

(51) International Patent Classification:
A61L 27/38 (2006.01) *A61L 27/50* (2006.01)(21) International Application Number:
PCT/IT2009/000488(22) International Filing Date:
28 October 2009 (28.10.2009)

(25) Filing Language: English

(26) Publication Language: English

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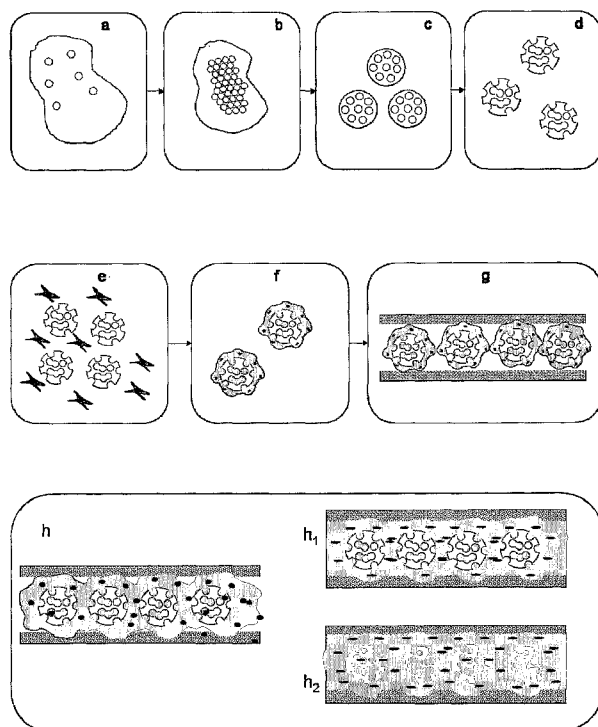
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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM,

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(54) Title: IN VITRO BIOENGINEERED ANIMAL TISSUE FIBER AND ITS USE IN THE TEXTILE INDUSTRY



(57) Abstract: Process for preparing a bioengineered fiber of tissue of animal origin comprising the steps of: a) seeding eukaryotic cells on biodegradable porous microbeads with a controlled and tunable degradation rate under dynamic seeding conditions, thereby obtaining microtissues; b) injecting the microtissues in a porous microtube suitable to guarantee nutrient supply to the cells and orientation of the synthesized tissue; and (c) culturing the microtube filled with the microtissues, thereby obtaining a bioengineered fiber. Furthermore, the present invention relates to a bioengineered fiber of tissue of animal origin obtainable by means said process. The invention also relates to the use of said bioengineered fiber as yarn in the textile industry.

Fig. 1



TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, — *with amended claims (Art. 19(1))*
ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

IN VITRO BIOENGINEERED ANIMAL TISSUE FIBER AND ITS USE IN THE TEXTILE INDUSTRY

Field of the Invention

The present invention relates to a bioengineered fiber of tissue of
5 animal origin and to a process for its preparation by means of *in vitro* cell
culture. Such fiber may particularly, but not exclusively, be used as yarn for
textile industry applications.

Background Art

In the textile field there is an increasing demand to develop technical
10 and innovative textiles capable of offering performances such as
impermeability, transpiration, antiallergenicity, fireproofing and mechanical
strength.

Currently, several materials having different origin may be
transformed into textile fibers, since both natural fibers (wool, silk, cotton,
15 flax) and artificial fibers (Rayon, glass fibers, synthetic fibers) lend
themselves to be spun and then woven to be used in the textile industry.

Against the low prices of the textile products coming from the extra-
European countries, the research in the industrialized countries aims to new
products with higher performances and multi-functionalities. The new
20 features requested for the textile products may be obtained by means of new
production processes and/or by means of novel fibers.

In this context the main advantage of the artificial fibers (i.e.
technofibers) lies in that they can be developed for the specific applications
that they will have to perform.

25 Most of the technofibers employed in the textile industry are of
synthetic origin. They are derived from organic compounds (monomers)
obtained from natural raw materials (oil) by means of polymerization
reactions, often conducted under polluting conditions, and further processed
to be spun by means of extrusion or cold drawing.

30 One of the most relevant application of the woven fibers, both natural

and synthetic, is their use in the textile market. Because of the availability of materials with a wide spectrum of functionalities and technological features, the fibers are used in several fields such as sport, clothing, furnishing, medical-surgical, automotive, and aerospace.

5 In a large number of applications in the above fields, leather is also currently used. Leather is obtained from natural animal pelts after a series of complex tanning treatments, giving the final product properties of preservability, softness, mechanical strength, flexibility and color. These properties allow leather to be useful in a large number of industrial
10 applications.

However, leather has problems such as limited supply, high prices and difficulty in obtaining a large quantity of leather of uniform quality.

Furthermore, due to the nature and to the structure of the raw materials used, tanning processes have some important technological limitations, some
15 of the main ones being the following:

- a low yield of the process of transformation of the raw skin, which consequently produces large amounts of waste material (substantially consisting of collagen) which must be disposed of;
- the high levels of pollutants in the water and gaseous effluents of
20 tanneries require the presence of suitable purifying treatments, so as to prevent the pollutants from being released into the environment;
- a low yield of the chemical processes for the stabilization of the raw materials require use of excess amounts of chemicals during
25 tanning, due to both the high compactness of the raw skin and the need to obtain diffusion of the chemicals inside the fiber structure of raw skin.

Furthermore, currently leather is produced in the form of sheets and it is not possible to produce leather as fibers suitable for use as yarn to weave,
30 as is instead possible with natural and synthetic fibers.

A further approach to produce textile industry products having properties close to leather consists in the use of bioengineering techniques.

It has been demonstrated that animal cells dissociated from their original tissue are capable of creating new tissues when cultured *in vitro* onto three-dimensional supports in an optimal environment. It has been shown to be possible, for example, to form tubular structures from dissociated endothelial cells (Montesano et al., J. Cell. Biol. 97:1648, 1983) or cartilage tissue from isolated chondrocytes (Kisiday et al., Proc. Natl. Acad. Sci USA. 99:9996, 2002). In suitable chemical-physical conditions and in suitable conditions of nutrients feed, when the dissociated cells are cultured on a three-dimensional scaffold acting as a temporary mechanical support for the same, the cells are capable of reconstructing their typical tissue because they retain *in vitro* also their ability to produce extracellular matrix (ECM) (Webb et al., Biomaterials. 24:4681, 2003).

The present invention aims to produce a bioengineered tissue yarn by using a new process in which particulate micro-carriers are seeded with eukaryote cells and the seeded micro-carriers are formed into a shaped assembly. By arranging in close mutual proximity under suitable culture conditions the cell seeded microcarriers are able to form a three-dimensional tissue equivalent due to their biological union. By employing appropriate techniques, the present invention aims to obtain a bioengineered tissue fiber suitable for textile industry.

Disclosure of the Invention

The present invention provides a process for preparing a bioengineered fiber suitable for the textile industry which does not require the production of a thin sheet of biological material to be subsequently processed into a shaped article.

In this context, the present invention aims at producing a bioengineered fiber-shaped product that can be used in the textile industry.

This aim, as well as these and other objects which will become better

apparent hereinafter, are achieved by a process for preparing a bioengineered fiber of tissue of animal origin comprises the steps of:

(a) seeding eukaryotic cells on biodegradable porous microbeads with a controlled and tunable degradation rate under dynamic seeding
5 conditions, thereby obtaining microtissues;

(b) injecting the microtissues in a porous microtube suitable to guarantee nutrient supply to the cells and orientation of the synthesized tissue; and

(c) culturing the microtube filled with the microtissues, thereby
10 obtaining a bioengineered fiber.

The aim and objects of the invention are also achieved by a bioengineered fiber of tissue of animal origin obtainable by means of said process.

Moreover, the aim and objects of the invention are also achieved by
15 the use of said bioengineered fiber of tissue of animal origin in the textile industry, in particular as textile yarn.

