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(54) **Title**: A DERMAL MATRIX AND PRODUCTION METHOD THEREOF HAVING SYNERGISTIC EFFECTS COMPRISING MICROPARTICLES WHICH PROVIDES TISSUE REPAIR

(57) **Abstract**: This invention is related to dermal matrices and production method thereof, having within its structure micro particles having antioxidant agents with synergistic effects, providing speedy repair of the dermal tissue, used in chronic wound treatments, basically comprising the steps of preparing the dermal matrix system (11), forming micro particles (12), combining the micro particles with the dermal matrix system.

DESCRIPTION

A DERMAL MATRIX AND PRODUCTION METHOD THEREOF HAVING SYNERGISTIC EFFECTS COMPRISING MICROPARTICLES WHICH PROVIDES TISSUE REPAIR

Technical Field

This invention is related to dermal matrices and production method thereof used in treating chronic wounds, which enables the speedy repair of the dermal tissue, and which comprises within its structure micro particles having antioxidant agents with synergistic effects.

Prior Art

Conventional products such as crèmes, gels, lotions and ointments are used in treating chronic wounds nowadays. In order for said products to be effective in treatment, they need to be applied frequently. Moreover the application dose must also be standardized.

There are however current treatments that could eliminate these problems which are a part of the conventional approach. In such treatment applications xenograft and graft transfer methods are used in order to replace new tissues over areas with large tissue losses. These methods are both painful and at the same time could have donor based disadvantages.

The most up to date final stage known in chronic wound treatments, comprise innovative formulations. Micro particle systems and matrix systems provide advantageous treatment methods when compared with the prior art methods. As a result of the literature research carried out, it has been observed that matrices comprising agents that help heal the area, and which provide the necessary mechanical support, compensating via and occlusive effect the lost tissue area as a result of various injuries, has been developed. Dermal matrices have been prepared especially comprising collagen-chitosan, collagen-gelatin, collagen-glycosaminoglycans, and collagen-hyaluronic acid compositions and by incubating fibroblast to these and healing of the wound tissue has been observed.

Dermal matrices are systems prepared with natural or synthetic polymers in order to effectively support cell growth and development and the usage of these in treating wounds have opened up a new horizon. The main aim of these structures is to re-develop the tissue that has been damaged and to carry different biomaterials and bioactive molecules to this region.

Nowadays commercial products that heal the epithelium having different characteristics are present. In said products wound patches prepared with chitosan which is a natural polymer is used, or polymers such as synthetic polyurethanes and PLGA are used and a medium where keratinocytes form small colonies in the epidermis is provided, and thereby a faster epithelium formation is obtained. In said products where epithelium is replaced, a two dimensional structure is provided. This dimension preferred in the formation of epithelium however is not sufficient in substituent treatments that require three dimensional structures.

The wound area is open to infection and similar complications and the most effective approach in preventing the infections are to provide effective treatment as soon as possible. The first six hours is the more important time span in terms of bacterial colonization formation after getting wounded. The most effective method in preventing infections are to seal off the wound by using sterile patches over the wounds and cleaning the wound under aseptic conditions before sealing it with a sterile patch, these conditions are even more effective than antibiotic treatment. For this reason, immediately covering the wound, increasing the self repair of the dermal layer, and developing fully layered tissues that shall replace the tissue area are some of the crucial subjects that need to be addressed in the current studies.

Conventional approaches (ointments, crèmes, gels, lotions) used in treating wounds, are not capable of overcoming basic problems such as infections of wounds, dehydration, heat loss, for live tissue to remain unprotected, protein, erythrocyte, leukocyte, immune agents and oligoelement losses.

One of the most important disadvantages of conventional formulations, is that the amount applied may change or vary and as a result the applied dose cannot be standardized and the application needs to be carried out frequently. Every time the application is carried out, the patient feels pain and this makes the treatment procedure more difficult. Moreover, allergic reactions could arise due to the surface active agent, or components such as preservatives and perfume used in these formulations. Besides conventional therapy, graft transfer, which is another treatment carried out in case of large tissue loss cases also has many disadvantages. These disadvantages can be diseases that are transmitted from the donor, or formation of new wounds at the donor area from which the graft was taken from. Although autografts are materials that are preferred, in some cases there may not be any suitable skin tissue that can be transferred in patients. In addition to this, a new wound is present at the area where the graft tissue is taken from. Another disadvantage is that these procedures may necessitate surgical operations. Innovative approaches were deemed necessary to shorten the treatment time, in order to prevent complications such as infections and in order to overcome said disadvantages.

