Preparation of sacran / collagen complex for cell adhesion gel

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1. Introduction

Polysaccharides sacran obtained from the extracellular matrix (ECM) of *Aphanothece sacrum* is a Japan-specific blue-green algae(Fig.1, 2)[1]. Sacran has the properties such as superabsorbency[2], antiallergic[3], metal adsorption[4], self-orientation[5] etc. It has been prepared using chemical cross-linking method[6] and physical crosslinking method the hydrogel of sacran in this laboratory[7]. Among them, a gel was prepared using the physical crosslinking method is found to have a specific anisotropy. It was considered whether it was possible to make a complex with the collagen from this research with sacran with these peculiar anisotropy and apply to a cell culture. Because sacran does not have an RGD sequence (Arg-Gly-Asp) is a cell adhesion factor, and mixed sacran and collagen to form a film by drying, and by performing a cell culture as a scaffold applicability it was examined.

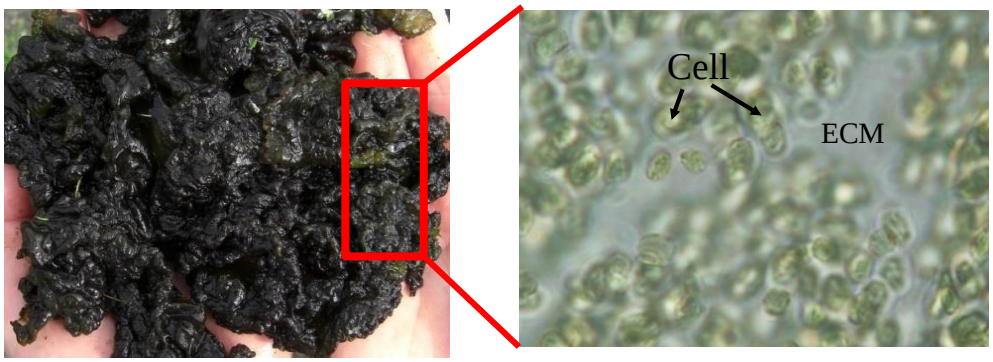

Fig.1 Microscope photograph of *Aphanothece sacrum*.

Fig.2 Sacran dry sample what extracted from *Aphanothece sacrum*.

2. Experimental

2-1 Preparation of sacran / collagen complex

The sacran was dissolved in water to prepare a 0.5wt% aqueous solution. Pour sacran solution in a container, after it has been sufficiently cooled in an ice-bath, respectively, it was gradually added to the collagen in the vessel containing the sacran aqueous solution, and mixed. Mixed solution was dried at 35℃ and to form a film.

2-2 Preparation of sacran / collagen complex in NaCl solution

The sacran was dissolved in water to prepare a 0.5wt% aqueous solution. Sacran and collagen solution were diluted with 0.6 M NaCl aqueous solution double. Pour sacran solution in a container, after it has been sufficiently cooled in an ice-bath, respectively, it was gradually added to the collagen in the vessel containing the sacran aqueous solution, and mixed. Mixed solution was dried at 35℃ and to form a film. Then, to remove the salt by immersing the film in water.

2-3 Measurement of the physical properties of sacran gels

2-3-1 Instrumentation of degree of swelling of sacran gels

Cast film and sacran gels were weighed and instrumentation of swelling degree by followingequation. I calculated the swelling degree, *q*, as a weight ratio.

$$q = \frac{W_{sw}}{W_d}$$

q : swelling degree （mg / mg）

W_{sw} : weight of gels （mg）

W_d : weight of film （mg）

2-3-2 Instrumentation of anisotropy of the sacran gels

The side of sacran gels and sacran film were measurement. And instrumentation of anisotropy by following equation.

$$Anisotropy = \frac{\alpha_Z}{\alpha_{x,\,y}}$$

$\alpha_{x,y}$: $\{(a_1-a_0)+(b_1-b_0)\}/2$

a_1 : short side of sacran gels (mm)

b_1 : long side of sacran gels (mm)

a_0, b_0 : side of sacran film (mm)

α_z : c_1 / c_0

c_1 : thickness of sacran gels (mm)

c_0 : thickness of sacran film (mm)

2-3-3 Measurement of mechanical modules of the sacran gels

Sacran gels was measured of mechanical modules by machine of mechanics compression test.

