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(54) **Title**: METHOD FOR PRODUCING A DECELLULARIZED TISSUE MATRIX

(57) **Abstract**: There is provided a method of decellularizing adipose tissue comprising the steps of: a) Laminating the adipose tissue;b) Treating the adipose tissue resulting from step a) with a lipoprotein lipase at 32-42 degrees Celsius and at a concentration of 10-55u/100mg; and c) Treating the adipose tissue resulting from step b) with a nuclease, at 32-42 degrees celsius and at a concentration of 709-1433u/mg; for the necessary period of time for the adipose tissue to have a total of DNA content equal to or less than 50 ng/mg. There is also provided the decellularized adipose tissue obtainable by the method, further products comprising the decelluarized tissue such as coatings and scaffolds, and its uses in tissue engineering and regenerative therapy.

Method for producing a decellularized tissue matrix

This application claims the benefit of European Patent Application EP15203113.4 filed on December 30th, 2015.The present invention relates to a method for producing a decellularized adipose tissue matrix, to the decellularized adipose tissue matrix obtainable by it and to its uses. The adipose tissue obtainable by the method has multiple applications in tissue engineering and regenerative therapy, especially in the topical treatment of wounds.

BACKGROUND ART

Skin ulcers are open lesions that usually involve the destruction of the epidermis and the dermis. In the worst of cases, the deep lesions also involve the destruction of the hypodermis, the lower layers of the skin. When the ulcers take more than 6 weeks to scar, they are classified as chronic. Chronic ulcers can be caused by a variety of different pathologies ranging from infections and local ischemia to cardiovascular dysfunctions. These have a high prevalence, inflict a heavy burden on the health systems worldwide and in many cases are not managed easily in the clinic.

Conventional topical treatments include bandages based on hydrogels, hydrocolloids, foams, alginates, active carbons, silicones and silver. However, even when there is an accurate diagnostic and the current standard treatment is administered, there are a high percentage of cases that remain resistant and do not respond positively.

One of the most promising last generation therapies for chronic cutaneous ulcers is based on the topical administration of acellular biological matrices. These are products that are derived from animal tissue, and which can be applied topically at the site of the wound to stimulate and replace the disrupted or missing extracellular matrix (ECM). When applied topically, they provide a scaffold rich in proteins, glycolipids and other ECM components to which cells migrate and proliferate, enabling tissue regeneration. Biological matrices are currently being explored in several applications, not only in regenerative therapy but also for tissue engineering such as internal implants and orthopedic devices. In particular, an interesting application, unrelated to

therapy, is the use of such matrices for *in vitro* culture devices. These acellular matrices resemble an actual tissue and therefore can be aptly used in any cell-culture device to promote the maintenance and growth of a cell culture.

There are currently a few commercial products exploiting such technologies in wound healing. They are obtained both from tissues of animal (Matriderm, Primatrix, Integra) and human (Apligraf, Epicel, Graftjacket) origin. However, all of them act at the level of the epidermis and dermis. Unfortunately, matrices that are capable of regenerating the hypodermis are still lacking, both because of their sub-optimal properties and because they are not obtained from subcutaneous adipose tissue.

The generation of a therapeutic acellular biological matrix from an animal tissue is not a straightforward process for several reasons. Any method for processing an initial tissue to yield an acellular matrix must ensure that the final product is endowed with an overall structure and mechanical properties that resemble that of the native ECM matrix, it must also preserve the proteins and other macromolecular components that enable the matrix to be used as a scaffolding for the regenerative processes to take place, and most crucially must be devoid of any nuclear components such as nuclear debris and DNA that would render it pro-inflammatory and immunogenic. Thus, any method of acellular matrix production must make sure that the series of treatments applied maximize the elimination of the immunogenic components of the original tissue and at the same time minimize the loss of structural characteristics that enable it to provide a barrier to infection and promote optimal cell activity, revascularization and tissue regeneration. This balance is not easily achieved.

A number of descriptions of methods for decellularizing a tissue to produce an acellular matrix are found in the prior art. Most are composed of a series of consecutive treatment steps where a variety of mechanical and/or chemical and/or biochemical (enzymatic) treatments are applied. Gilbert TW., et. al. “Strategies for tissue and organ decellularization” J. Cell. Biochem. 2012, vol. 113, pp. 2217-2222, summarizes the typical treatments implemented in the decellularization of tissues in regenerative medicine. Typical enzymatic treatments include trypsin, DNAse, lipase and α-galactosidase. They are usually combined with a variety of physical and chemical treatments to

facilitate removal of undesirable components and to boost the effects of the degrading enzymes. The chemical treatments fall into different categories such as detergents, organic solvents, acidic and alkaline solutions, etc. As it is disclosed in this reference, it is extremely rare to only rely in a single chemical treatment to decellularize a tissue, and it is generally regarded as more advantageous to combine numerous chemicals and biochemicals in a series of short washes to ensure the efficiency of the treatments and hence a proper decellularization. However, the use of chemicals implies a risk in terms of contamination with undesirable residues which are not apt for biopharmaceutical applications in the final product. Ideally, it would be desirable to find methods which do not imply the use of any chemical treatments that may raise safety concerns.

In US2012/0264190 it is disclosed a decellularized and delipidized extracellular matrix produced from adipose tissue, and methods for producing it, which can be composed of a series of treatments including delipidation with lipases, protein digestion with a range of proteases such as pepsin, papain, matrix metalloproteases (MMPs) and trypsin, nucleic acid digestion with endonucleases, exonucleases, DNAses and RNAses. However, this method is characterized by a step where detergents such as sodium dioxycholate, sodium docecyl sulphate or Triton X-100 are used.

US2011/0151011 discloses a method for decellularizing adipose tissue comprising subjecting the adipose tissue to a series of enzymatic digestions and a series of solvent extractions so that the final acellular adipose tissue has a well preserved 3D structure for regenerative therapy. The methods described can involve protocols where a series of treatments can be combined, including treatments with proteases, DNAse and RNAse, chelating agents such as EDTA, detergents such as Triton-X100 and lipases.

US2013/0202563 discloses a method of producing a cell growth scaffold from adipose tissue comprising a washing step with n-propanol, isopropanol or a mixture thereof. The treatment does not imply the use of enzymes for the degradation of the original tissue components, and is mostly based on the use of alcohols.

WO2011/132089 discloses a method for decellularizing tissues which

comprises contacting the original tissue with different chemical solutions and surfactants and then treating the resulting surfactant-treated tissues biochemically with a series of degrading enzymes such as nucleases.

The promise of the use of biological matrices for regenerative therapy warrants the development of new, safer and more efficient production methods. The development of methods where minimal disruption of the original overall structure and maximal elimination of pro-inflammatory and immunogenic components is achieved is still an active area of research.

SUMMARY OF THE INVENTION

Inventors have devised a simple and yet very efficient method of decellularizing adipose tissue. The method of the invention comprises a reduced number of steps that render it substantially simpler than many methods found in the art, thanks to the particular conditions that have been found to be optimal in terms of performance. The method involves the use of only two biochemical (enzymatic) treatment steps, namely lipoprotein lipase and a nuclease of high (biopharmaceutical) quality.

Remarkably the method does not involve a series of lengthy, serial chemical treatments, which ensures that it can be applied without raising any concerns related to the safety of the final decellularized matrix. The two enzymatic treatments are carried out under very specific conditions of concentration, duration and temperature, and only with enzymes of biopharmaceutical quality, which guarantee the quality of the decellularized matrix obtained. Of note, the method will usually entail the use of resected tissue (although it could be applied to lipoaspirates as well), it does not feature a combination of thorough chemical treatments and does not involve the use of reagents of animal origin, organic solvents or any aggressive mechanical treatments.

Thus, a first aspect of the present invention is a method of decellularizing adipose tissue comprising the steps of: a) Laminating the adipose tissue; b) Treating the adipose tissue resulting from step a) with a lipoprotein lipase at 32-42 degrees Celsius and at a concentration of 10-55u/100mg; and c) Treating the adipose tissue resulting from step b) with a nuclease, at 32-42 degrees Celsius and at a concentration of 709-1433u/mg; for the necessary

period of time for the adipose tissue to have a total of DNA content equal to or less than 50 ng/mg.

In the present invention the DNA content is referred to the ng of DNA per mg of dry weigth decellularized human adipose tissue, as described in the examples.

The decellularized matrix obtained by the method is endowed with properties expected for therapeutic applications as it has a very low DNA content (usually far below 50ng/mg, which is the threshold above which *in vivo* immune reactions have been described according to Crapo, PM., et. al. "An overview of tissue and whole organ decellularization processes" Biomaterials 2011, vol. 32, pp. 3233-3243), a desirable percentage of extracellular matrix protein (in particular perlecan, elastin, collagen type I and IV) and a surprisingly well preserved overall morphology. This last point is key. The matrix has a global structure resembling that of the original tissue, which maximizes the likelihood of success in regenerative medicine. Therefore, inventors have found a method that neatly strikes a balance between elimination of unwanted components such as nuclear debris and other immunogenic DNA and preservation of structural integrity of the original tissue.

Of note, the method can be applied with subtle variations that allow obtaining a decellularized matrix with the desired qualities but with varying degrees of triglyceride content, which is an advantage since depending on the final application the matrix might need a higher or lower content in lipids.

Thus, a second aspect of the present invention is a decellularized adipose tissue matrix obtainable by the method of the first aspect of the invention.

