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(71) Applicants (*for all designated States except US*): **UNIVERSIDAD TÉCNICA SANTA MARÍA** [CL/CL]; Av. España, No. 1680, Valparaíso, V Region (CL). **UNIVERSIDAD DE VALPARAISO** [CL/CL]; Av. Errazuriz No. 2190, Valparaiso, V. Region (CL). **FUNDACION INSTITUTO DE SEGURIDAD DEL TRABAJO** [CL/CL]; Medio Oriente No. 1175, Vina del Mar, V. Region (CL).

(71) Applicant (*for TT only*): **ROMERAL CABEZA, Angel** [ES/ES]; Alcala 35, E-28014 Madrid (ES).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **YOUNG ANZE, Manuel Eduardo** [CL/CL]; Av. España, 1680, Valparaíso (CL). **ACEVEDO GUTIÉRREZ, Cristian Andrés** [CL/CL]; Av. España, 1680, Valparaíso (CL). **ALBORNOZ MARQUEZ, Fernando Antonio** [CL/CL]; Av. España, 1680, Valparaíso (CL). **WEINSTEIN OPPENHEIMER, Caroline Ruth** [CL/CL]; Av. Errazuriz No. 2190, Valparaiso, V. Region (CL). **ACEITUNO ALVAREZ, Alexis Roobins** [CL/CL]; Av. Errazuriz No. 2190, Valparaiso, V. Region (CL). **BROWN GONZALEZ, Donald Irving** [CL/CL]; Av. Errazuriz No. 2190, Valparaiso, V. Region (CL). **TAPIA MURUA, Sergio Miguel** [CL/CL]; Av. Playa Ancha No. 850, Valparaiso, V. Region (CL).

(74) Agent: **CARPINTERO LÓPEZ, Francisco**; Herrero & Asociados, S.L., Alcalá, 35, E-28014 Madrid (ES).

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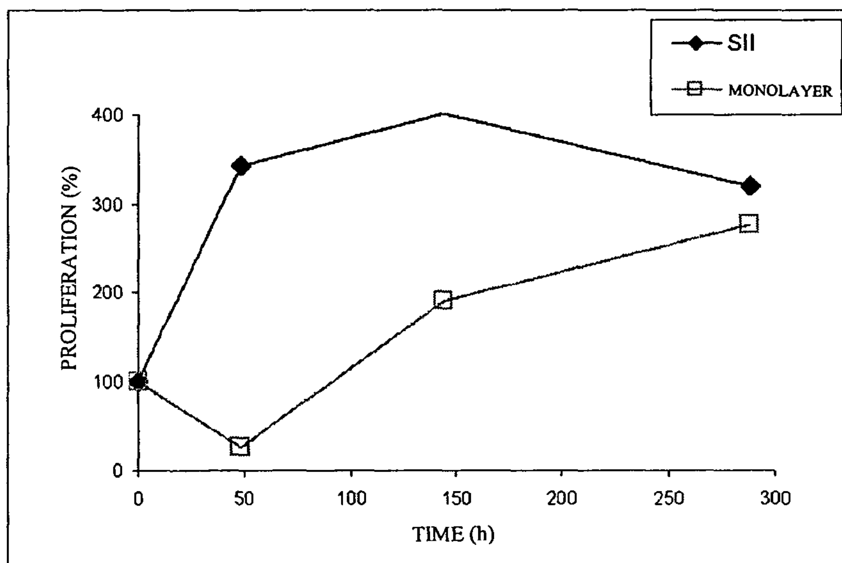
(54) Title: INTEGRATED IMPLANT SYSTEM (IIS) BIOCOMPATIBLE, BIODEGRADABLE AND BIOACTIVE, COMPRISING A BIOCOMPATIBLE STERILE POROUS POLYMERIC MATRIX AND A GEL, INTEGRATING IN SITU THE TRIDIMENSIONAL MATRIX STRUCTURE



(57) Abstract: The present invention refers to an Integrated Implant System constituted as a gel-matrix-cells integrated system, that allows providing implants in a brief time period, for covering a great skin extension to be treated, with a successful acceptance in patients with burns, chronic damage or wound skin, needing of a skin grafting.

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INTEGRATED IMPLANT SYSTEM (IIS) BIOCOMPATIBLE, BIODEGRADABLE AND BIOACTIVE, COMPRISING A BIOCOMPATIBLE STERILE POROUS POLYMERIC MATRIX AND A GEL, INTEGRATING IN SITU THE TRIDIMENSIONAL MATRIX STRUCTURE

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FIELD OF THE INVENTION

The present invention is related to an Integrated Implant System easy to use and handle for being applied in surgical
10 grafting. It is constituted as a gel-matrix-cells integrated system, a feature that allows providing implants in a brief time period, for covering a great skin extension to be treated, with a successful acceptance in patients with burns, chronic damage or wound skin, needing of a skin
15 grafting.

BACKGROUND OF THE INVENTION

Skin is the largest organ of the body, covering the body's
20 outer surface area. Skin is composed of two main layers: the surface epithelia or epidermis, containing epidermal cells as keratinocytes, and the underlying connective tissue layer or dermis, containing dermal cells as fibroblasts. Several and important functions are associated
25 to the skin, for instance, the protection against injury and dehydration, acting as a barrier against infections, perceiving or detecting environmental stimuli, excreting diverse substances, regulating the body temperature, and helping to maintain the hydric balance. Substantially
30 intact and healthy skin is needed , not only for the body welfare, but for its survival.

The skin health and integrity may be affected by congenital or acquired pathological conditions, either acute or
35 chronic, for which the natural skin repair and regeneration

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processes may be insufficient. These conditions include burns, wounds, ulcers, infections, congenital diseases and/or abnormalities. Therefore, a non-appropriate condition and state of the skin is extremely harmful, since it affects the health condition and the organism existence. Those patients that have been affected by burns in a vast surface area of their bodies often require an immediate and extensive replacement of skin. In addition, some less life threatening conditions can be present, however considered as chronic conditions of skin, for instance as occurs for passive hyperemia, the diabetic ulcers or decubitus ulcer, which may result in more severe conditions, if no treatment is applied, particularly since those patients exhibiting these conditions suffer an underlying pathology. On the other hand, the morbidity and mortality drop-off in this kind of patients depends of the appropriate and effective restoration of the skin structure and function.