2-3-4 Instrumentation of M_c of the sacran gels[8]

M_c is molecular weight between the cross-point in gels. M_c was instrumented by following equation.

$$M_C = 3\frac{d}{q}\frac{RT}{K}$$

M_c : molecular weight between the cross-point (g /mol)

d : density of gel (10^3 g / m^3) [Approximated by the density of water]

q : degree of swelling of sacran gels (mg / mg)

R : gas constant (8.31 m^2 kg s^{-2} K^{-1} mol^{-1})

T : room temperature (27 ºC, 300 K)

K : mechanical modules of sacran gels (kPa)

2-3-5 FT-IR

Spectra were measured prepared gel between 400-4000 cm^{-1}.

2-4 Cell culture

Sacran / collagen composite was immersed for 24 hours in 70% ethanol and sterilized. Sterile film 24 hour immersion in 10% fetal bovine serum-containing Dulbecco's medium (DMEM), punching the swelling after 20mmΦ, were transferred to 24-well culture plates. Human mesenchymal stem cells (hMSC) were seeded 50000, 24 hours of culture the medium is added 1mL, stained living cells CalceinAM, and observed under a fluorescence microscope.

3. Results and discussion

3-1 sacran / collagen complex

The sacran / collagen mixed solution was observed with a polarizing microscope(Fig.3). Aggregates of fibrous has been confirmed in the figure on the arrow. This is considered to be a complex of sacran and collagen, it is believed to have been formed into fibers by shear stress due to stirring immediately after mixing.

Further, drying the solution, a film was observed with similarly polarizing microscope that allowed to absorb water(Fig.4). Substantially, it can be confirmed that not formed oriented it can be confirmed that it could be confirmed that different portions of the alignment direction inside the film (in the circle) are present uniform plane orientation direction of the arrow . It can not aggregates of the too large fibrous fall, presumably because that could not be exhibited domino effect. Further, aggregates of fibrous could be confirmed as shown in the figure in the red line. Therefore, the orientation of the gel, orientation of some fibrous aggregate produced by the shear stress at the time of mixing and stirring is thought to be due to deposited in the plane direction by drying and concentration.

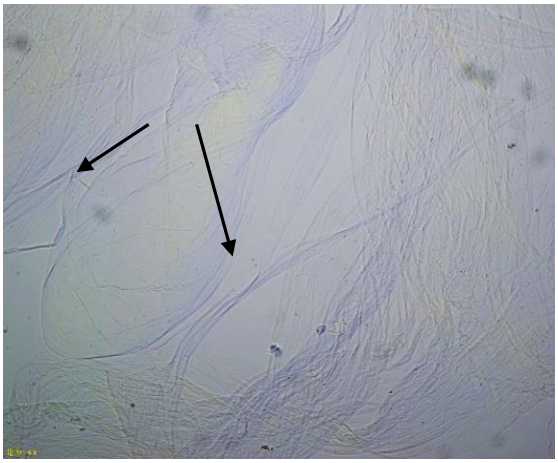

Fig.3 Polarized light microscope image of the mixed solution.