The formulations that have been prepared in order to reach these aims, having collagen, chitosan and gelatin, are two dimensional systems, and said two dimensional systems were not sufficient enough to provide physical support. In order to overcome this problem, and the methods used to ensure that the dermal matrices prepared using collagen were three dimensional comprising chemical materials, ruin the conditions that need to be present for cell growth. In the present technique, the products developed to reach said aims do not comprise micro particles and they act like a wound patch wherein cells can grow a little bit faster.

Brief description of the Invention

The aim of the present invention is to provide a dermal matrix which may increase the efficiency of chronic wound treatments, decrease the time of healing and/or ensure tissue repair.

Another aim of the invention is to provide a dermal matrix not only comprising collagen and laminin but also comprising micro particles which carry antioxidant agents that may help to speed up tissue repair speed by showing synergistic effects.

Another aim of the invention is to obtain a dermal matrix production method that may enable tissue repair, wherein agents formed of proteins located inside the natural structure of the dermis and other components are used.

Another aim of the invention is to provide a dermal matrix production method which may support cell growth and tissue repair, by means of using a cross linking method which is not a chemical method.

Another aim of the invention is to provide a dermal matrix production method which may enable tissue repair and prevent heat sensitive bio materials from degrading by applying a lyophilisation procedure.

Detailed description of the invention

A dermal matrix production method in accordance with the present invention has been shown in the attached figure, wherein said figure illustrates the following;

Figure 1 – Is the flow chart of the method subject to the invention. In particular, the flow chart shows a dermal matrix production method (10) characterized in that it comprises the steps of:

- –Preparing the dermal matrix system (11),
- - preparing the collagen solution system (111),
- -adding laminin onto collagen solution and mixing (112),
- -freezing and storing the mixture (113),
- -Lyophilization of the stored mixture (114),
- -Cross linking the lyophilisate (115),
- -Forming micro particles (12),

- -Preparing aqueous solution of the polymer (121),
- -Preparing the phospholipid, antioxidant agent and alcohol mixture (122),
- -adding the aqueous solution onto the alcohol mixture and mixing (123),
- -spray drying the whole mixture obtained (124),
- -combining the micro particles with a dermal matrix system (13).

The method (10) subject to the invention is principally based on preparing a dermal matrix system and a micro particle system separately and following this spraying the micro particle onto the dermal matrix thus combining these two systems physically.

According to the method (10) subject to the invention, first of all a collagen solution is prepared (111) by adding collagen to acidic acid solution in order to prepare a dermal matrix system (11). In the preferred application of the invention bovine collagen is added (111) at a concentration of 0,1% to 0.1 M acidic acid solution.

Following this laminin is added onto the collagen solution (112). In a preferred embodiment of the invention, 2.5ml of the collagen solution that has been prepared is taken and 20µl laminin is added, and all of it is mixed in an ice bath (112). When using the ice bath, it is aimed to prevent the deterioration of the protein structures that are present inside the mixture. In a preferred embodiment of the invention, mixing is carried out at a speed of 3000 cycles/minute, for 1 minute, using a 10-G probe.

The mixture comprising collagen and laminin is left to wait for 12 hours at a temperature of -20±1 °C and is frozen (113). Following this, said mixture which has been frozen is subjected to lyophilization for 24 hours (114). Thereby it is ensured that the deterioration of the heat sensitive biomaterials inside said mixture is prevented.

The lyophilisate structure comprising collagen and laminin that has been obtained, is cross linked (115) under 254nm wavelength using an ultraviolet cross binder (UV-cross linker) device. In another place, an aqueous polymer solution is prepared (121) in order to provide the formation of micro particles (12). In order to ensure this 0.2g hyaluronic acid sodium salt (HA) is used and it is dissolved in 150ml pure water. In the meantime,

dipalmitoylphosphatidylcholine (DPPC) and resveratrol is mixed into alcohol and is dissolved (122). In a preferred embodiment of the invention, 0.7-0.75 g DPPC and 0.05-0.1 g resveratrol 350 ml is added into ethanol and is mixed (122). Usage of resveratrol (antioxidant) shows synergic effects with collagen and laminin that forms the protein structure, and as a result the efficiency of the end product regarding tissue repair is increased. Besides this, it is ensured that the matrix integrity of the end product is retained for a long time.