Skin substitutes may be used for treating these or other conditions. The desirable properties for skin substitutes are availability and handling, low rejection degree, high adherence degree, highly independent from a skin donor, relative easiness for being produced from a skin biopsy of a minimal size, and the feature of a cost-effective production and use. Several methods for elaborating skin substitutes have been assayed, which satisfy some or all these requirements, but with varied success degrees.

However, a predominant design for a skin substitute, which satisfactorily is able of regenerating all the skin structures and functions, is not available yet. Only, a full thickness skin graft is able to virtually restoring all the normal non damaged skin structures and functions. Large and extensive information is available about commercial devices or technical solutions for solving this

skin graft problem, nevertheless these do not provide the qualities and advantages of the development described in the present invention.

5 US 2003/0170892, describes a device comprising a cell- free biocompatible reticulated matrix, wherein a first (layer) of dermal cells are cultured, and over these cells, epidermal cells are grown. Further, it describes the method for producing said device, useful for applications in skin
10 wounds, in therapeutic treatment and/or for the *in vitro* assay of human skin. Providing similar structures and functions to a normal healthy skin, as a barrier function.

The above development exhibits clear differences and
15 disadvantages regarding the present invention, since the described implant corresponds to a device comprising cells grown in the surface and in clearly differentiated layers. Furthermore, due to the design and the method for producing said device, the required time for applying the same is
20 longer than 24 hours, considered from the moment when the dermal cells culture is available for being seeded over the matrix.

CN 1562392 describes a method for preparing an artificial
25 skin in an active bi-layer supported on a collagen sponge, which is cultured in a bioreactor with a fibroblasts suspension for generating the dermis and on top of this, epidermal cells are seeded. Structural differences with the corresponding graft of the present invention, can be
30 clearly distinguished since this document mentions a layer type of graft exhibiting cells on the surface, wherein the seeding method is less efficient, more complex, more expensive and takes longer time for reaching similar results. The same differences regarding the present
35 invention are observed for the patent application CN

1468634, wherein an artificial skin implant with a double film is described, with superficial growth of epidermal cells over a fibroblasts-PGA structure.

5 US 6,733,530 describes a device wherein autologous keratinocytes are grown over a biocompatible substrate, which is pre-seeded with allogenic or autologous dermal fibroblasts, which can be applied over an artificial skin substrate grafted to the receptor patient. The layered and 10 superficial structure of the device, and the method for preparing the same, described in this document, makes the present invention a different device with clear advantages regarding the applications of the same.

15 In a same way to the preceding cases, in the patent CA 2497895 is described a fibrin cell support to form cell cultures. It is possible to establish cell cultures on this support, such as keratinocytes cultures. In a disclosed embodiment, it is possible to incorporate cells in the 20 fibrin gel structure. The above description considerably differs from the present invention device, since the present invention corresponds to a gel-matrix-cells integrated device, which exhibits better handling characteristics as it comprises a biocompatible support 25 available as the porous matrix, which contributes to device strength, resistance and flexibility. In fact, the presence of a biocompatible support does not form part of what is described in the document, since the fibrin gel is used in combination with a preliminary treatment of the wound with 30 artificial skin (Integra™). Furthermore, in the document the product is applied as a paste or spray on the wound, which implies great difficulties for fixing the fibrin gel to the wound and for being properly accepted by the grafted patient. Accordingly, combined additional or supplementary 35 treatments are needed, in order to allow the graft fixing

to the wound, since the fibrin gel slides from the application site. In the present invention no fixing or additional treatments are required in order to maintain the device on the application site and to start the regeneration of the damaged skin. Although in the reference is mentioned the incorporation of polymers, these function only as fibrin carriers, furthermore these are not incorporated into the fibrin gel, as in the present invention. The above provides a different support with a closer *in situ* integration of the matrix-gel, corresponding to one of the present invention contributions.

In the same way the patent application WO02078721 describes a system for providing an autologous *in situ* implant, wherein a keratinocytes and fibroblasts suspension containing fibrin, are mixed with a thrombin, fibrinogen and collagenase solution. As in the latter case, the reference differs from the present invention, as it is not related to an integrated device comprising a biocompatible support as a porous matrix. At the same time, in the present invention the strength, handling and adherence properties are improved and optimized, regarding the technical solution disclosed in the document WO02078721. Furthermore, the physical qualities of strength and the *in situ* preparation, makes this product difficult to handle and store, since it corresponds to a cell suspension which is applied in the site to be treated (wound or damaged tissue), exhibiting adherence difficulties such as described for the previous document.

The main purpose of the skin substitutes is to save the patient's life, providing a coating or barrier, which prevents the skin dehydration and infection. A second objective is to allow for a functional healing, which additionally should be cosmetically acceptable.

Different cell types and material combinations, which have been evaluated for producing cutaneous substitutes, are nowadays commercially available. Most of said commercial products are formed by allogenic cells-containing matrix, generally from neonatal foreskin, which exhibits the advantage of containing a higher number of stem cells for keratinocytes, potential mitogenic properties, an exacerbated metabolism and minimal antigenicity [1,2].

One of the known most simple cutaneous substitutes is EPICEL ®, which is composed by autologous keratinocytes arranged on a paraffined gauze, which is a non biodegradable material. Said cells are obtained from a full thickness biopsy from the injured subject, the cells are subsequently grown over a layer of murine fibroblasts, which have previously been irradiated. Thus, stratified keratinocytes layers are achieved, containing from two to eight layers[3]. In this system, rejection is totally avoided and the cells are permanently incorporated into the tissue. However, it is fragile and with poor cosmetic ability of maintaining an acceptable appearance in the patients. Some available studies indicate that implanting stratified keratinocytes (differentiated) is not the most suitable approach, since the higher differentiating degree and the lower proliferating ability. It is preferable, such as is provided by the present invention, to implant cells with the least number of *in vitro* passages.

A retrospective study carried out in a 30 patients group, exhibiting burns that cover 78% of the body surface, treated with EPICEL®, an extremely high survival rate (90%) was informed[4]. Nevertheless, the higher difficulty of this treatment is related to its cost and logistics.