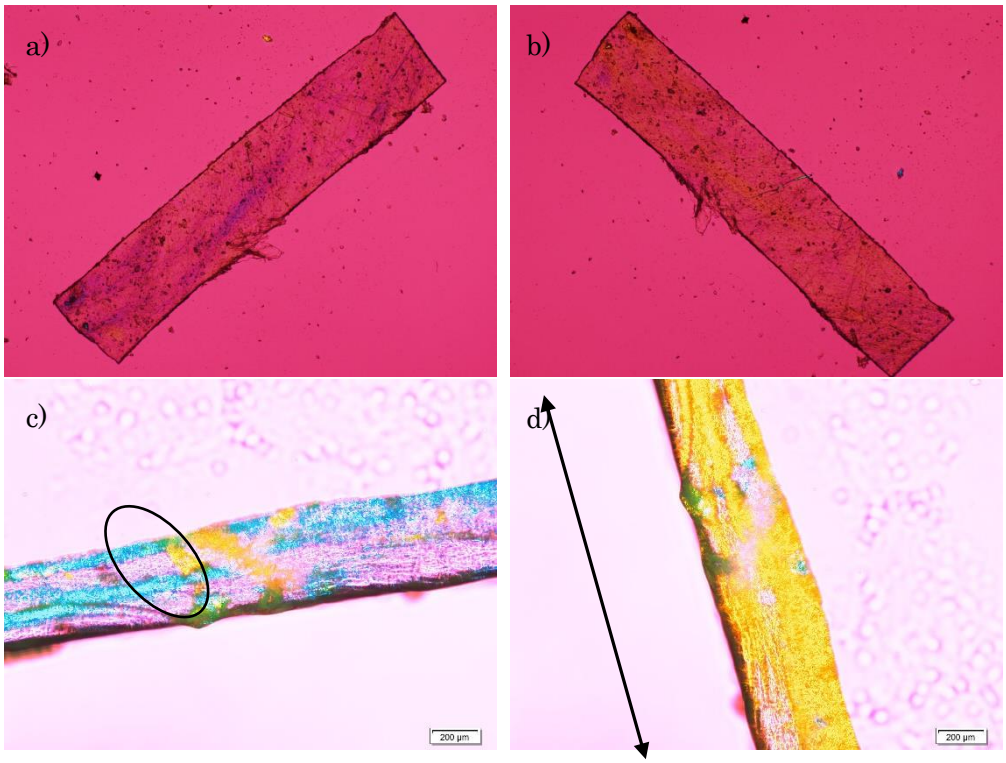

Fig.4 Polarization microscopy image of sacran / collagen film (1 hour after water absorption).
a) Top1, b) Top2, c) Side1, d) Side2
(OLYMPUS BX51)

3-2 sacran / collagen complex in NaCl

It was added and mixed salts for controlling the formation of the above aggregates. The results are shown below (Fig.5). Aggregates in 0.1 M and 0.2 M has been confirmed. On the other hand, agglomerates in the 0.3 M and 0.4 M could not be confirmed. It is considered complex was not formed to ions generated by the dissolution of the NaCl was cancel the charge of sacran and collagen[9]. Further, collagen had salted out in 0.5 M or more.