Because of the low content of undesirable components and its overall morphological structure, the matrix obtainable by the method can find a myriad of different applications, including the most restrictive and demanding ones such as the healing of deep wounds and ulcers that affect the hypodermis. The maximized conservation of the original morphology together with the final composition of the treated matrix ensures a maximal performance for regenerative purposes. The matrix obtainable by the first

aspect is characterized by an absence of organic solvents, low residual content in terms of immunogenic components ensuring a minimal host response when applied in therapy, remarkable structural and functional similarities with the endogenous tissue, rich in basement membrane proteins and preserved overall architecture.

A third aspect of the present invention is a powder, foam, particle or hydrogel comprising the decellularized adipose tissue according to the second aspect of the invention.

A fourth aspect of the invention is a biocompatible scaffold or biocompatible coating comprising the decellularized adipose tissue according to the second aspect of the invention or the powder, foam, particle or hydrogel of the third aspect of the invention.

A fifth aspect of the invention is the decellularized adipose tissue according to the second aspect of the invention, the powder, foam, particle or hydrogel according to the third aspect of the invention, or the biocompatible scaffold or biocompatible coating according to the fourth aspect of the invention for use in tissue engineering and regenerative therapy.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1: H&E stained sections of normal and decellularized hAT (hAT and dhAT-LM4). Adipocyte (column a), fibrillary regions (column b, asterisk) and vascular structures (column c, arrows) are shown. Scale bar represents 200 µm.

FIG. 2: IHQ analysis of Collagen type I (fibrillary region, column a), collagen type IV (adipocyte region, column b), collagen type IV (vascular structures, column c), laminin (column d) and HSPG2 (column e) of normal and decellularized hAT (hAT and dhAT-LM4). Vascular structures have been identified (arrow). Scale bar represents 200 µm.

FIG. 3: H&E staining (column a) and IHQ analysis of collagen type I (column b), collagen type IV (column c), laminin (column d) and HSPG2 (column e) of dhATs obtained by different lipase treatment (LM3 and LM5). Scale bar

represents 100µm or 200 µm.

FIG. 4: H&E staining and IHQ analysis of collagen type-I of dhATs treated and untreated with the high quality nuclease Benzonase (LM3 and M8 respectively). Column a, adipocyte region. Column b, fibrillary region. Column c, collagen Type-I.
Scale bar represents 200 µm.

FIG. 5: H&E staining and IHQ analysis of collagen type I (a), collagen type IV (b), laminin (c) and HSPG2 (d) of dhATs untreated with trypsin/triton-x100 (M7). Adipocyte (I), fibrillary regions (II, asterisk) and vascular structures (III, arrow) are shown. For comparative purposes with treated dhAT see Figure 3 (LM3 dhAT). Scale bar represents 200 µm.

FIG. 6: Macroscopic images and relative triglyceride content (%) of original hAT and dhATs obtained by different Lipase incubation conditions in the decellularization process (LM1-LM5, conditions described in Table 1). Results are shown as relative triglyceride content (%) considering original hAT as a 100%.

FIG. 7: Macroscopic images and relative triglyceride content (%) of original hAT and dhATs obtained by the decellularization process with the lack of trypsin/triton-x100 pretreatment (M7) or Benzonase treatment (LM8). Results are shown as relative triglyceride content (%) considering original hAT as a 100%.

FIG. 8: Macroscopic and microscopic images of the processed dhAT of the invention: a) macroscopic image of a powder obtained by micronization, b) microscopic image of powder obtained by Scanning Electron Microscopy. Scale bar 100 µm, c) macroscopic image of a porous scaffold obtained by freeze drying, d) microscopic image of a porous scaffold obtained by Scanning Electron Microscopy, e) macroscopic image of the application of plastic compression, f) macroscopic image of a sheet obtained by plastic compression, g) macroscopic image of rolled compressed sheet and h) macroscopic image of a cylindrical form obtained by rolling the compressed sheet.

DETAILED DESCRIPTION OF THE INVENTION

For the sake of understanding, the following definitions are included and expected to be applied throughout description, claims and drawings.

The terms “acellular” and “decellularized” are herein used interchangeably.

The term “decellularization” as used herein refers to a process by which a tissue is submitted to one or more treatments in order to maximize the removal of cells present in it, leaving only the extracellular matrix (ECM) that is rich in structural proteins such as collagens, elastin, growth factors, and glycolipids.

The term “biocompatible scaffold” as used herein refers to a substance with sufficient structural stability to provide a substrate to support, foster and promote the growth of living cells which make up a tissue. Such a scaffold can be used for recovery of a damaged tissue. The scaffold fills in the gap left by a wound, giving a structure to be colonized by cells and new blood vessels, ultimately leading to tissue regeneration.

As mentioned above, a first aspect of the present invention is a method of decellularizing adipose tissue comprising the steps of: a) Laminating the adipose tissue; b) Treating the adipose tissue resulting from step a) with a lipoprotein lipase at 32-42 degrees Celsius and at a concentration of 10-55u/100mg; and c) Treating the adipose tissue resulting from step b) with a nuclease, at 32-42 degrees Celsius and at a concentration of 709-1433u/mg; for the necessary period of time for the adipose tissue to have a total of DNA content equal to or less than 50 ng/mg;

In a particular embodiment of the first aspect of the invention, the method of decellularizing adipose tissue is a method wherein the lipase treatment b) is carried out either: at a concentration of 10-30u/100mg for 39-49 hours at 32-42 degrees Celsius or at a concentration of 45-55u/100mg for 18-28 hours a 32-42 degrees Celsius; and the nuclease treatment c) is carried out either at a concentration of 709-719u/mg for 67-77 hours at 32-42 degrees Celsius or at a concentration of 1423-1433u/mg for 35-45 hours at 32-42 degrees Celsius or at a concentration of 1423-1433u/mg for 67-77 hours at 32-42 degrees

Celsius.

In another particular embodiment of the first aspect of the invention, the method of decellularizing adipose tissue is a method wherein in step b) the treatment is at a concentration of 25u/100mg for 44 hours at 37 degrees Celsius or at a concentration of 50u/100mg for 23 hours at 37 degrees Celsius, and step c) is carried out either at a concentration of 709-719u/mg for 67-77 hours at 32-42 degrees Celsius or at a concentration of 1423-1433u/mg for 35-45 hours at 32-42 degrees Celsius or at a concentration of 1423-1433u/mg for 67-77 hours at 32-42 degrees Celsius.

In another particular embodiment of the first aspect of the invention, the method of decellularizing adipose tissue is a method wherein step b) is carried out either at a concentration of 10-30u/100mg for 39-49 hours at 32-42 degrees Celsius or at a concentration of 45-55u/100mg for 18-28 hours a 32-42 degrees Celsius, and in step c) the treatment is at a concentration of 714u/mg for 72 hours at 37 degrees Celsius or at a concentration of 1428u/mg for 40 hours at 37 degrees Celsius or at a concentration of 1428u/mg for 72 hours at 32-42 degrees Celsius.

In another particular embodiment of the first aspect of the invention, the method of decellularizing adipose tissue is a method wherein in step b) the treatment is at a concentration of 25u/100mg for 4 hours at 37 degrees Celsius.

In another particular embodiment of the first aspect of the invention, the method of decellularizing adipose tissue is a method wherein in step b) the treatment is at a concentration of 50u/100mg for 23 hours at 37 degrees Celsius.

In another particular embodiment of the first aspect of the invention, the method of decellularizing adipose tissue is a method wherein in step c) the treatment is at a concentration of 714u/mg for 72 hours at 37 degrees Celsius.

In another particular embodiment of the first aspect of the invention, the method of decellularizing adipose tissue is a method wherein in step c) the treatment is at a concentration of 1428u/mg for 40 hours at 37 degrees

Celsius.

In another particular embodiment of the first aspect of the invention, the method of decellularizing adipose tissue is a method wherein in step c) the treatment is at a concentration of 1428u/mg for 72 hours at 37 degrees Celsius.

In another particular embodiment of the first aspect of the invention, the method of decellularizing adipose tissue further comprises a step between steps a) and b), wherein the adipose tissue resulting from step a) is treated with trypsin and triton-X100. In another particular embodiment, this last step is characterized by the fact that the trypsin-tritonX100 treatment further comprises EDTA and it is carried out at 37 degrees Celsius overnight and Triton-X100 is at 1% in v/v (1ml/100ml).

This further step is not compulsory as can be seen in the experimental data found below, where the decellularized matrix with the best properties is obtained without it (M7). However, if the final product is desired to have a lower content in lipids (such as that found for LM3 or LM4), then this optional treatment step of tripsin+triton-X100 may be implemented.

In another particular embodiment of the first aspect of the invention, the method further comprises a step d) comprising freezing or liofiphilizing and sterilizing the adipose tissue resulting from step c).

In another particular embodiment of the first aspect of the invention, the sterilization is carried out with ethylene dichloride. In another particular embodiment the sterilization is carried out with ultraviolet light. In another particular embodiment the method is carried out under aseptic conditions.

In another particular embodiment of the first aspect of the invention, the method of decellularizing adipose tissue is a method wherein in steps b) and c) the treatments are treatments under vacuum and with stirring at 100-150 rpm.

In another particular embodiment, the stirring at 100-150 rpm is orbital stirring.

In another particular embodiment of the first aspect of the invention, the method of decellularizing adipose tissue is a method wherein after steps a), b) and c), there is a washing step with buffer at room temperature, under vacuum and under stirring at 100-150 rpm, and wherein the washing step after step a) the buffer further comprises at least one antibiotic, at least one antimycotic and at least one protease inhibitor, in the washing step after step b) the buffer comprises at least one antibiotic, at least one antimycotic, at least one protease inhibitor and at least one lipase inhibitor and wherein the washing step after step c) the buffer further comprises at least one antibiotic, at least one antimycotic ,at least one protease inhibitor and at least one nuclease inhibitor.

