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(54) **HYDROGEL THIN FILM CONTAINING EXTRACELLULAR MATRIX COMPONENTS**

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(57) **ABSTRACT**

The thin film of the invention comprises a hydrate of a vitrified gel containing one or more extracellular matrix components, which can be integrated with a retainer as required. A hydrogel thin film containing one or more extracellular matrix components such as thin-film collagen hydrogel thin film, which is useful for a cell culture substratum and for preventing organ adhesion, can be easily prepared, and is excellent in expediency.

Fig. 1

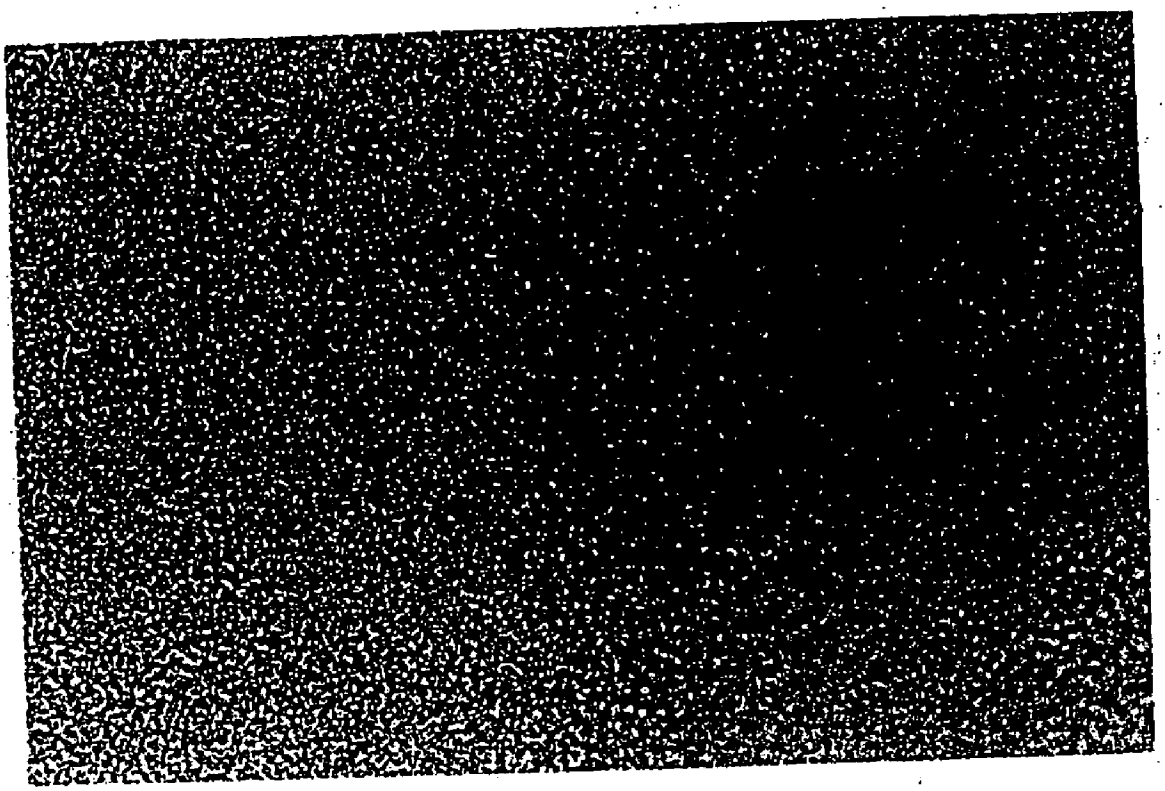


Fig. 2

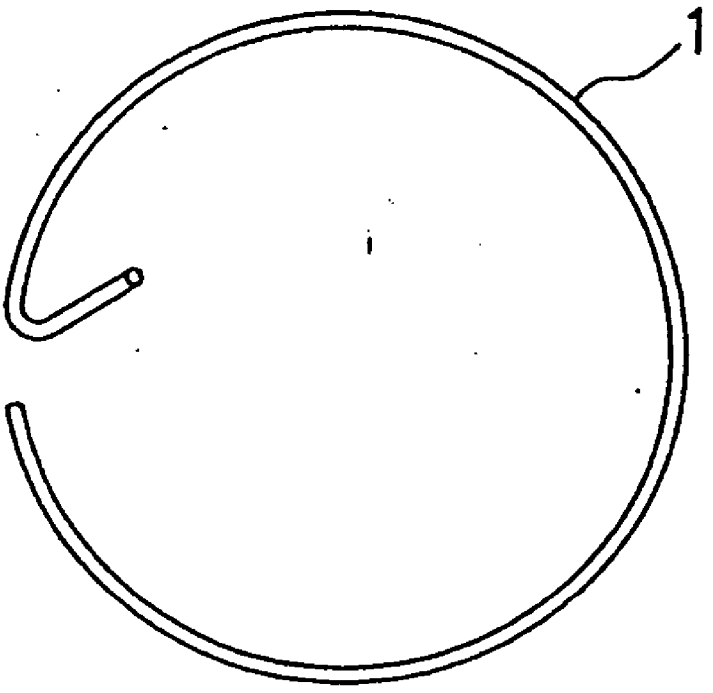


Fig. 3

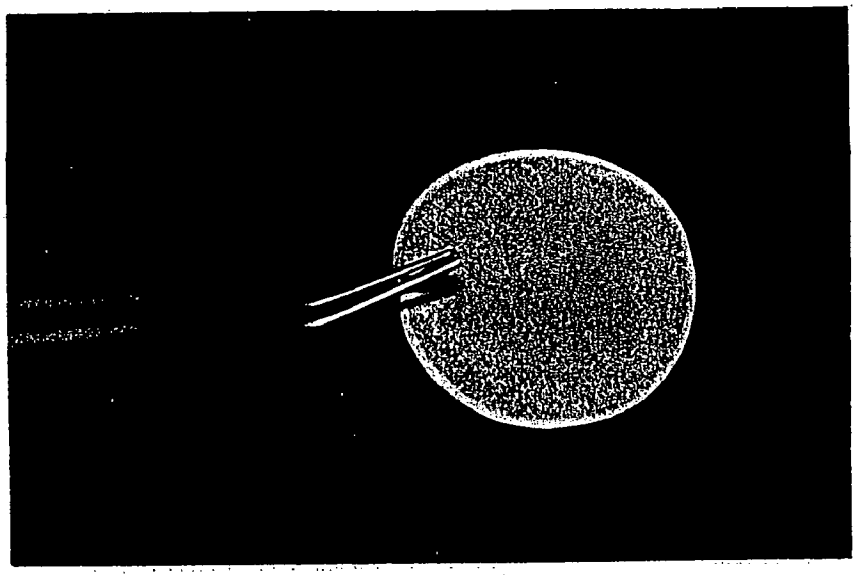


Fig. 4

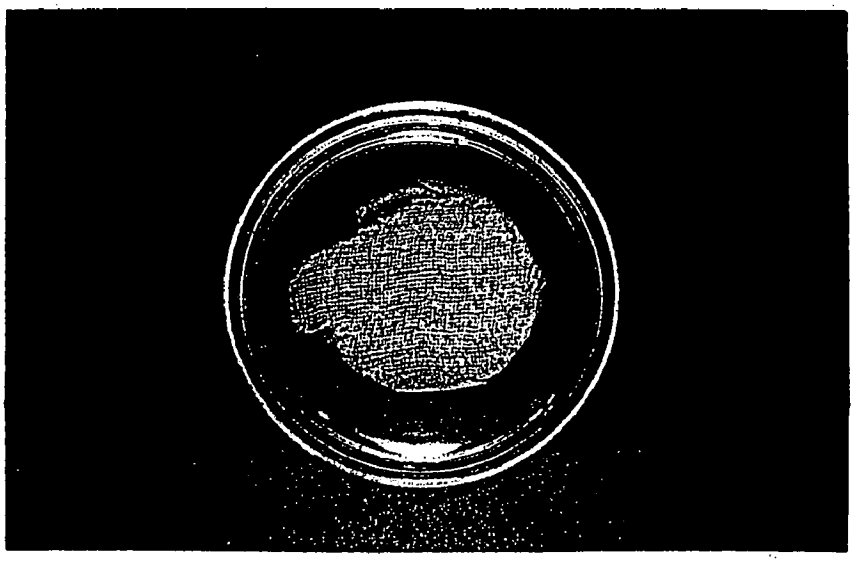


Fig. 5

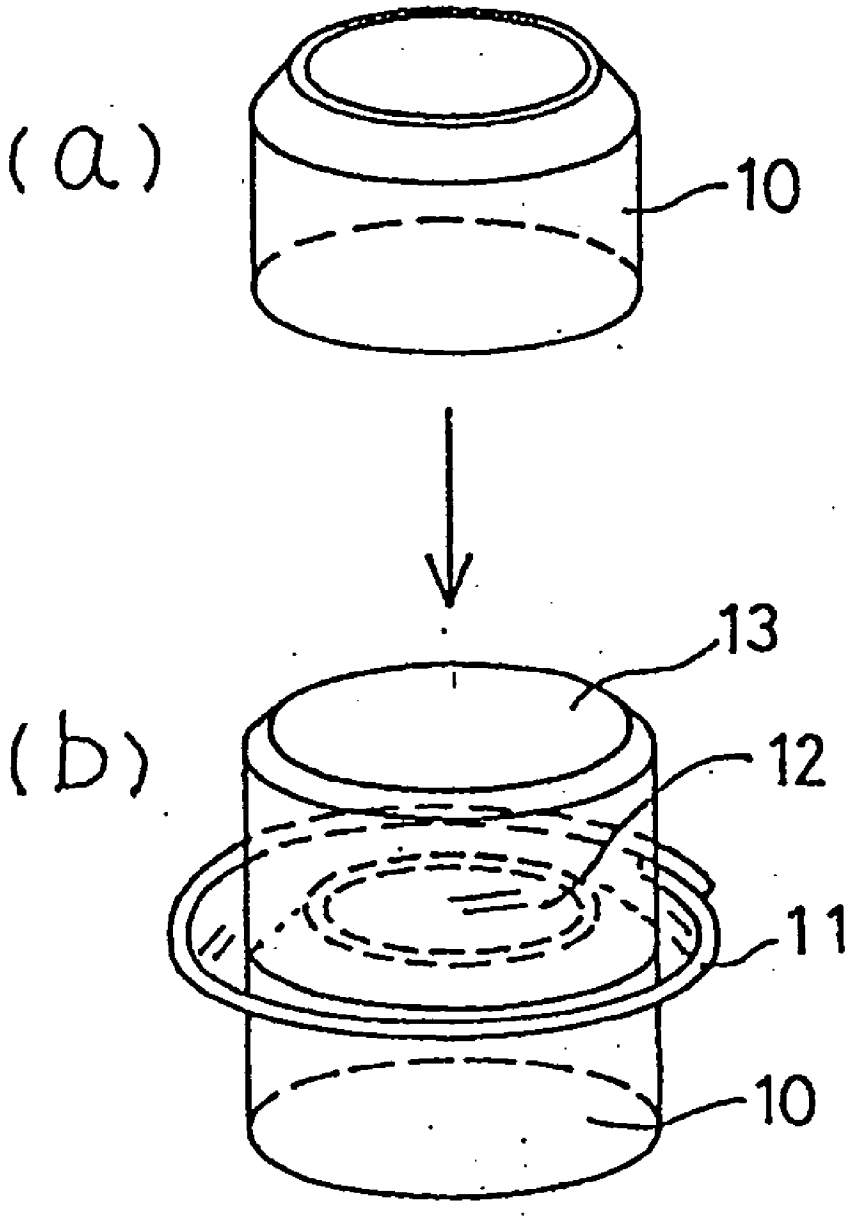
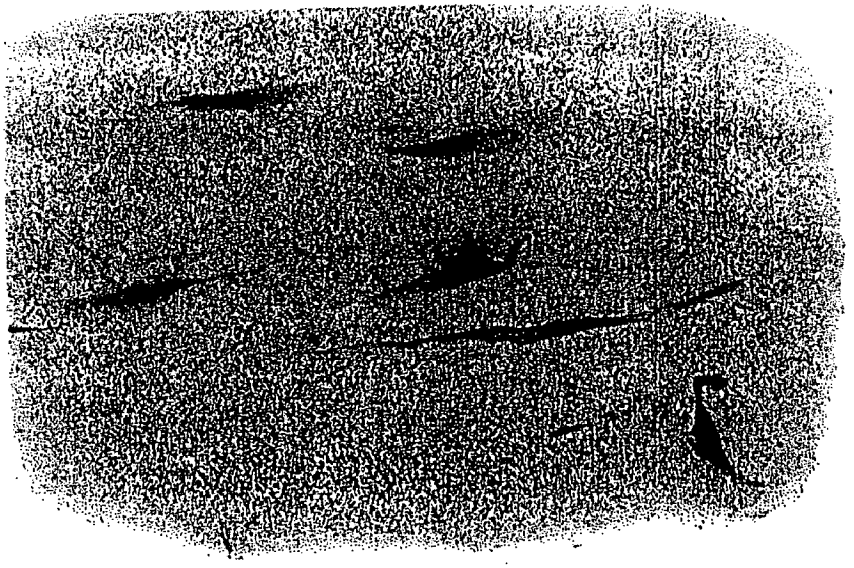