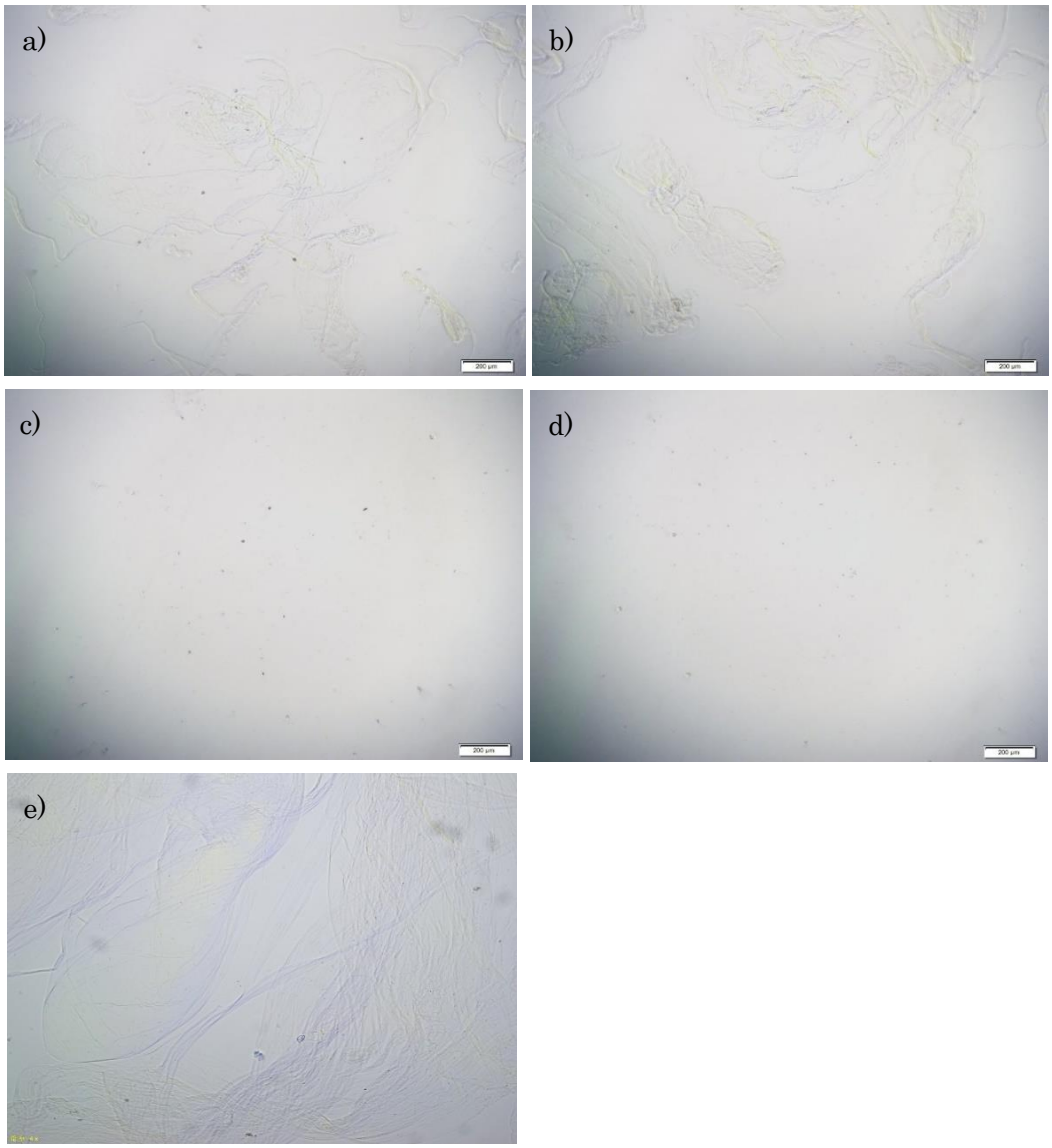

Fig.5 Polarization microscopy image of sacran / collagen mixed solution.
a) 0.1 M NaCl, b) 0.2 M NaCl, c) 0.3 M NaCl, d) 0.4 M NaCl, e) non NaCl
(OLYMPUS BX51)

After leaving the dry film it was confirmed oriented in a polarization microscope(Fig.6). By sample is also rotated, the change in hue of the transmitted light of a portion of the upper surface was confirmed. Therefore, the surface of the complex was confirmed to be partially oriented. Further, the change in hue as well transmitted light even with the side surface was confirmed. However, since it does not have any change in the uniform color, it is considered that disturbed slightly oriented. It can be said that was successful in the control of the aggregate by salt added since the aggregates of fibrous can not be confirmed from the image.