Other known product, based in autologous cells is Laserskin®, which is indicated for the treatment of second-degree deep burns and for chronic ulcer. This product consists of a biodegradable estherified hyaluronic acid matrix, with laser made micro-perforations in order to allow the keratinocytes settlement and proliferation. Said autologous keratinocytes are obtained from a biopsy and are directly cultured in the mentioned matrix. Laserskin®, is only available in Europe. The efficiency in treating the diabetic foot ulcer has been demonstrated for this product; however no controlled clinical trials are available. This device can be used combined with Hyaff™ a support containing dermis fibroblasts, wherein the fibroblasts can be autologous or allogenic. These products correspond to devices wherein cells can be grown within their matrix, not only in the surface, nevertheless, these do not correspond to an *in situ* integrated system formed by matrix-gel cells as the one of the present invention.

On the other hand, Celaderm® is a commercially available product, this product contains metabolically active foreskin-derived heterologous keratinocytes which however are unable of proliferating. This product has been used for treating chronic ulcer; and as a further advantage, it can be cryo-preserved[6]. Some available studies reveal the effectiveness of this product in burns, however not compared with autografting[7]. This product exhibits significant differences regarding the present invention, as it does not involve an integrated system.
Other known product is Dermagraft®, an approved substitute for treating diabetic foot ulcer. In this case, fibroblasts are obtained from neonatal foreskin and cultured on a polyglactine matrix, during approximately three weeks. In this term, cells secrete matrix proteins, providing a tridimensional *in situ* matrix which serves as a dermis

substitute. The product is delivered cryo-preserved and requires thawing and washing to be used[8].

Other product is Transcyte® (originally known as Dermagraft-TC®) containing non-viable fibroblasts on a silicone-covered nylon net. Said product is prescribed as a temporary cover or coating for burn wounds which have been split by surgery, as an alternative for cadaver skin[9,10].

Other allogenic product is the one known as LSE, primary used in the treatment of the diabetic foot and the venous leg ulcers[11]. The LSE corresponds to a cutaneous substitute composed by a collagen matrix with viable fibroblasts and a stratified epidermal layer. One difference regarding the present invention is that it corresponds to an integrated device without forming stratified or differentiated epithelia.

The OrCel® product is very similar to the previous one with the difference that the dermal matrix is a porous, crosslinked type II collagen sponge, instead of a gel. The sponge is non symmetrical, in a way that a side is covered by a layer of an acid soluble atelocollagen gel, for sealing the macroscopic pores. Fibroblasts are grown on or within the porous side of the collagen sponge, while the keratinocytes are cultured in the non-porous side, covered by the gel of this collagen matrix. The cells seeded matrix is kept submerged for inhibiting differentiation and stratification of keratinocytes. The time and ratio for the fibroblasts and keratinocytes seeding are designed for controlling the cellular density and the cytokines expression in the final product. It has been seen that the co-culture of fibroblasts and keratinocytes exhibits synergic effects over the production levels of some cytokines and growth factors[12-13].

The PolyActive™ product, uses polyethylene oxide/polybutylphtalate (PEO/PBT) and may use the own patient fibroblasts for the dermis, and the own patient keratinocytes cultured for the epidermis. Finally, Integra™ which uses a collagen-glycosaminoglycan matrix (GAG) providing a non cellular or acellular dermal component, and which may use a thin autograft or other devices containing cells.

The tissue engineering discipline, intended to the generation of a solution for burned patients, or with disability scars or cutaneous ulcers, is an area of great scientific interest. Numerous scientific publications can be found, wherein products are described, which contain autologous or heterologous cells, in combination with some different nature matrix.

In general, the market known and existing products exhibit differences and disadvantages regarding the present invention. The main differences are that said devices do not correspond to an *in situ* integrated device with a fibrin gel and a biocompatible polymer. Among the disadvantages of the state of the art devices, the content of bovine nature components can be found; therefore, it should be considered that these products soon will no longer be used, due to the appearance of prion contamination cases in USA, the need of long incubating or preparation periods, and the differentiation degree (stratification) for the cells, as keratinocytes, which are non favorable factors for these devices.

In the literature, there are many studies carried out in animals, which show that the presence of cells in an implant is beneficial for the healing of a cutaneous injury and that this benefit is higher if the cells are

autologous. Most of the commercial products use heterologous fibroblasts from neonatal foreskin. The selection of this type of cells is due to the easiness for growing them *in vitro*. However, these cells are heterologous, what additionally implies a rejection risk, the needing of carrying out expensive analysis for guarantying that they are not contaminated with microorganisms, such as HIV or with C hepatitis virus, among others. Additionally, the products comprising only fibroblasts, may influence on a non desirable healing, from an aesthetic point of view as for the fibroblasts may be differentiated into myofibroblasts, responsible of the non desirable contraction of the wound.

Besides products are known wherein autologous dermal and epidermal cells are used. Nevertheless, these are products that distribute the cells in different layers, on a same support, in different faces of the support or in different supports. Generally, said products, are difficult of handling and the availability timing for applying the device is of more than 24 hours, since it is necessary to seed, attach and culture the cells over the surface of the supports or over the cultured dermal cells.

A clear disadvantage of the devices for grafting is the need of adhesives for fixing the implants onto the injuries. Mechanical devices or organic polymers can be used as attachers (stapples or gauze), wherein the main objective of these is to achieve the graft take, being this understood as the fixing of the device to the damaged skin, achieving the placement in the site to be treated.

Lyophilized pigskin has been used to immediate treatment of patients after the occurrence of a traumatic event. This is a transitory solution, since it only allows extending the

patient life, while it is possible to apply definitive solutions. In addition, solutions have been developed, on the base of materials that help for burns healing. However, they are cell- free solutions, which unlikely can be compared with the complexity and with the contribution and efficiency in the wound healing, which implies an integrated system with cellular components and a biocompatible matrix developed as part of the present invention.

Therefore, the present invention provides an integrated implant system, being this understood as an intimate link among cells-gel-support, wherein can be combined more than one type of dermal or epidermal, autologous, allogenic, xenogenic or chimeric cells, exhibiting favourable: handling characteristics, time for obtaining the same, application availability, adherence, almost no rejection by the patient, better cellular development and easy conservation. Further, it is informed an easy and fast process for obtaining these integrated implants, wherein the cells are incorporated in the support matrix, providing a device for immediately being applied or conserved.

BRIEF DESCRIPTION OF THE FIGURES.

Figure 1 shows a photography of the biocompatible cell free, microporous matrix, which is used in the present invention as the support for the integrated implant. Photograph A corresponds to the polymerized matrix and photograph B corresponds to the cross-linked matrix.