Fig. 6

Fig. 7



HYDROGEL THIN FILM CONTAINING EXTRACELLULAR MATRIX COMPONENTS

FIELD OF THE INVENTION

[0001] The present invention relates to a hydrogel thin film containing an extracellular matrix components More particulary, the present invention relates to a novel hydrogel thin film containing an extracellular matrix components, which is useful for a cell culture substratum and prevention of organ adhesion, has an appropriate elastic strength, can be easily prepared and can be used simply.

PRIOR ART AND PROBLEMS

[0002] Cell culture has conventionally been carried out in various manners for various purposes including development of various medical techniques and various medical drugs.

[0003] A method using an extracellular matrix components such as collagen is known as a method for cell culture. In this method, in the case of collagen for example, various trial efforts have been made to ensure a three-dimensionality as closest as possible to forms of biological tissues or functional expression by alleviating restrictions imposed as a two-dimensional planar culture for ordinary cell culture.

[0004] Regarding the culturing methods using collagen or the like attracting the general attention as to usage thereof, however, in the case of collagen hydrogel for example, there is a problem of difficulty in handling the collagen hydrogel itself because of the softness. It is not therefore easy to prepare a cell culture substratum, and a more simple method for utilization has not as yet been established.

[0005] Under such circumstances, the present inventors have carried out studies from various points of view regarding utilization of an extracellular matrix components such as collagen. The object of these studies was to improve the conventional culturing method, and to achieve a method for using a new matrix substance, which would permit easier preparation of a cell culture substratum, be simply applicable. Provide satisfactory performance as a culture substratum, and be applicable also for preventing organ adhesion.

SUMMARY OF THE INVENTION

[0006] As means to solve the foregoing problems, the present invention provides a hydrogen thin film containing an extracellular matrix components, which is a thin film comprising a hydrate of a vitrified matrix gel containing an extracellular matrix components (claim **1**).

[0007] The present invention provides also embodiments wherein the hydrogel thin film contains a plurality of extracellular matrix components (claim **2**), wherein the extracellular matrix components is collagen (claim **3**), wherein the hydrogel thin film has a cell culture medium components (claim **4**), and wherein the thin film is integrated with a retainer (claim **5**). There are provided embodiments of the retainer, wherein the retainer is an annular or a mesh shape (claim **6**), wherein the retainer comprises a biological absorbing substance(S) (claim **7**), and wherein the retainer has a circular opening which comprises a cylinder retaining and integrating the thin film (claim **8**).