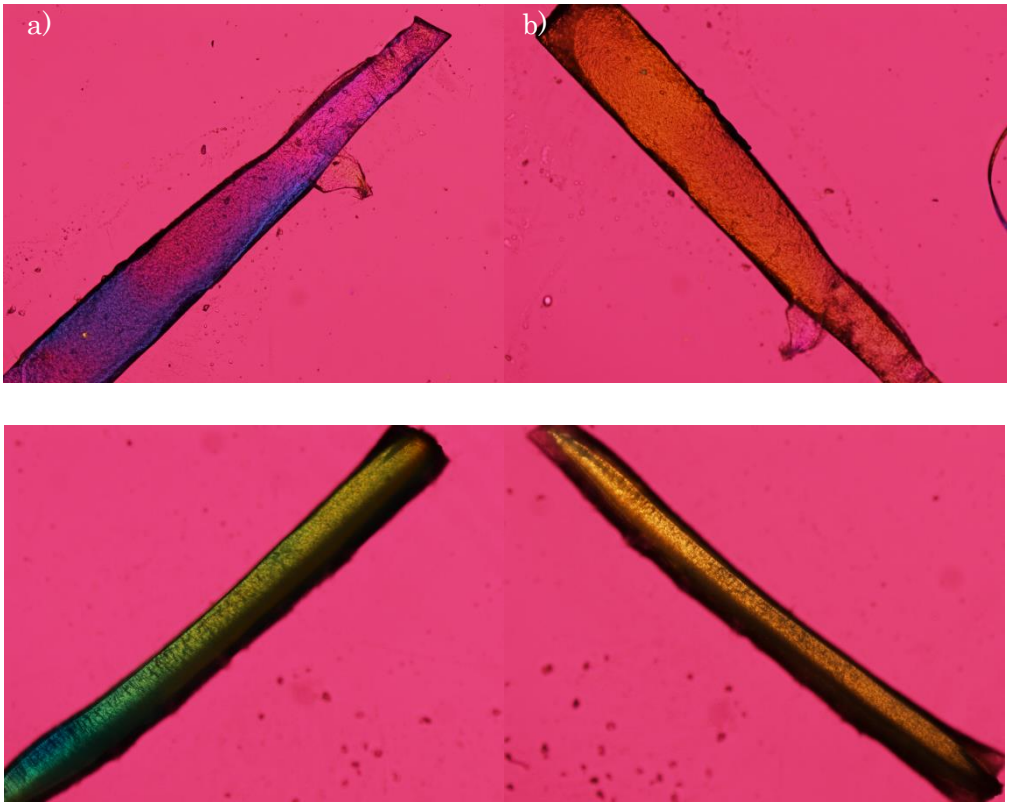

Fig.6 Polarization microscopy image of sacran / collagen complex (sacran: collagen ratio of 1: 1).
a) Top1, b) Top2, c) Side1, d) Side2

3-3 Physical properties

The measurement results of physical properties are shown below (Table.1).The degree of swelling was significantly increased when the sacran amount is reduced. Further it was confirmed that greatly reduce the degree of swelling without heating by adding collagen when compared with sacran single physical gel. Further, sacran: collagen 2: 1, and sacran single physical gel at low gel strength due to the high degree of swelling, anisotropy, compression modulus of elasticity, it was not possible to measure between crosslinking points molecular weight. Large swelling because the feature is lost that contribute carboxyl group and sulfuric acid group in the water absorbing capacity by the composite and collagen is considered to have reduced.

Table.1 The physical properties of the complex

	swelling degree	anisotropy	mechanical modules /KPa	molecular weight between the cross-point /g·mol^{-1}
sacran：nativecollagen 1:1	10	9.3	108	7.5
sacran：atelocollagen 1:1	61	46	5	29
sacran：nativecollagen 2:1	305	—	—	—
sacran：atelocollagen 2:1	304	—	—	—
sacran single physical gel 60℃	1110	—	—	—