In another particular embodiment of the first aspect of the invention, the method of decellularizing adipose tissue is a method wherein in step b), the lipoprotein lipase treatment comprises Triton-X100 at 0.5% and a cofactor in phosphate buffer. In another particular embodiment of the first aspect of the invention, the method of decellularizing adipose tissue is a method wherein in step b), the lipoprotein lipase treatment comprises Triton-X100 at 0.1% and a cofactor in phosphate buffer.

In another particular embodiment, steps b) and c) are carried out in the presence of a cofactor. In another particular embodiment, the cofactor is magnesium (Mg+2).

In another particular embodiment of the first aspect of the invention, the method of decellularizing adipose tissue is a method wherein in step a), the lamination of the adipose tissue is manual. In another particular embodiment of the first aspect of the invention, the method of decellularizing adipose tissue is a method wherein in step a), the laminated block sizes are 1-15 cm long, 0.5-8 cm wide and 0.3-2 cm thick.

In another particular embodiment of the first aspect of the invention, the method of decellularizing adipose tissue is a method wherein in step b), the lipoprotein lipase is selected from the group consisiting of lipase and phospholipase from bacterial origin (genus Pseudomonas, Staphyloccus, Bacillus, etc.), yeast origin (genus Candida aldicans, Candida Antarctica, Candida rugosa, Geotrichum asteroids, Geotrichumcandidium,,

Trichosporonfermentans, Saccharomycopsis lipolytica, Yarrowia lipolytica), fungal origin (genus Penicillum, Rhizopus, Rhizomucor, etc), and mammalian origin (porcine, bovine, horse, human, etc.). In this latter group there are included the lipoprotein lipase, acylglicerol lipase, triacylglicerol lipase, hormone sensitive lipase, pancreatic lipase, bile salt activated lipase, pancreatic lipase related protein 1, pancreatic lipase related protein 2, phospholipase A1, phospholipase A2, calcium independent phospholipase A2, endothelial lipase, phosphatidylinositol phospholipase A2, endogenous phospholipase C, phosphoinosositide phospholipase C, phospholipase C, lysophospholipase D and phospholipase D.

In another particular embodiment of the first aspect of the invention, the method of decellularizing adipose tissue is a method wherein in step c), the nuclease is Benzonase®. Benzonase is an endonuclease with very high pharmaceutical quality provided by Merck-Millipore, which states in its technical specifications: Benzonase® is a unique, genetically-engineered endonuclease that is only available from Merck Millipore. Produced in *E.coli*, this non-specific, recombinant endonuclease cleaves all kinds of DNA and RNA variants into fragments that comprise < 8 soluble base pairs. This leads to an utmost minimum of nucleic acid load.

In another particular embodiment of the first aspect of the invention, the method of decellularizing adipose tissue is a method wherein in step c), the nuclease is selected from the group consisiting of nucleases from bacterial origin (Serratia marcescens, Clostridium), yeast origin, fungal origin (Asperguillus), and mammalian origin (porcine, bovine, horse, human, etc.) including deoxyribonuclases (DNases) and ribonucleases (RNases) such as DNase I, DNase II, DNase V, micrococcal nuclease, type I site-especific DNase, RNase I, RNase H, RNase III, RNase L, RNase P, nuclease S1, yeast RNase, RNase U2, RNase T2, RNase T1, RNase P4, RNase M5, RNase IX, RNase E, RNase D, RNase alpha, RNase PH, RNase AS, RNase Phyb, RNase J1, Pancreatic RNase, nanoRNase.

As mentioned above, a second aspect of the present invention is a decellularized adipose tissue matrix obtainable by the method of the first aspect of the invention.

In a particular embodiment of the second aspect of the invention, the adipose tissue to be treated is of subcutaneous origin, such as mammary or abdominal.

In a particular embodiment of the second aspect of the invention, the adipose tissue to be treated is obtained from lipoaspirate.

In another particular embodiment of the second aspect of the invention, the adipose tissue to be treated is obtained from resection.

In another particular embodiment of the second aspect of the invention, the adipose tissue derives from the epiplon or the omentum.

In a particular embodiment of the second aspect of the invention, the adipose tissue is mammal adipose tissue.

In a particular embodiment of the second aspect of the invention, the adipose tissue is human adipose tissue.

In a particular embodiment of the second aspect of the invention, the adipose tissue obtainable by the method of the first aspect has a total DNA content of 0.015-0.306 ng/mg. This range of DNA content is considered as very acceptable by the inventors as it is far below the 50ng/mg threshold described above. Thus, the first aspect of the invention allows obtaining a decellularized matrix that, although it is characterized by a highly preserved morphology when compared to the original tissue, it has a nuclear residual content which minimizes the likelihood of *in vivo* adverse immune reactions.

In a particular embodiment of the second aspect of the invention, the adipose tissue matrix further comprises foreign bioactive molecules. In a particular embodiment of the second aspect of the invention, the natural or synthetic bioactive molecules are selected from the group consisting of growth factors, hormones, vitamins, antioxidants, anti-inflammatories, antibacterial, antifungal, wound healing accelerators/healing promoting agents, or mixtures thereof.

In a particular embodiment of the second aspect of the invention, the adipose tissue matrix further comprises i) synthetic polymers (PEG, poly(α-hydroxy

esters), polystyrene, polyurethane and copolymers and blends) and ii) natural polymers (protein-based elastin, collagen, fibrin; or polysaccharide-based collagen, chitosan, hyaluronic acid, beta-glucans, gelatin, micro- and nano-cellulose and their derivatives).

In a particular embodiment of the second aspect of the invention, the adipose tissue matrix further comprises human or animal live cells (inside or on top of the material): somatic cells (adipocyte, fibroblast, osteocyte, osteoblast chondrocyte, chondroblast, myo-epithelium, bone marrow, macrophages, etc.), endothelial, stem cells or induced pluripotent stem cells.

In a particular embodiment of the second aspect of the invention, the decellularized adipose tissue has a total protein content of 100-931 µg/mg and a triglyceride content of 25-65% of the original tissue (w/w).

Depending on the eventual application, the second aspect of the invention can be further processed by micronization, plastic compression, dissolution, freeze drying, self-assembling, chemical crosslinking and/or induced physical interactions, electrospinning, spin coating or 3D printing.

As mentioned above, a third aspect of the present invention is a powder, foam, particle or hydrogel comprising the decellularized adipose tissue according to the second aspect of the invention.

In a particular embodiment of the third aspect of the invention, the powder, foam, particle or hydrogel comprising the decellularized adipose tissue is mixed with further bioactive components such as growth factors, foreign structural proteins or scarring agents. The resulting composite mixture has additional advantages in terms of tissue regeneration.

As mentioned above, a fifth aspect of the invention is the decellularized adipose tissue according to the second aspect of the invention, the powder, foam, particle or hydrogel according to the third aspect of the invention, or the biocompatible scaffold or biocompatible coating according to the fourth aspect of the invention for use in tissue engineering and regenerative therapy

In a particular embodiment of the fifth aspect, the use is in combination with a

matrix selected from the group consisting of Matriderm, Primatrix, Integra, Apligraf, Epicel and Graftjacket.

In a particular embodiment of the fifth aspect, the use is in combination with any other matrix available in regenerative medicine.

In a particular embodiment of the fifth aspect, the therapy is a therapy of wound healing. In particular, the manifold applications of the first aspect of the invention comprise: wound healing, tissue engineering, regenerative medicine, additive therapies, and cell carriers for the treatment of disease or damaged tissue and organs, healing or prevention of disease and restoration, correction or alteration of physiological functions of: i) connective tissue (adipose, skin, blood vessel, cartilage, bone etc.), ii) epithelial and endothelial tissue (epidermis, intestine epithelium, vessel endothelium etc.), iii) muscle tissue and iv) nervous tissue.

In a particular embodiment, the wound healing is wound healing of deep wounds (type III and IV) involving the hypodermis. In a particular embodiment, the wound healing is wound healing of diabetic foot ulcer, venous ulcer or pressure ulcer.

In a particular embodiment of the fifth aspect, the use is an autologous use, that is, the adipose tissue is extracted from a patient, the method of the invention is applied to said adipose tissue, and then the resulting decellularized matrix is applied to the same patient for regenerative therapy or tissue engineering purposes.

In a particular embodiment of the fifth aspect, the use is an allogenic use, that is, the adipose tissue is extracted from a first donor, the method of the invention is applied to said adipose tissue, and then the resulting decellularized matrix is applied to a second subject for regenerative therapy or tissue engineering purposes.

In a particular embodiment of the fifth aspect, the use is a xenogenic use, that is, the adipose tissue is extracted from a first animal donor, the method of the invention is applied to said adipose tissue, and then the resulting decellularized matrix is applied to a second subject which belongs to a

different species as the first animal donor.

In a particular embodiment of the fifth aspect, the use in tissue engineering is for an orthopedic device.

It is also part of the invention, the use of the second aspect of the invention for *in vitro* culture of cells. The matrix obtainable by the first aspect of the invention can be used as a support in any in vitro cell-culture device, thus offering a biocompatible support which fosters cell growth and differentiation.