Brief Description of the Drawings

Further characteristics and advantages of the invention will become better apparent from the following detailed description, illustrated in the
20 accompanying drawings, wherein:

Figure 1 shows the steps of a preferred embodiment of the process of the invention; in particular:

figure 1a-1d show the preparation of macroporous gelatin microbeads (a- gelatin-toluene emulsion; b- saturated toluene droplets in gelatin
25 solution; c- formation of gelatin microbeads containing droplets of toluene; d- porous gelatin microbeads obtained after toluene washing with ethanol);

figure 1e shown the dynamic fibroblast seeding on the gelatin porous microbeads;

figure 1f shows the microbeads covered with a tissue layer
30 (microtissue);

figure 1g shows the injection and molding step of the microtissues in the porous microtube;

figure 1h shows the tissue growth into the microtube;

figure 1h1 shows the cell alignment and tissue deposition along tube
5 axis;

figure 1h2 shows the synchronous microbeads degradation and neotissue formation;

figure 2a shows a Scanning Electron Microscope (SEM) picture of a biodegradable porous gelatin microbead (bar is 10 μm);

10 figure 2b shows a Scanning Electron Microscope (SEM) picture of a biodegradable porous gelatin microbead (bar is 20 μm);

figure 3 shows a graph of the concentration of cells in the culture medium in function of time as a result of dynamic seeding;

figure 4a shows an optical microscope picture of a microbead
15 colonized by fibroblasts 2 hours after seeding and subjected to MTT assay;

figure 4b shows an optical microscope picture of a microbead colonized by fibroblasts 5 hours after seeding and subjected to MTT assay;

figure 4c shows an optical microscope picture of a microbead colonized by fibroblasts 24 hours after seeding and subjected to MTT assay;

20 figure 4d shows an optical microscope picture of a microbead colonized by fibroblasts 48 hours after seeding and subjected to MTT assay;

figure 4e shows an optical microscope picture of a microbead colonized by fibroblasts 96 hours after seeding and subjected to MTT assay;

figure 5a shows a picture of a hematoxylin/eosin stained 20%
25 crosslinked microbead after 4 days of culturing of the fibroblasts seeded therein;

figure 5b shows a picture of a hematoxylin/eosin stained 10% crosslinked microbead after 4 days of culturing of the fibroblasts seeded therein;

30 figure 6a shows a SEM picture of a microbead after 4 days of culture

of the fibroblasts seeded therein (bar is 100 μm);

figure 6b shows an enlargement of the SEM picture of a microbead after 4 days of culture of the fibroblasts seeded therein (bar is 10 μm);

figure 7a shows a SEM picture of the diameter of the PTFE
5 microtube;

figure 7b shows a SEM picture of the porosity of the PTFE microtube;

figure 8 shows a scheme of the apparatus for injecting the seeded microbeads into the microtube;

10 figure 9 shows a picture of a bioengineered fiber of tissue of animal origin obtained after 1 week of culture of the microbeads injected into the microtube;

figure 10a shows a picture of a hematoxylin/eosin stained fiber obtained after 3 weeks of culturing of the 20% crosslinked microbeads
15 within the microtube;

figure 10b shows an enlargement of the picture of a hematoxylin/eosin stained fiber obtained after 3 weeks of culturing of the 20% crosslinked microbeads within the microtube;

figure 11a shows a picture of a hematoxylin/eosin stained fiber
20 obtained after 3 weeks of culturing of the 10% crosslinked microbeads within the microtube;

figure 11b shows an enlargement of the picture of a hematoxylin/eosin stained fiber obtained after 3 weeks of culturing of the 10% crosslinked microbeads within the microtube;

25 figure 12a shows a picture of the ultrastructural analysis of the fiber obtained after 3 weeks of culture (bar is 100 nm); and

figure 12b shows a picture of the ultrastructural analysis of the fiber obtained after 3 weeks of culture (bar is 500 nm).

Ways of carrying out the Invention

30 In the present description the term "bioengineered fiber" refers to a

fiber made by biological tissue obtained by means of *in vitro* culturing of eukaryotic cells, particularly fibroblasts, of animal origin.

Furthermore, in the present description the term “microtissue” refers to a complex consisting of a microcarrier surrounded by eukaryotic cells and
5 a micrometric layer of their extracellular matrix. Particularly the cells can be fibroblast of animal origin and the microcarriers can be porous microbeads.

The process herein described comprises several steps suitable to produce a bioengineered fiber. The first step consists in seeding eukaryotic cells on biodegradable porous microbeads with a controlled and tunable
10 degradation rate under dynamic seeding conditions, thereby obtaining microtissues.

Particularly, in the dynamic cell seeding step the cells and the microbeads are maintained in condition of intermittent stirring followed by continuous stirring.

15 The dynamic cell seeding may be carried out by subjecting the cells and microbeads in a suspension to stirring phases having a duration time ranging from 3 to 15 minutes, preferably 5 minutes, alternated with rest phases having a duration time ranging from 15 to 60 minutes, preferably 30 minutes. The alternating phases (stirring/rest) may be carried out for a total
20 time of 5 to 12 hours, preferably 6 hours, at the end of which the cells and microbeads may be maintained under continuous stirring for a time ranging from 72 to 160 hours, preferably 90 hours.

Preferably, the stirring rate may be set in the range of 20-40 rpm, more preferably 30 rpm, in order to maintain the microbeads and cells in
25 suspensions while avoiding the damage induced by mechanical stress due to the stirring.

The dynamic cell seeding allows a better distribution of the cells onto the surface of the microbeads, creates an homogeneous culturing milieu, avoids microbeads aggregation and improves gas and nutrient exchange
30 between the cell seeded microbeads and the culture medium.

Preferably in the process of the invention the eukaryotic cells are fibroblasts, more preferably the fibroblasts are primary fibroblasts. In a non-limitative example, the fibroblasts may be bovine fibroblasts.

Before seeding on the microbeads, the primary fibroblasts are
5 amplified, generally until the semi-confluence stage is reached. Fibroblasts seeding may be carried out in MEM Eagle–Earle BSS culture medium enriched with a solution of essential and non-essential amino acids (2x), 20% Fetal Bovine Serum (FBS) and glutamine 2mM.

Once the semi-confluence stage is reached, the fibroblasts are
10 trypsinised and 5×10^5 cells per mg of microbeads are seeded on the microbeads according to the procedure herein described.

The microbeads used in the process herein described act as a support for cell adhesion and further offer to the cells a protection against mechanically induced damage.

15 In an embodiment of the present invention, the process herein described may further comprise a step of preparing the porous microbeads prepared having controlled and tunable degradation rate before the step (a).

The microbeads may be for example gelatin microbeads with a controlled degradation rate, obtained by means of the “oil-in water-in oil”
20 (O/W/O) double emulsion technique, by mixing an aqueous solution of gelatin containing a first surface-active agent, with a hydrophobic organic solvent containing a second surface-active agent.

The O/W/O emulsion consists of a three-phase system wherein a water phase, containing a first hydrophobic phase, is dispersed in a second
25 hydrophobic phase. In the process according to the invention, the O/W/O emulsion is prepared by mixing an aqueous solution of gelatin containing a first surface-active agent, such as a polyethoxylated sorbitol ester, with an hydrophobic organic solvent, for example toluene, containing a second surface-active agent, for example a sorbitol ester. The first surface-active
30 agent may be TWEEN 85 (sorbitan trioleate ethoxylated with 20 moles of

ethylene oxide). The second surface-active agent may be SPAN 85 (sorbitan trioleate). Addition of the hydrophobic organic solvent to the aqueous phase leads to formation of a hydrophobic phase in the form of organic solvent droplets trapped within the aqueous phase. When addition of the organic
5 solvent leads to saturation of the aqueous solution, the latter becomes dispersed in an external hydrophobic phase of the solvent. Gelatin microbeads with entrapped organic solvents droplets are thus formed.

In the next step, the microbeads are washed with a solvent miscible with the hydrophobic solvent used for the preparation of the double emulsion
10 which is not capable of dissolving gelatin, for example ethanol may be used when toluene is used in the emulsion. Accordingly, the droplets of hydrophobic organic solvent trapped within the microbeads are removed, thereby forming an extended network of micropores in the microbeads.

In a preferred embodiment of the process, the biodegradable porous
15 microbeads may have a diameter ranging from 75 μm to 150 μm , more preferably 150 μm .

In a further embodiment, the gelatin porous microbeads may be stabilized by means of a treatment with a cross linking agent, whose amount influences the rate of degradation of the microbeads. It is thus possible to
20 modulate the rate of degradation of the microbeads by varying the concentration of the crosslinking agent during the stabilization, thereby synchronizing the rate of degradation of the gelatin microbeads with the rate of extracellular matrix synthesis of the cells seeded within the microbeads.

The cross linking agent, which may be for example glyceraldehyde,
25 may be used at concentration ranging from 10% w/w to 20% w/w of the microbeads weight.

Preferably, the gelatine porous microbeads may be subjected to sterilization, so as to provide for a sterilized environment for the cells which will be seeded into the microbeads. Preferably, sterilization may be carried
30 out by means of autoclaving or gamma-ray irradiation. For example,

sterilization may be carried out in an autoclave at a temperature of 121°C for 15 minutes.

After the seeding of the cells onto the microbeads during step (a) and before step (b), the cell seeded microbeads (microtissues) may be optionally
5 subjected to a further cell culturing step in suspension, so as to increase the extracellular matrix deposition within the microbeads.

