dermaOXY skin assay: effect and evidence

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- an assembly of knowledge
on an advanced cosmetic treatment
aiming at facial skin improvement,
anti-aging, and damage compensation

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Introduction

This text is a report on the activity conducted for dermaOXY (a brand by MedicTinedic ApS, Varde, Denmark). It involves two dermaOXY products: dermaOXY HYALURON SERUM and dermaOXY SYN SERUM. These are applied to the facial skin in combination with a 90 percent pure oxygen gas stream. Occasionally, the treatment is supported by low-level light exposure, prepared by mechanical microporation of skin or both. The dermaOXY skin improvement approach is used in treatments by clinics spread across 23 countries [1]. This text also includes an assessment of the instrument set DermaLab® Combo, which is used for the physical characterization of skin status after treatment.

The report consists of four main parts, dedicated to

1. the properties of human skin
2. the anti-aging methods applied by the dermaOXY treatment
3. the analytical methods applied by dermaOXY to characterize the effects of the dermaOXY treatment
4. an evaluation of the above

Selected aspects of the human skin are described in order to provide a general background of knowledge. In more detail: the three layers of skin (epidermis, dermis and hypodermis) and their constituents are described. This knowledge is important for assessing the dermaOXY approach to slow down (or better yet inhibit) the phenotypical signs of aging. Professor Beate Klösgen and B.Sc. Lasse Menov performed the study and wrote this report. Lars Melgaard, COO of dermaOXY, provided the information on the dermaOXY approach. Doris Westphal from Westphals Fabrikker A/S, Sønder-Omme, Denmark kindly supplied data about the sera and their constituents. Additional information was acquired during an open discussion among the above mentioned persons and Lars L. Kristensen from Lambert Kristensen ApS, Esbjerg, Denmark. This exchange of information was organized by L. Melgaard and hosted by D. Westphal.
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1. Human skin

The integumentary system is the outermost organ protecting the human organism. It comprises skin and its appendages, namely hair, glands and nails [2]. Skin is the largest human organ. It accounts for about 15 % of the total adult body weight [3]. It is the barrier between the human organism and the hostile surroundings, protecting against pathogens (e.g. bacteria) as well as excessive water evaporation [4]. The average human adult consists of about 60 wt%¹ water for men and 50 wt% for women. The amount of muscle has an influence on the body water content, as muscles contain as much as 75 wt% water. Thus, old people contain less water, as muscle mass tends to decrease with aging [5]. The body water content in infants can be as high as 75 wt% whereas in obese people it can be as little as 45 wt%. Adipose tissue – i.e. tissue comprising mostly large cells (~0.1 mm) known as adipocytes that contain fatty acids as an energy reserve [3] – holds as little as 10 wt% water [6]. Besides protecting the body from water loss, the skin cushions the body and it maintains vital functions as thermoregulation, insulation and protection from chemical and radiation damage as well as mechanical injuries [3, 7].

In the cosmetic market, MedicTinedic ApS presents itself with a menu of advanced methods for skin care, combining the application of specifically assembled chemical cocktails in the form of serums with delicate efficiency enhancers. Examples of enhancers are oxygen massage and LED photosensitizing. The analysis of these tools requires insight into the relevant properties of skin. Therefore, and as a first step, some selected features of skin are presented in the following. These comprise the skin morphology, the cellular composition of the skin and some essential natural polymers like keratin, elastin, collagen and especially hyaluronic acid. Skin deficiencies, such as selected diseases and the aging of the skin, are addressed in order to evaluate the dermaOXY approach to treat these.

1.1. Skin structure and function

The skin is anatomically divided into three main functional layers (Figure 1)². The innermost layer is hypodermis, also referred to as the subcutaneous layer or fatty tissue. It is made up of adipocytes and holds the blood vessels supplying the skin with nutrition and providing waste removal. On top of it lies the dermis. This is where the connective tissue is produced. Connective tissue is an extracellular matrix that supports, connects, or separates different types of tissues and organs in the body. All connective tissues have three main components: ground substance, fibers and cells. The typical appearances of connective tissue in skin are all described in this chapter. Dermis is responsible for the tensile strength and the elastic properties of skin. The outermost layer is called the

¹ percentage by weight, abbreviated wt%

² Hypodermis might not be considered as part of the skin, though there is no skin without it. Hypodermis is always found directly below the dermis of skin.
Human skin

epidermis. It serves as a shell, protecting the human organism from dehydration through evaporation and against pathogens.

