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Thin Micro-Porous Scaffold Layer on Metallic Substrate
금속기질에 얇은 마이크로 다공질 스케폴드 코팅에 관한 연구

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주요용어: 티타늄(Titanium), 표면 교정(Surface Modification), 내피 세포(Endothelial Cells), 생체 적합성 (Biocompatibility)

요 약 : 티타늄과 티타늄 합금은 재료적 특이성 때문에 심장 혈관 임플란트에서 일반적으로 사용되어 왔다. 일찍이 적용된 예로는 인공심장판막, 심박조율기의 보호케이스, 혈액 순환 장치 등이 있다. 하지만 물질유도 혈전증(Material-induced thrombosis)은 혈전폐색에 의해 기인한 기능 손실로 심장혈관 임플란트 장치의 주된 합병증으로 존재하고 있으며, 심장혈관 임플란트의 혈전유전자는 심장혈관장치의 발달에 주된 난관 중 하나로 남아있다. 그리고 텍스처 혈액 접합 물질(Textured blood-contacting material)은 1960년대 초반 이후부터 혈액순환 보조 장치의 임상실험에 사용되고 있다. 접합 물질에 내장된 텍스처 섬유조직 표면은 형성, 성장, 안정적 부착, 생물학적 내벽(neointimal layer) 등 유도 혈액(entrapping blood) 성분에 의해 형성된다. 공동(cavity) 형상의 용해 가능한 미립자를 사용하는 SCPL법(Solvent casting/particulate leaching method)은 티타늄 기질 이전에 형성된 폴리우레탄 위에 텍스처(texture)를 생성하기 위해 사용되었다. 또한 콜라겐의 부동화(不動化)에 의한 공동(cavity)은 혈액 접합면에 잔존하기 위한 내피세포를 고정할 수 있는 효과가 있다. cpTi로 충화된 PU 기소공성(microporous)은 구조적 특성과 혈전증 감소를 위한 생물학적 내벽 사용의 잠재성을 평가하기 위한 세포 공동체 실험을 통해서 평가되었다.

1. Introduction

Titanium and titanium alloys are common in cardiovascular implants because of their unique properties. Early application examples were prosthetic heart valves, protective cases in pacemakers, and circulatory devices. However, material-induced thrombosis remains a primary complication of cardiovascular implant devices, leading to the loss of function by thrombotic occlusion. Cardiovascular devices made entirely of titanium have in general not been very successful clinically mainly due to problems with blood-clotting occurring on the device surface[1]. Hence, an important method in the design of hemocompatible materials is the formation of surfaces that control protein adsorption by means of biological macromolecule coatings, such as heparin, albumin, thrombomodular, and endothelial cells.

The growth of an endothelial cell (EC) layer on a blood-contacting surface has been suggested for the long term use of VADs, and it is believed that the metabolically active endothelial lining plays a major role in preventing blood thrombosis. A technical hurdle to successful endothelialisation on blood contacting surfaces that still exists is the inability of seeded cells to resist shear stress due to post operative blood flow. There have been many studies on the use of an EC layer to improve the blood compatibility of ventricular assist devices[2]. Although a lining layer of endothelial cells (ECs) on a blood contacting surface has shown less thrombogenicity[3], a

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possible problem with the endothelial cell lining strategy is that the originally formed monolayer of ECs may be partially washed away by the blood flow and lead to the loss of endothelium[4].

To improve the adhesion and viability of the endothelial cell layer, different methods were proposed to have a textured topology for cell attachment and a functional layer for cardiovascular devices. Textured blood-contacting materials have been used clinically in circulatory assist devices since the early 1960s[5),6)]. An enhanced SCPL (Solvent casting/particulate leaching) method, using dissolvable particles to form the cavities, was used to create texture on polyurethane prelayered titanium substrate. These cavities, if modified by the immobilization of collagen, are able to anchor endothelial cell to remain on blood contacting surface. The three-dimensional surface topography of blood-contacting materials is a major contributing factor in the formation of a stable endothelial layer upon the material under fluid-induced shear stress[5),6)]. The porous phase is expected to facilitate the 3D-culture of ECs. The PU microporous layered cpTi was evaluated via structural characterisation and cell culture experiments to assess its potential use for endothelial lining to reduce thrombosis.