[0008] The present invention provides also a glass-like substance of a arled gel containing an extracellular matrix components as a precursor of the foregoing hydrogel thin film containing an extracellular matrix components.

[0009] The present invention further provides, regarding the foregoing hydrogel thin film containing an extracellular matrix components, a method comprising the steps of preparing the gel from a solution containing the extracellular matrix components, drying the resultant gel for vitrification thereof, and hydrating the vitrified gel (claim **10**), and a method in which a hydrogel thin film containing an extracellular matrix components is used as the cell culture substratum (claim **15**).

[0010] The present invention further provides a method of culturing cells using the foregoing substratum.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] **FIG. 1** shows a phase-contrast microphotograph illustrating the surface of a collagen hydrogel thin film as a reference;

[0012] **FIG. 2** shows a perspective view illustrating a circular wire serving as a retainer;

[0013] **FIG. 3** shows a photograph showing a collagen hydrogel thin film substratum integrated with a circular wire retainer;

[0014] **FIG. 4** shows a photograph showing a collagen hydrogel thin film embedded gauze;

[0015] **FIG. 5** shows a process diagram illustrating preparation of the substratum of the present invention using a cylinder as the retainer;

[0016] **FIG. 6** shows a microphotograph showing formation of a colony for MEC; and

[0017] **FIG. 7** shows a microphotograph. showing the case of HDF.

[0018] In the drawings, the reference numerals represent the following components:

[0019] **1**: Circular wire retainer

[0020] **10**: Cylindrical appliance

[0021] **11**: Circular wire retainer

[0022] **12**: Collagen hydrogel thin film

[0023] **13**: Cover

DETAILED DESCRIPTION OF THE INVENTION

[0024] As described above, the hydrogel thin film containing an extracellular matrix components of the present invention, as the thin film itself and as that integrated with the retainer, has an appropriate elastic strength and a thin film shape permitting easy handling, and enables achievement of a biochemical composition as a cell culture substratum, thus providing easy preparation for culturing and an excellent expediency.

[0025] Collagen is a representative extracellular matrix components and its application is attracting the general attention. Applicable extracellular matrix components other than collagen include fibronectin, vitronectin, laminin, proteoglycan, glycosaminoglycan, and MATRIGEL (brand name) and may appropriately be used.

[0026] There is no particular restriction on the cell culture medium components, and optimum salt composition, concentration and pH are selected.

[0027] An annulus made of a wire or a metal line, or a mesh comprising gauze or other woven stuff may be appropriately used as a retainer. A biological absorbing substance may also be applicable. It suffices to select a shape, a size and a material in response to the manner of use. The retainer may be a cylinder having a circular opening, or a container as a precursor thereof.

[0028] Manufacture of a cell culture substratum comprises the steps of first mixing an aqueous solution of a matrix such as collagen with a composition having a medium or serum, placing any of various retainers as described above into this mixed solution, and incubating it at a suitable temperature for its gelation.

[0029] The resultant gel is vitrified by drying by air for example. This vitrification phenomenon, utilization of the thus vitrified gel after further modification, and use of the modified gel after vitrification as a cell culture substratum are not known. but disclosed for the first time in the present invention.

[0030] More particularly, in the manufacturing method of the present invention, the vitrified gel containing an extracellular matrix components such as collagen is hydrated. This provides a hydrogel thin film containing an extracellular matrix components having a satisfactory elastic strength, which serves as a cell culture substratum and is useful for preventing organ adhesion.

[0031] For vitrification, in the present invention, it is the general practice to slowly and completely dry the gel containing the extracellular matrix components having a terminal concentration in an aseptic manner (for example, through aseptic air drying), thereby vitrifying the gel. Hydration of the vitrified gel can easily be effected by treating it by PBS, for example.

[0032] The construction and the functions of the present invention will now be described below further in detail by means of examples involving collagen.