1.1.1. The hypodermis

The hypodermis is the innermost layer of the skin (see “subcutaneous layer”, Figure 1). It is around 2 mm thick on average, and it is mostly composed of adipocytes [8]. The thickness varies widely across the body, e.g. between buttocks and scalp. The hypodermal thickness is a sexual characteristic, since adipose tissue tends to accumulate differently in men and women. This phenotypic effect is triggered as a hormonal response and it results in fat deposits on the abdomen of men, whereas for women it occurs below the waist, i.e. around the thighs, hips and buttocks. The hypodermis also accommodates blood vessels and nerves that extend upwards through the dermis. Here they form capillaries and sensory receptors that provide the upper layers of the skin with nutrition, removal of waste products, and with sensational responses, such as soft touch, temperature changes and itch. The adipose tissue functions as an insulator and as well cushions the body, protecting the tissue below the skin from mechanical stress [3, 7].

Figure 1: Human skin – The three main functional layers of the human skin, and the locations of sweat glands, hair follicles and their glands, the blood vessels and the sensory system. The appendages arise from the dermis-hypodermis junction and extend across the epidermis [9].
1.1.2. The dermis

The dermis is the middle layer of skin. It is around 2-4 mm thick and it consists primarily of an extracellular matrix with a sparse population of cells. Dermis is anatomically divided into two regions: stratum papillare and stratum reticulare. The stratum papillare is the uppermost region of dermis that displays papillae into epidermis in Figure 1. The stratum reticulare is the bottom part of dermis and here it is anchored to the hypodermis by matrix proteins, such as collagen and elastin. The dermis and its extracellular matrix give the skin its tensile strength [7, 10].

The most abundant cell type in dermis is the fibroblast. Its function involves the synthesis and as well the degradation of the extracellular matrix (see Figure 2). This matrix of the connective tissue is a complex structure composed of the highly organized polymer networks of collagen and elastin. Besides the matrix and fibroblasts, the connective tissue of the dermis contains another complex mixture referred to as the ground substance. It is composed mainly of proteoglycans which are negatively charged macromolecules that maintain the tissue hydration via osmotic pressure. Dermis also contains glycoproteins, glycosaminoglycans, and water [7, 11]. A peculiar glycosaminoglycan of special interest to this project is hyaluronan or hyaluronic acid (see 1.3.4). Figure 2 illustrates this coexistence of the fibers and the different types of cells in the connective tissue of dermis. The fibroblasts are drawn to be closely connected to the collagen fibers, the elastic fibers and the reticular fibers. The network of these fibers makes up for the extracellular matrix that supports the various types of immune cells and some adipocytes that exist in the ground substance. Nerve fibers and capillaries extend into the dermal papillary (or: stratum papillare) from hypodermis. The capillaries supply epidermis with nutrition and waste removal by diffusion.

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3 There are different types of white blood cells in dermis. They represent the immune system and are beyond the scope of this project.
Figure 2: The connective tissue – The three different fibers of dermis’ connective tissue are depicted in relation to one another. The big, sturdy collagen fibers are in close contact with the fibroblasts. They provide tensile strength to the skin. The elastic fibers provide elasticity to the skin and the reticular fiber meshwork provide support to the capillaries and the nerve fibers. Some adipocytes, four types of white blood cells (macrophage, lymphocyte, neutrophil, and plasma cell) and the mast cell (part of the immune system) are also found within the ground substance [12]. (Only the fibroblasts are in the scope of this project).

Dermis also houses the appendages (see Figure 1) which are structures penetrating the skin. The appendages comprise the eccrine glands (sweat glands) and the hair follicles. The latter are connected to the sebaceous and to the apocrine glands. The apocrine glands (not depicted in Figure 1) are found mainly in the axilla (armpits) and groin but also in beard and areola areas (around nipples) [13]. They are stimulated by hormonal changes during puberty to function throughout adult life. The appendages extend from their roots at the deep dermis (superficial hypodermis) through the basement membrane and epidermis (see 1.1.3).
1.1.3. The epidermis

The epidermis is anchored to the underlying connective tissue in dermis by the basement membrane (Figure 3, “lamina basale”). At the same time, the basement membrane supports the epidermis. It is a thin layer of specialized extracellular matrix, different from the extracellular matrix of the connective tissue in dermis. The major molecular constituents of the basement membrane are collagen IV, laminin-entactin complexes and proteoglycans. Together they form the membrane by self-assembly [14]. The basement membrane appears to be only 0.5-1 µm thick but electron microscopy images suggest a thickness that is no more than 35 nm at certain places of the body [15]. This is a thousand times narrower than suggested by results from optical microscopy.