3-4 FT-IR

The measurement results of FT-IR is shown below(Fig.7). 3300 cm^{-1} and 3088 cm^{-1} peak of NH. 1637 cm^{-1}(Amide Ⅰ),1545 cm^{-1}(Amide Ⅱ),1237 cm^{-1}(Amide Ⅲ) is a unique peak in collagen. Amide Ⅲ peak is a peak derived from the triple helix structure of collagen. Normally, when a triple helix structure of collagen in the thermal denaturation has been destroyed, this peak is reduced or lost. Triple helix structure of collagen since it alone and neither decrease was observed by comparing the composite are held(Fig.8). Collagen peak was confirmed to 1537 cm^{-1}. Complexes were confirmed to be red-shifted about 8 ~ 9 cm^{-1}(Fig9, 10). The results show that sacran and collagen are complexed.

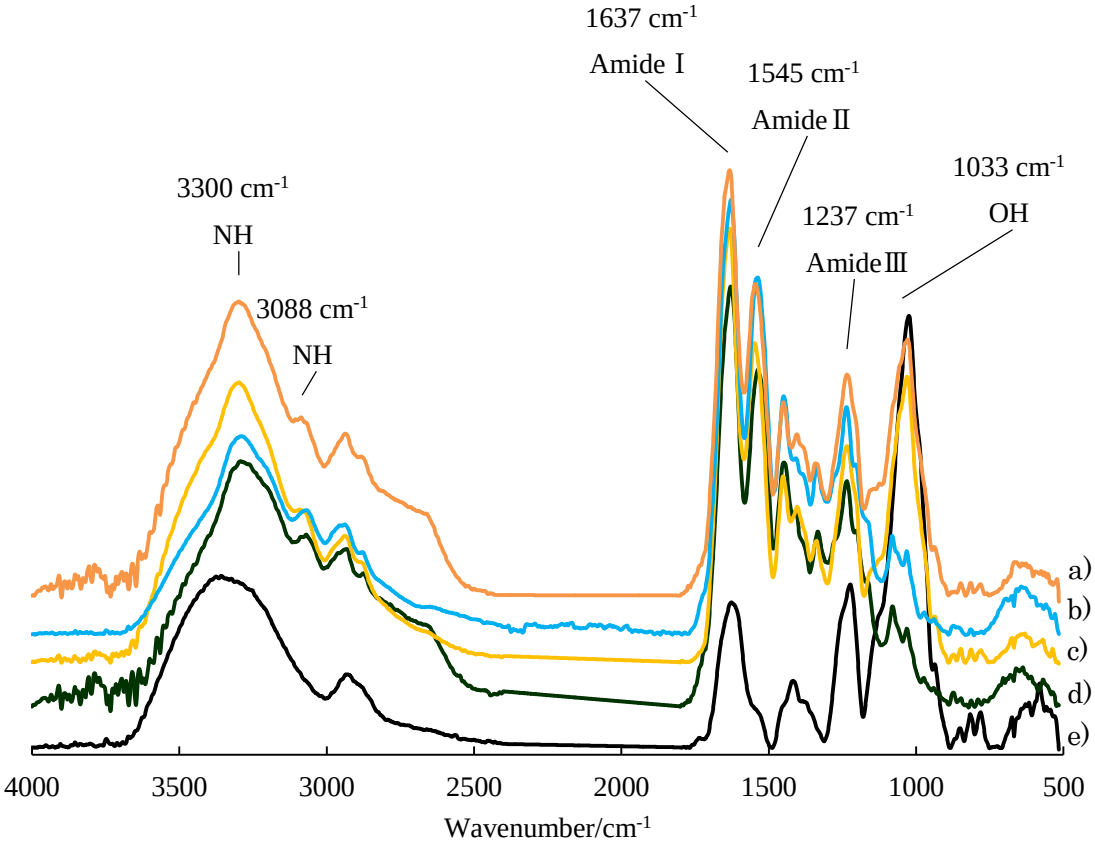

Fig.7 IR spectrum of sacran/collagen.

a) sacran:nativecollagen, b)nativecollagen, c) sacran:atelocollagen, d)atelocollagen, e)sacran