2. Materials and methods

2.1 Preparation of specimens

Commercially pure titanium specimens were fabricated into disks (Ø 13 mm x 5 mm). They were polished with 600 grit SiC abrasive, washed with acetone, and rinsed with distilled water in an ultrasonic bath. The disks were dried in an oven at 40 °C. Alkali heat treatment was performed by immersing CpTi specimens in 100 mL of 5M NaOH aqueous solution at 60°C for 24 hours. After that, the titanium specimens were gently washed with distilled water, and dried in the air. The alkali- treated specimens were put in an electric furnace, and then heated to 500°C for one hour. They were heated up at a rate of 5 °C/min and cooled down in the furnace. The specimens were immersed in a 30 vol % H_2O_2 solution at room temperature for 30 minutes.

2.2 Fabrication of microporous scaffold on titanium substrate

The microporous layer was fabricated by dipping the specimens into a polymer solution prepared by dissolving 15g polyurethane pellets (Zytar® Z1A1, Biomer Technology Ltd) into 10 ml of methylformamide (Sigma)/ tetrahydrofuran (Sigma) (volume ratio: 50:50) solution at room temperature. Subsequently, the specimens were centrifuged at room temperature at 1 500 rpm for one minute. Salt particles were then deposited onto the polyurethane layer. The formed thin-mixture layer was then air-dried or vacuum-dried, followed by salt leaching in distilled water, resulting in a microporous thick layer on the titanium substrate. Collagen-coating (Fig. 1.) on the microporous layer was prepared by immersing the specimens into a bovine collagen Type I acidic solution (Type I, pH 3.2, 0.3 wt%, Cellmatrix, Nitta Gelatin, Osaka); subsequently, the specimens were treated by air gun, followed by vacuum-drying.

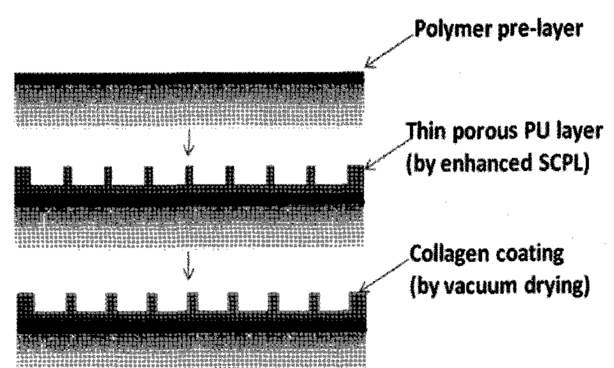

Fig. 1 Fabrication of microporous PU coating on titanium substrate

2.3 Structural Characterization

The structural characterization as done by scanning electron microscopy (SEM). Samples were examined using an FEI Quanta 200 Environmental SEM, in high-vacuum mode, with

a secondary electron detector. The specimens were mounted on stubs using double-sided carbon tape and then sputter-coated (Bio-Rad SC500 coater) with gold, except for bare cpTi samples.

2.4 In vitro studies

Cell retention of HAECs on bare titanium and thin micro porous scaffold coated titanium with or without collagen coating were investigated using cell culture methods. Triplicate samples were arranged in separate culture plates for evaluations of retention and cell morphology. Samples were hydrated in culture medium for 4 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, and were then placed in the well of a 24-well tissue culture plate. For the majority of experiments conducted, HAECs (ScienCell Research Laboratories, Carlsbad, CA) between passage 9 to 12 were seeded in endothelial medium (ECM) (ScienCell Research Laboratories, Carlsbad, CA).

2.4.1 Cell retention assay

HAECs were seeded at a density of $\sim 4\times10^5$ cells/mL in endothelial medium (ECM). Seeded HAECs on substrates were cultured under static conditions for 24 h in the ECM medium under a 5% CO2 atmosphere at 37°C. HAECs were then exposed to fluid shear stress in a 250 mL spinner flask (ProclutureTM, Corning) featured with grooves on the flask wall acting as baffles and an impeller. The spinner flask was autoclaved before use. The substrates were then transferred around the wall of the baffled spinner flask. Mixing was done by a magnetic stirrer at the bottom of the vessel. When used for the retention test, the flask was filled with 100 ml of culture medium and stirred at ~80 rpm for 2 min.