The epidermis proper is devoid of blood and nerve supplies. Anatomically, it exhibits four regions (Figure 3). Formally, it is often sub-divided into two regions, namely the viable epidermis, containing the three layers of viable keratinocytes, and the non-viable cornified region of the stratum corneum. The total thickness of the epidermis varies from about 5-100 µm, depending on the location on the body, and it can even reach 600 µm on the palms and the soles. There are various cells present in epidermis, and they can be divided into two types, namely the keratinocytes and the non-keratinocytes. Amongst the non-keratinocytes are numerous dendritic cells, e.g. the Langerhans’ cells. Other non-keratinocytes are melanocytes and Merkel cells [15]. They are described further in 1.2. More than 95 % of the epidermal volume consists of keratinocytes. These cells, once generated from the stratum basale at the basement membrane, mostly produce keratin while continuously exploiting their start-up material until they finally end up as nonviable corneocytes.

The four regions of the epidermis (Figure 3) are divided according to the differentiation state of the keratinocytes [7]. Stratum basale is a single layer of columnar metabolically active cells known as basal keratinocytes. This layer of cells is referred to as the basal layer. It is attached to the basement membrane. The basal layer consists primarily of the basal keratinocytes that bear the potential to divide. Apart from the keratinocytes, also melanocytes and Merkel cells are found in the basal layer (see 1.2). As the basal keratinocytes divide, they produce more keratinocytes such that layers of different generations of keratinocytes are formed. The keratinocytes in these layers between the basal layer and the surface of the skin undergo a continuous transformation to finally end up as the dead cornified cells (corneocytes) in the stratum corneum.

---

4 proteins making up a network foundation for cells

5 proteins that are heavily glycosylated, i.e. connected to carbohydrates, commonly referred to as sugars

6 Dendritic cells are antigen-presenting cells.

7 A fifth region, named stratum lucidum, is in humans present only in palms and soles.
During their development, the keratinocytes while still adjacent to the basal layer do not only produce keratin but as well generate intercellular contacts by developing desmosomes (described below). Roughly, these are compact proteinaceous structures that grow across cell membranes into neighbouring cell membranes and thus connect the adjacent cells. With the desmosomes, a net-like protection for the basal layer is formed. These connections exhibit a spinous histological appearance, giving rise to the name stratum spinosum for the three-to-four cell thick layer above the basal layer [16]. Stratum spinosum consists of irregularly formed polyhedral keratinocytes with increasingly decaying capacity for cell division. The stratum spinosum also accommodates the Langerhans’ cells, i.e. the antigen-presenting cells of the epidermis.

Figure 3: The epidermal layers – The four layers are situated on top of the non-cellular lamina basale (in this text: basement membrane) and they are linked together by desmosomes. Irregular polyhedral keratinocytes are found in stratum spinosum and in stratum granulosum, they are flattened and produce keratohyalin granules. The terminally differentiated keratinocytes are called corneocytes and they make up the stratum corneum [17].

From layer to layer, the cell machinery is increasingly depleted from energy supply and material and hence the cells continuously degenerate, losing intracellular volume and as well elasticity.

Stratum granulosum contains flattened, polyhedral keratinocytes that are non-dividing as the cell nuclei are gradually lost. The keratinocytes of this layer are characterized by the
presence of keratohyalin granules, polyribosomes\textsuperscript{8}, large Golgi bodies and rough endoplasmic reticulum. The granules in this layer gives rise to the name \textit{stratum granulosum} \textsuperscript{16}. The keratohyalin granules continue increasing in size and number until they finally constitute most of the left-over cell volume. Finally, the terminal differentiation of keratinocytes\textsuperscript{9} results in the formation of corneocytes, i.e. dead cells that form the horny surface layer of the skin called \textit{stratum corneum}.

