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Collagen immobilized PVA hydrogel –hydroxyapatite composites for keratoprosthesis

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Because of the shortage of corneal grafts, the artificial cornea is one of the effective treatments to recover the corneal blindness. But the reliable artificial cornea is not yet developed. In this study, we are developing reliable artificial cornea by PVA hydrogel as a base polymer. We previously found that the poly(vinyl alcohol) (PVA) hydrogel prepared by low-temperature crystallization method had high transparency and excellent mechanical properties in spite of the high water content. However, the PVA hydrogel ranks most bioinactive polymer and has lowest affinity for supporting cell growth and adhesion. In order to improve the bioaffinity, the collagen-immobilized PVA hydrogel(PVA-COL) was synthesized. Moreover, PVA-COL-hydroxyapatite(PVA-COL-HAp) composite was also synthesized to improve the bioadhesion of the corneal device.

Key words: artificial cornea, poly(vinyl-alcohol), hydroxyapatite, collagen, composites

1. INTRODUCTION

Although more than twenty thousand people are estimated to require corneal transplantation per year in Japan, only fifteen to sixteen hundred patients can actually receive transplantation. The main reason for this gap is the shortage of donor eyes for transplantation. In the past 40 years, many researchers have attempted the development of keratoprosthesis, but the trials have frequently failed because of poor biocompatibility especially due to the weak adhesion between the host cornea and the prosthesis. To solve the problem, we have been developing a poly(vinyl alcohol)(PVA) hydrogel based keratoprosthesis[1-4]. During the course of this study, it was found that the optical part of the hydrogel keratoprosthesis should be covered by corneal epithelium to prevent bacterial invasion and to maintain a healthy tear film. We have found that the immobilization of type I collagen on the surface of the PVA hydrogel was effective in supporting adhesion and growth of rabbit corneal epithelium. However, the adhesion was not strong enough to prevent the down growth of the corneal epithelium. Hydroxyapatite(HAp) is well known as one of the best biocompatible materials and it has been applied for percutaneous devices. The result was very promising and the device can prevent epithelial down growth[5].

In this study, we try to synthesize the collagen-immobilized Poly(vinyl alcohol) hydorgel-hydroxyapatite(PVA-COL-HAp) composite on the periphery of the hydorgel disc to achieve the firm adhesion between the host corneal tissue and the keratoprosthesis. The preparation method, characterization, and the results of the corneal cell adhesion and proliferation on the composite material were studied

2. MATERIALS AND METHODS

2.1. Preparation of the collagen immobilized PVA (PVA-COL) hydrogel

The substrate PVA hydrogel was prepared as follows. PVA powder(Mw. 77,000, 99.9% saponification, purchased from Wako Pure Chemical Industries, LTD) was dissolved in a dimethyl sulfoxide-water (80-20) mixed solvent, and the resulting viscous solution was allowed to stand at –20 degree C for 24hr for setting a gel. The resulting PVA hydrogel was dehydrated by series ethanol and dried under the vacuum at 80 degree C for 1day for the further surface modification reaction. Isocyanate groups were first introduced onto the surface by the reaction between the surface OH groups of the PVA and the isocyanate group of hexamethylene diisocyantate(HMDI). The surface activated PVA immersed in the type I collagen solution(0.5mg/ml) to immobilized the collagen

on the surface of the PVA hydrogel. (Fig.1) Amount of collagen immobilized was determined by BCA protein assay kit to measure the absorbance(562nm) by using multi-plate Reader, GENios, TECAN Japan Co.Ltd, Japan.

2.2. Preparation of PVA-COL-HAp composite

According to the method of Taguchi et. al[5], hydroxyapatite was precipitated on the peripheral surface of the collagen-immobilized PVA hydrogel disc by the alternate soaking in two kinds of solutions, $CaCl_2$ (0.2M) and $NaHPO_4$ (0.12M), respectively.(Fig.1)

2.3. Characterization of the PVA-COL-HAp composite

The spatial distribution of the hydroxyapatite formed on the collagen-immobilized PVA hydrogel was analyzed using Scanning Electron Microscopic SEM(JSM5600LV, JEOL Co., Japan) with an energy-dispersion X-ray analyzer(EDX: JED2200,JOEL Co., Japan). Distribution of the calcium and phosphorous on the samples were measured by element mapping mode.

The crystal structure was determined with X-ray diffraction(XRD-PW1700, Philips Ltd., USA) using CuK α radiation generated at 40kV and 40mA; the range of diffraction angle 2 θ was 10.01-69.99deg. Composition of organic (collagen-immobilized PVA) and inorganic(hydroxyapatite) in the composites was determined with thermo gravimetric analysis(TGA) and differential thermal analysis(DTA)(Tg8120, Rigaku Co., Japan)

FT-IR diffuse reflectance spectra(Spectrum 2000, Perkin-Elmer Co. USA) was measured under nitrogen atmosphere after the samples were dried in vacuum chamber.