The step (b) of the process is carried out following the seeding of the microbeads or after the optional step of culturing in suspension the microtissues obtained by seeding the cells onto the microbeads. In particular,
10 step (b) consists of injecting the microtissues in a porous microtube that allows nutrient flow to the cells and directionality (geometrical guidance) for the neotissue growth.

The porosity of the microtube allows the exchange of nutrients and waste products of the cellular metabolism between the culture medium and
15 the cells and vice versa. Furthermore, the geometry of the microtube guides cell growth, inducing the cells to grow along the longitudinal axis of the microtube and leading to formation of the desired fiber structure. Indeed, it has been demonstrated that directional cell locomotion plays a crucial role in many physiological processes underlying tissue development and
20 organization (Chun-Nin et Al. Biophys. J. Vol 79:144-152, 2000).

Preferably, the microtube may have an inner diameter from 300 μm to 500 μm . Furthermore the microtube may be made with a polymeric material, for example polytetrafluoroethylene (PTFE). In particular, PTFE is suitable since it is nonreactive, chemically and thermally stable, so that it
25 may be also subjected to sterilization by autoclaving.

Once the microtissues are injected in the microtube, said microtube is cultured under cell culture conditions in order to allow the cells in the microtissues to continue their growth, extracellular matrix d formation of the bioengineered fiber. The culture conditions employed in step (c) may be
30 dynamic or static conditions, depending on whether the microtube is

subjected to stirred or not.

During the cell culturing, the cell growth and extracellular matrix deposition lead to the biological union of the microtissues within the microtube, thus leading to the formation of a continuous structure of biological material shaped as a fiber.

The amount of extracellular matrix deposited by the cells depends upon the cell type, the cell culture conditions (presence of stimulating factors), nature of the substrate and number of cells. Therefore, in an embodiment of the present invention, the rate of synthesis of the extracellular matrix may be further enhanced by addition of specific biochemical factors, such as ascorbic acid, preferably in an amount of 50 mg/ml can be used.

Formation of the bioengineered tissue may be monitored during time by means of histological analysis, for example by staining with hematoxylin/eosin (figures 5a and 5b).

The bioengineered fibers of tissue of animal origin obtainable by means of the process described herein may be used in the field of textile industries.

Examples

The invention will be further described by means of examples which are to be considered non-limitative of the scope of the present invention

Example 1

Porous biodegradable microbeads have been prepared according to the double emulsion technique (O/W/O) by dissolving in water gelatin (type B Sigma Aldrich Chemical Company, Bloom 225, Mw=176654 Dalton) at a concentration of 8% w/v (weight/volume). The solution was kept at 60°C. Subsequently, to 10 ml of aqueous gelatin solution containing an amount of 6% w/v Tween 85 - sorbitan trioleate polyethoxylate - (Sigma Aldrich Chemical Company) is added a hydrophobic solution consisting of toluene containing SPAN 85 - sorbitan trioleate - (Sigma Aldrich Chemical

Company), as shown in figure 1a. Following addition of toluene the formation of droplets of hydrophobic phase within the aqueous phase is observed (figure 1b). Toluene is continuously added till a final volume of 40 ml is reached, whereupon the dispersion of the aqueous phase containing the toluene droplets within the external hydrophobic phase is observed (figure 1c).

The emulsion is cooled below 20°C and 30 ml of ethanol are added to remove toluene and thus obtain the porous gelatin microbeads. The microbeads are then further washed with ethanol and after a final wash acetone they are dried at room temperature (figure 1d). The microbeads are sieved and the fraction between 75 and 150 µm is recovered. Such fraction of microbeads is stabilized by means of chemical treatment with glyceraldehyde, so as to render the gelatin insoluble in water at 37°C.

The stabilized microbeads have been examined by means of Scanning Electron Microscopy and the presence of a large number of cavities having about 20 µm diameter was observed (figure 2).

Example 2

The degradation rate of the gelatin microbeads may be modulated by variation of the degree of crosslinking, i.e. the amount of crosslinks introduced during the stabilization step. Using two different amounts of glyceraldehyde (10% and 20% w/w of the microbeads) it was possible to obtain microbeads with two different crosslinking degrees. No morphological differences were observed between the two crosslinked types of microbeads during the SEM analysis. The microbeads crosslinked at 20% have been sterilized by autoclaving and the microbeads crosslinked at 10% were sterilized by irradiation with gamma-rays. The microbeads were then placed in contact with the culture medium containing serum before the cell seeding step.

Primary fibroblasts were amplified in Petri dishes having a 100 mm diameter and were seeded on both types of microbeads at a concentration of

5×10^5 cells/mg (figure 1e). Cell seeding was conducted under dynamic conditions by subjecting the suspension of cells and microbeads to stirring, so as to achieve a uniform distribution of the cells on the microbeads.

The dynamic seeding has been carried out in a spinner flask (100 ml Bellco) by using an intermittent stirring for 5 hours in which 5 minutes of stirring were alternated with 30 minutes of static culture, then using continuous stirring for 90 hours. The stirring rate employed was 30 rpm.

Subsequently, 2 ml of each suspension of microbeads were collected so as to evaluate cell adhesion kinetics and cell vitality during the first hours following seeding. 1 ml of suspension was used to evaluate the number of cells not adhered to microbeads and 1 ml was used for an MTT assay. MTT is a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide) which is converted into a blue formazan by the dehydrogenases present in living cells.

The count of the number of cells not adhered present in the culture medium showed a decrease of the number of cells not adhered to the microbeads over time, both for the 10% crosslinked microbeads and the 20% crosslinked microbeads. In particular, figure 3 depicts a graph of the decreasing concentrations of cells in the culture medium over time. The concentration of cells (ρ) for each time point is normalized to the initial concentration of cells (ρ_0).

The MTT assay revealed a low number of live cells in the microbeads after 2 hours from seeding (figure 4a) which grows over time after 5 hours (figure 4b), 24 hours (figure 4c), 48 hours (figure 4d) and 96 hours (figure 4e), as evidenced by the increasingly deeper blue color of the microbeads. It is thus evident that the gelatine microbeads represent a good substrate for fibroblast adhesion and growth.

Furthermore, histological analysis with hematoxylin/eosin staining have been performed on samples taken from cultures after 4 days of seeding. The samples were fixed in formalin (24 hours), sequentially dehydrated in

alcohol (75°, 85°, 95°, 100°) and embedded in paraffin. Sections having a 5 µm thickness were obtained with a microtome (Zeiss) and stained with hematoxylin/eosin. The staining revealed that the fibroblasts, owing to the production of extracellular matrix proteins, are capable of establishing cell-to-cell, cell-to-material and cell-to-extracellular matrix contacts, thereby leading to the assembly of compact and homogeneous structures between the microbeads and the cells (figures 5a and 5b). This finding is further supported by the SEM pictures of figures 6a and 6b which show that the cells have completely colonized the microbeads filling the porosity within the gelatin with extracellular matrix.

Example 3

After 96 hours of suspension culture, the fibroblasts-seeded microbeads were transferred into the porous PTFE microtubes (Markel Corporation). The microtubes have an inner diameter of 430 µm, an outer diameter of 860 µm and porous surface wherein the pore size is about 6 µm (figures 7a and 7b).

The seeded microbeads were transferred to the microtubes by means of an apparatus as shown in figure 8. This apparatus comprises a chamber (1) containing a suspension of the fibroblast-seeded microbeads (2); the chamber is connected to a pump (3) by means of a valve (4) and has an exit (5) connected to a microtube (6) having micropores (7). A stirring device (8), such a magnetic stirrer, may be placed in correspondence of the chamber (1).

By opening the valve (4), the pump (3) transfers the suspension of seeded microbeads (2) from the chamber (1) to the microtube (6) through exit (5). The end of the PTFE microtube is closed to maintain the microbeads inside the microtube while allowing the medium to flow through the porosity of the microtube.

The microtubes filled with the seeded microbeads were then kept under static culture conditions in the presence of ascorbic acid to stimulate the extracellular matrix deposition.

After 7 days of culturing it was possible to remove from the PTFE microtube a bioengineered fiber having a length of 6 cm and a diameter of 400 μm . The fiber consists of a structure of extracellular matrix, fibroblasts and microbeads (figure 9). The assembly of the seeded gelatin microbeads (microtissues) and the extracellular matrix deposition processes led to the establishment of cellular interactions between the fibroblasts and to the assembly of the desired bioengineered fiber.