EXAMPLES

Example 1

Preparation of Collagen Hydrogel Thin Film

[0033] 2 ml of quintuple-concentration Dulbecco's Modified Eagle Medium (GIBCO #31600-034), 0.1 ml of 10,000 units/ml penicillin and 10,000 μg/ml streptomycin (GIBCO #15140-031), 0.2 ml of 1M HEPES (GIBCO #15630-023), 0.493 ml of 7.5% sodium bicarbonate solution (GIBCO #25060-011), 1.407 ml of distilled water, and 1 ml of fetal bovine serum were put in a sterilized conical tube (Falcon #2070) chilled on ice, having an inner volume of 50 ml, and mixed. Then 4.8 ml of 0.5% aqueous type-I collagen solution -(CELLGEN I-AC or I-PC, made by Koken Company) was added into the tube and uniformly mixed. After placing 2 ml of the mixed collagen solution having a final concentration of 0.24% in a culture dish made of hydrophobic Polystyrene (ϕ35 mm; Falcon #1008), the solution was held in a humidified incubator at 37° C. in the presence of 5% CO_2/95% air in 3 hours for its gelation.

[0034] This collagen gel of the final concentration of 0.24% was vitrified by completely air-drying slowly in an aseptic manner in the covered dish. The vitrified collagen gel was hydrated by adding 2 ml of PBS. The collagen gel thus hydrated was rinsed with 2 ml of PBS several times. The hydrated collagen gel was peeled off from the dish and collected by tracing the inner wall of the dish with a sharp-end pincette along. the periphery, in the form of a thin-film collagen hydrogel having a satisfactory elastic strength, as shown in the phase-contrast microphotograph in **FIG. 1**.

Example 2

Preparation of Collagen Hydrogel Thin-Film with Peripheral Wire Retainer

[0035] A circular retainer (**1**) with a knob as shown in **FIG. 2** was made with a stainless steel wire (size: #20; 0.9 mm), and 2 ml of the mixed collagen solution having a final concentration of 0.24% prepared in Example 1 was placed, together with this wire retainer, into a hydrophobic polystyrene culture dish (ϕ35 mm; Falcon #1008). In the same manner as in example 1, the colagen solution with the retainer was vitrified ater its glation. The vitrified collagen gel was hydrated by adding 2 ml PBS. Further. the collagen gel thus hydrated was rinsed several times with 2 ml of PBS. The hydrated collagen gel could be peeled off and collected from the dish by slowly lifting the stainless steel knob, in the form of a thin-film collagen hydrogel having a satisfactory elastic strength w1th a wire retainer surround it as shown in **FIG. 3**.

Example 3

Preparation of Gauze Embedding Type Collagen Hydrogel Thin Film

[0036] A sterilized type-III gauze as prescribed in the Japanese Pharmacopoeia (K-Pine made by, Kawamoto Hotai Zairyou Company) was cut in a circular shape with a knob in an aseptic manner, and immersed the cut gauze in a cell culture medium (Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, 20 mM HEPES, 100 units/ml penicillin and 100 μg/ml streptomycin). The gauze was put, together with 5 ml of the mixed collagen solution having a final concentration of 0.24% prepared in Example 1, in a hydrophobic polystyrene culture dish (ϕ60 mm; Falcon #1007). In the same manner as in example 1, the collagen solution with the gauze was vitrified after its gelation.

[0037] Furthermore, the vitrified collagen gel was hydrated by adding PBS in the same manner as in Example 1. The hydrated collagen gel could be peeled off and collected from the dish by slowly lifting the knob of the gauze, in the form of a thin-film collagen hydrogel having a satisfactory elastic strength embeddfng the gauze as a whole as shown in **FIG. 4**.

Example 4

Preparation Cylinder-Retained Collagen Hydrogel Thin-Film

[0038] A sterilized conical tube having an inner volume of 50 ml (Falcon #2070) was cut to prepare a cylindrical

appliance (**10**) capable of fixing a collagen hydrogel thin-film as a cell culture substratum as shown in **FIG. 5**(*a*).