Figure 2 corresponds to a table wherein different assays are identified, which are carried out in rabbits (column 1). The animal was surgically prepared, and then the integrated implant of the present invention was applied

with different amounts of cells per implant area unit (column 2). In each case the ratio between keratinocytes (column 3) and fibroblasts (column 4) that are seeded in the implant varies, with the purpose of observing the differences in the wounds response as function of different proportions of skin cells of the implant.

Figure 3. Correspond to microphotography of histological, longitudinal sections of an integrated implant. Wherein, photograph A corresponds to a layer of the IIS upper surface, observed with magnification (scale bar = 100 μm) wherein is observed the more homogeneous fibrin matrix (blue) and the reticular polymer weave (red) and photograph B corresponds to a higher magnification of photograph A (scale bar = 20 μm). Cells mainly located at the fibrin matrix are observed (arrows) and also in the reticular polymer weave (arrow tip). These microphotographs are notable as evidence of the closer integration among the system components: matrix-gel-cells. The above results support and clarify the components integration, which turns the present invention as different regarding the state of the art.

Figure 4. Presents a plot showing the kinetics of cellular proliferation in an integrated implant system (IIS) like the one of the present invention and the proliferation in a monolayer system within a culture flask, for fibroblasts, at $4x10^4$ cell/cm^2, as the initial concentration. The high proliferation, up to 300% in the first 48 hours, turns this development in an effective tool at 24 hours, wherein the growth is of 200%, an advantageous situation for the treatment of critical patients.

DETAILED DESCRIPTION OF THE INVENTION

The present invention corresponds to an integrated implant system (IIS), wherein into a matrix or porous support a gel or polymer components is incorporated and get absorbed into the matrix and *in situ* clots.

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In a preferred modality, the present invention corresponds to an integrated implant system (IIS), wherein into a matrix or porous support are incorporated the components of a gel or polymer with an active agent or with the desired
10 cells, which are absorbed in the inner part of the matrix, coagulating *in situ*. Wherein the active agent can be selected from the group consisting of: plants, animals or microorganisms natural extracts; chemicals, pharmaceuticals, cosmetics or polymers, their salts or
15 derivatives; macromolecules or microorganisms.

IIS applications may be many and multiple, depending on the presence of active agents and/or cells. In the case it contains cells, the applications will depend of the
20 features and the cell type to be applied in the IIS, the type of porous matrix and the polymer type to be integrated. Even, the SII could be used as a carrier, for therapeutic evaluation, for safety or efficiency of natural, chemical, pharmaceutical, cosmetic products,
25 polymers or macromolecules. Nevertheless, in a preferred modality the application will be related to treating, curing, or delivering a benefit for pathologies o for chronic or acute injuries, affecting the skin of a mammal, preferably humans. Additionally, the invention comprises a
30 method for preparing the IIS from isolated cells, which will be integrated into the matrix forming the gel-matrix-cells IIS.

In a preferred modality, the applications will be intended
35 to problems related with burns, wounds, ulcers, infections,

surgeries, diseases and/or congenital abnormalities of the skin. Particularly, the main objective and application of the invention is to be used as a surgical skin implant for burned people, chronic diseases or for regenerative treatments. Also the IIS can be used on a non damaged surface, a minimally damaged surface, or a surface that has surgically been prepared, and which for other reasons requires an IIS graft.

The IIS is composed by a biocompatible cell-free reticulated matrix, known as support or scaffold, providing an easy to handle support. Said scaffold may be acquired from those commercially available or may be prepared according to a production protocol, as is described below in the present invention or by other production protocol for preparing biocompatible polymers.

For preparing the IIS, it is possible to use a matrix composed of a full length natural or synthetic protein, or a polypeptide, as well as inorganic or organic polymers or their mixture. For example, a lyophilized collagen sponge can be used, either alone or combined with a carbohydrate (a mucopolysaccharide, such as a glycosaminoglycan (GAG), particularly chondroitin-6-sulphate). The collagen can be bovine tissue collagen, from bovine tendon, or from other bovine sources (bone or muscle), other xenogenic sources (for example from swine, sheep, goat, etcetera), of human origin, recombinant or a combination of any of the previous. Other proteins such as elastin or reticulin, or natural or synthetic amino acid polymers, may also be used.

One preferred embodiment for the matrix used in the present invention is composed by gelatin-chitosan-hyaluronic acid. Particularly, the matrix can be commercially available or it can be obtained according to the following process. In a

preferred modality a matrix containing a concentration between about 0.5 to 5%, preferably between about 1 to about 3% of gelatin is used, a concentration between about 0.5 to 3%, preferably between about 1 to about 2% of chitosan is used, and a concentration between about 0.5 to 2%, preferably between about 0.8 to about 0.5% of hyaluronic acid is used, and preferably these components are used in a weight ratio of 7:2:1, respectively.

In one embodiment of the invention, the polymeric matrix may be prepared according to the following state of the art derived procedure (Haifeng L. *et al.* 2004):

-a gelatin solution (1% w/v) is mixed with a chitosan (2% w/v) solution, in 1% v/v acetic acid solution, together with a hyaluronic acid (0,01% w/v) solution. Said mixture is homogenized by stirring at 50°C, during 30 minutes, subsequently the mixture is poured in a container or device for shaping it, for example a Petri dish until the desired height. Subsequently, the plate containing the mixture is cooled at 4°C, until a gel is formed, which may subsequently be frozen at -20°C, during 8 hours, or at -80°C during 6 hours. Said frozen gel is carefully immersed in liquid nitrogen, during 2 to 5 minutes, and finally is lyophilized during 24-48 hours.

Then, the lyophilized polymer is submerged in 20 mL of a 50 mM 2-morpholine-ethane sulfonic acid (MES) solution, 90% ethanol, during 30 minutes at room temperature. Subsequently, the cross-linking is achieved submerging the obtained matrix in 20 mL of a cross-linking solution which is composed by 50 mM MES, 30 mM 1-ethyl-(3,3-dimethyl-aminopropyl)carbodiimide (EDC) and 8 mM N-hydroxysuccinimide (NHS), in a 1:9 water:ethanol mixture. Then, the matrix is allowed to stand for about 2 hours, washed with ethanol, frozen and submerged in liquid

nitrogen for about 2 to 5 minutes, finally lyophilizing the obtained cross-linked polymeric matrix.