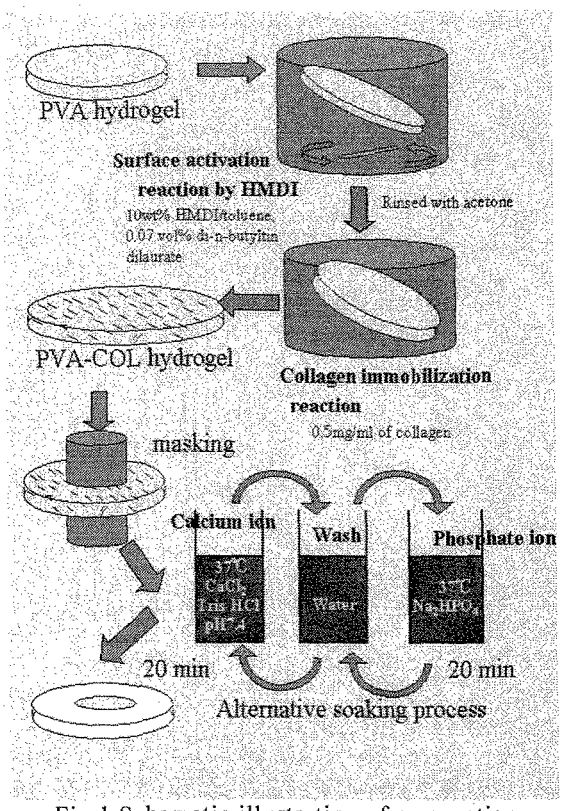

Fig.1 Schematic illustration of preparation method for PVA-COL-HAp

2.4. Cell attachment assay and proliferation assay

Cell attachment assay and proliferation assay were carried out using a chick embryonic keratocyte-like cell and an sv-40-immortalized human corneal epithelial cell line. 4-6 x 10^4cells/well and 1 x 10^4cells/well of each cell was seeded in each well of the 24-well cell culture dishes for cell attachment assay and proliferation assay, respectively.

3. RESULTS and DISCUSSION

The PVA hydorgel prepared by the low temperature crystallization method using DMSO/Water mixed solvent was transparent and had excellent mechanical properties. From the results of BCA protein assay, it was found that about 0.5 μ g/cm^2 of collagen was covalently immobilized on the surfaces of PVA hydrogel. Even after the collagen immobilization reaction, the PVA hydrogel was kept optically clear. The transmittance of visible light was more than 99%.

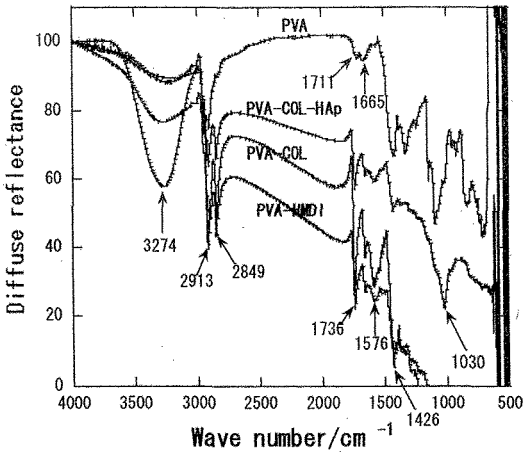

Fig.2 FT-IR spectra of PVA, PVA-HMDI, PVA-COL, and PVA-COL-HAp.

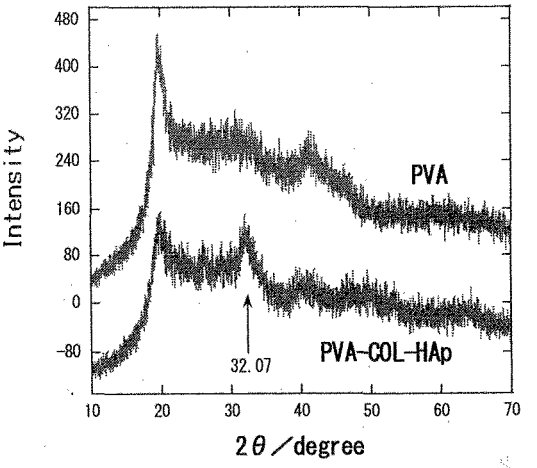

Fig.3 XRD pattern of PVA and PVA-COL-HAp

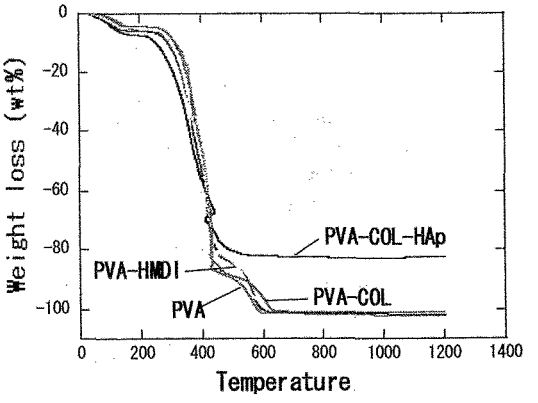

Fig.4 Measurement of the inorganic contents of PVA, PVA-HMDI, PVA-COL, and PVA-COL-HAp using TG-DTA.