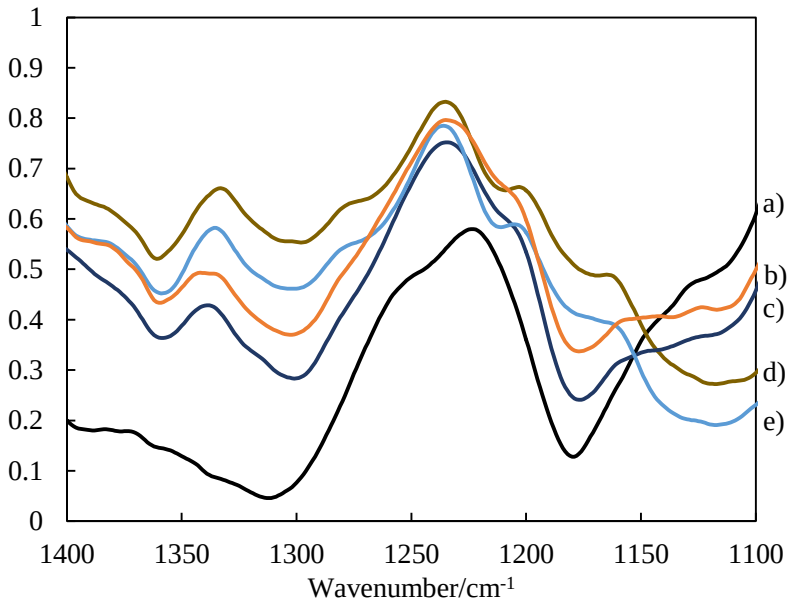

Fig.8 IR spectrum of sacran/collagen. (Amide Ⅲ peaks)

a)sacran, b) sacran:nativecollage, c) atelocollagen, d) sacran:atelocollagen, e)nativecollagen

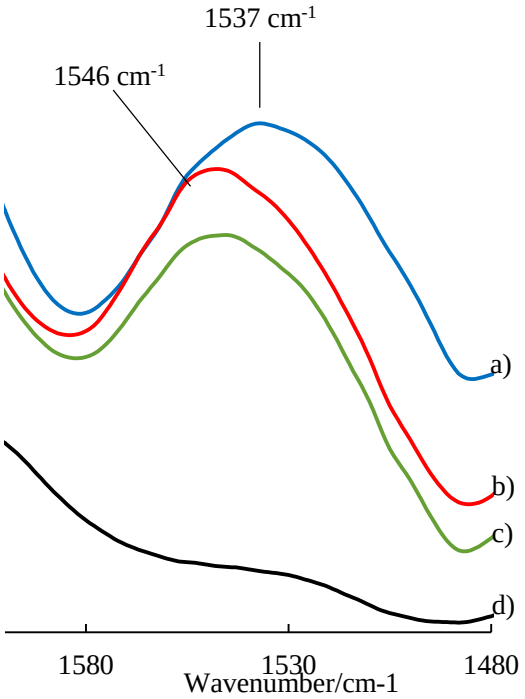

Fig.9 IR spectrum of sacran/atelocollagen

a) atelocollagen, b) sacran:ateloecollagen 1:1, c) sacran:atelocollagen 2:1, d) sacran