The HA solution (121) that has been prepared, is added onto the solution (122) of DPPC and resveratrol in alcohol and is mixed at 40 ± 1°C (123). The whole mixture that has been prepared is placed inside a spray dryer and micro particles are obtained by spraying (124). In the preferred embodiment of the invention; the spray dryer has been adjusted such that the initial temperature is 180°C, and the spraying speed is 400 L/h.

HA micro particles loaded with resveratrol and prepared with a spray dryer are sprayed onto the previously prepared dermal matrix (11) as dry powder and said two systems are combined (13). According to the preferred embodiment of the invention, the sprayed micro particles comprise 50 µM resveratrol. These matrices that have been obtained, especially are used in chronic wound treatments and they significantly increase the speed of healing of the wound.

In order to determine the diameter and thickness of the dermal matrixes obtained using the method subject to the invention, automatic compass has been used and the diameter and the thicknesses of the matrices have been evaluated. As a result of the analysis that has been carried out, the average diameter of the obtained dermal matrix is 2.24 ± 0.05 cm and their thicknesses have been measured as 0.23 ± 0.04 cm.

It has been determined that the pore sizes of the dermal matrices that have been obtained using scanner electron microscope and confocal microscope in order to determine surface morphology were around at most 100 µm. In addition to this, it has been observed that three dimensional collagen fibers were present and micro particles were cross linked.

The matrices that have been prepared for water retention capacity, have been left to wait at 37 ± 1°C water, for 20seconds after their weights have been determined. By calculating the percent rate of the initial weight and comparing it to the weight measured after the test is completed, the water retention capacity has been determined to be % 84 ± 1.5.

A TA-XT plus texture analysis device has been used in order to determine the mechanical characteristics of dermal matrices. As a result of the analysis, the hardness 26-21 N, compression 23-18 N/mm, cohesion 0.93-1 and elasticity values 0.8-1, have been calculated by way of power, time curves that belong to the matrix. As result of the humidity content determination, the humidity amount of the dermal matrix subject to the invention has been calculated as 0.2%. The sizes of the micro particles located inside the dermal matrix, have been calculated to be between 15-30 μm using the laser diffraction method.

As it can be seen in the images obtained with SEM, the formulations are spherical and have smooth surfaces. The particles sizes observed through SEM, have been compared with the results of the previously carried out laser diffraction distribution method, and the sizes have been found to be consistent.

The drug loading capacity of the micro particles that have been prepared, has been calculated as 0.5-1mg and the encapsulation efficiency have been calculated to be between %97-%98.7.

In order to determine the release amount of resveratrol from micro particles, active agent release studies have been carried out from micro particle formulations prepared with different resveratrol concentrations. The results of the analysis showed that the released resveratrol amount from the formulations for 24 hours were between %73-85.

The effect of hyaluronidase, collagenase, lipase and phospholipase enzymes in relation to release of resveratrol from micro particles was examined. Separate enzymatic degradation studies have been carried out with hyaluronidase and collagenase enzymes

in order to examine the enzyme effect to the dermal matrix. While the dermal matrix which did not comprise micro particles were degraded enzymatically with collagenase in 30 minutes, the degradation of the dermal matrix comprising resveratrol loaded micro particles took twice the amount of time. Resveratrol was not only advantageous in that it supported wound healing but also it was advantageous because it protected the matrix integrity against collagenase for a longer time.

Confocal microscope 3D software program has been used in order to determine if the micro particles were homogenously distributed in the dermal matrix and where they were localized. The confocal study was renewed and the data have been evaluated in this program. The morphological studies carried out showed that the micro particles were distributed much more homogeneously at the top surface of the dermal matrix and that said micro particles were in complete contact with the matrix.