Thus, the second aspect of the invention can find applications for in vitro normal and diseased 2D and 3D cell culture to obtain: i) an analogous environment to *in vivo* tissue; ii) cell carriers and iii) organ synthesis for research, diagnostic, drug screening or implantation. It can also find applications in dressing materials, cell carriers, coatings, fillers, flowable powdered particles for drug delivery, bioinks, scaffolds (foams, micro- and macro-porous hydrogels, thermal inducing hydrogels), sheets, , patch, cylindrical or tubular form, spray.

Throughout the description and claims the word "comprise" and variations of the word, are not intended to exclude other technical features, additives, components, or steps. Furthermore, the word “comprise” and its variations encompass the term “consisting of”. Additional objects, advantages and features of the invention will become apparent to those skilled in the art upon examination of the description or may be learned by practice of the invention. The following examples are provided by way of illustration, and they are not intended to be limiting of the present invention. Furthermore, the present invention covers all possible combinations of particular and preferred embodiments described herein.

EXAMPLES

Materials and Methods

1 Donation of human adipose tissue

Human adipose tissue (hAT) was donated by patients who have undergone plastic surgery at Policlínica Gipuzkoa Hospital according to the Spanish

Ministry act 1301/2006, of 10 November, which lays down standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of cells and human tissues. The investigation was carried out previous approbation by the Basque Country Ethical Commission (Comité Ético de Investigación Clínica, CEIC).

Tissues were cleaned with ultrapure distillated water (Millipore), fractionated manually by a blade in approximately 25 cm^3 and frozen at -30ºC until use. hATs were treated for the decellularization processes as described below. All the processes were carried out under aseptic conditions.

2 Decellularization of human adipose tissue

Tissue fractions were defrosted at room temperature (RT) and sliced manually by a blade in pieces of approximately 0.3 x 0.5 x 1 cm and treated with Trypsin and Triton-x100, Lipoprotein lipase and DNAse for tissue decellularization.

In order to eliminate remnant reagents, washing steps were carried out between treatments with a phosphate buffer (PBS – phosphate buffered saline) composed of Disodium hydrogen phosphate dehydrate (1.2 mg/l, EMPROVE® bio, Merck Millipore), Sodium dihydrogen phosphate dehydrate (0.885 mg/l, EMPROVE® bio, Merck Millipore), 10ml/l penicillin-streptomycin (in accordance with cGMP, LONZA), 10ml/l fungizone (Fungizone® Antimycotic, ThermoFisher Scientific) and 1.25 ml/l protease inhibitor cocktail III (EDTA-free, Merck Millipore). For washing, fresh 5 ml phosphate buffer was added to each tissue fraction and maintained under vacuum and orbital shaking (100 rpm) at RT for 10min. This rinse step was repeated at least 5 times and could include cofactors for the inactivation of the enzymes.

In order to fine-tune the decellularization process and to analyze the effect of each treatment on the decellularization effectivity and native ECM protein conservation, tissues were treated under several conditions: i) avoiding one of the treatments (trypsin and triton-x100, lipase or DNAse), ii) applying several enzyme concentration and incubation times (both lipase and DNase) and iii) applying a lower quality enzyme (DNAse for research use). A description of decellularization treatment conditions for each sample is described in Table 1 (found below).

ID	DECELLULARIZATION CONDITIONS		
	TRYPSIN + TRITON-X100	LIPASE	DNASE
BM1	30 min. Trypsin/EDTA 37°C And 1% Triton-x overnight.RT	50u./100mg 23h 37°C	UNTREATED
BM2			Benzonase EMPROVE® bio 714 u./mg 40h. / 37°C
BM3			Benzonase EMPROVE® bio 714 u./mg 72h./ 37°C
BM4			Benzonase EMPROVE® bio 1428 u./mg 40h./ 37°C
BM5			Benzonase EMPROVE® bio 1428 u./mg 72h./ 37°C
BM6		50u/100mg 23h 37°C	DNAse for research use 1428 u./mg 72h. / 37°C
LM1		UNTREATED	Benzonase EMPROVE® bio (Merck Millipore) 1428 u./mg 72h. / 37°C
LM2		25u/100mg 23h 37°C	
LM3		25u/100mg 44h 37°C	
LM4		50u/100mg 23h 37°C	
LM5		50u/100mg 44h 37°C	
M7	UNTREATED	25u/100mg 44h 37°C	Benzonase EMPROVE® bio 1428 u./mg 72h. / 37°C
M8	30 min. T/E 37°C And 1% Triton-x o.n.RT		UNTREATED

Table 1: Description of decellularization process conditions and sample ID.

Most but not all of the tissues were subjected to a pretreatment with trypsin (CTS™ TrypLE™ 1X For Use in Manufacturing Tissue-based Products, Thermofishcer Scientific) and triton-X100 (EMPROVE® bio Merck Millipore). To do so, trypsin (3 ml/tissue fraction) was added and maintained for a short period of time (30min) stirred with magnetic bars (50 rpm) at 37ºC. After rising, 1% triton-x100 in the buffer described earlier was added (8 ml/tissue fraction) and maintained overnight at RT. As indicated in Table 1, some tissues were untreated with trypsin and tryton-x100 (M7) in order to analyze the effect of

this protease and detergent treatment in the conservation of the tissue morphology and ECM proteins.

After rinsing, tissues were treated with a Lipoprotein lipase (IVD quality, Roche) under several enzyme concentration and incubation time (25-50u/100mg during 23h or 44h at 37ºC, 100rpm. orbital shaking and vacuum, Table 1). The enzyme was dissolved in the buffer described earlier (3 ml) within 0.5% triton-x100 (EMPROVE® bio, Merck Millipore).

After rinsing, tissues were treated with a DNase (Benzonase EMPROVE® bio Merck Millipore) under several enzyme concentration and incubation time (714u-1428u/mg during 23h or 44h at 37ºC, 100rpm. orbital shaking and vacuum). The enzyme was dissolved in the buffer described earlier (3 ml) with the addition of 2mM Magnesium chloride hexahydrate (EMPROVE® Merck Millipore) as an enzyme cofactor. Some tissues were treated with a research quality DNAse I (Sigma Aldrich Chemical) under one specific Lipase and DNAse conditions (50u/100mg and 714u/mg 72h respectively).

dhATs were used freshly or lyophilized before freezing for characterization of the treatments effect on residual DNA, tissue morphology, cell-nuclei observation (Hematoxylin & Eosin) and ECM protein expression and arrangement (Immunohistochemistry). Finally some dhATs were subjected to total triglyceride and protein content quantification. Protein composition was analyzed by chromatography in one of the dAT (LM4).

3 Quantification of the residual DNA

Remnant single and double strain DNA was analyzed by quantitative Real Time Polymerase Chain Reaction (qRT-PCR). The DNA was extracted from the lyophilized dhATs by the QiAmp kit (Qiagen) according to the manufacturer's instructions. For the absolute quantification, a standard curve of known concentration DNA samples was obtained from human blood. To do so, DNA extraction was done by the Qiamp DNA Blood MiniKit (Qiagen) according to the manufacturer's instructions. A 50 ng/ml DNA solution was obtained and analyzed by ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) and serial dilutions were prepared. Detection was carried out by the specific sequence of the human gene hemoglobin, beta (HBB, 11p.15.5) and analyzed using LightCycler 480II (Roche).

Reactions were performed in triplicate; data were shown as remnant DNA (ng) for dry weight dhAT (mg) and subjected to statistical analysis as described later. The detection limit of the technique was estimated in approximately 20pg of DNA. The DNA quantification was carried out by DNAdata, a Genetic Disease Diagnostic Centre authorized by The Basque Country Government.

4 Histologic analysis

Freshly conserved dhATs were subjected to histological analysis of tissue morphology and cell nuclei observation by Hematoxylin-Eosin (H&E) staining and inmunohistochemical analysis of ECM proteins.

To do so, dhATs were introduced in formalin (Bellés Diagnóstic i Investigació), included in paraffin (Histowax LEICA) by an automatic tissue processor (ASP300, Leica Microsystems) and sectioned in 3.5µm. Some of the sections were stained by H&E following own procedures. Briefly, sections were treated with xylene (Bellés Diagnostics, S.L), alcohol (Citoscan, Bellés Diagnostics) and running water previous to 4-7 min Hematoxylin treatment (Haematoxylin Harris GURR® mercury free, VWR). After that, sections were treated with running water and acidic alcohol (99:1, etanol: hidroclorhidric acid 37%, VWR) and 5-30 seg. Eosin (Giemsa's azur eosin methylene blue solution, VWR). Finally sections were treated with alcohol, xylene and DPX mounting media (Casa Alvarez).

The immunohistochemistry was carried out for the detection of collagen type-I, collagen type-IV, laminin and heparan sulphate proteoglycan-2 (HSPG2 or perlecan) proteins by the automatic Benchmark XT (Roche Diagnostics) system. The methodology consisted on the following steps: i) deparaffination ii) antigenic recuperation, iiI) dilution and incubation with appropriate monoclonal antibody, iv) amplification, detection and visualization by Amplification Kit (Roche) and Ultraview Universal DAB Detection Kit, (Roche). Staining was optimized for each protein using kidney, skin, colon, placenta and adipose tissue as controls and specific conditions were detailed in Table 1. Samples were visualized by BX-51 (Olympus) microscope and digitalized using a Nanozoomer 2.0 RS scan (Hamamatzu).The Specific IHQ conditions for each protein is described in Table 2 (found below).