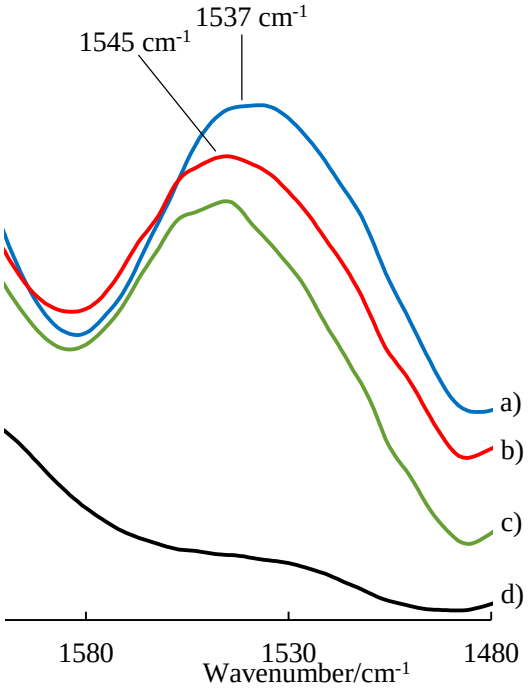

Fig.10 IR spectrum of sacran/nativecollagen.

a) nativecollagen, b) sacran:natinecollagen 1:1, c) sacran:nativecollagen 2:1, d) sacran

3-5 Cell culture

The results of the cell culture is shown below (Fig.11).Even compared the culture dish and sacran / collagen gel was no difference in the adhesion and spreading. Also, when compared to the sacran single physical gel, sacran single physical gel although cells are adhesion growth, it has been confirmed those are many that are not spreading. This is considered a result of the introduction of the RGD sequence by collagen, complexation with collagen was confirmed to be effective in cell culture.

The orientation of the cells was not confirmed even in a complex and, single physical gel.

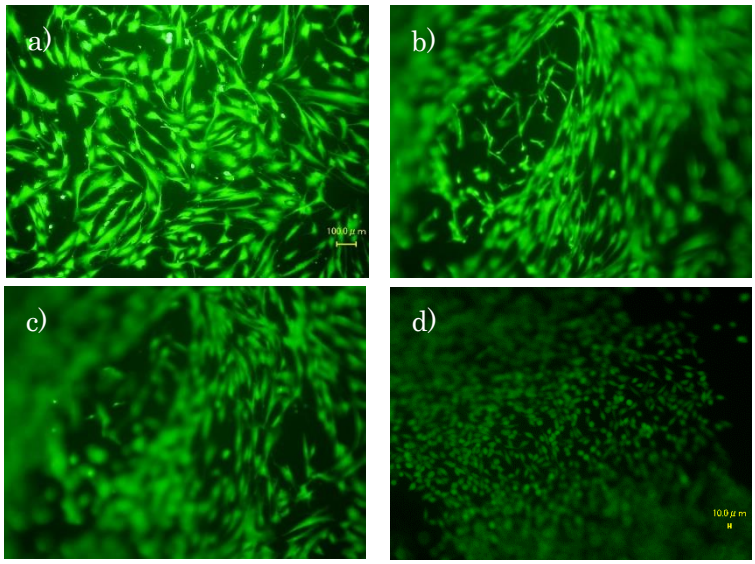

Fig.11 Observation image of the cell culture

a) Culture dish, b) sacran/collagen gel (Focus:left), c) sacran/collagen (Focus:right), d) sacran single physical gel(100℃）

4. Conclusion

In this study, the aim of the preparation of cell scaffold, was tried to prepare for use cell adhesion gel the electrostatic interaction of sacran and collagen. Thus, we succeeded in creating had orientation gel. In addition, it was found that it is possible to control the initial gelation behavior by the addition of salt. This gel by reducing the amount of sacran, swelling is reduced. This is because the effect of contributing to the carboxyl group and sulfate group to the degree of swelling sacran is lost by compounding collagen. In addition, sacran and collagen has been found that it is complexed from FT-IR results. In addition, it was found that the triple helix structure of collagen by composite is held. As a result of the cell culture with sacran / collagen gel. Culture dish similar adhesion and spreading could be confirmed. From this fact, it was found that can be applied to the cell scaffold. Further, results of comparison with sacran single physical gel, differences in adhesion was observed. However, sacran single physical gel is extensibility was inferior than sacran / collagen gel. From this fact, it is a composite of the sacran and collagen was found to be effective in cell culture. Also, it could not be confirmed with respect to the orientation of the cell.

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