\textit{Stratum corneum} is the uppermost layer of the skin, constituting its outer surface. This layer exclusively consists of corneocytes, i.e. terminally differentiated keratinocytes. They have lost their nuclei and as well all capacity for metabolic activity, lacking cytoplasm and functional cytoplasmic organelles. Corneocytes are “cornified” dead keratinocytes containing abundant keratin. Each corneocyte is enclosed in a protein-rich cornified cell envelope. The envelope also provides covalent linkage sites for the so-called mortar described in part 1.1.4. \textsuperscript{16}

The \textit{stratum corneum} usually consists of 15-20 cell layers. It provides the essential barrier function of the skin. It prevents invasion by infectious agents, and it reduces trans-epidermal water loss (TEWL) from the dermis \textsuperscript{18}. When stripping off the \textit{stratum corneum} a breakdown of the barrier function results in a dramatic increase in the permeability to water and as well other compounds \textsuperscript{19, 20}.

The transformation from basal keratinocytes into corneocytes capable of desquamation is often referred to as the cell renewal factor (CRF) or cell turnover rate. Estimates of the CRF range from two weeks in infants to 40-56 days in middle-aged adults. The CRF also depends on the detailed anatomical site, and it is commonly quantified to 28 days as an averaged rule of thumb \textsuperscript{16, 21, 22}. The extent of CRF is tightly connected to material and energy supply from the blood vessels in the hypodermis and thus capillaries in the dermis.

Phenotypical changes of the keratinocyte morphology are evident in the different layers of the epidermis. The morphology changes from columnar shapes close to the basement membrane to stacks of flattened cells, with an increased amount of desmosomes \textsuperscript{23}, higher up. The protein filaments that connect the final corneocytes are called corneodesmosomes \textsuperscript{17}.

Throughout the epidermis, adjacent cells are linked by layer-specific desmosomes (\textbf{Figure 2}). Desmosomes are transmembrane proteins that establish bridges to connect adjacent keratinocytes. They as well serve as anchoring platforms for keratin filaments inside the cells. The basal keratinocytes are anchored to the basement membrane by

\textsuperscript{8} clusters of ribosomes, all of them utilizing the same mRNA to synthesize the same protein, in this case keratin

\textsuperscript{9} Not to be confused with apoptosis (programmed cell death), though similar molecular mechanisms are involved in both processes.

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hemi-desmosomes. These are only one half part of a desmosome [15] and they also differ from regular desmosomes in the composition of adhesion proteins [8, 24]. In *stratum corneum*, the desmosomes linking adjacent corneocytes are known as corneodesmosomes.

There is a continuous decrease of the pH value of the epidermis towards the skin surface (see Figure 4). It changes from normal physiological pH to pH ~4 [25]. This pH change passively triggers the chemical activity of human kallikrein-related serine peptidases (KLKs). The KLKs degrade the desmosomes at a pH below 5.3 [26]. The result is corneocytes with little or no connection to each other at the surface of the skin. This allows a desquamation process without causing wounds that would extend into the depth of skin.

**Figure 4: The desmosomes – As the pH decrease toward the skin surface, peptidases (KLKs) initiate the degradation of corneodesmosomes to allow for corneocyte desquamation [26].**

At normal physiological dermal pH, above pH 6 [25], this degradation process is hampered by the presence of an inhibitor called LEKTI\(^\text{10}\). This is illustrated in the bottom right-hand corner of Figure 4. It ensures that the keratinocyte cells are successfully linked by the desmosomes in the layers below *stratum corneum* [26].

\(^{10}\) LEKTI is an abbreviation of lympho-epithelial kazal-type related inhibitor.
The cell interstitium of the epidermis is constituted by the so-called epidermal mortar. It serves as another cohesion factor between adjacent cells and is described in the following.

1.1.4. The epidermal mortar

The programmed cell death of the keratinocytes during their transformation process in the epidermis culminates in the apoptotic secretion of a humectant that consists of intercellular lipid [8, 15, 27]. The lipid within this humectant amounts to no more than 7-10 wt% [18]. In an accepted model, the stratum corneum is morphologically mimicking a brick and mortar structure. This is illustrated in Figure 5. The corneocytes make up the “bricks”. The “mortar” is a multiple bilayer structure of lipid and an aqueous solution in between the bricks. From here on, the intercellular substance is referred to as mortar.