Example 4

The fiber removed from the microtubes which were subjected to injection and culturing of the microtissues were histologically examined following fixation with formalin. Hematoxylin/eosin staining revealed the a conspicuous presence of extracellular matrix which allowed the fusion of the initially isolated microbeads. In particular, figures 10 and 11 show histological sections of the fibers obtained respectively from the 20% crosslinked microbeads and the 10% crosslinked microbeads after 3 weeks of culture. From said figures it is possible to notice the extracellular matrix distribution and the orientation of the cells along the microtube axis. Further SEM analysis shows (figure 12) that collagen is correctly assembled in fibrils having a characteristic banded pattern (figure 12a). The fibril diameter is of the same order of magnitude of the one found in native tissues, i.e. about 20-30 nm for a length of several micrometers (figure 12b).

Furthermore, after 3 weeks of culture it is possible to observe that degradation of the gelatin microbeads occurs, with a consequent change in the morphology of the microbeads. Degradation is more pronounced in the microbeads with lower degree of crosslinking (figure 11) when compared to microbeads with a higher degree of crosslinking (figure 10). These findings indicate that by modulating the amount of crosslinking agents employed in the stabilization step it is possible to modulate the rate of degradation of the microbeads.

In practice it has been found that the process according to the

invention fully achieve the intended aim and objects, since it allows to obtain bioengineered fibers of tissue of animal origin from microbeads seeded with suitable cells.

Moreover, it has been observed that the process according to the
5 invention allows to obtain structures in the form of fibers of tissue of animal origin without the need for subsequent assembly, since the microtubes allow to obtain a biological tissue formed by the cells having the desired shape.

The process thus conceived is susceptible of numerous modifications and variations, all of which are within the scope of the appended claims;
10 moreover, all the details may further be replaced with other technically equivalent elements.

In practice, the materials used, as well as the shapes and dimensions, may be any according to requirements without thereby abandoning the scope of the protection of the appended claims.

CLAIMS

1. Process for preparing a bioengineered fiber of tissue of animal origin comprising the steps of:

(a) seeding eukaryotic cells on biodegradable porous microbeads
5 with a controlled and tunable degradation rate under dynamic seeding conditions, thereby obtaining microtissues;

(b) injecting the microtissues in a porous microtube suitable to guarantee nutrient supply to the cells and orientation of the synthesized tissue; and

10 (c) culturing the microtube filled with the microtissues, thereby obtaining a bioengineered fiber.

2. The process according to claim 1, wherein the dynamic seeding conditions consist of seeding cells under a condition of intermittent stirring followed by continuous stirring.

15 3. The process according to claim 2, wherein the condition of intermittent stirring followed by continuous stirring consist of:

stirring the porous microbeads and the cells at 20 to 40 rpm for periods of time between 3 and 15 minutes followed by periods of time between 15 and 60 minutes without stirring for a total amount of time of 5 to 12 hours;
20 and

subsequently stirring continuously for a period of time between 72 and 160 hours.

4. The process according to one or more of the preceding claims, wherein the eukaryotic cells are primary fibroblasts.

25 5. The process according to one or more of the preceding claims, wherein the porous microtube is a polytetrafluoroethylene (PTFE) microtube.

6. The process according to one or more of the preceding claims, wherein the microtissues are subjected to cell culturing in suspension before injection in the microtube during step (b).

30 7. The process according to one or more of the preceding claims,

further comprising the step of preparing the porous biodegradable microbeads before step (a), said preparation of the porous biodegradable microbeads comprising the steps of :

(i) preparing an oil-in water-in oil (O/W/O) double emulsion mixing
5 an aqueous solution of gelatin comprising a first surface active agent with a hydrophobic organic solvent comprising a second surface active agent, thereby obtaining gelatin microbeads;

(ii) removing the hydrophobic organic solvent from the gelatin microbeads by washing with a solvent miscible with said hydrophobic
10 organic solvent, thereby obtaining porous gelatin microbeads.

8. The process according to claim 7, wherein said preparation of the porous biodegradable microbeads further comprises the step of:

(iii) treating the porous gelatin microbeads with a crosslinking agent.

9. The process according to claim 8, wherein the crosslinking agent is
15 glyceraldehyde.

10. The process according to claim 8 or 9, wherein the crosslinking agent is in amount of 10% to 20% w/w of the microbeads weight.

11. A bioengineered fiber of tissue of animal origin obtainable by the process of one or more of claims 1 to 10.

20 12. Use of the bioengineered fiber according to claim 11 as yarn in the textile industry.

AMENDED CLAIMS

received by the International Bureau on 25 February 2011 (25.02.2011)

1. A process for preparing a bioengineered fiber of tissue of animal origin comprising the steps of:

(a) seeding eukaryotic cells on biodegradable porous microbeads with a controlled and tunable degradation rate under dynamic seeding conditions, thereby obtaining microtissues, said dynamic seeding consisting of seeding cells under a condition of intermittent stirring followed by continuous stirring, said dynamic seeding lasting for 5 to 12 hours;

(b) injecting the microtissues in a porous microtube suitable to guarantee nutrient supply to the cells and orientation of the synthesized tissue; and

(c) culturing the microtube filled with the microtissues, thereby obtaining a bioengineered fiber.

2. The process according to claim 1, wherein the dynamic seeding conditions consist of seeding cells under a condition of intermittent stirring followed by continuous stirring.

3. The process according to claim 2, wherein the condition of intermittent stirring followed by continuous stirring consist of:

stirring the porous microbeads and the cells at 20 to 40 rpm for periods of time between 3 and 15 minutes followed by periods of time between 15 and 60 minutes without stirring for a total amount of time of 5 to 12 hours; and

subsequently stirring continuously for a period of time between 72 and 160 hours.

4. The process according to one or more of the preceding claims, wherein the eukaryotic cells are primary fibroblasts.

5. The process according to one or more of the preceding claims, wherein the porous microtube is a polytetrafluoroethylene (PTFE) microtube.

6. The process according to one or more of the preceding claims, wherein the microtissues are subjected to cell culturing in suspension before injection in the microtube during step (b).

7. The process according to one or more of the preceding claims, further comprising the step of preparing the porous biodegradable microbeads before step (a), said preparation of the porous biodegradable microbeads comprising the steps of:

(i) preparing an oil-in water-in oil (O/W/O) double emulsion mixing an aqueous solution of gelatin comprising a first surface active agent with a hydrophobic organic solvent comprising a second surface active agent, thereby obtaining gelatin microbeads;

(ii) removing the hydrophobic organic solvent from the gelatin microbeads by washing with a solvent miscible with said hydrophobic organic solvent, thereby obtaining porous gelatin microbeads.

8. The process according to claim 7, wherein said preparation of the porous biodegradable microbeads further comprises the step of:

(iii) treating the porous gelatin microbeads with a crosslinking agent.

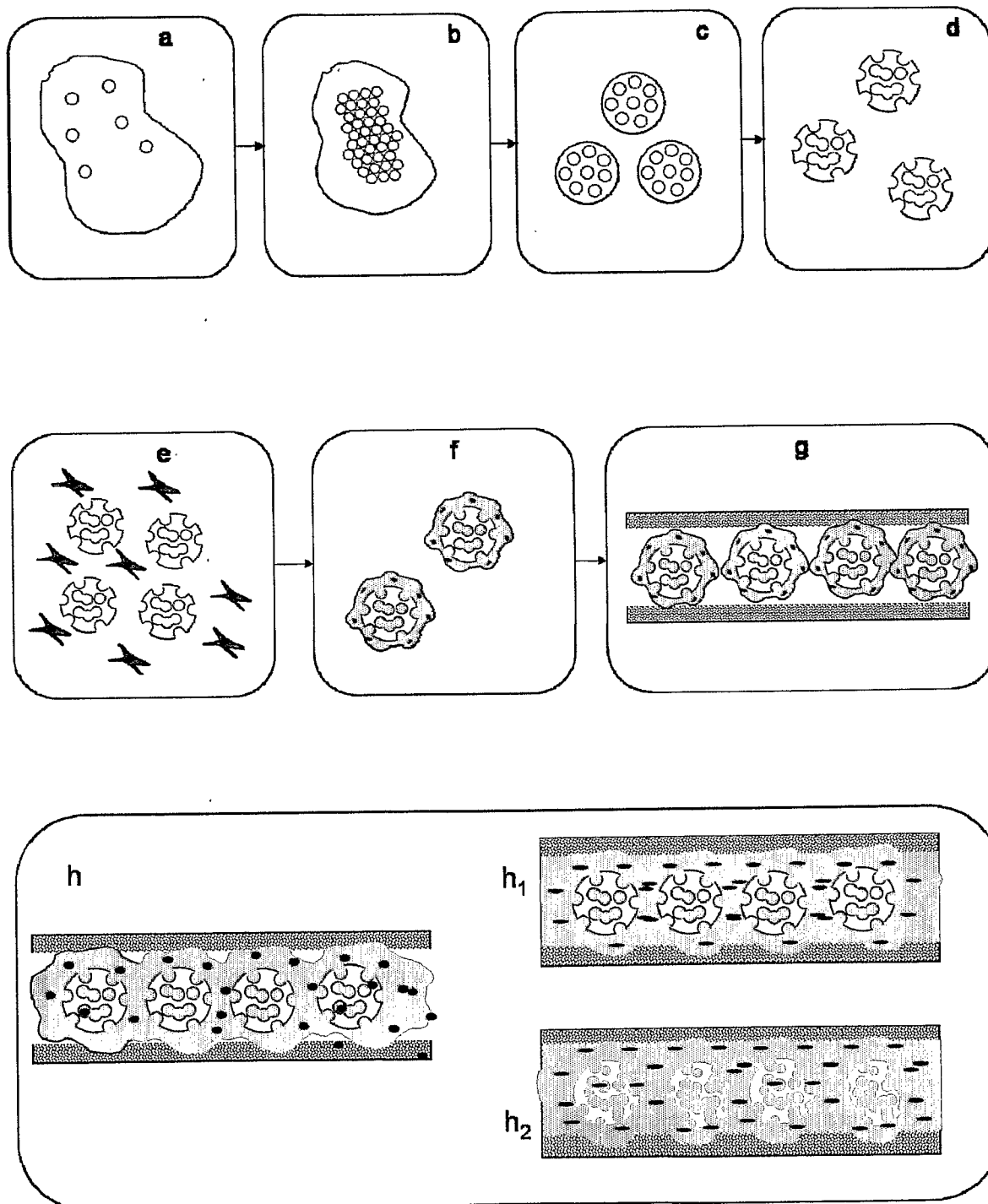
9. The process according to claim 8, wherein the crosslinking agent is glyceraldehyde.