IHQ conditions and controls				
Protein	**Antigenic recuperation**	**Antibody, Dilution and incubation**		**Positive control**
Collagen type-I	Cell conditioning solution1 (CC1, Roche) ph=8.3 30 min.,	Antibody	Mouse monoclonal Collagen type-I clon COL-I (Abcam)	Kidney
		Dilution	1:200	
		Incubation	37ºC/32min	
Collagen type-IV	CC1 (ph=8.3) 30 min.,	Antibody	Mouse monoclonal collagen type-IV clon CIV22 (Roche)	Kidney
		Dilution	No dilution required	
		Incubation	37ºC/32min	
Laminin	Pepsin-HCl 0.2M (Sigma) RT 30 min.	Antibody	Mouse monoclonal Anti-Human laminin clon 4C7 (Dako),	Kidney
		Dilution	1:10,	
		Incubation	RT/60min	
HSPG2/Perlecan	CC1 (ph=8.3) 30 min.,	Antibody	mouse monoclonal Anti-Heparan SulfateProteoglycan 2, clon A74, (Abcam)	Colon
		Dilution	1:10	
		Incubation	37ºC/32min	

Table 2: Specific IHQ analysis conditions for collagen type I, collagen type-IV, laminin and HSPG2.

These histological evaluations were performed by Althia Health, S.L. (Althia), a company accredited by the Spanish National Entity of Accreditation (ENAC) for the testing of Pathology and Molecular Pathology and as technical competence according to the criteria of the standard UNE-EN-ISO 15189: 2007 (laboratory reference number 1029 / LE2012).

5 Quantification of the triglyceride content

Triglyceride quantification was carried out by the Triglyceride Quantification Kit (Biovision) according to the manufacturer's instructions. Briefly, 100mg dhAT samples were prepared by homogenization in 1 ml solution containing 5% NP-40 (Biovision) in water and slowly heated to 80-90ºC in a water bath for 5 min. After cooling down at room temperature, the heating process was repeated

one more time and insoluble material was removed by a speed centrifugation for 2min. Extracted samples were diluted 10 fold with deionized water. Absorbance (570nm) was measured in triplicates of samples and triglcyeride standards in a multiwell spectrophotometer (Power Wave Xs, Biotek). Triglyceride concentration was obtained from the standard curve and shown as relative percentage considering the non-treated original hAT as 100% triglyceride content.

6 Quantification of total protein content

For the total protein quantification, dhATs were firstly digested by cold acidic pepsin. 1% pepsin (Sigma Aldrich) in 0.5 M Acetic acid (Panreac) solution was prepared and 1ml/mg was added to the dhATs. The digestion was obtained under orbital shaking for 48h at RT. The acidic pH was neutralized and digested solutions were centrifuged at 4000rpm for 5min. Supernatants were stored at -30ºC until use.

Total protein content was analyzed by Pierce™ BCA Protein Assay Kit (Thermoficher Scientific) according to the manufacturer´s instructions. A standard curve was obtained with bovine serum albumin (BSA) standards included in the kit. Absorbance (570nm) was measured in triplicates of samples and standards in a multiwell spectrophotometer (Power Wave Xs, Biotek). Results showed as protein content (µg) for dry dhAT (mg).

7 Liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis.

Protein composition of one dhAT (LM4) was analyzed by LC-MS/MS. For protein extraction the dhAT was homogenized in 8M urea using a Precellys®24 homogenizer (Bertin Technologies) with 1.0 mm diameter zirconia/silica beads (BioSpec). The homogenate was sonicated for 3 min to reduce viscosity and the crude extract was then clarified by centrifugation at 16 000 × g for 10 min, transferred to new tubes and stored at -20 °C. After that, protein digestion was carried out diluting sample 5 fold with 50 mM NH4HCO3. Briefly, proteins were reduced (5 mM DTT, room temperature, 25 min), alkylated (15 mM iodoacetamide, room temperature, 30 min) and digested with trypsin (0.01 µg/µl, 37 °C, 16 hours, Roche Diagnostics). Resulting peptides were desalted using C-18 Micro SpinColumns (Harvard Apparatus).

LC-MS/MS analysis was performed using a Q Exactive (Thermo Scientific) interfaced with an Easy-nLC 1000 nanoUPLC System (Thermo Scientific). Digested peptides were loaded onto an Acclaim PepMap100 precolumn (75 µm x 2 cm, Thermo Scientific) connected to an Acclaim PepMap RSLC (50 µm x 15 cm, Thermo Scientific) analytical column. Peptides were eluted directly onto the nanoES Emitter (Thermo Scientific) with a 45 min linear gradient from 3% to 30% of acetonitrile in 0.1% of formic acid at a flow rate of 300 nl/min. The Q Exactive was operated in FullMS/dd-MS2 (Top10) Data Dependent Acquisition mode. Survey scans were acquired at a resolution of 70000 (m/z 200) and fragmentation spectra at 17500 (m/z 200). Peptide selection was carried out with an isolation window of 2.0 Th and normalized collision energy of 28 was applied for peptide fragmentation. The maximum injection time was 120 ms for survey and MS/MS scans and AGC target values of 3E6 for survey scans and 5E5 for MS/MS scans were used. Raw files were processed and searched with MaxQuant1 (version 1.5.3.17) software and Andromeda2 search engine. Precursor and fragment mass tolerances were set to 4.5 and 20 ppm respectively and up to 2 missed cleavages were allowed. Carbamidomethylation of Cys was set as fixed modification, oxidation of Met and protein N-term acetylation as variable modifications and a human UniProtKB-SwissProt database (version 2015_09) was used. A false discovery rate (FDR) of 0.01 for peptides and proteins and a minimum peptide length of 7 amino acids were required. Obtained results were exported into Microsoft Office Excel (Microsoft) for further analysis.

The mass spectrometry analysis was performed in the Proteomics Core Facility-SGIKER (member of ProteoRed-ISCIII) at the University of the Basque Country.

8 Statistical analysis

Quantitative results are reported as mean ± standard deviation and statistical analysis was performed on the remnant DNA data using the t student test ($p > 0.05$, $p \geq 0.01$ and $p \geq 0.001$).

Results and Discussion

1 Quantification of the remnant DNA

All the assayed dhATs except the BM1 (untreated with Benzonase) showed very low remnant DNA (Table 3, found below) and meet the general decellularization criteria (DNA ≤ 50 ng/mg), a threshold beyond which *in vivo* immune reactions have been described (Crapo PM., et.al., *ibid*). BM1 showed a 365.506 ± 78.105 ng/mg dry weight while dhATs obtained by different incubation conditions of the Benzonase showed a remnant DNA between 0.004-0.613 ng/mg dry weights (BM2-BM5). The remnant DNA in de dhATs was significantly lower both at higher enzyme concentration (BM2 against BM4) and incubation time (BM2 against BM3 and BM4 against BM5). The differences were low (0.5-0.6 ng/mg dry weight) but up to the detection limit of the technique (20pg).

REMNANT DNA			
ID	**AVERAGE ± STANDARD DEVIATION ng DNA/mg dhAT dry weight**	**t Student against BM1**	**t Student against BM2**
BM1	356.506 ± 78.102		*
BM2	0.613 ± 0.050	*	
BM3	0.004 ± 0.002	*	**
BM4	0.109 ± 0.015	*	**
BM5	0.047± 0.005	*	**
BM6	4.150 ± 0.187	**t Student against BM3**	
		**	
LM1	0.0002 ± 0.0003	**t Student against LM1**	
LM3	0.306 ± 0.005	**	
LM4	0.045 ± 0.017	*	
LM5	0.212 ± 0.023	**	
M7	0.015 ± 0.004	*	

Table 3: Results of the quantification of remnant DNA in dhATs. Statistical t student analysis of the significant differences between remnant DNA obtained with different decellularization conditions *(p>=0.01) and **(p>=0.001).

Higher differences in remnant DNA were found when reactive qualities were changed. A research quality DNAse (BM6) and Benzonase Emprove BIO quality (BM3) treatments where compared and BM6 showed a statistically significant lower remnant DNA of 4.146 ng/mg dry weight (Table 3).

Remnant DNA in dhATs showed differences also depending on the lipase treatment. LM1 (untreated with Lipase) showed statistically significant lower remnant DNA (0.2-0.3ng/mg) in comparison with LM3 and LM5 (treated during 44h with 25u/100mg and 50u/100mg respectively). This effect could be attributed to interactions between the enzymes in the decellularization processes. When a 23h lipase treatment was applied (LM4), the effect of the enzyme on the remnant DNA was irrelevant (0.045 ng/mg).

The dhAT untreated with trypsin and triton-x100 (M7) showed an statistically lower remnant DNA (0.015 ng/mg) than the one obtained with the trypsin and triton-x100 pretreatment (LM3, 0.306ng/mg).The differences were low (0.29 ng/mg) but up to the detection limit of the technique (20pg).

2 Histologic and IHQ analysis

Morphologically, H&E staining of dhATs showed a mature adipose tissue in which highlights the absence of cell nuclei, but where preservation of tissue structure is observed: the cytoplasmic space of adipocytes and more dense fibrous tissue are recognized as well as vascular structures. FIG. 1 shows a comparative H&E staining of hAT and dhAT (as an example, LM4).

hAT contains various ECM and Basement Membrane (BM) proteins, including collagen type I–VI, laminin, fibronectin, elastin, and glycosaminoglycans (GAGs). In this investigation, collagen type-I, collagen type-IV, laminin and a MB-specific heparan sulfate proteoglycan (HSPG2) have been analyzed by IHQ. FIG. 2 shows the conservation of a very similar pattern expression of the hAT ECM and BM proteins in processed dhAT (LM4 as an example). Collagen type-I was expressed mainly in fibrotic region, collagen type-IV and laminin were observed mainly surrounding adipocytes (laminin was also observed in fibrillary region, not collagen type-IV). The specific HSPG2 protein expression pattern have also been conserved during the processing, HSPG2 expressed in the fibrillary region and less heterogeneously in the adipocyte regions. Finally, vascular structures have been also conserved and expressed BM proteins similarly to the original hAT.

