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Polymeric membranes modulate human keratinocyte differentiation in specific epidermal layers

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ABSTRACT

In vitro models of human bioengineered skin substitutes are an alternative to animal experimentation for testing the effects and toxicity of drugs, cosmetics and pollutants. For the first time specific and distinct human epidermal strata were engineered by using membranes and keratinocytes. To this purpose, biodegradable membranes of chitosan (CHT), polycaprolactone (PCL) and a polymeric blend of CHT-PCL were prepared by phase-inversion technique and characterized in order to evaluate their morphological, physico-chemical and mechanical properties. The capability of membranes to modulate keratinocyte differentiation inducing specific interactions in epidermal membrane systems was investigated. The overall results demonstrated that the membrane properties strongly influence the cell morpho-functional behaviour of human keratinocytes, modulating their terminal differentiation, with the creation of specific epidermal strata or a fully proliferative epidermal multilayer system. In particular, human keratinocytes adhered on CHT and CHT-PCL membranes, forming the structure of the epidermal top layers, such as the corneum and granulosum strata, characterized by withdrawal or reduction from the cell cycle and cell proliferation. On the PCL membrane, keratinocytes developed an epidermal basal lamina, with high proliferating cells that stratified and migrated over time to form a complete differentiating epidermal multilayer system.

1. Introduction

The skin is the largest organ of the body and it is the first physiological barrier against external agents for humans. As a mechanical and waterproof barrier, it has the function of protection against infections, environmental pollutants, and ultraviolet rays. It is also an excretory organ, and many waste products are secreted through the skin in sweat solution. Advances in tissue engineering led to the realization of human bioengineered skin substitutes for *in vivo* skin regeneration and *in vitro* applications. Notably, *in vitro* skin models are useful tools, in addition to biological and pathophysiological research, for testing the adsorption, accumulation, effect and/or toxicity of drugs, cosmetics, chemicals and environmental pollutants. Many drugs are administered through the skin by percutaneous adsorption and a large amount of drug metabolites are secreted in sweating solution.

The first barrier of the skin in contact with external agents is the epidermis, which is constituted of several distinct layers of keratinocytes undergoing a differentiation process with a migratory phenotype from the basal lamina to the outermost layers. Artificial human epidermis models are commercially available and currently used for testing of phototoxicity, corrosivity, irritancy and drug penetration. These models consist of human keratinocytes cultured on a matrix acting as an artificial dermis, i.e. polycarbonate filters in SkinEthic®, a type-I bovine collagen matrix in Episkin®, and a silicone gel in Epiderm® [1]. These models are currently the closest to the human epidermis, with an extensively proven accuracy and reproducibility [1–4], they represent the entire epidermis. To date, information about the creation of specific and distinct epidermal strata, important for the understanding of the interactions with external agents along the epidermis layers, is still missing. External agents could have different effects on the epidermal strata in terms of adsorption, permeation, accumulation, activity and/or toxicity. Although it has been demonstrated that the keratinocyte proliferation and differentiation can be experimentally manipulated by changing media conditions [5,6], the guiding of their differentiation in distinct epidermal strata by specific cell-material

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interactions has never been reported. It is noteworthy that the morphological and physico-chemical surface properties of polymeric membranes, i.e. porosity, roughness, wettability, charge and chemical groups, play a pivotal role in the interactions with cells, influencing their adhesion and triggering specific pathways that govern the cell morpho-functional behaviour and differentiation [7–9]. In addition, membrane mechanical properties, exerting specific tension on the focal adhesions, which represent the anchorage points of cells on the membrane surface, may influence and guide the cell fate and differentiation [10]. Taking in account these considerations, the combination of polymeric membranes with human keratinocytes could be a promising strategy to engineer epidermal membrane layers. Among the different polymers investigated for skin tissue engineering chitosan (CHT), polycaprolactone (PCL) and a polymeric blend of CHT-PCL have a great potential to support the adhesion and proliferation of keratinocytes. CHT, owing to its notable biodegradability together with its anti-inflammatory, antimicrobial and haemostatic properties, has been extensively used as a cell-free wound dressing and for wound healing, as well as for skin grafting [11,12]. In combination with other natural polymers or peptides (i.e. laminin, gelatin, collagen, glycosaminoglycans) it has been used for the development of epidermal grafts [13,14] and as dermal and skin equivalent models [15,16]. PCL, since it exhibits good mechanical properties, a slow degradation rate and easy processing ability, has been blended with natural polymers (i.e. gelatin, collagen) in order to tune the desired mechanical properties and biodegradation rate for the realization of epidermal and bi-layer skin substitutes [17,18]. CHT-PCL blends have been tested for skin applications, as a potential wound dressing and to heal burns and cuts [19–21].

Despite the *in vitro* investigations performed on such polymers that highlighted the cell viability and proliferation in a limited period of time (3–7 days) [17,19,20], their ability to promote the formation of isolated epidermal layers is still unexplored.