[**0039**] By the use of a collagen hydrogel thin film (**12**) with a wire peripheral retainer (**11**) prepared in Example 2, the cover (**13**) prepared from the conical tube was pressed as shown in **FIG. 5**(*b*), and then the wire retainer (**11**) was removed. By doing so, it was possible to transfer and fix easily the collagen hydrogel thin film onto an opening of the appliance (**10**). In this state, the resultant product could be used for cell culture. The gauze embedding type collagen hydrogel thin film shown in Example 3 could be fixed onto the appliance in the same manner as above.

Example 5

Cell Culture on Collagen Hydrogel Thin Film

[**0040**] 10 ml of the cell culture medium was placed in a hydrophilic polystyrene culture dish (ϕ60 mm; Falcon #3002) , and the collagen hydrogel fixed onto the appliance in Example 4 was put into the dish. 2 ml of the cell suspension (3×10^4/ml) was seeded in the interior of the appliance and cultured in a humidified incubator at 37° C. in the presence of 5% CO_2/95% air. Human dermal fibroblasts (HOF) and human cholangio-adenocarcinoma cell line (MEC) were used as cells. After culturing the cells for five days, the cells were fixed with formalin and stained directly with hema-toxylin-eosin (HE). In the case of MEC, the cells formed several colonies on the collagen hydrogel thin film as shown in the photograph in **FIG. 6**. In the case of HDF, some cells seemed to invade the collagen hydrogel thin film (photo in **FIG. 7**). A frozen cross-section of the gel was therefore prepared and subjected to HE staining: while MEC showed no invasion into the gel, HOF revealed an apparent invasion into the gel.

[**0041**] It is needless to mention that the present invention is not limited in any manner by the examples shown above. Various embodiments are possible as to cells capable of being cultured, composition of culture medium, and culturing conditions as well as kinds of an extracellular matrix components such as a collagen hydrogel thin film, composition of substratum, thickness and elastic strength of the thin film.

[**0042**] According to the present invention. as described above in detail, there are available a culture substratum and an organ adhesion preventive substance of a hydrogel thin film containing an extracellular matrix components such as collagen, which can be easily prepared and provides an excellent expediency.

1-17. (canceled)

18. A method of making a hydrated and vitrified matrix gel consisting essentially of one or more extracellular matrix components, which comprises:

incubating an aqueous solution consisting essentially of one or more extracellular matrix components to form a matrix gel,

drying the matrix gel for vitrification thereof, and

hydrating the vitrified matrix gel to make the hydrated and vitrified matrix gel.

19. The method according to claim 18, wherein one of the extracellular matrix components is collagen.

20. The method according to claim 18, wherein the hydrated and vitrified gel is retained within a retainer, which method further comprises incubating the aqueous solution consisting essentially of one or more extracellular matrix components within a retainer to form a matrix gel in a retainer prior to the drying of the matrix gel.

21. The method according to claim 20, wherein the retainer is an annulus, a mesh, a cylinder or a container.

22. A cell culture substratum comprising the hydrated and vitrified matrix gel produced by the method of any one of claims 18 to 21.

23. A cell culturing method comprising seeding and culturing cells on the substratum of claim 22.

24. A method of making a hydrated and vitrified matrix gel consisting essentially of one or more extracellular matrix components and one or more cell culture medium components, which comprises:

incubating an aqueous solution consisting essentially of one or more extracellular matrix components and one or more cell culture medium components, to form a matrix gel,

drying the matrix gel for vitrification thereof, and

hydrating the vitrified matrix gel to make the hydrated and vitrified matrix gel.

25. The method according to claim 24, wherein one of the extracellular matrix components is collagen.

26. The method according to claim 24, wherein the hydrated and vitrified gel is retained within a retainer, which method further comprises incubating the aqueous solution consisting essentially of one or more extracellular matrix components and one or more cell culture medium components within a retainer to form a matrix gel in a retainer prior to the drying of the matrix gel.

27. The method according to claim 26, wherein the retainer is an annulus, a mesh, a cylinder or a container.

28. A cell culture substratum comprising the hydrated and vitrified matrix gel produced by the method of any one of claims 24 to 27.

29. A cell culturing method comprising seeding and culturing cells on the substratum of claim 28.

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