The percent retention was determined with the PicoGreen DNA assay (Invitrogen). Medium was removed and the substrates were washed with PBS. 100 μL of PicoGreen at 1:200 dilution of reagent A in TE buffer (10 mmol/Tris-HCl, 1 mmol/liter EDTA, pH 8.0) was added to substrates and incubated in the dark at room temperature for 5 min and the cells were lysed by freeze-thawing. A standard curve was constructed using the 2 μg/mL DNA stock. Levels of DNA in each sample were calculated using the standard curve. Percentage retention was calculated as the quantity of DNA from attached cells under static conditions. The fluorescence of each sample was measured with a POLARstar OPTIMA multidetection microplate reader (BMG Labtech).

2.4.2 Cell morphology assay

HAECs were seeded at a density of $\sim 1\times10^6$ cells/mL in endothelial medium (ECM) to allow dense endothelial cell layer formation for confocal microscopy assay. After three days of culture, cells were fixed with 4% fresh paraformaldehyde for 20 minutes at room temperature, washed for three times with PBS, and permeabilised for five minutes with 0.2 % Tween in PBS. Cells were incubated with 0.1 mg/ml of propidium iodide (Sigma-Aldrich P4170) in PBS for five minutes, and washed for three tiems with PBS. These were then incubated for 15 - 20 minutes with Alexa Fluor 488 (A-12379, Molecular Probes, Eugene, OR, USA; stock dissolved in methanol and stored at - 20°C). The fluorescently labelled samples were examined using a Leica TCS 4D confocal laser scanning microscope (Leica Laser- technik GmbH, Heidelberg, Germany) with a Leitz DMIRB inverted microscope (Leica).

3. Results and discussion

3.1 Cauterisation of micro porous layer on titanium substrate

It can be hypothesized that the integration of endothelial cells into the pores of the particle-cast surface is responsible for adherence due to low shear stress. The endothelial cells spread into the underlying pores and becomes entrapped within these pores. This entrapment results in increase cell attachment and retention rate between the endothelial cells and the underlying micro porous surface. The sandblast cpTi, PU coated cpTi, and micro porous layered cpTi specimens were shown in Fig. 2. The topology of micro porous surface

was fabricated by using the enhanced SCPL with 90~212 μm particles size.

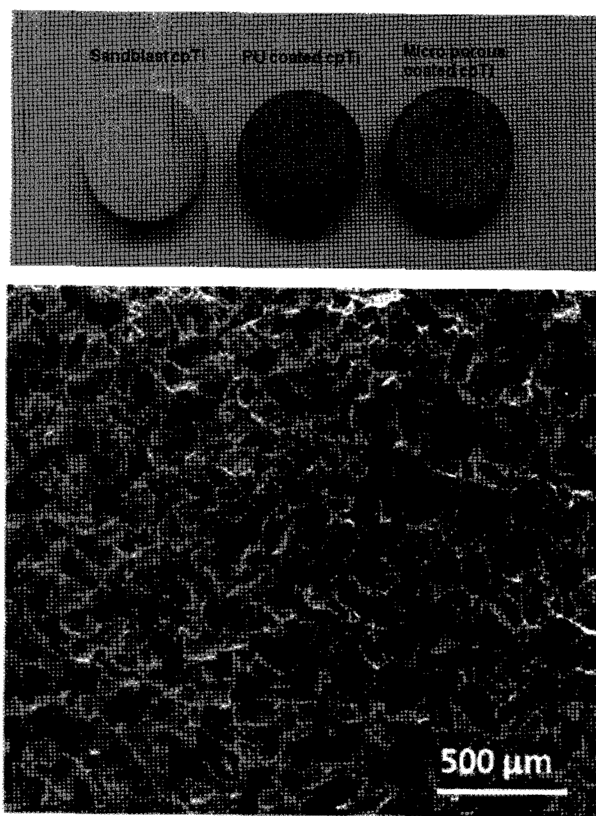

Fig. 2 Photograph of samples: scadblast cpTi, PU coated cpTi, and PU microporous film coated cpTi. Right side is SEM image of microporous coated cpTi surface

An undesirable skin layer (closed pores) formed on the surface of 3D scaffold was routinely observed from previous publications, when classic SCPL was used to fabricate 3D scaffold. In contrast to the sample prepared by enhanced SCPL with vacuum drying (as shown in Fig. 2), the surface was highly porous without showing a surface skin. The increase of pore sizes which increase height of wall reduce fluid induced shear stress inside the near the corner of wall. However, endothelial cells are unable to bridge pores greater than a cell diameter. Therefore, 90~212μm of salt size to fabricate a PU porous layer on titanium was chosen.