Alternatively, the formed IIS is a biocompatible, cells-free, reticulated matrix, and which provides an easy to handle support, for a cells population to be integrated, previous to the implanting, comprising the components of a polymer or gel which is *in situ* polymerized. Wherein said matrix may comprise the above indicated components, and may be obtained according to the above disclosed embodiments.

In this embodiment of the invention, the cells used for being integrated within the matrix, embedded therein in order to form one embodiment of the SII, may be obtained from the patient to be treated (autologous), may be obtained from other human subject (allogenic), or may be obtained from other species (xenogenics), or obtained from many other sources (chimeric). In one preferred embodiment of the invention, the cells used in the development of the IIS are autologous skin cells, either from dermis and/or epidermis. Then, for developing the present invention, can be used epidermal cells, which may be selected, for example, from keratinocytes, melanocytes, immunocytes, stem cells or others; and/or dermal cells, selected, per example, from fibroblasts, endothelial cells, immunocytes, nervous cells, myocytes, stem cells or others.

The cells which will form the IIS may be grown as a pure culture or as a mixed culture. Once a suitable number of cells are obtained, the cellular populations are harvested for their inclusion within the matrix. In the embodiments of the invention, the cells may be integrated into the matrix, in solutions containing a concentration of up to 8 $x\ 10^6$, preferably between $2\ X\ 10^2$ to $4\ X\ 10^6$, more preferably between $3.5\ X\ 10^2$ to $4\ X\ 10^6$, and the most

preferred about 1 X 10^5 cells/mL. In one embodiment of the invention, the used cells are dermal and/or epidermal cells, the ratio of the cells used for inoculating the matrix is comprised within the range of about 20:1 to 1:20 of dermal cells:epidermal cells, in a preferred manner in ratios from 1:1 to 1:10, more preferably between 1:2 and 1:5, considering the usual ratios as from 1:2 to 1:4.

In other preferred embodiment of the invention, the IIS is used as a temporary skin substitute. In this embodiment, the matrix may be seeded with cells having non autologous genotypes.

Further, the invention is intended as a method to prepare said IIS for surgical grafting on skin wounds. On a biocompatible and porous matrix, as those previously described, one of the gel components is deposited, therefore incorporating it easily and quickly into the matrix, after which the next component is added causing the *in situ* gel formation. Said gelified matrix is incubated under appropriate conditions for forming the IIS of the invention. In a preferred embodiment, on a biocompatible, porous matrix, as those described in the present invention, a desired cells suspension is deposited, contained in one of the gel component, in order to easily and quickly incorporate the cells into the matrix, after which the next component is added, causing the *in situ* gel formation. Said inoculated and gelified matrix is incubated under proper conditions for obtaining the integrated implant system (IIS) of the invention.

In one preferred embodiment of the present invention, the IIS comprises dermal cells and/or epidermal cells population cultured under suitable conditions and which are obtained in the conventional manner, as described in the

state of the art, which are deposited as a part of a cells suspension in a thrombin solution, onto the matrix. Then, the integration of said cellular suspension within the matrix is carried out, preferably through the use of a thrombin and fibrinogen system, in such a way that a fibrin polymer is produced in situ resulting in an gel like environment, which allows the cells to get embedded within the matrix.

Alternatively, in the present invention, other system could be used for generating a gel with adhesive properties, such as the combinations of cyanoacrylate esters/water or amine, gelatin-resorcinol/aldehyde, natural bioadhesives/enzymes. Once incubated this IIS, it is considered in condition or ready for being surgically grafted to the patient, which can be carried out during the first day, i.e. in a period no longer than 24 hours, in one embodiment of the invention, from the moment when the desired cells, available in a culture, are integrated into the matrix. The availability of cultured cells highly reduces the need of a skin donor, in order to complete the closing of the extended and full thickness skin wounds.

The cells suspension has a concentration of up to 8 x 10^6 cells/mL, preferably between 2 X 10^2 to 4 X 10^6, more preferably between 3,5 X 10^2 to 4 X 10^6, and in a preferred modality about 1 X 10^5, wherein the cells are suspended in a thrombin solution at a concentration comprised between 25 to 750 NIH/mL (NIH: Enzymatic Activity Units), more preferably 125 to 500 NIH/mL and in a preferred manner about 250 NIH/mL.

The described fibrinogen solution can be used at a concentration comprised between 10 to 90 mg/mL, preferably

between 10-50 mg/mL, preferably 15-30 mg/mL and more preferably about 20 mg/mL.

In order to produce the IIS, between 50-500 μL, preferably between 50-250 μL and more preferably about 100 $\mu L/cm^2$ of a thrombin solution are applied into the matrix. Once the previous solution is absorbed, a fibrinogen solution at a ratio of 50 to 500 $\mu L/cm^2$, preferably between 50-250 $\mu L/cm^2$ and more preferably of about 100 $\mu L/cm^2$ is applied onto the matrix, producing the fibrin gel formation, which gets incorporated, i.e. it gets integrated, into the matrix, providing a IIS wherein matrix and gel are closely integrated in the matrix depth. The thrombin:fibrinogen ratio may fluctuate from 1:0.5 to 1:5, preferably 1:0.5 to 1:2.5 and in a more preferred ratio 1:1.

In one preferred embodiment for structuring the IIS, with the thrombin solution, suspended cells can be added in said solution, in such a way that when the fibrinogen is added, a fibrin gel is formed, wherein the gel gets integrated into the matrix and together to this the applied cells, such that in the IIS, the components are closely related, in the full depth of the matrix, the gel, the cells with the matrix, as can be appreciated in figure 3. Finally, the gel-matrix-cells IIS is submerged in a suitable media, which can be selected from DMEM/F-12, DMEM, RPMI or MEM, among others; preferably it is submerged in DMEM/F-12 culture media.

In one preferred modality, the IIS and the method of the invention exhibit a great contribution and advantage, since they incorporate both the main skin cellular populations, i.e. fibroblasts and keratinocytes. For an appropriate restoration of the injured or damaged skin area, said condition results ideal. In said preferred embodiment, both

cells types are integrated, simultaneously, within the gel-matrix-cells IIS. Preferably, said cells are autologous and the keratinocytes are not differentiated, therefore, they have a great potential for proliferation.