However, variations in tissue structures and protein expression were observed in dhATs obtained under different conditions of the decellularization process. Related to the lipase treatment, certain lipase concentration and incubation

time affected the morphology and proteins of the resultant dhATs. In particular, the dhAT LM5 (treated with 50u/100mg and 44h incubation at 37ºC) showed alterations in tissue morphology: no visible vascular structures and destruction of ECM structure in fibrillary regions observed by H&E staining and a diminished of collagen type-I expression (FIG. 3, comparative between LM3 and LM5). No significant alterations were observed on the collagen type IV, laminin and HSPG2 expression. These results showed a limit in the lipase treatment related to the conservation of the original tissue morphology and protein expression which could be considered as the decellularization conditions lower than the LM5 dhAT.

Some differences were also observed related to the nuclease (Benzonase) treatments. In dhAT M8 (untreated with Benzonase) some cell nuclei were observed both adipocyte and fibrous regions. As shown with the lipase treatment, Benzonase also produces alterations in the fibrillary structures of the tissue and a diminishing of collagen type-I expression (FIG. 4). No significant alterations were observed on the collagen type IV, laminin and HSPG2 expression. These results showed that the Benzonase treatment must be determined by the remnant DNA (in our investigations the showed limit in the Benzonase treatment was conditions of the dhAT BM3).

The effect of the trypsin/triton-x 100 pretreatment was analyzed by histologic observation of dhATs obtained with and without the pretreatment (LM3 vs. M7). The M7 dhAT showed a clearly higher histologic preservation of the original tissue morphology and protein expression being the most preserved dhAT analyzed in this investigation (FIG. 5). Although the trypsin treatment was very short and the triton x-100 was added in a very low percentage (1%) these results showed a considerable adverse effect of the trypsin and non-ionic detergent treatments on the conservation of tissue morphology and ECM proteins in decellularization of hAT.

3 Quantification of triglyceride content

Triglyceride quantification showed the efficacy of the lipase treatment on the delipidation of hAT observed also macroscopically (FIG. 6). Increasing concentration and incubation time of the lipase showed dhATs with decreasing triglyceride content (ranging from 70±10% to 15 ± 10%) in comparison with original hAT (considered as 100% triglyceride content).

Results also showed differences between dhAT untreated with trypsin/triton-x 100 pretreatment and Benzonase treatment. Absence of these treatments results on lower delipidates dhATs (LM3 vs. M7).

4 Quantification of total protein content

Results of total protein content of dhATs are shown in Table 4 (found below) and showed differences between investigated dhATs. Some differences were observed in the dhATs obtained by different lipase treatments. When non-treated LM1 protein content was compared with lipase treated LM2 and LM4 dhATs a lower total protein content was observed. However in those obtained by longer lipase incubation time (LM5), a higher total protein content was observed and could be attributed to a more delipidated dhAT. More specific characterization is necessary to determine the effect of the treatments on protein content which could depend on the delipidation range.

TOTAL PROTEIN CONTENT	
ID	AVERAGE ± STANDARD DEVIATION (µg protein/mg dhAT dry weight)
LM1	759.1 ± 17.0
LM2	565.2 ± 22.3
LM3	931.5 ± 21.5
LM4	693.6 ± 6.5
LM5	820.6 ± 14.5
M7	796.3 ± 20.6
M8	824.12 ± 12.5

Table 4: Results of the total protein content of some dhAT obtained during the investigation.

5.- LC-MS/MS analysis

Specific protein analysis has been carried out for the LM4 dhAT. Results showed a composition of 136 human proteins conserved after the decellularization process (false discovery ratio: 1% and discarding the proteins detected by 1 peptide).

Table 5 (found below) showed some of the proteins conserved after the decellularization process with special relevance on composition, interactions and protein assembly of ECM and BM. Results include collagens (I, IV, VI, XV and XVIII), glycoproteins (laminin, fibulin, emilin, tenascin and fibrin protein families), proteoglycans (lumican, decorin, mimecan and biglycan) and a specific BM sulphated proteoglycan (HSPG2). Other ligands, anchoring, phosphorylation proteins have also been identified (Nidogens, Periostin, Prolargin, protein s-100).

Collagens are implicated in essential biological process such as angiogenesis, basement membrane organization, blood vessel morphogenesis, cell adhesion, cell migration, collagen biosynthetic process, collagen catabolic process, endodermal cell differentiation, endothelial cell morphogenesis, epithelial cell differentiation, extracellular fibril organization, extracellular matrix disassembly, extracellular matrix organization, patterning of blood vessels, regulation of cellular component organization, skin development, wound healing, spreading of epidermial, osteoblast differentiation, homeostasis of number of cells within a tissue, single organismal cell-cell adhesion, organ morphogenesis, positive regulation of cell proliferation, response to hydrostatic pressure.

Glycoproteins (laminin, fibulin, emilin, tenascin and fibrin protein families) are implicated in biological processes including actin cytoskeleton organization, cell adhesion, cell-matrix adhesion, collagen fibril organization, collagen metabolic process, elastic fiber assembly, endoderm development, endodermal cell differentiation, extracellular fibril organization, extracellular matrix disassembly, extracellular matrix organization, fatty acid metabolic process, regulation of cell adhesion, regulation of cell migration, regulation of embryonic development, positive regulation of epithelial cell proliferation, substrate adhesion-dependent cell spreading, epidermal growth factor receptor signaling pathway, protein localization to cell surface, regulation of cell growth, regulation of removal of superoxide radicals, secretion, triglyceride metabolic process.

Proteoglycans (lumican, decorin, mimecan and biglycan) are implicated in biological processes including carbohydrate metabolic process, cartilage

development, chondroitin sulfate biosynthetic process, chondroitin sulfate catabolic process, chondroitin sulfate metabolic process, collagen fibril organization, dermatan sulfate metabolic process, extracellular matrix disassembly, extracellular matrix organization, glycosaminoglycan metabolic process, keratan sulfate biosynthetic process, keratan sulfate catabolic process, keratan sulfate metabolic process, organ morphogenesis, peptide cross-linking via chondroitin 4-sulfate glycosaminoglycan, positive regulation of transforming growth factor beta 1 production, response to growth factor, response to mechanical stimulus, skeletal muscle tissue development small molecule metabolic process, wound healing.

The specific BM Heparan sulphate proteoglycan-2 (HSPG2) is implicated in angiogenesis, brain development, carbohydrate metabolic process, cardiac muscle tissue development, cartilage development involved in endochondral bone morphogenesis, chondrocyte differentiation, chondroitin sulfate metabolic process, embryonic skeletal system morphogenesis, endochondral ossification, extracellular matrix disassembly, extracellular matrix organization, glycosaminoglycan biosynthetic process, glycosaminoglycan catabolic process, glycosaminoglycan metabolic process, lipoprotein metabolic process, phototransduction, visible light, protein localization, retinoid metabolic process, small molecule metabolic process.

Nidogens, Periostin, Prolargin, protein s-100 are ligand, anchoring, phosphorylation proteins implicated in basement membrane organization, carbohydrate metabolic process, cell aging, cell adhesion, cell-matrix disassembly, establishment of protein localization to plasma membrane, extracellular matrix organization, glycosaminoglycan metabolic process, keratan sulfate biosynthetic process, keratan sulfate catabolic process, keratan sulfate metabolic process, membrane budding, membrane raft assembly, positive regulation of binding, positive regulation of cell-substrate adhesion, positive regulation of focal adhesion assembly positive regulation of GTPase activity, positive regulation of stress fiber assembly, positive regulation of substrate adhesion-dependent cell spreading, protein heterotramerization, small molecule metabolic process skeletal system development, tissue development.

PROTEIN IDS	PROTEIN NAMES	GENE NAMES	PEPTIDES	UNIQUE PEPTIDES
P98160	Basement membrane-specific heparan sulfate proteoglycan core protein	HSPG2	21	21
P21810	Biglycan	BGN	5	5
P27797	Calreticulin	CALR	2	2
P02452	Collagen alpha-1(I) chain	COL1A1	12	12
P02462-2;P02462	Collagen alpha-1(IV) chain;Arresten	COL4A1	4	4
P12109	Collagen alpha-1(VI) chain	COL6A1	13	13
P39059	Collagen alpha-1(XV) chain;Restin;Restin-2;Restin-3;Restin-4	COL15A1	2	2
P39060-2;P39060-1;P39060	Collagen alpha-1(XVIII) chain;Endostatin	COL18A1	2	2
P08123	Collagen alpha-2(I) chain	COL1A2	6	6
P08572	Collagen alpha-2(IV) chain;Canstatin	COL4A2	4	4
P12110;P12110-3;P12110-2	Collagen alpha-2(VI) chain	COL6A2	10	10
P12111-4;P12111-2;P12111;P12111-3;P12111-5	Collagen alpha-3(VI) chain	COL6A3	52	52
P07585;P07585-4;P07585-2;P07585-3	Decorin	DCN	10	10
Q12805-2;Q12805-4;Q12805;Q12805-3;Q12805-5	EGF-containing fibulin-like extracellular matrix protein 1	EFEMP1	9	9
Q07507	Dermatopontin	DPT	4	4
Q9Y6C2;Q9Y6C2-2	EMILIN-1	EMILIN1	4	4
P35555;P35556	Fibrillin-1	FBN1	31	31
Q9UBX5	Fibulin-5	FBLN5	2	2
P02675;CON__P02676	Fibrinogen beta chain;Fibrinopeptide B;Fibrinogen beta chain	FGB	3	3
P09382	Galectin-1	LGALS1	6	6
Q16363-2;Q16363	Laminin subunit alpha-4	LAMA4	5	5
P07942	Laminin subunit beta-1	LAMB1	3	3
P55268	Laminin subunit beta-2	LAMB2	6	6
P11047	Laminin subunit gamma-1	LAMC1	10	10
P51884;CON__Q05443	Lumican	LUM	9	9
P55083;P55083-2	Microfibril-associated glycoprotein 4	MFAP4	2	2
P20774	Mimecan	OGN	4	4
P14543-2;P14543	Nidogen-1	NID1	9	9
Q14112;Q14112-2	Nidogen-2	NID2	12	12
Q15063-4;Q15063-2;Q15063-3;Q15063-5;Q15063;Q15063-7;Q150636;CON__Q2KJC7	Periostin	POSTN	6	6
P02545-2;P02545-6;P02545;P02545-3;P02545-5;P02545-4	Prelamin-A/C;Lamin-A/C	LMNA	11	11
P51888	Prolargin	PRELP	5	5
P60903	Protein S100-A10	S100A10	2	2
P22105;P22105-3;P22105-4	Tenascin-X	TNXB	2	2