Compressive tests were performed to evaluate mechanical characteristics of the PU micro porous scaffolds produced by the enhanced SCPL method with a 15% (w/v) polymer solution and a 99 - 212 μm salt size. The typical stress-strain curve of the polyurethane scaffold is shown in Fig. 3. The mechanical characteristics of the PU scaffold were summarized in Table 1. The high porosity which increases the large surface area/volume ratio is necessary to facilitate cellular attachment. In general, if engineering tissues in vitro using any of the cell types studies, scaffolds with the high porosity (90%) are likely to support greater cellular metabolism. The level of the compression modulus obtained with different salt particle sizes were about 5.49 kPa and a high porosity (~92 %) was obtained. These preliminary results also suggest that the porous PU scaffold with a high porosity (~90%) can be formed on the titanium surface.

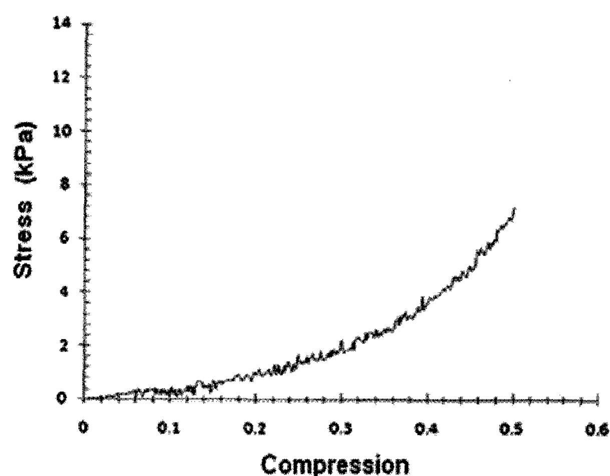

Fig. 3 Typical stress-strain curve of the polyurethane scaffold produced by the enhanced SCPL with 99~212 μm salt size and vacuum dried

Table 1 The mechanical characterizations of the PU scaffold

Polymer	Compressive Modulus (KPa)	Porosity (vol %)	Particle .Size (μm)
Polyurethane	5.49±0.87	92.45±0.95	99~212

The surface shown in Fig. 4(a, c) was fabricated by using particles retained between the 90 and 212 μm sieves, whereas the surface shown in Fig. 4(b, d) was fabricated by using particles retained

between the 70 and 90 μm sieves. The use of larger particles resulted in larger pore areas.

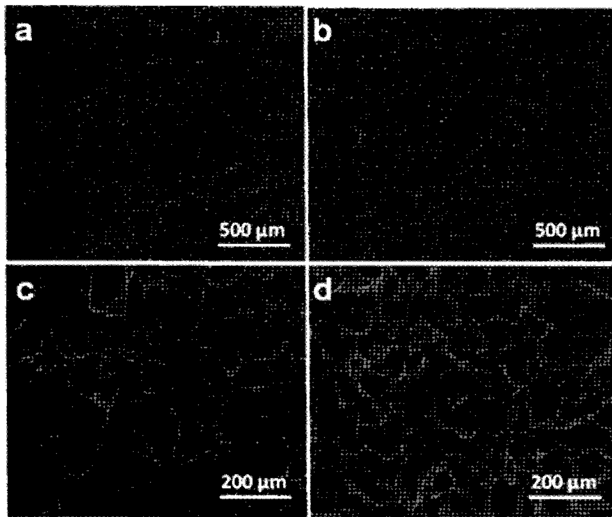

Fig. 4. SEM images of the top view of micro porous surfaces with enhanced SCPL using a salt size of 99~212 (a,c) and a salt size of 70~99 µm (b,d) with 15% (w/v) polymer solution and air-dried

3.2 In vitro test

Cell retention analysis was measured with HAECs seeded onto different substrates in baffled spinner flask with shear stresses generated at 80 rpm for 2 mins. Baffled spinner flask with a triangular impeller design was previously utilized for dynamic cell culture[7]. The Reynolds number of the bulk flow can be calculated depending on the diameter of the impeller Di (cm), and the agitation rate N (in rev/s) of the stirring rod.

$$Re = \rho D_i^2 N / \mu \tag{1}$$

where ρ denotes density of the growth medium (g/cm^3) and μ denotes viscosity of the growth medium (g/cm•s) (approximated to be equal to that of water[8]). The geometry and operating conditions yield an impeller Reynolds number of 3,822 suggesting the presence of a fully turbulent hydrodynamic regime, defined by Re > 1,000[9]. Using the Kolmogorov's theory of turbulent eddies and the Nagata correlation, the equivalent maximum shear stress for agitation rates of 80 rpm was calculated to be 2.4 dyne/cm^2, which is higher than mean shear stress (< 1 dyne/cm^2) of the large vein[10]. Data showing the amount of DNA of each substrate that was covered by HAECs after static culture and retention test are tabulated in Table 2.