Other advantageous factor of the IIS of one of the embodiments of the invention, as it has been mentioned, is that the cells are included, i.e. embedded within the IIS. The IIS performs as a carrier system, adapts to the shape of the wound, with an appropriate adjustment, which does not require of methods, devices or additional adhesives for fixing the IIS to the skin wound to be treated. Furthermore, it is 100% biodegradable, having been identified the *in vitro* ability of the cells integrated into the IIS of moving to the IIS surroundings (data not shown). The carrier components are non-inert and exhibit antibacterial, chemotactic and proangiogenic properties, helping to an earlier wound healing. From a handling point of view, the IIS does not require of additional supports neither of adhesives, which is highly beneficial since with a single and direct application the graft "starting" can be achieved, i.e. it gets adhered almost immediately, without the need of additional components or products for adhering it.

Once the integrated implant system has been grafted to the patient, and once the biodegradable matrix has been reabsorbed by the body, the cells get organized in order to form a functional skin tissue. The device comprises many of the properties and structures that are found in the normal non-damaged skin, and functions as in the usual way as does a non damaged skin, in order to protect the subject from the fluids loss and against bacterial infections. The integrated implant system establishes a basement membrane, and maintains the same anatomical configuration for the

layers or cell populations in the same way as usually occurs in the non-damaged skin.

The following examples and the detailed description of the invention, are related to the most preferred embodiments for carrying out the invention, however, these are not intended to restrict the scope of the invention.

EXAMPLE 1. Construction of the Integrated Implant System (IIS).

For manufacturing the IIS the following solutions must be prepared:

a) Calcium chloride and sodium chloride solution.
About 3 to 12 g of di-hydrate calcium chloride are dissolved in 1 liter of sterile milli-Q water (Millipore). Sodium chloride is added until a solution with an osmolarity comprised between 280 -320 mOsm/L, is obtained. Preferably 4,5 g of di-hydrate $CaCl_2$ and 6,1 g of NaCl in 1 liter of water (300 mOsm/L), are used.

b) Fibrinogen solution
An amount between 10 and 90 mg of fibrinogen in 1 mL of sterile Milli-Q water, is dissolved. Preferably, 20 mg of a lyophilized product is used, with a minimal concentration of 65% (13 mg).

c) Thrombin solution
In 1 mL of sterile calcium chloride and sodium chloride solution, between 250 and 500 NIH thrombin are dissolved. Preferably, 1 mg of a lyophilized product with an activity of 258 NIH/mg-lyophilized product (258 NIH) is used.
d) Integrated implant system preparation

The IIS preparation is always carried out in a sterile environment. Thus, a polymeric matrix portion is provided, which is either commercially available or prepared according to the above indicated process, in an appropriate size for the required application. The matrix portion is sterilized, through methods usually known in the state of the art, as for instance, through irradiation, UV treatment or with and alcohol solution, without being restricted to said options. In one preferred embodiment, the matrix portion is submerged for a suitable time period, preferably for about 1 hour, in an aqueous alcohol solution, wherein said alcohol corresponds to a 50% to 90% alcohol solution in sterile Milli-Q water, preferably a 70% aqueous alcohol solution, wherein the alcohol is selected from the group consisting of ethanol, propanol and iso-propanol. Then, the matrix is removed and dried over a sterile absorbent paper. The matrix is placed in a suitable sterile container (for example, a Petri dish or other sterile container with appropriate dimensions for the selected matrix portion) and over the matrix portion a thrombin solution is spread at a ratio of about 100 μL solution, to be applied over about 1 cm^2 of the polymer. Subsequently, the absorption of the solution is allowed, which usually takes place within the first three minutes, preferable before 2 minutes. Then, about 100 $\mu L/cm^2$ of fibrinogen is added over the matrix containing the absorbed solution. Subsequently, the clot formation is visually verified, which means that the IIS can be immediately used or conserved for later applications.

EXAMPLE 2. Construction of the Integrated implant system (IIS) containing cells.

In a complementary manner to the description of example 1, in one preferred embodiment of the invention, it is

possible to prepare an IIS containing the desired cells integrated into the matrix-gel system. For such a case, the matrix is prepared as described above and additionally a cellular suspension is prepared, for which the cells from a cell culture obtained through trypsinization, are centrifuged in order to obtain a pellet, which is suspended in 1 mL of a $CaCl_2$ and NaCl-containing thrombin solution.

The IIS preparation is always carried out in a sterile environment. Therefore, a polymeric matrix portion is provided, which can be either commercially available or prepared according to the above-indicated process, in an appropriate size. The matrix is submerged during a suitable time period, preferably for about 1 hour, in an aqueous alcohol solution, wherein said alcohol corresponds to a 50% to 90% alcohol solution in sterile Milli-Q water, preferably it corresponds to an aqueous 70% alcohol solution, wherein the alcohol is selected from the group consisting of ethanol, propanol and iso-propanol. Then, the matrix is removed and dried over sterile absorbent paper. Subsequently, the dried matrix is submerged in DMEM/F12 (10% FBS) media, for about 18 to 30 hours, preferably during less or about 24 hours. The sterility is checked through microscopy observation, by the media turbidity and the appearance of change in color. Subsequently, the polymer is removed, and once more dried over sterile absorbent paper.

The matrix is placed in a suitable sterile container, for example, a Petri dish or other sterile container with appropriate dimensions for the selected matrix portion. Over said matrix portion, 100 μL of a thrombin and cells solution are spread, containing a maximum of up to 8×10^6, preferably between 2×10^2 to 4×10^6, more preferably between 3.5×10^2 and 3.5×10^6 cells/mL of final solution,

preferably a suspension containing about 2 x 10^5 cells/ mL of solution, for being applied onto about 1 cm^2 of the matrix. Then, the absorption of the cellular suspension is allowed, which generally occurs within the first three minutes, preferably before 2 minutes. Subsequently, over the matrix containing the absorbed suspension, 100 $\mu L/cm^2$ of fibrinogen are added. Later, the clot formation is visually verified, and the obtained implant system is submerged in DMEM/F-12 (10% FBS) culture media.

Wherein, the cells suspension corresponds to allogenic cells, autologous cells or stem cells, wherein the cells come from a cellular culture for each of said cellular types, and which have been isolated from biopsies obtained according to standard procedures described in the state of the art. In one embodiment of the present invention, said cellular cultures correspond to an autologous fibroblasts cellular culture, or to an autologous keratinocytes cellular culture, or to a stem cell culture, or a mixture thereof, in a calcium chloride (30 mM) and sodium chloride (100 mM) solution. In a preferred embodiment of the present invention, the cells suspension contains an autologous fibroblasts and autologous keratinocytes combination.