The aim of this work was the realization of specific and distinct epidermal strata by using human keratinocytes and membranes of CHT, PCL and CHT-PCL, which have different morphological, physico-chemical and mechanical properties. To this purpose, the membranes were prepared by phase-inversion technique and used to trigger the differentiation of human keratinocytes through their properties. Thus, we challenged the development of specific and distinct epidermal membrane layers, which were maintained functionally active up to 3 weeks.

2. Materials and methods

2.1. Membrane preparation

The membranes of CHT, PCL and a blend of CHT-PCL were prepared in flat configuration by phase-inversion technique by the solvent evaporation process [10]. The polymeric solutions were cast on a glass plate and moulded as thin films by a handle-casting knife (Elcometer, gap set at 250 μm). The solvent evaporation was allowed in a controlled atmosphere at 20 °C, until the cast solution became a solid membrane. The casting solution for CHT membrane was obtained by dissolving 4% (wt/v) CHT (75% deacetylated, Sigma, Milan, Italy) in acetic acid solution 2% (v/v), adding polyethylene glycol (PEG, MW = 6000 Da) (Merck-Schuchardt, Hohenbrunn, Germany) at a 4:1 ratio and stirring for 2 h until complete dissolution. The casting solution for PCL membranes was achieved by dissolving 10% (wt/wt) PCL with medium MW (Mn ~ 45,000 by GPC, Sigma, Milan, Italy) in 1.4-Dioxane (100%) at 50 °C until complete dissolution. For the preparation of the CHT-PCL blend, 3% CHT (wt/v) in 0.5% acetic acid solution (v/v) and 0.5% PCL (wt/v) with low MW (Mn ~ 14,500–10,500 by GPC, Sigma, Milan, Italy) in 100% acetic acid solution were mixed in a 4:1 volume ratio where neither polymers precipitated, stirring until a single phase solution was attained.

After the solvent evaporation, the CHT and CHT-PCL membranes were immersed in a neutralization bath solution of 1% NaOH, needed in order to free all the chitosan domains from the acetylation caused by the action of the solvent. All the membranes were repeatedly washed with distilled water, before the final drying process.

2.2. Membrane characterization

After preparation, the membranes were characterised in order to evaluate their morphological, physico-chemical and mechanical properties.

The topography and roughness of the membrane surfaces were evaluated by using atomic force microscopy (AFM), NanoScope III (Digital Instruments, VEECO Metrology Group). Tapping Mode™ AFM operated by scanning a tip attached to the end of an oscillating cantilever across 9 mm^2 of sample surface, for 512 points, at a rate of 2.54 Hz. The cantilever was oscillated at or near its resonance frequency with amplitude typically from 20 to 100 nm. Silicon probes with resonance frequency 200–300 kHz, nominal tip radius of curvature 5–10 nm and cantilever length 125 mm, were used. Surface roughness was estimated with respect to the mean absolute value difference, Ra, and the root mean squared difference, RMS, between the actual surface height and that of the line dividing the surface of the investigated profile into two equal areas. The reported roughness values are the average of 30 measurements on different membrane samples.

The cross-section thickness of the membranes was measured with Carl Mahr 40E digital micrometer (Germany). The mean pore size was determined by a Capillary Flow Porometer CFP 1500 AEXL (Porous Materials Inc., PMI, Ithaca, New York, USA).

The wettability properties were characterized by using water contact angle (WCA) measurements obtained by the sessile drop method and water sorption at room temperature using a CAM 200 contact angle meter (KSV Instruments, Helsinki, Finland).

The mechanical properties of the membrane were assessed via a Zwich/Roell Z2.5 tensile testing machine (Germany). A pre-load of 0.05 MPa was applied before starting tensile tests at constant elongation rate of 4 mm/min. Real-time longitudinal deformation measurements were acquired and analysed by testXpert® testing software. Tensile tests were carried out at 20 °C for dry and wet membranes considering the different cross-section thickness. For each membrane, at least 10 double clamped strips (1 × 5 cm) were used. The Tensile Modulus E, evaluated from the slope of the linear portion of the stress-strain curve, the Ultimate Tensile Strength Rm and the elongation at break ε were determined.

The degradation properties of the membranes were investigated by treatment with an enzymatic solution, by using 1 mg/mL of lysozyme in PBS, as previously reported [22]. In particular, samples of 1.5 × 1.5 cm^2 were precisely weighed, immersed in the enzymatic solution and incubated at 37 °C, with refreshing media every 7 days. After that, three samples per each group were washed with distilled water and dried at room temperature to constant weight. The dissolution index was calculated as $\%S = \frac{Wi-Wd}{Wi} \times 100$ where Wi is the sample weight before incubation in enzymatic solution and Wd is the dried sample weights after dissolution test. Each test consisted of four replicate measurements.

2.3. Cell cultures

Human Keratinocytes HaCaT, immortalized non-tumorigenic cell line isolated from a 62-year-old male donor and cryopreserved (CLS, Cell Line Service, Eppelheim, Germany) were used. Cells