Table 5: ECM and Basal Membrane proteins conserved in dhAT (LM4) and

identified by LC-MS/MS.

As can be seen in the analyses presented above, the composition of the obtained decellularized matrices is rich in all the components that ensure a successful exploitation in all the applications sought.

Finally, it should be added that inventors have started preliminary studies of different materials processing techniques in order to find out whether the decellularized matrices of the present invention are easily amenable to manipulation with the goal of producing different final products for a variety of applications. As can be seen in FIG. 8, the matrices permit their processing with several technologies: they could be successfully dissolved and freeze dryed, micronized in liquid nitrogen, compressed and rolled to obtain a porous scaffold, powder, sheet and cylindrical formats which will ideally permit to apply the material for the described applications.

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CLAIMS

1. A method of decellularizing adipose tissue comprising the steps of:

a) Laminating the adipose tissue;

b) Treating the adipose tissue resulting from step a) with a lipoprotein lipase either:
at a concentration of 10-30u/100mg for 39-49 hours at 32-42 degrees Celsius
or
at a concentration of 45-55u/100mg for 18-28 hours a 32-42 degrees Celsius; and
c) Treating the adipose tissue resulting from step b) with a nuclease, either:
at a concentration of 709-719u/mg for 67-77 hours at 32-42 degrees Celsius
or
at a concentration of 1423-1433u/mg for 35-45 hours at 32-42 degrees Celsius
or
at a concentration of 1423-1433u/mg for 67-77 hours at 32-42 degrees Celsius;

for the adipose tissue to have a total of DNA content equal to or less than 50 ng/mg.

2. The method of decellularizing adipose tissue according to claim 1, wherein said method does not comprise the use of organic solvents.

3. The method of decellularizing adipose tissue according to any one of claims 1-2, wherein:
in step b) the treatment is carried out either at a concentration of 25u/100mg for 44 hours at 37 degrees Celsius or at a concentration of 50u/100mg for 23 hours a 37 degrees Celsius, and step c) is carried out either at a concentration of 709-719u/mg for 67-77 hours at 32-42 degrees Celsius or at a concentration of 1423-1433u/mg for 35-45 hours at 32-42 degrees Celsius or at a concentration of 1423-1433u/mg for 67-77 hours at 32-42 degrees

Celsius.

4. The method of decellularizing adipose tissue according to any one of claims 1-2, wherein:
step b) is carried out either at a concentration of 10-30u/100mg for 39-49 hours at 32-42 degrees Celsius or at a concentration of 45-55u/100mg for 18-28 hours a 32-42 degrees Celsius, and in step c) the treatment is carried out at a concentration of 714u/mg for 72 hours at 37 degrees Celsius or at a concentration of 1428u/mg for 40 hours at 37 degrees Celsius or at a concentration of 1428u/mg for 72 hours at 37 degrees Celsius.

5. The method of decellularizing adipose tissue according to any of the claims 1 to 4, further comprising a step between steps a) and b), wherein the adipose tissue resulting from step a) is treated with trypsin and triton-X100.

6. The method of decellularizing adipose tissue according to any one of claims 1 to 5, further comprising a step d) comprising freezing or lyophilizing and sterilizing the adipose tissue resulting from step c).

7. The method of decellularizing adipose tissue according to any one of claims 1 to 6, wherein in steps b) and c) the treatments are carried out under vacuum and with stirring at 100-150 rpm.

8. The method of decellularizing adipose tissue according to any one of claims 1 to 7, wherein after steps a), b) and c), there is a washing step with buffer at room temperature, under vacuum and under stirring at 100-150 rpm, and wherein the washing step after step a) the buffer further comprises at least one antibiotic, at least one antimycotic and at least one protease inhibitor, in the washing step after step b) the buffer comprises at least one antibiotic, at least one antimycotic, at least one protease inhibitor and at least one lipase inhibitor and wherein the washing step after step c) the buffer further comprises at least one antibiotic, at least one antimycotic, at least one protease inhibitor and at least one nuclease inhibitor.

9. A decellularized adipose tissue matrix obtainable by the method according to any one of claims 1 to 8.

10. The decellularized adipose tissue according to claim 9, wherein the adipose tissue has a total DNA content of from 0.015 to 0.306 ng/mg.

11. The decellularized adipose tissue according to any one of claims 9 to 10, wherein the adipose tissue has a total protein content of 100-931 ug/mg and a total triglyceride content of 25-75% in weight.

12. A powder, foam, particle or hydrogel comprising the decellularized adipose tissue according to any one of claims 9-11.

13. A biocompatible scaffold or biocompatible coating comprising the decellularized adipose tissue according to any one of claims 9-11 or the powder, foam, particle or hydrogel according to claim 12.

14. The decellularized adipose tissue according to any one of claims 9 to 11, the powder, foam, particle or hydrogel according to claim 12, or the biocompatible scaffold or biocompatible coating according to claim 13 for use in tissue engineering and regenerative therapy.