10. The process according to claim 8 or 9, wherein the crosslinking agent is in amount of 10% to 20% w/w of the microbeads weight.

11. A bioengineered fiber of tissue of animal origin obtainable by the process of one or more of claims 1 to 10.

12. Use of the bioengineered fiber according to claim 11 as yarn in the textile industry.

1 / 10

*Fig. 1*

2 / 10

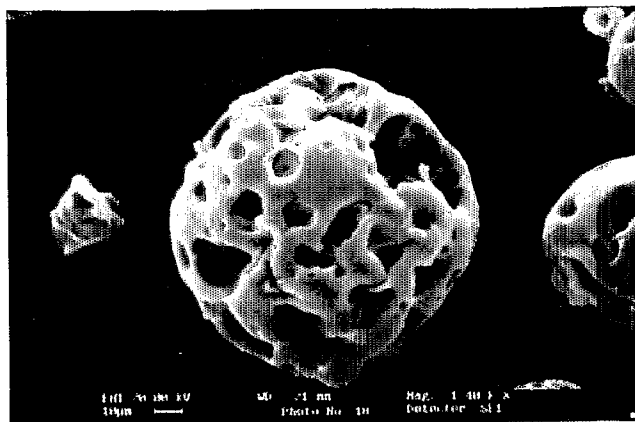
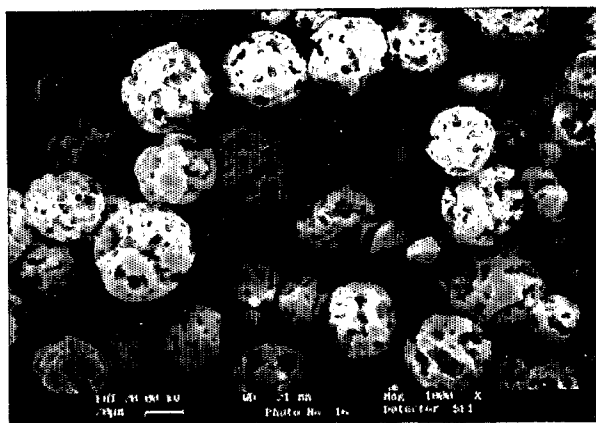
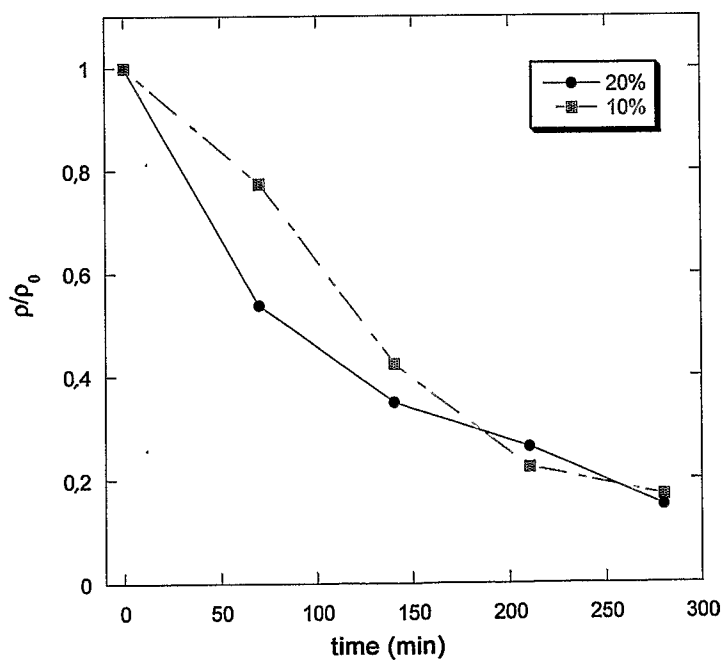
*Fig. 2a**Fig. 2b**Fig. 3*