![Figure 5: Brick and mortar model – The corneocytes lie like bricks within the mortar. Two transport routes can be attributed to the structure: the mortar makes up the intercellular route while the transcellular route requires crossing of both mortar and bricks[16].](image)

The mortar accounts for approximately 15 % of the dry weight of stratum corneum or 20 % of its volume [8]. It consists of approximately equimolar concentrations of free fatty acids, cholesterol, cholesterol esters and ceramides. All of these molecules are amphipathic, i.e. they consist of a hydrophilic and a hydrophobic part. This gives rise to formation of a multiple bilayer structure in the mortar, as shown in Figure 5, with a thin aqueous film in between the bilayers of the multilayer. The multilayer mortar has a gel-like consistency. Within its aqueous film, it allows the passage of small water-soluble molecules like ruthenium red (a dye that stains the hydrophobic parts of mortar) [15], giving evidence to an intercellular route as shown in Figure 5. The routes of topical percutaneous absorption are described next.
1.1.5. The routes for percutaneous absorption

The surface of the skin is covered by a waxy layer of sebum mixed with sweat, bacteria and dead cells. The sebum is excreted from the sebaceous glands and it consists mostly of triglycerides and wax esters [28]. The sebum layer is thin (0.4-10 µm), irregular and discontinuous. Thus it cannot serve as an effective protectant against transepidermal absorption [29]. The possible effects of a chemical compound, when applied to the surface of skin, depend on its physico-chemical and bio-active properties as are weight, size, polarity, charge status, and chemical reactivity. Most small water-soluble non-electrolytes diffuse into the capillary system of the upper dermis a thousand times more rapidly when the stratum corneum is absent, damaged or diseased. In one of the following routes described, namely the poration route, the epidermis is temporarily damaged thus opening direct access to the dermis via the micropores.

There are four alternative routes through the stratum corneum and deeper into the skin that a chemical, after application to the skin surface, may take (schematically shown in Figure 6):

a) the intercellular route – The chemical diffuses around the corneocytes through the mortar.

b) the appendageal route – The chemical enters the shunts provided by the hair follicles, sweat glands and sebaceous glands.

c) the transcellular route – The chemical diffuses through the keratin-packed corneocytes and their protein-rich, cornified envelopes.

d) the poration route – The chemical enters the skin through pores thus bypassing the stratum corneum and even the entire epidermis, depending on the depth of the pore. Such pores may arise from accidental wounding, or they are intentionally created e.g. by micro needle poration.
There are four routes through for chemical agents to cross the epidermis and reach the dermis. a) The intercellular route, b) the appendageal route, c) the transcellular route and d) the poration route [30].

The physicochemical nature of a compound does not only dictate the route to be taken but also the final absorption in the tissue and the physiological effect of a chemical there. Particularly essential properties are solubility and size, since small molecules penetrate more easily and as well faster than large ones. This is due to the general nature of passive transport through **stratum corneum**. Once chemicals pass the horny layer they may be distributed and permeated rapidly within and through the living tissue of the viable epidermis and dermis [29].

### 1.1.5.1. The appendageal route

The appendages cover no more than 0.1-1 % of the total surface area of the skin. Therefore, they do not play an essential role in the absorption of most chemicals. However, the surface area of the shunts can be as high as 10 % of the surface in some areas of the body such as the scalp. The appendageal route might therefore be an option for treatments of the scalp. An optimum size for penetration through the follicles was found to be around five µm (the size of a typical bacterium). Larger objects (>10 µm) do not penetrate into the follicular orifices [31]. Thus, the contribution of the appendageal route of percutaneous absorption is mostly negligible [32]. The extent to which hair may extend into the deeper parts of skin differs. For example, vellus hair of the face extends 1 mm into dermis whereas terminal hair (the thick, long and dark hairs) goes as far as about 3 mm, all the way into hypodermis [16, 31]. The hair follicles facilitate the transport of lipophilic molecules since they are filled with sebum from the sebaceous glands. The sebum itself contains bacteriostatic and fungistatic components. Therefore it has an inhibiting effect on growth and reproduction of bacteria and fungi. It is rich in free fatty acids, squalene, wax esters, triglycerides, cholesterol, and cholesterol esters [28, 31].
1.1.5.2. The transepidermal routes

The transepidermal routes are displayed in Figure 6 and were shortly mentioned in 1.1.4. Whether a chemical is absorbed through the mortar (Figure 6, a) or through the protein-rich corneocytes (Figure 6, c), depends on the solubility, the partition coefficient, and the diffusivity of that chemical within these lipid or protein phases. The absorption may vary from site to site and as well depend on individual conditions as gender, age, and race. The absorption variability of this route is estimated to be 30-40% [32].