The human dermal fibroblast cells, (Invitrogen C-013-5C) were cultured in humid conditions comprising 37±0.5 °C and 5% CO_2 according to the instructions of the producer. Resveratrol solution, has been incubated with formulations that did not comprise resveratrol and micro particle formulations that comprised resveratrol for 24 hours and the effects of all samples regarding cell vitality were examined. As the resveratrol solution, or formulations that are empty or do contain active agents were observed not to have cytotoxic potential, and they have shown cell proliferation increasing effects in concentrations that are corresponding to 50 µM resveratrol content. Oxidative stress parameters such as total glutation, malondialdehyde, and superoxide dismutase and glutation peroxidase have been examined inside the cells that have been analyzed. The results that were obtained, were an indication that the dosage type developed for resveratrol comprised oxidative activity at a molecular level.

In conclusion, the studies carried out in the recent years, showed that wound healing were dependent on the components selected inside the formulation and that the innovative formulations comprising proteins that form the natural structure of the dermis provided efficient treatment and treatment time could be shortened. It is obvious that new drug carrying systems need to be prepared for long term wound treatments.

There are no new generation formulations that comprise the selected combination. The synergistic effect of the dermal matrices with resveratrol in relation to tissue repair has been evaluated for the first time with this invention.

It will be understood that the term "comprise" and any of its derivatives (eg comprises, comprising) as used in this specification is to be taken to be inclusive of features to which it refers, and is not meant to exclude the presence of any additional features unless otherwise stated or implied.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A dermal matrix production method comprising:

(i) Preparing the dermal matrix system by a process comprising the steps of:

- preparing a collagen solution,
- adding laminin onto the collagen solution and mixing to form a collagen-laminin mixture,
- freezing and storing the mixture,
- lyophilizing the stored mixture, and
- cross linking the lyophilisate,

(ii) Forming micro particles by a process comprising the steps of:

- preparing an aqueous polymer solution by dissolving hyaluronic acid (HA) in pure water,
- preparing a mixture of phospholipid, antioxidant agent and alcohol by mixing dipalmitoylphosphatidylcholine (DPPC) and resveratrol into alcohol such that they are dissolved,
- adding the aqueous polymer solution to the phospholipid, antioxidant agent and alcohol mixture and mixing, and
- spray drying the whole mixture obtained,

(iii) Combining the micro particles with the dermal matrix system by spraying the micro particles in a dry powder form onto the dermal matrix system.

2. A dermal matrix production method according to claim 1, wherein the step of preparing a mixture of phospholipid, antioxidant agent and alcohol comprises mixing 0.7-0.75 g dipalmitoylphosphatidylcholine (DPPC) and 0.05-0.1g resveratrol into 350 mL alcohol such that they are dissolved.

3. A dermal matrix production method according to claim 1 or 2, wherein the step of adding the aqueous polymer solution to the phospholipid, antioxidant agent and alcohol mixture is conducted at 40 ± 1°C.

4. A dermal matrix production method according to any one of the preceding claims, wherein the step of preparing the collagen solution comprises adding bovine collagen at a concentration of 0.1% to 0.1M acidic acid solution.

5. A dermal matrix production method according to any one of the preceding claims, wherein the step of adding laminin onto the collagen solution comprises taking 2.5ml of the collagen solution that has been prepared and adding 20µl laminin into this, and mixing them in an ice bath.

6. A dermal matrix production method according to any one of the preceding claims, wherein the step of freezing and storing the mixture involves the collagen and laminin mixture being left for 12 hours overnight at a temperature of -20 ± 1 ºC.

7. A dermal matrix production method according to any one of the preceding claims, wherein the step of lyophilizing the stored mixture comprises lyophilisation for 24 hours to prevent deterioration of the biomaterials sensitive to heat, present inside said mixture.

8. A dermal matrix production method according to any one of the preceding claims, wherein the step of cross linking the lyophilisate comprises cross linking under 254 nm wavelength using an ultraviolet cross linker (UV-cross linker) device.

9. A dermal matrix production method according to any one of the preceding claims, wherein the step of preparing an aqueous polymer solution comprises mixing 0.2 g hyaluronic acid (HA) in 150 mL pure water.

10. A formulation of a dermal matrix produced by a method according to any one of the preceding claims, wherein the formulation comprises antioxidant-loaded hyaluronic acid (HA)-dipalmitoylphosphatidylcholine (DPPC) micro particles in a collagen-laminin scaffold.

