of porcine pericardium and decellularized porcine pericardium. Data are expressed as the mean ± S.D. ** $p < 0.01$, where the values for the modified porcine pericardium samples are compared with dP, respectively. The numbers 2,4,8 are the PEG arms, dP: decellularized porcine pericardium, 1,2: NHS ratio. Scale: 100 μm.

3.2. Surface Characteristic of Modified Decellularization Porcine Pericardium

Figure 2a shows the amine index of the non-modification and modification samples. The amine index was reduced to 40%, indicating that PEG bound to the free amine of the

dP. The chemical binding between the dP and PEG is shown in Figure 2b. The C-O-C bond of modified samples was significantly increased compared to the dP. Figure 2c shows the result of anti-PEG antibody application. A brown color was observed in all modified samples, while it was not observed in the dP. This result indicates that PEG was successfully introduced to the dP.

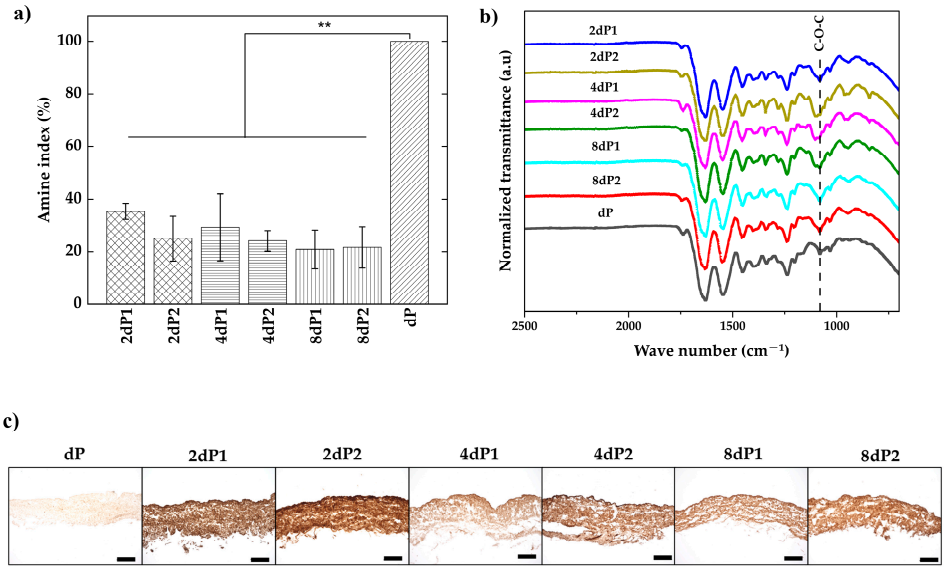

Figure 2. (**a**) The amine index; (**b**) ATR-FITR spectra (**c**) Immunohistochemistry staining of the non-modification and modification decellularized porcine pericardium. Data are expressed as the mean ± S.D. ** $p < 0.01$, where the values for the modified porcine pericardium samples are compared with dP, respectively. The numbers 2,4,8 are the PEG arms, dP: decellularized porcine pericardium, 1,2: NHS ratio. Scale: 200 μm.

3.3. Inflammatory Response and Cell Adhesion Ability

During in vitro analysis, HiBiT tagged THP-1 with the assistance of phorbol 12-myristate 13-acetate (PAM), differentiated to the macrophage, and reacted with the membrane to secrete interleukin-1β (IL-1β), which was detected by the luminescence level. Figure 3a shows the inflammation response of the macrophage to the samples. The inflammatory level of the modified samples was comparable to the TCPSs and dP. The inflammatory level was also significantly low compared to the TCPSs with LPS. There was no significant difference between the dP and the modified samples. Therefore, PEG introduction to the dP did not cause any inflammation.

Figure 3b shows the fibroblast density adherence to the non-modification and modification dP samples. The cell density on the dP on day 1 was 3.82×10^3 cells/cm^2 and this increased to 2.12×10^4 cells/cm^2 on day 7, comparable to the TCPSs (tissue culture polyethylenes). The cell density significantly decreased once PEG was introduced to the dP. On days 1 and 3, the level of cell density was similar in all modification conditions. On day 7, the increase in cell density was observed as higher in the 4-arm sample, followed by the 2-arm and 8-arm PEG-modified samples.