The diffusion coefficients of commonly used drugs across skin range from $10^{-6}$ cm$^2$/s to $10^{-13}$ cm$^2$/s. Lag times of chemical appearance across skin can range from a few minutes to several days [32]. During the past 20 years, chemical penetration enhancers and other ways to enhance percutaneous absorption have been widely investigated, in order to be able to control, trigger, and enhance permeation. The chemical enhancers are usually small molecules that increase the fluidity of the mortar. Examples of commonly investigated enhancers include amides (including urea), and amphiphiles like alkanols, bile salts, chelating agents, cyclodextrins, dimethylsulfoxide (DMSO), essential oils\textsuperscript{11} [33], ethanol, fatty acid derivatives, oleic acid and other surfactants. Many of these work better in the simultaneous presence of ethanol or propylene glycol in the formulation. Water itself is an effective penetration enhancer [32]. As an example, the stratum corneum swells several-fold when immersed in water, thus “widening” the aqueous films within the mortar [29]. The diffusive transport of all small water soluble molecules into the dermis is thus enhanced. Other ways of improving the absorption includes the use of prodrugs. These are compounds that are first absorbed as they are and then transformed to the desired final chemical agent by the enzymatic activities within the skin. Also nanocarriers, such as micelles and liposomes [34, 35] are being used. Nanocarriers consist of nanoscopic objects self-assembled from amphiphiles with an inner volume. The inner volume can comfort and deliver the molecules to be transported. Other ways of enhancing the absorption into the dermis is achieved by weakening or by-passing the barrier function of the epidermis by ablation methods, such as mechanical peeling, the use of laser light, or by microporation [31].

1.1.5.3. The route through microporated skin

Skin microporation creates impermanent microscopic holes in the skin that allow transport of small molecules and even macromolecules. Depending on the pore depth and its filling, the chemicals can pass directly into dermis and even hypodermis.

A micropore is schematically depicted in Figure 6, d. It may arise from several methods including chemical etching, laser ablation and mechanical damage, such as the poration of skin by microneedles.

\textsuperscript{11} e.g. eucalyptus, anise, chenopodium, ylang ylang, etc.
There are many types of microneedles varying in material, size, shape and depending on the purpose, e.g. dissolving, hollow, solid and coated microneedles. In this report, focus is on the solid microneedle device known as a dermalRollings, described in more detail in 2.2, Figure 16.

When applied, the poration depth is usually less than the microneedle length. This is attributed in part to the elasticity of the skin and in part to the deformation of the skin that may happen before the dermalRollings is applied.

The density of the needles on the dermalRollings is most essential: the needles will not penetrate the skin if the needle-to-needle spacing is too narrow. This can be explained by the “bed of nails”-effect that allows a person to actually lie on a bed of sharp nails without them penetrating the skin. Since the body weight is distributed over a large number of needles the resulting force per needle lies below the force needed to penetrate the skin.

The application of a dermalRollings with 500 µm long needles (model: CIT8) results in the formation of 16 micropores/cm² of skin with an average effective depth of around 150 µm and with the average diameter of the pores at the skin surface around 82 µm [31, 36]. The pores obtained extend well through the epidermis and may allow properly selected chemicals to come in direct contact with the dermis, to be absorbed there.

The TEWL increases [31] when porations are made. This initiates, within ~ 3-4 hours after poration of the skin, a barrier recovery cascade as a response to the damage. The time slot of at least 3 hours before restoration of the epidermal barrier properties can be used for the successful application of chemicals via the micro-pore transport route. The complete closure of the pores may take up to 15 hours. The healing process is artificially prolonged by occluding the pores by a non-breathable film or solution. An occlusion limits the TEWL, thus delaying the barrier recovery response [31]. This delays the pore closure to between 72 and 120 hours [31].