Fig. 4a

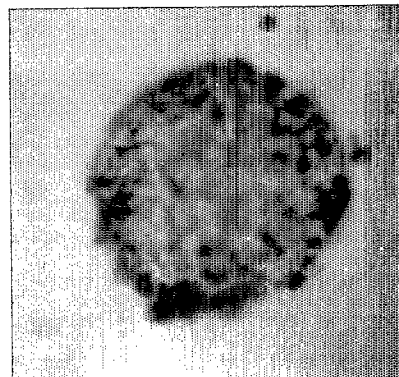


Fig. 4b

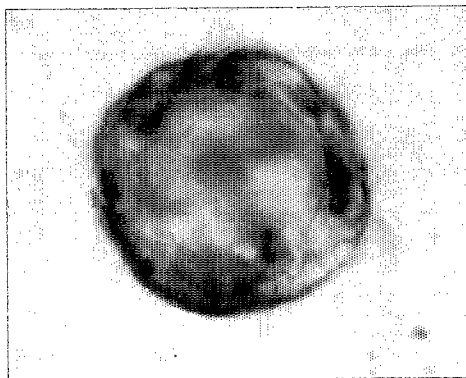


Fig. 4c



Fig. 4d

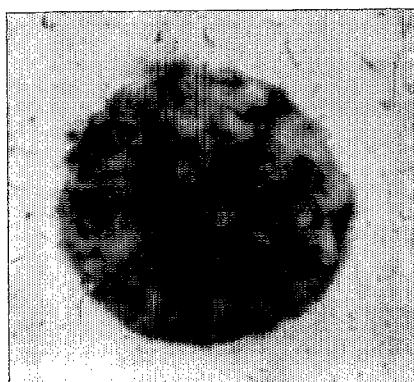


Fig. 4e

4 / 10

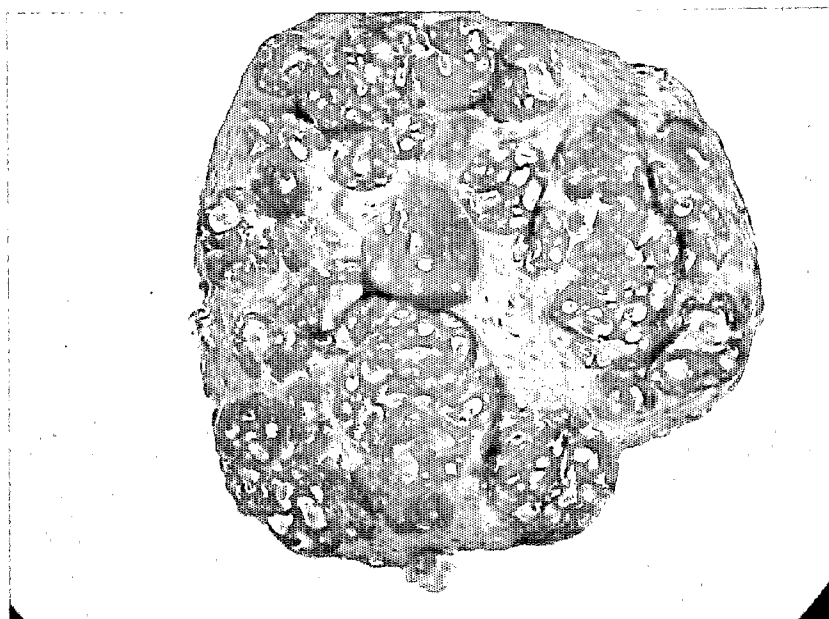


Fig. 5a

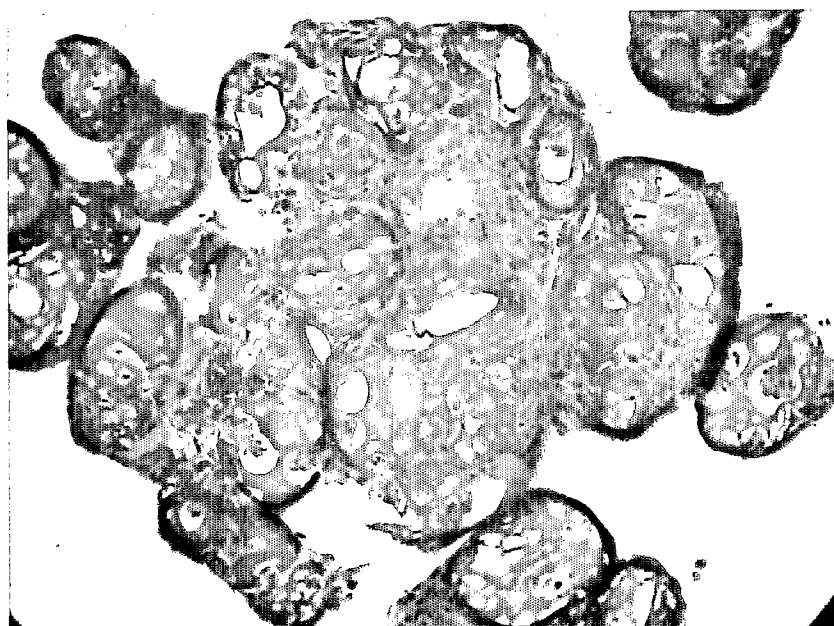