EXAMPLE 3. Growth characteristics of the cells incorporated into the IIS (Figure 4).

The ability of growing of those cells incorporated into an SII, prepared according to example 2, was evaluated. The existence of a time, wherein cells are in their maximum proliferative ability, was determined.

Comparing the growth behaviour on a monolayer system, as it takes place in the state of the art devices, wherein the cells grow on the polymers surface or on mechanical

supports, in regard to the ISS of the present invention, it was found that the IIS shows clear and outstanding advantages, which could result in better healing and recovery ability of those patients treated with the device of the present invention.

The cell cultures in the IIS show that during the first 50 hours of culture, an outstanding increase of the proliferation is achieved, in regard to the monolayer system (Figure 4). The latter is a relevant advantage of the present invention, insofar as 24 hours from the assembly and seeding of the IIS, it is possible to proceed to implanting it onto the cutaneous lesions, wherein the IIS comprises cells in an increasing and active curve of a maximum proliferation ability, which may provide better qualities and lower recovery time in the lesion to which the IIS is applied.

EXAMPLE 4. Preclinical IIS application on rabbits.

Rabbits were surgically prepared, in order to receive the IIS, this corresponds to the extraction of circular zones 2.5 cm in diameter of dermis and epidermis from the animal's dorsal area.

Those animals that were not treated showed a critical clinical condition, resulting in 33% of deaths. On the contrary, those rabbits that were immediately treated with different IIS, survived the trial in excellent conditions (n=8).

The dorsal section of the animal being curve, makes it difficult to maintain implant devices on place. However, the application of the IIS did not show those difficulties, being expedite, of easy handling and adhesion in different

zones of the assayed animal. On the other hand, when a fibrin gel with cells was applied, as occurs in some state of the art devices, it resulted complicated in handling, difficult to maintain on place in the lesion and with poor adhesion.

The animals treated with the IIS, showed very low infections incidence and when these occurred, they shared the feature of spontaneously disappearing. This may mean that the IIS rapidly reconstitutes the functional skin systems that provide recovery qualities to the damaged area.

No clinical evidences of rejection against any of the components of the graft were observed during the trials. Additionally, a re epithelization in the damaged area was noticed, within a 25 days period, which was confirmed through a clinical and histological evaluation.

The set of examples and the invention description, without the intention of restricting, provide evidences about the differences and advantages of the present invention regarding the currently known and disclosed devices, which are powerful and enough qualities, in the sight of any person skilled in the art, which further allow differentiating the present invention of the matter known in the state of the art, and certainly, cannot be deduced or obviously derived from said background.

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15 Concepts in Pediatric Burn Care.

CLAIMS

1. Biocompatible, biodegradable and bioactive integrated implant system (IIS), which at least comprises an sterile porous, biocompatible polymeric matrix and a gel, which are closely associated, since the gel gelification occurs in the inner part of the matrix, becoming *in situ* integrated to the matrix tridimensional structure.

2. The integrated implant system, of claim 1, comprising a sterile porous, biocompatible polymeric matrix, a gel and additionally active agents and/or cells, which are closely associated, since the gel containing the active agents or the cells, is incorporated within the inner part of the matrix, becoming *in situ* integrated to the matrix tridimensional structure.

3. The integrated implant system, according to claim 2, wherein the active agent can be selected from the group consisting of plants, animal cells or microorganisms extracts; chemicals, pharmaceuticals, cosmetics or polymers, their salts or derivatives; macromolecules or microorganisms.

4. The integrated implant system, according to claims 2 to 3, wherein the cells can be from the patient to be treated (autologous), they may be from another subject of the same species (allogenic), from other species (xenogenic), or from multiple sources (chimeric), which can be normal or recombinant.

5. The integrated implant system, according to claim 4, wherein the cells can be selected from epidermal cells, and which can be selected from keratinocytes, melanocytes, immunocytes, stem cells or others, and/or dermal cells,

selected from the group consisting of fibroblasts, endothelial cells, immunocytes, nervous cells, myocytes, stem cells or others.

5 6. The integrated implant system, according to claims 4 to 5, wherein the cells can be present at concentrations of up to $8x10^6$, preferably between $2x10^2$ to $4x10^6$, more preferably between $3.5x10^2$ to $4x10^6$, and the most preferable of about $1x10^5$ cells/mL.

10

7. The integrated implant system, according to claims 4 to 6, wherein the used cells are selected from dermal and/or epidermal cells, wherein the ratio of the used cells for inoculating the matrix is comprised in the range of about
15 20:1 to 1:20 among dermal:epidermal cells, in a preferred way in ratios from 1:1 to 1:10, more preferably between 1:2 to 1:5, considering as the more usual the 1:2 to 1:4 ratios.

20 8. The integrated implant system, according to claims 4 to 7, wherein the used cells are keratinocytes and/or fibroblasts.

9. The integrated implant system, according to any of the
25 preceding claims, wherein the IIS matrix can be acquired from those that are commercially available, or can be used as a component of the matrix at least one natural or synthetic full length protein, or a polypeptide, as well as inorganic, organic polymers, or a mixture thereof.

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10. The integrated implant system, according to any of the preceding claims, wherein as the matrix a lyophilyzed collagen sponge can be used, either alone or combined with a carbohydrate, as a mucopolysaccharide, such as a
35 glycosaminoglycan (GAG), particularly chondroitin-6-

sulfate; where the collagen can be bovine tissue collagen, or bovine tendon collagen, or from other bovine sources, such as bone or muscle, from other xenogeneic sources, as for example from swine, sheep or goat, from human origin, it may be recombinant or a combination of any thereof.

11. The integrated implant system, according to any of the preceding claims, wherein as the matrix can be employed other proteins such as elastin or reticulin, or synthetic or natural amino acid polymers can also be used.

12. The integrated implant system, according to any of the preceding claims, wherein the used matrix is composed by gelatin, chitosan and hyaluronic acid, wherein the gelatin is found in an amount of up to about 1%, chitosan up to about 2% and the hyaluronic acid up to about 1%, preferably in a weight ratio of about 7:2:1, respectively.

13. The integrated implant system, according to any of the preceding claims, wherein the used gel is a fibrin polymer obtained through a thrombin and fibrinogen system.