15. The decellularized adipose tissue for use according to claim 14, wherein the therapy is a therapy of wound healing.

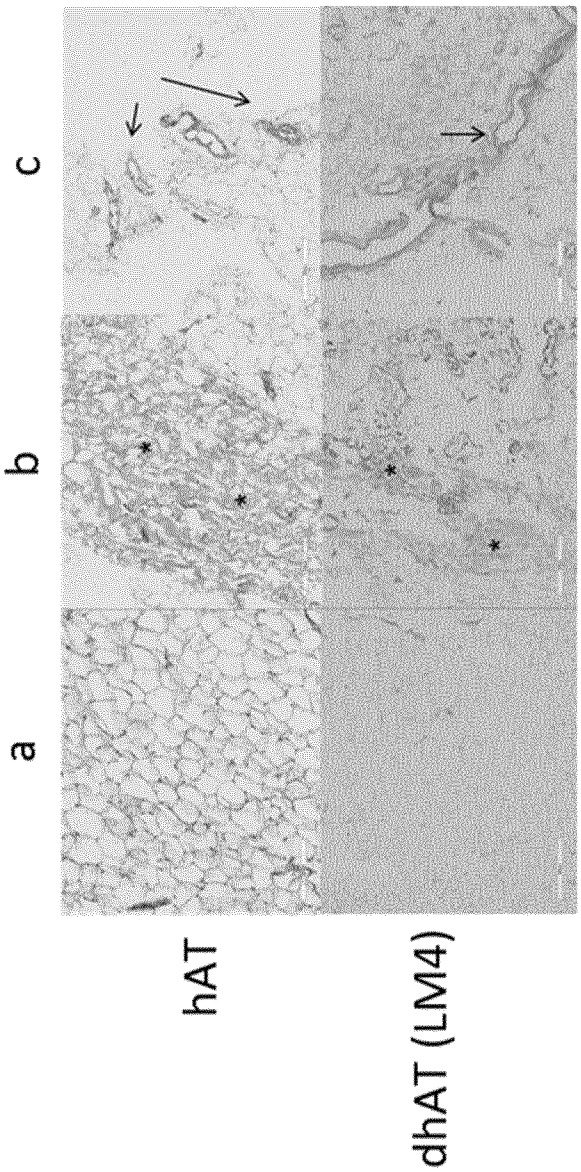


FIG.1

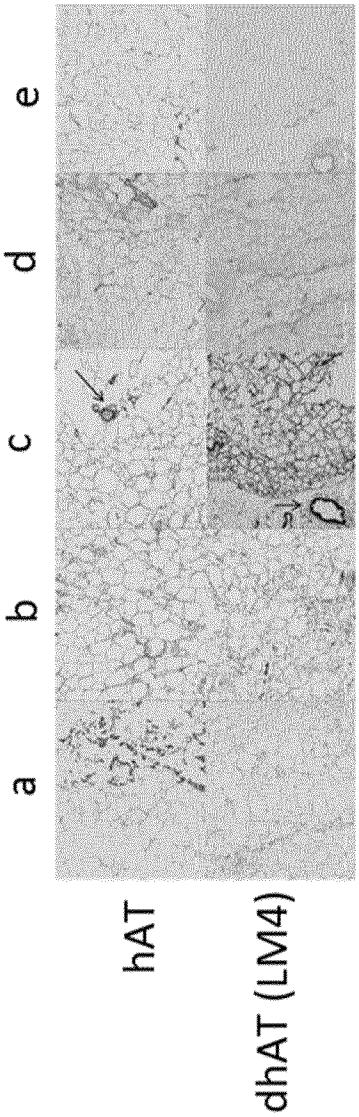


FIG.2

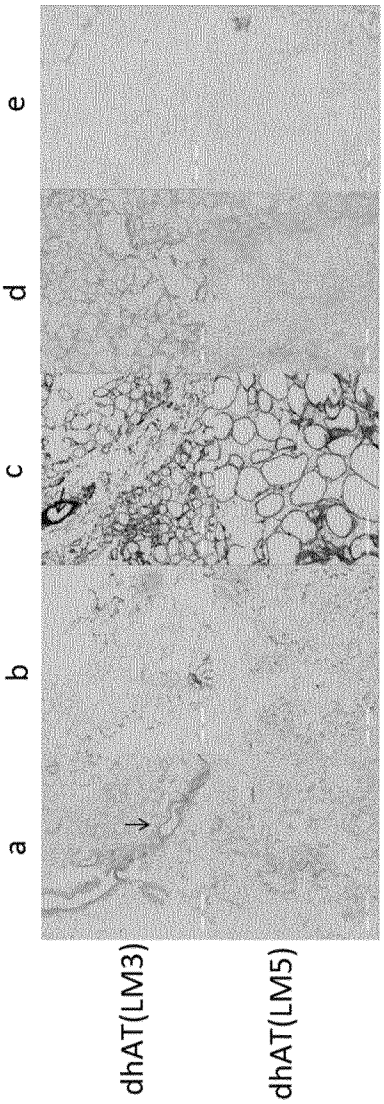


FIG.3

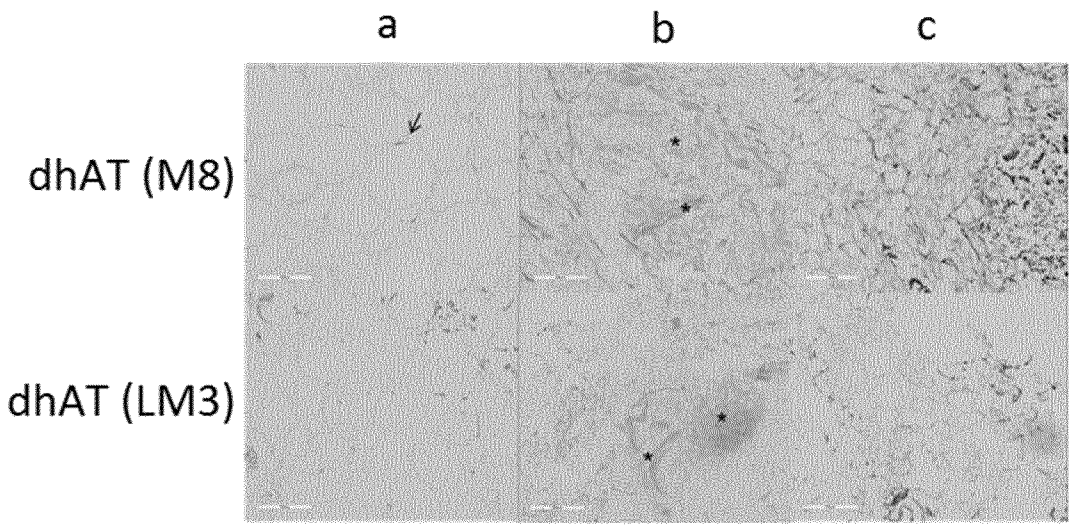


FIG. 4

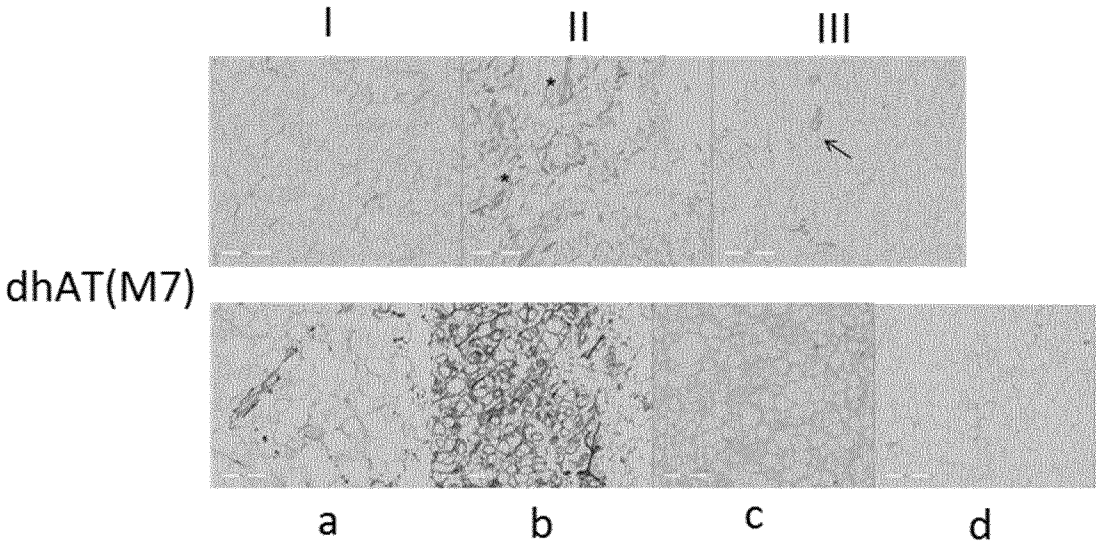
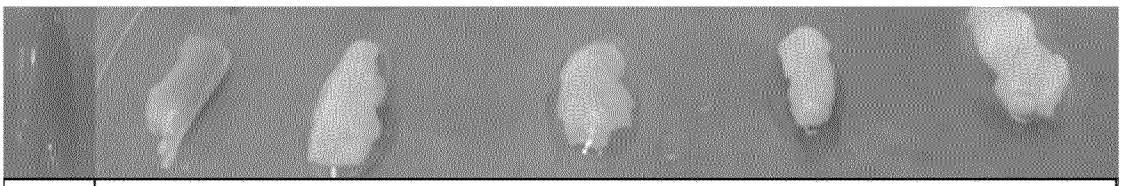
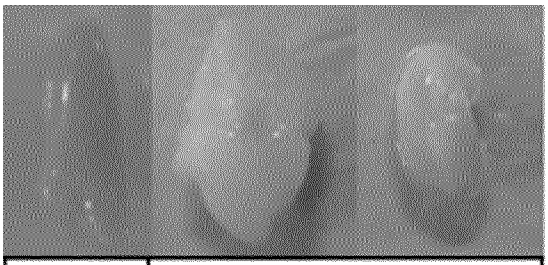


FIG. 5



hAT	dhATs				
	LM1	LM2	LM3	LM4	LM5
100%	85±5%	70±10%	55±10%	35±10%	15±10%

FIG.6



hAT	dhATs	
	M7	M8
100%	65±10%	70±10%

FIG. 7

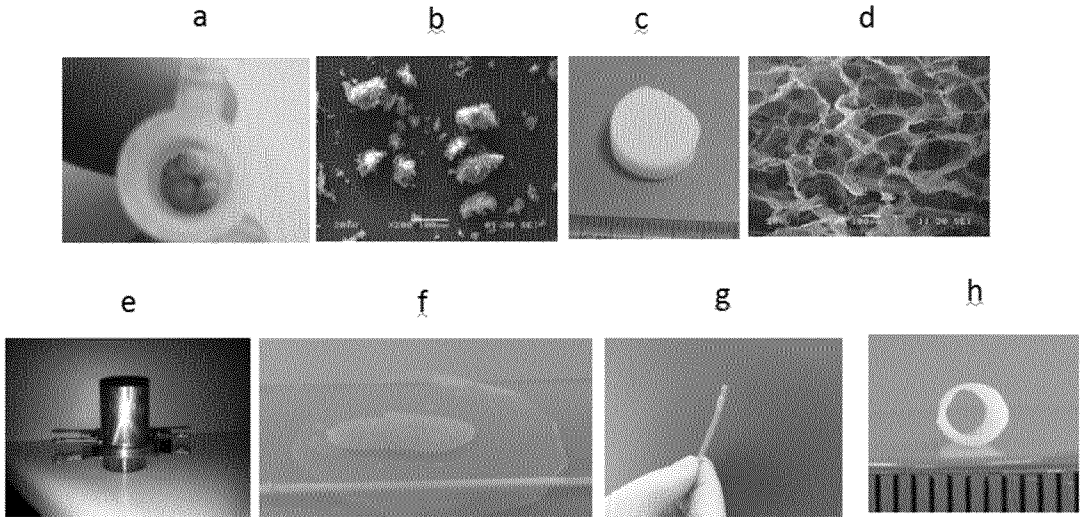


FIG. 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/082849

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K35/35 A61L27/36 A61P41/00
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)
A61K A61L

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 practicable, search terms used)
EPO-Internal, EMBASE, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	Matherials and methods; page 28, left-hand column	12
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 March 2017	13/03/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Escolar Blasco, P

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Form PCT/ISA/210 (second sheet) (April 2005)

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A	page 1048, paragraph 1-3 abstract	6,14,15

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	WO 96/32905 A1 (ST JUDE MEDICAL [US]; BISHOPRIC NANETTE H [US]; DOUSMAN LINDA [US]; YA) 24 October 1996 (1996-10-24) page 13, paragraph D; example 1 -----	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/082849

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