Fig.2 shows the FT-IR spectra of PVA, PVA-HMDI, PVA-COL, and PVA-COL-HAp. For the PVA spectra, very strong and wide absorption band was observed around 3274 cm^{-1}which was assigned to hydroxyl group of PVA. On the contrary, the absorption bands become weak for the spectra of PVA-HMDI, PVA-COL, and PVA-COL-HAp. It seems to be reasonable that hydroxyl group of the PVA surface decreased with the proceed of the surface modification. At the same time, the absorption bands assigned to methylene(-CH_2- ;observed 2913, 2849cm^{-1})group and amide I(C=O; 1665 cm^{-1}) and amide II(-NH; 1556 cm^{-1}) groups increased for the spectra of PVA-HMDI and PVA-COL. For the spectrum of PVA-COL-HAp, the absorption bands assigned to methylene and amide I and amide II become weak, and the new absorption band appeared at 1030 cm^{-1} assigned to low crystalline hydroxyapatite. These results suggested that the PVA-COL and PVA-COL-HAp were successfully synthesized.

The results of EDX analysis by element mapping mode(the results did not show), calcium and phosphorous was clearly observed on the periphery of the PVA-COL hydrogel disc. This result suggested that the calcium phosphate was successfully introduced just on the periphery part of the PVA-COL. Fig.3 shows XRD pattern of PVA and PVA-COL-HAp. The results of XRD also suggested that hydroxyapatite was successfully introduced on the periphery of the PVA-COL hydrogel, and the degree of crystallinity was relatively low. The result was consistent with the results of FT-IR measurement.

The TGA-DTA measurements of the PVA, PVA-HMDI, PVA-COL, and PVA-COL-HAp were carried out using the samples dried up. The results were shown in Fig.4. It was determined that the inorganic (hydroxyapatite) content of the PVA-COL-HAp composite was about 20wt %/ PVA-COL dry weight. The water content of the PVA-COL hydrogel is about 80%. Therefore the inorganic content of the PVA-COL-HAp composite hydrogel is calculated roughly at 4wt% in wet state.

Cell attachment and proliferation assays were carried out. The results of Keratocyte-like cells seeding after 3days were shown in Fig.5. No cell attachment and proliferation was observed on the PVA and PVA-HMDI. In contrast, keratocyte-like cells were attached on the PVA-COL and PVA-COL-HAp disc. And after 3days, the seeded cells proliferated well on the PVA-COL and PVA-COL-HAp. This result suggested that both PVA-COL and PVA-COL-HAp have an affinity toward the corneal stroma. An sv-40 immortalized human corneal epithelial cell was also well attached and proliferated on the

PVA-COL and PVA-COL-HAp(results were not shown). This result suggested that the both PVA-COL and PVA-COL-HAp composite are compatible for the corneal epithelial cells.

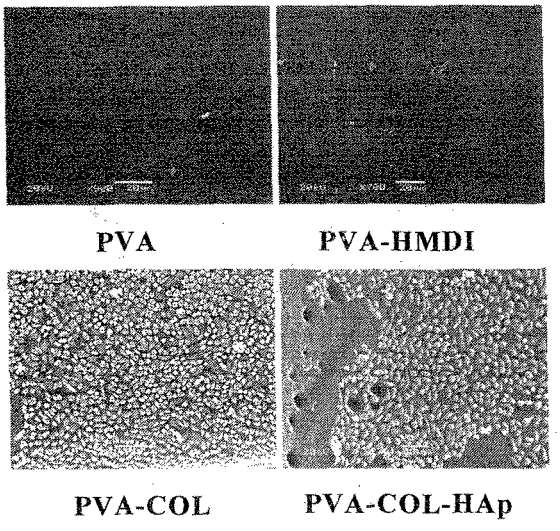

Fig5. Scanning electron microscopic observation of Keratocyte like cells adhesion on the PVA, PVA-HMDI, PVA-COL, and PVA-COL- HAP. 3 days after cells seeding.

4. CONCLUSION

Collagen immobilized Poly(vinyl alcohol) hydrogel-hydroxyapatite composite was successfully synthesized. This composite shows an affinity toward the corneal cells such as stromal cell and epithelium in vitro. Therefore we concluded this material have a great potential for keratoprosthesis application. Further study such as long term animal study is required.

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