14. The integrated implant system, according to any of the preceding claims, wherein alternatively other system for producing the gel could be used, which fulfills the conditions of the present invention, such as combinations of cyanoacrylate esters/water or amine, gelatin-resorcinol/aldehyde, natural bioadhesives/enzymes.

15. The integrated implant system, according to any of the preceding claims, wherein the fibrinogen solution may contain a concentration between 10 to 90 mg/ml, preferably between 10-50 mg/ml, more preferably 15-30 mg/ml and most preferably of about 20 mg/mL; and the thrombin solution contains a concentration between 25 to 750 NIH/mL, more

preferably 125 to 500 NIH/mL and the most preferably of about 250 NIH/mL.

16. The integrated implant system, according to any of the preceding claims, wherein the system components are non inert and exhibit antibacterial, chemotactic, and proangiogenic properties, further, the system does not require of additional supports neither adhesives for binding and remaining in the application site.

17. Use of the implant system, according to claims 1 to 16, which is useful for preparing a medical application product, suitable for treating, curing, or providing a benefit to pathologies or chronic or acute injuries, affecting the skin of a mammal, preferably humans.

18. The use of the implant system, according to claims 1 to 16, since it is useful for preparing a product to be used as an application carrier, for therapeutic evaluation, for the safety or efficiency of natural chemical products, pharmaceutical products, polymers, cosmetics or macromolecules.

19. The use of the implant system, according to claim 17, wherein the problems affecting the skin are related with burns, wounds, ulcers, infections, surgery, diseases and/or congenital abnormalities.

20. The use of the implant system, according to any of the preceding claims, wherein it is preferably used in skin surgical implants for burned people, for chronic diseases or regenerative treatments.

21. The use of the implant system, according to any of the preceding claims, wherein it may also be applied over a non

damaged surface, a minimally damaged surface, or a surgically prepared surface.

22. Method for preparing an implant system, according to claim 1, wherein said method is carried out in a sterile environment, comprising the steps of providing on a porous and biocompatible matrix, as those described in the preceding claims, one of the gel components, for incorporating it easy and quickly into the matrix, after what the remaining component is provided in order to cause the *in situ* gelification, and subsequently it is incubated under proper conditions for obtaining the IIS.

23. Method for preparing an implant system, according to claim 2, wherein said method is carried out in a sterile environment, comprising application to a porous and biocompatible matrix, as those described in the preceding claims, a desired cells suspension in one of the gel components, in order to incorporate easy and quickly the cells into the matrix, after which the remaining component is added, resulting in the *in situ* gelification of the gel, subsequently said matrix inoculated and gelified is incubated under proper conditions for obtaining the integrated implant system.

24. The method for preparing an implant system, according to claim 23, wherein the desired cells are suspended in thrombin and wherein the remaining component which achieves the gelification is fibrinogen.

25. The method for preparing an implant system, according to the preceding claims, wherein said method is carried out in a sterile environment and comprises the steps of:

a) providing a polymeric matrix portion of an appropriate size;

b) applying to said polymeric matrix a suitable volume of a solution comprised of thrombin and cells;
c) allows the solution to be absorbed in the polymeric matrix, during an appropriate time period and adding the
5 fibrinogen solution;
d) verifying that the material is absorbed and the fibrin gel formation in the inner part of the matrix.

26. The method for preparing an implant system, according
10 to the preceding claims, wherein said method is carried out in a sterile environment and comprises the steps of:
a) providing a polymeric matrix portion of a suitable size;
b) sterilizing said polymeric matrix portion, preferably immersing it in an alcoholic solution, during one hour;
15 c) removing said polymeric matrix portion and drying it over a sterile absorbent paper;
d) submerging said dried polymeric matrix portion, in DMEM/F12 medium during a time period comprised between about 18 to about 30 hours; preferably during 24 hours;
20 e) controlling the sterility of said polymeric matrix portion, through microscopy observation regarding the turbidity and the medium colour change appearance;
f) removing the polymeric matrix portion and drying it over sterile absorbent paper;
25 g) applying to the polymeric matrix a suitable volume of a solution comprised of thrombin and cells;
h) allowing the solution to get incorporated in the polymeric matrix, during a suitable time period;
i) adding the fibrinogen solution;
30 j) visually verifying the fibrin clot formation;
k) Submerging the formed skin implant system in DMEM/F-12, DMEM, RPMI or MEM medium, preferably in DEM/F-12, during the appropriate time period.

27. The method for preparing the implant system, according to the preceding claims 25 to 26, wherein the indicated sterilization can be achieved through irradiation, UV light treatment or alcohol treatment, wherein the alcoholic solution consists in a solution comprising sterile water and an alcohol selected from the group consisting of ethanol, propanol or isopropanol.

28. The method for preparing the implant system, according to claim 27, wherein the solution comprises alcohol between about 50% and about 90%, preferably 70%.

29. The method for preparing the implant system, according to the preceding claims, wherein the alcohol corresponds to 70% ethanol.

Figure 1.

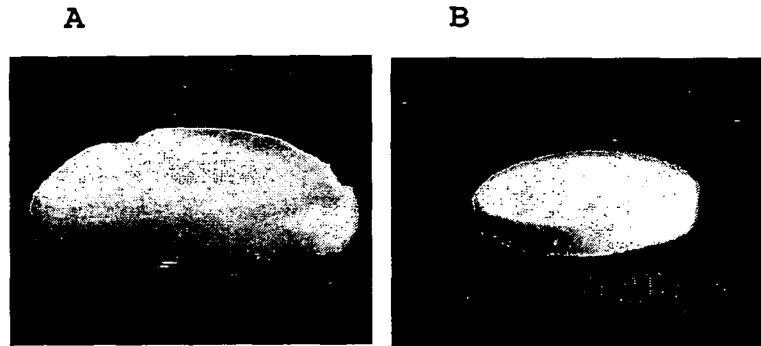

Figure 2.

Rabbit	Cell/cm^2	% keratinocyte	% Fibroblastes
C2	$6{,}8 \times 10^4$	67	33
C4	$1{,}6 \times 10^4$	43	57
C5	$1{,}9 \times 10^4$	Natural Coculture	
C6	$4{,}0 \times 10^4$	67	33
C7	$3{,}8 \times 10^4$	8	92
C9	$1{,}4 \times 10^4$	30	70
C11	$1{,}6 \times 10^4$	63	37
C13	$1{,}5 \times 10^4$	31	69
C14	$2{,}9 \times 10^4$	9	5

Figure 3.

Figure 4.