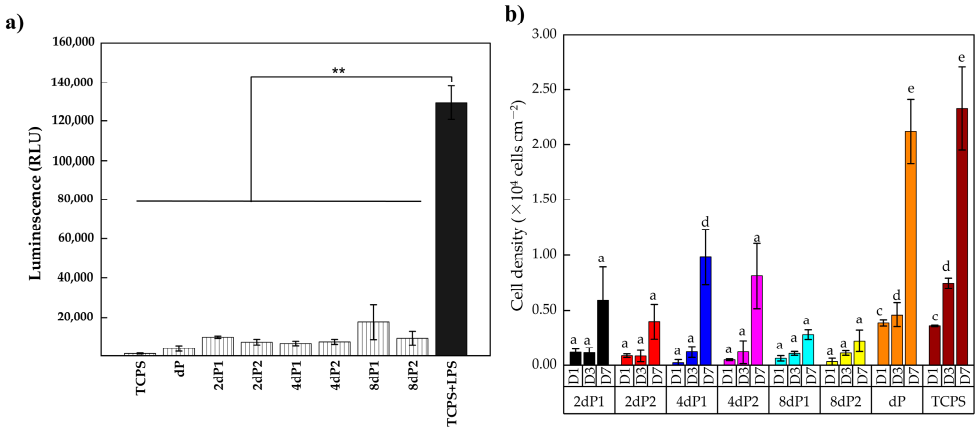

Figure 3. (**a**) The inflammatory response of the macrophage differentiated from the THP-1 tagged HiBiT; (**b**) Fibroblast density adherence to the non-modification and modification dP samples, $n = 3$. Data are expressed as the mean ± S.D.** $p < 0.01$, where the values for the modified porcine pericardium samples are compared with the dP, respectively. The numbers 2,4,8 are the PEG arms, dP: decellularized porcine pericardium, 1,2: NHS ratio. a,b,c,d,e denote statistically significant differences.

4. Discussion

dP has been used as a scaffold, tissue replacement, wound dressing, and so on [7,18,24,25] in biomaterial applications due to its function in preserving the extracellular matrix (ECM) after the decellularization process, exhibiting minimal immunogenicity, and having favorable biocompatibility. Through the HHP decellularization process, we prepared a dP sample with a well-preserved ECM structure and low DNA residue.

This study investigated the new application of dP in a cell-repelling membrane. To switch the original property of dP from cell-adhering to cell-repelling, a multi-arm PEGNHS was introduced to modify the surface of the dP by forming an amine bond between the NH_2 of the dP and the NHS of PEG. Decreasing the free amine was confirmed through a ninhydrin assay. PEG consists of a repeated C-O-C functional group, respecting molecular weight. The significantly increased C-O-C bond on the modified pericardium's surface confirmed PEG's presence. The brownish color of the tissue section also confirmed the presence of PEG. With this result, we successfully confirmed the introduction of the PEG molecule to the decellularized porcine pericardium.

In the early stage of implantation, macrophage cells play an important role in reacting with the implanted membrane. Minimizing this reaction by maintaining low inflammation in the original decellularized porcine pericardium (dP) after the modification process is necessary to avoid rejection. With the proof of the HiBiT assay, our modified membrane has a similarly low inflammation level to the dP and could be implanted into the body without worry of rejection.

The fibroblast is essential for the maintenance, repair, and remodeling of cells in the wound-healing process. It secretes ECM components and forms the ECM structure, resulting in adhesion between the implant and the wound area. For producing a cell-repelling membrane, a primary test of the membrane with the fibroblast was conducted. A low level of fibroblast adherence was achieved. Different levels of cell recovery on day 7 were observed, indicating that we could use this membrane in a variety of applications, such as long/short periods of cell-repelling, if required. Therefore, this membrane could be used as a cell-repelling membrane and wound cover without worry about sticking of the implant and wound.

5. Conclusions

In this study, a cell-repelling membrane fabricated from the decellularized porcine pericardium with different multi-arm polyethylene glycols was developed. A low inflammatory response of the dP was maintained, even after PEG was introduced. Difference durations

of cell-repelling correlating to different multi-arm applications were achieved, highlighting PEG's potential for a variety of applications, such as long or short application periods.

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Conflicts of Interest: The authors declare no conflicts of interest.

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