Fig. 5b

5 / 10

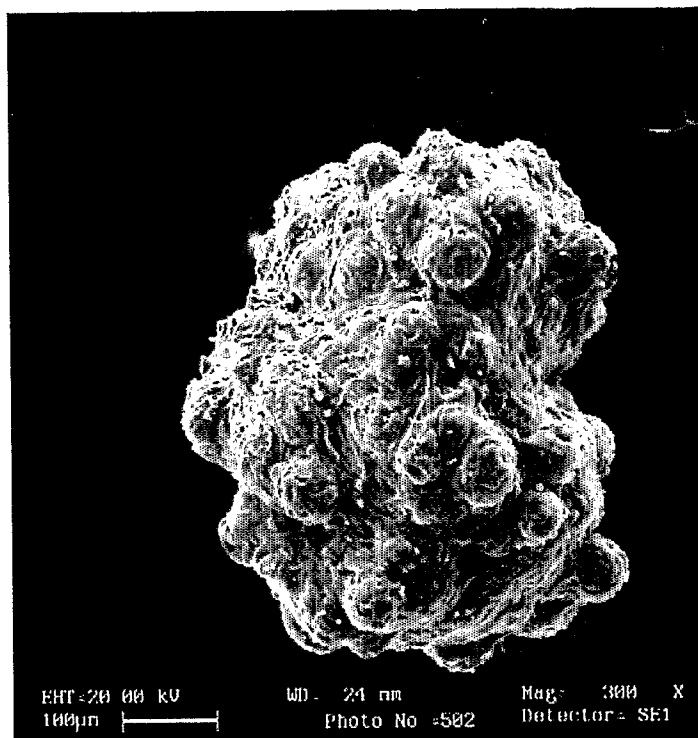


Fig. 6a

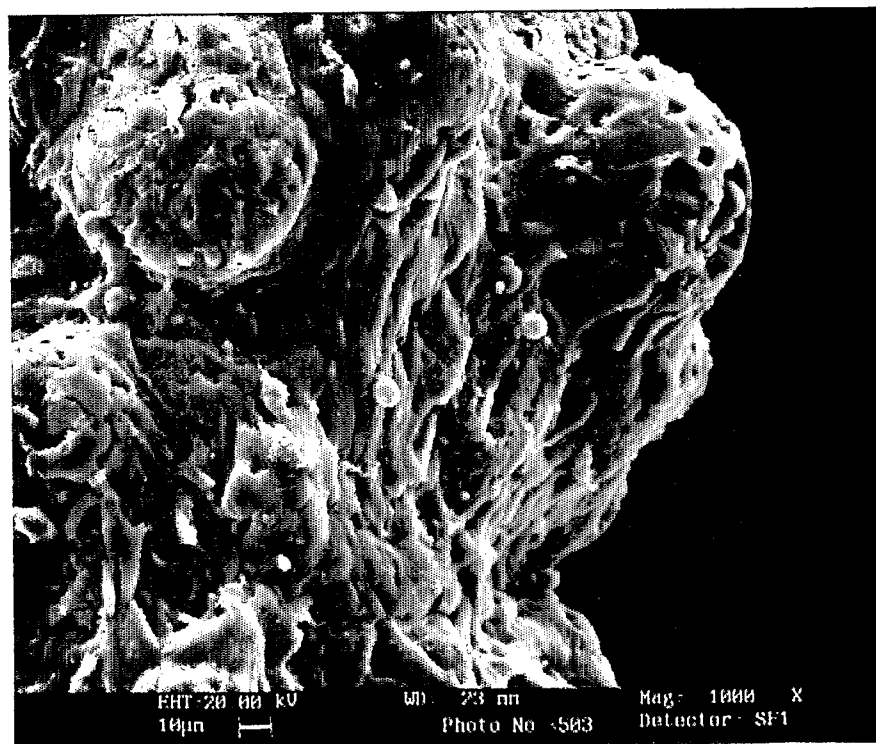
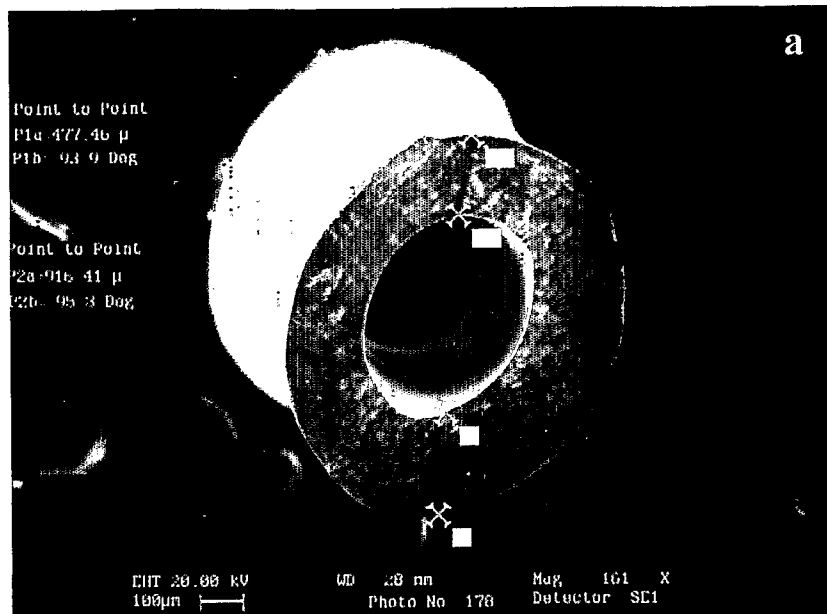
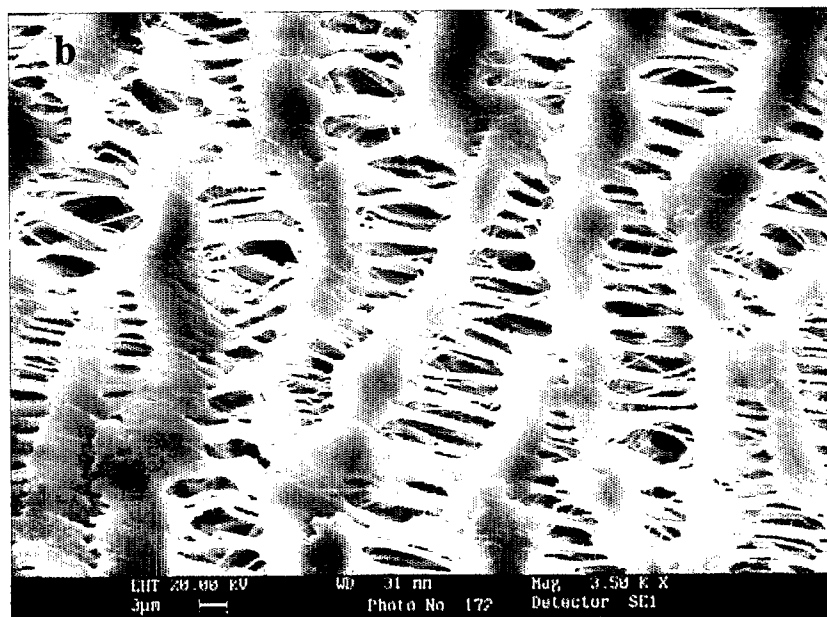
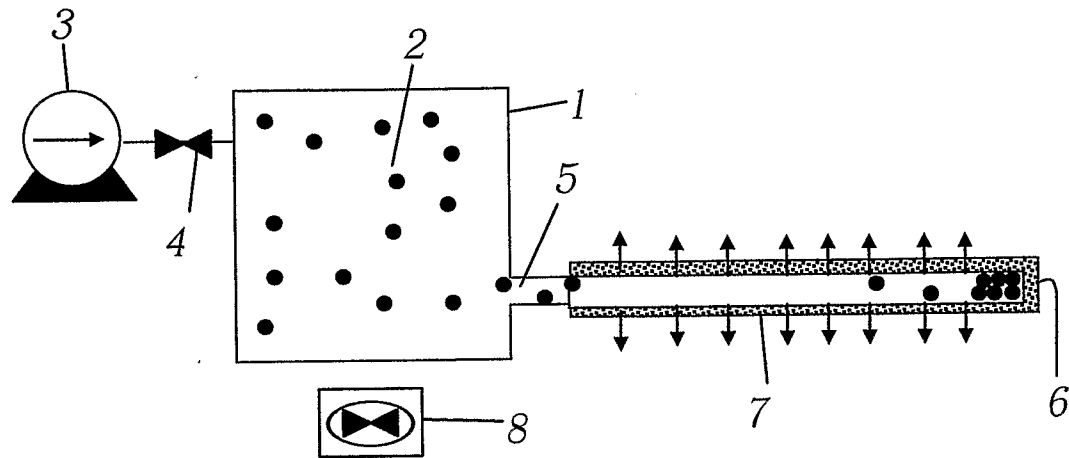
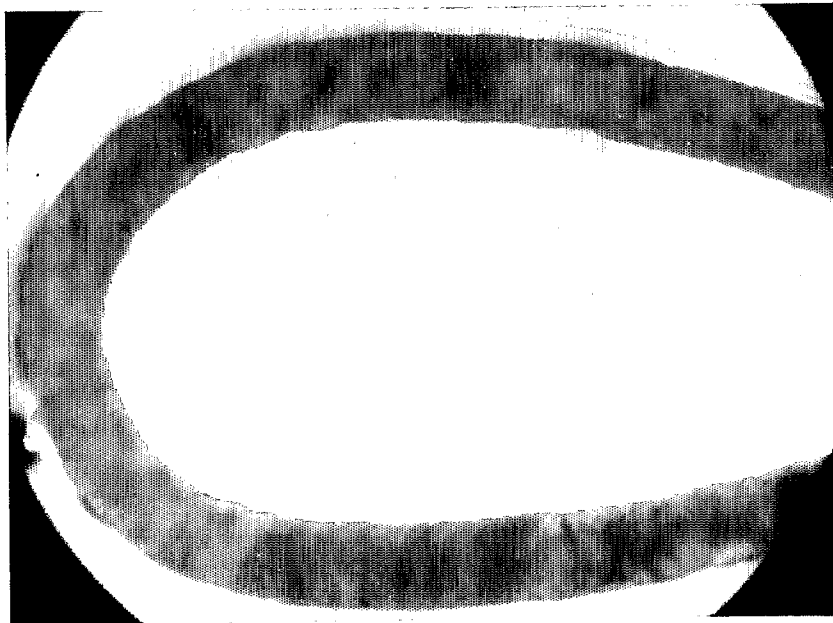