Table 2 DNA content of the EC layer on substrates after static culture and shear test

Sample	Static culture (ng/mL)	After exposure to shear stress (ng/mL)
Sandblast cpTi	13.16±0.96	10.98±0.62
Miroporous PU Coated cpTi	17.36±0.92	15.40±0.31
Collagen and microporous PU coated cpTi	17.56±0.91	15.17±0.76

* values shown are means±one standard deviation.

ECs on sandblast cpTi substrates tended to detach rapidly when exposed to the fluid induced shear stress. However, the microporous topology, which might reduce the shear stress, resulted in high cell retention rate. Though cell retention was

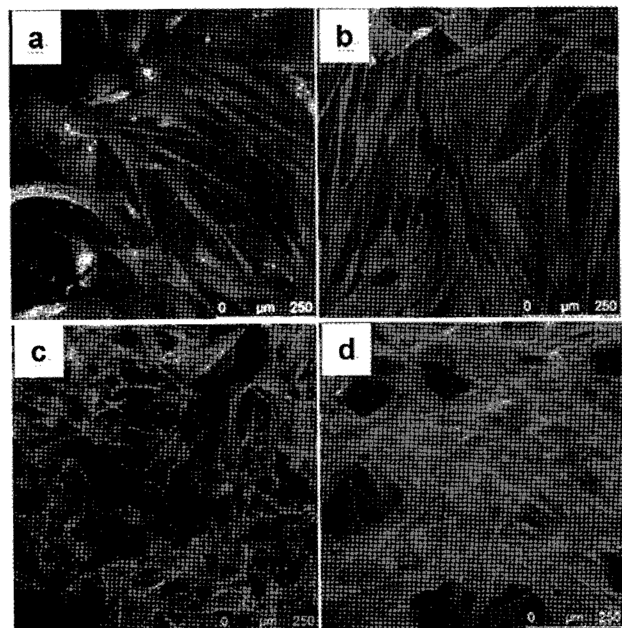

Fig. 5. Confocal microscopy images of HAECs cultured on each surfaces for 3days on sandblast cpTi (a), PU prelayered cpTi (b), PU microporous scaffold on cpTi without (c), and with collagen coating (d)

higher than sandblast cpTi in all microporous cases examined, there was no increased effect on percentage retention when comparing the collagen-coated surface to the uncoated surface. This is probably because many cells attached with collagen coating on the top wall zone where high shear stress occurs or weak bonding between collagen and porous PU layer.

After HAECs were seeded on each surface of sandblast cpTi, PU perlayered cpTi, and PU microporous layered cpTi without or with collagen coating according to the method previously mentioned, HAECs were cultured for 3 day in ECM medium. The morphology of HAECs was observed by confocal microscopy (Fig 5).

Confocal observation revealed that cells were distributed in the microporous layer, with more cells being observed compared to sandblast cpTi. The integration of endothelial cells into the pores of the porous surface is responsible for cell seeding efficiency after washing with medium due to low shear stress. Therefore, more HAECs were seeded on the PU microporous surface comprared to sandblast cpTi. It was shown that the endothelial cells spread into the underlying pores and became entrapped within these pores and formed a dense ECs layer.

4. Conclusions

Enhanced SCPL method was used to fabricate a microporous layer on the PU prelayered cpTi substrate. These thin scaffolds have shown open pores and a high porosity (~93%). Moreover, a dense surface skin layer, often observed in other solvent casting/salt leaching methods, was not found on the surface of the present porous layer. In the present study, the effectiveness of the microporous surface for cell retention was confirmed. The concept of combining a synthetic polymer scaffold with metallic substrate is very attractive and seems effective in forming an endothelial layer due to a high degree of cell attachment and cell retention compared to sandblast cpTi; however, the development of scaffolds allowing good cell proliferation remains a challenge.

Acknowledgement

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