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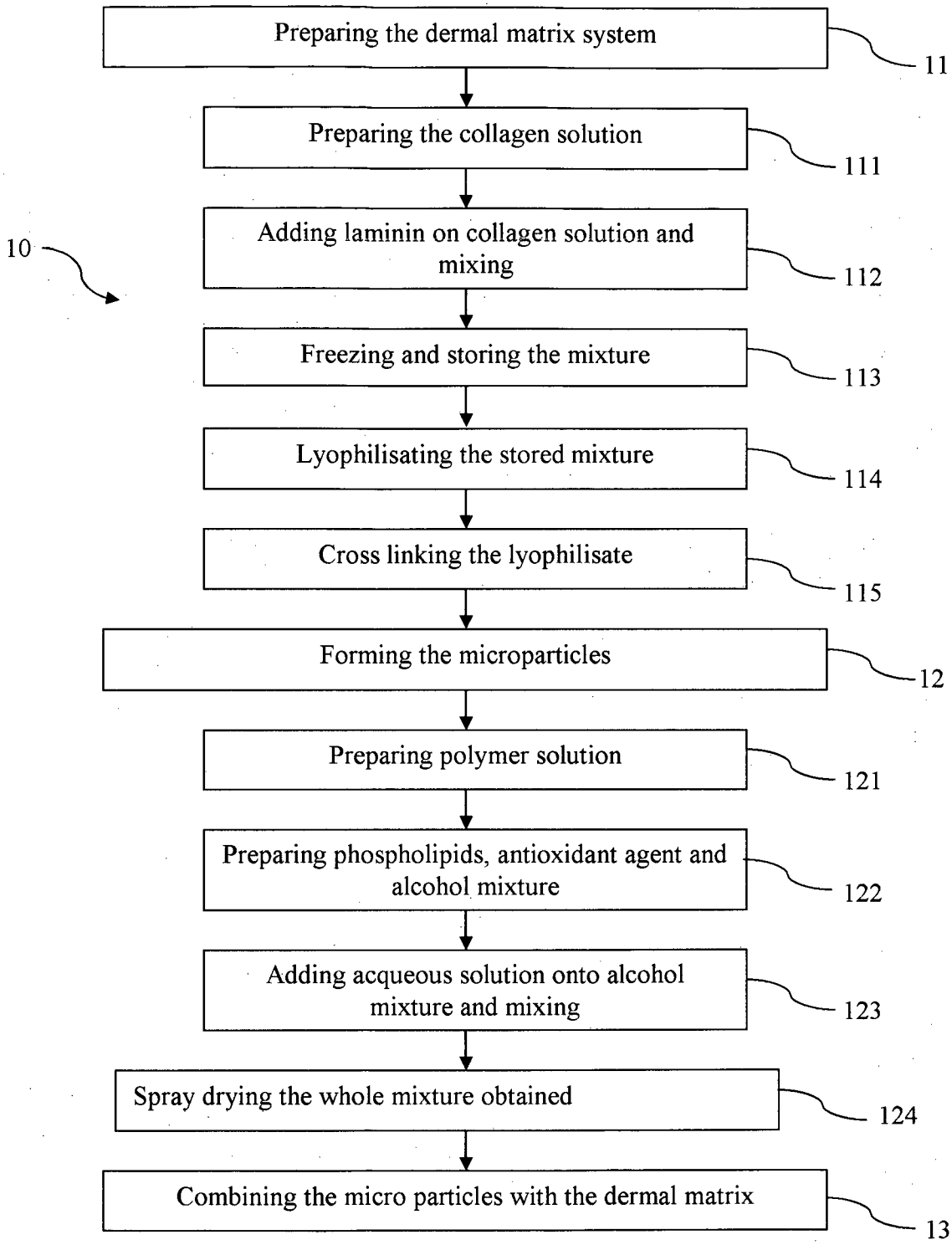
11. A dermal matrix formulation according to claim 10, wherein the formulation is biodegradable.

12. A dermal matrix formulation according to claim 10 or 11, for use in chronic wound treatments.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Adding acqueous solution onto alcohol mixture and mixing
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Spray drying the whole mixture obtained
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Combining the micro particles with the dermal matrix
13

Figure 1