Fig. 6b

6 / 10

*Fig. 7a**Fig. 7b*

7 / 10

*Fig. 8**Fig. 9*

8 / 10

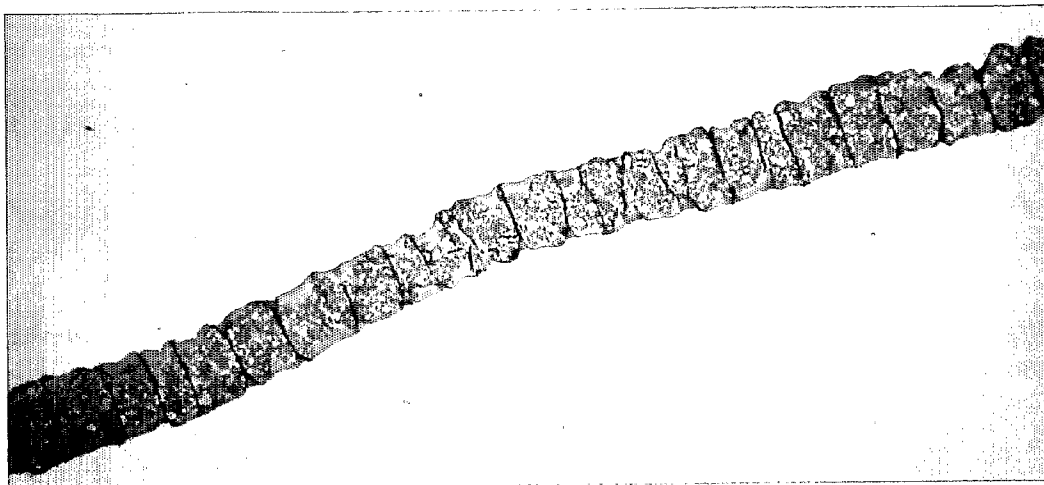


Fig. 10a

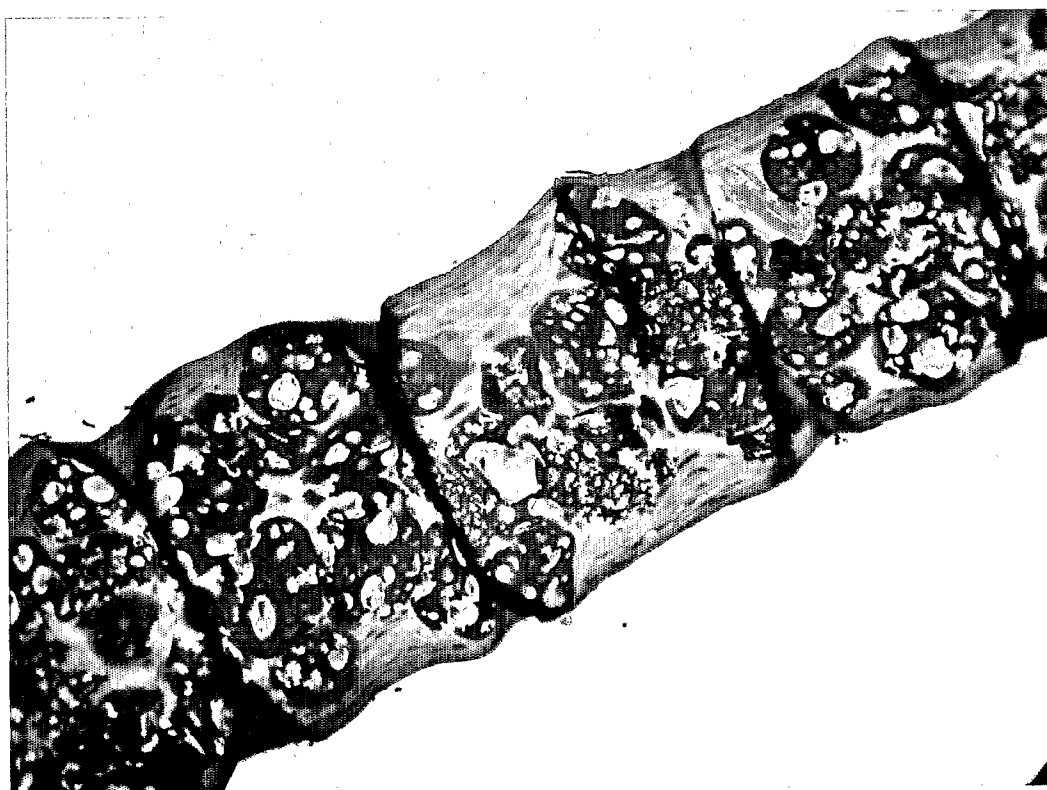


Fig. 10b

9/10

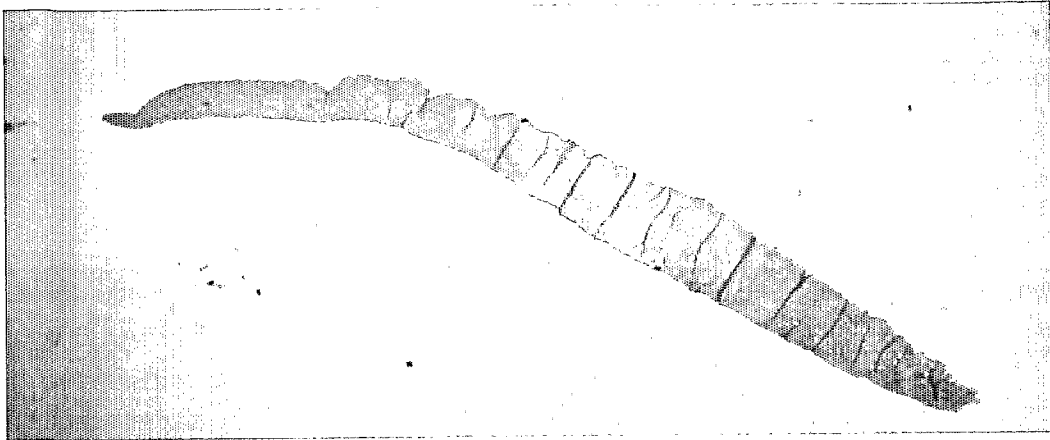


Fig. 11a

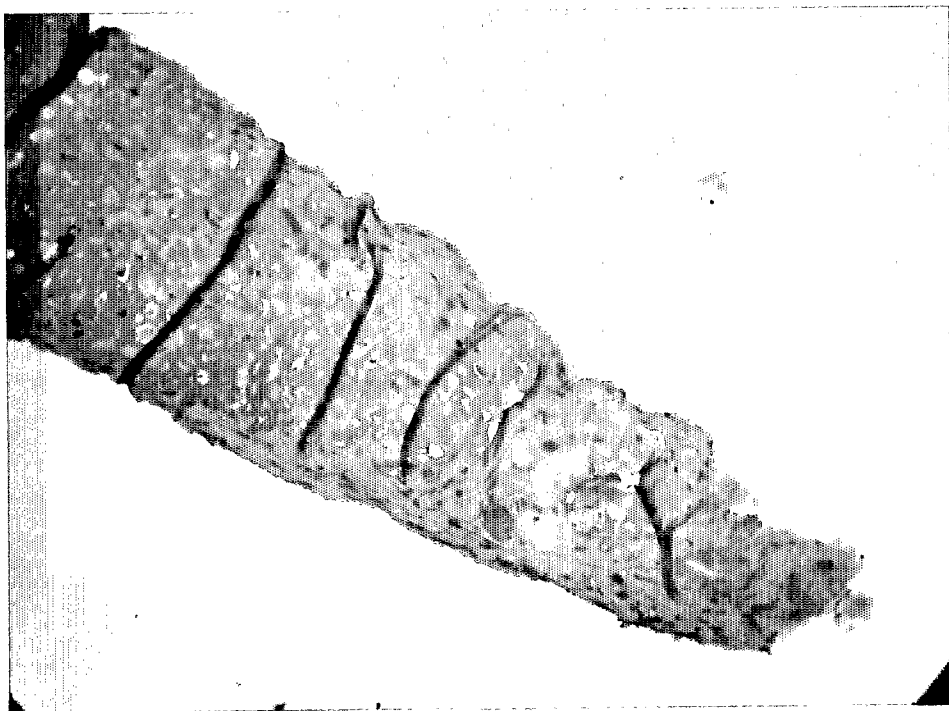


Fig. 11b

10 / 10

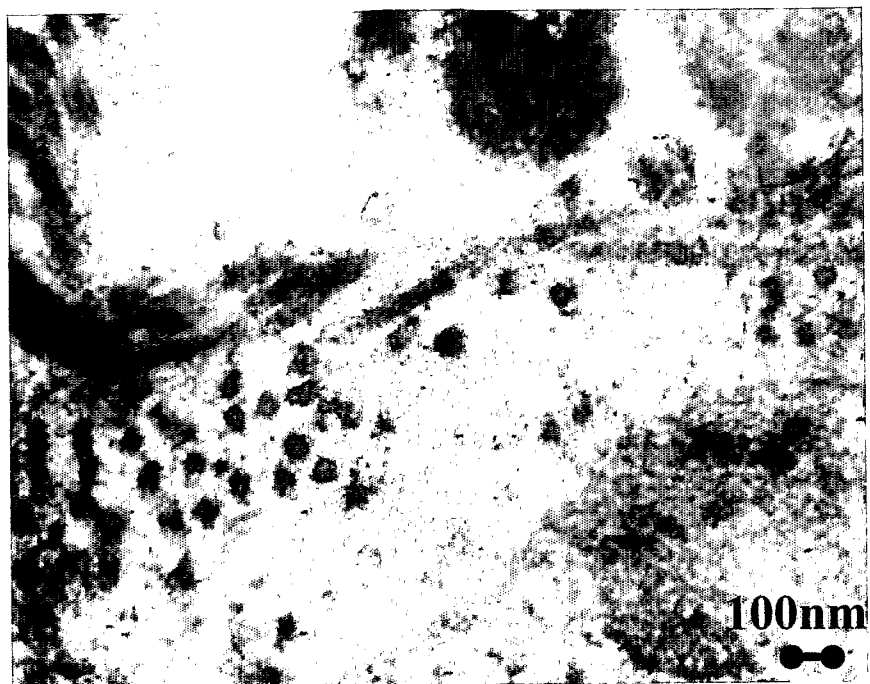


Fig. 12a

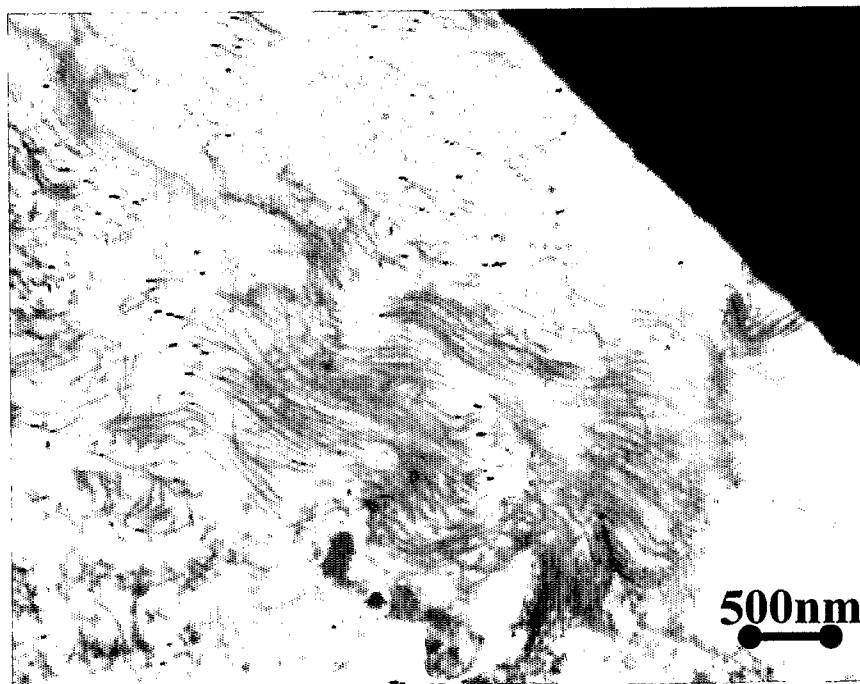


Fig. 12b

INTERNATIONAL SEARCH REPORT

International application No

PCT/IT2009/000488

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61L27/38 A61L27/50
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61L D02G

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2008/061261 A2 (CYTOGRAFT TISSUE ENGINEERING I [US]; L HEUREUX NICOLAS [US]; MCALLISTE) 22 May 2008 (2008-05-22) paragraph [0010] - paragraph [0018]	1-12
A	US 2003/100108 A1 (ALTMAN GREGORY H [US] ET AL ALTMAN GREGORY H [US] ET AL) 29 May 2003 (2003-05-29) figure 5; examples 1-3	1-12
A	WO 02/062357 A1 (COMMW SCIENT IND RES ORG [AU]; IND TECH RES INST; WERKMEISTER JEROME A) 15 August 2002 (2002-08-15) claim 34 examples 8,17,21-36	1-12

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Further documents are listed in the continuation of Box C.

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See patent family annex.

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- *O* document referring to an oral disclosure, use, exhibition or other means
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Date of the actual completion of the international search

21 July 2010

Date of mailing of the international search report

28/07/2010

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Messemanne, Jasmine

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IT2009/000488

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