The microporation treatment is painless since there are no nerves in epidermis. The nerve endings are located around the capillaries in the upper part of dermis. Needles of length corresponding to the depth of the superficial dermis are thus expected to cause pain. However, the treatment with microneedles of as much as 430 µm in length was reported to be painless [31]. Most probably, the entire length of the needle did not penetrate the skin in that case. The depth of penetration is more important for the skin treatment and for the pain response of the patient than the length of the microneedles. In turn, the entry depth is affected by the needle-needle spacing.

1.2. The cell types in the skin

The dermaOXY method of skin treatment aims at the cosmetic improvement of skin appearance by a combination of methods that do not only target the external surface of skin but as well the internal volume of the skin layers, intending to rejuvenate the tissue
or at least aiming at prolonged lifetime by supporting healthy conditions within the dermis. The methods and serums applied seek to specifically address several appearances of skin aging. In order to understand these, more details about skin and its vital constituents are going to be shortly addressed in the following.

The skin layers and their cells are entered into Table 1, from skin surface to the inner layers of the skin. In layers with several cell types, the most abundant cell type is listed first. Especially, keratinocytes are found in all the sub-layers throughout epidermis though having slightly different appearances and functions from sub-layer to sub-layer. These differences are described in 1.1.3 and 1.2.2.

Table 1: The skin cell types – The cell types and functions are listed according to their location in the skin. When there are various cells in one layer the most abundant cell of that layer is listed first.

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<td>corneocytes</td>
<td>protection against pathogens and TEWL</td>
</tr>
<tr>
<td>stratum granulosum</td>
<td>granulated keratinocytes</td>
<td>cornification begins, i.e. production of keratin granules and lipid [17]</td>
</tr>
<tr>
<td>stratum spinosum</td>
<td>irregular, polyhedral keratinocytes</td>
<td>production of keratin and desmosomes (intracellular connections)</td>
</tr>
<tr>
<td></td>
<td>Langerhans’ cells</td>
<td>immune response</td>
</tr>
<tr>
<td>stratum basale</td>
<td>basal keratinocytes</td>
<td>cell division for skin formation</td>
</tr>
<tr>
<td></td>
<td>melanocytes</td>
<td>melanin production (sun blocker)</td>
</tr>
<tr>
<td></td>
<td>Merkel cells</td>
<td>sensation response (light touch)</td>
</tr>
<tr>
<td>basement membrane</td>
<td>no cells, only proteins</td>
<td>separation and linkage of epidermis to dermis</td>
</tr>
<tr>
<td>dermis(^{12})</td>
<td>fibroblasts</td>
<td>production of extracellular matrix, wound healing</td>
</tr>
<tr>
<td>hypodermis</td>
<td>adipocytes</td>
<td>energy storage, cushioning, insulation</td>
</tr>
</tbody>
</table>

\(^{12}\) Cells of the immune system are omitted.
The adipocytes of hypodermis are cells that are specialized in energy storage by use of fat. These cells are not addressed in further detail as the scope of this text is the dermaOXY treatment, which addresses the top layers of skin, namely epidermis and dermis.

1.2.1. The corneocyte

The corneocyte is the main constituent of the stratum corneum. Fully 85 % (dry mass) of the stratum corneum is protein, mostly associated with corneocytes. The corneocyte contains a core of keratin that is surrounded by an envelope (see below). The proteins are very stable to chemical treatment and proteases, and high temperatures are required for their denaturation (the transition temperature is around 95°C [37]). All in all, the corneocyte exhibits considerable resistance to chemical and physical denaturation [8].

The core of the corneocyte is composed of highly organized densely packed keratin molecules (main keratin types here are K1 and K10 [8]). Keratins account for about 80 % of the dry mass of a corneocyte. Native functional keratins are insoluble in water but may be transferred into water by the application of denaturants such as sodium dodecyl sulphate (harsh detergent) or urea (a chaotropic agent; that is a molecule capable of disturbing the molecular order of water and modifying the hydrophobic interaction that is stabilizing a protein structure) [8].

During the cornification\textsuperscript{13} process of the keratinocytes, certain proteins such as involucrin and loricrin are expressed. They are cross-linked by enzymes of the transglutaminase (TGase) family that are located in the cell membrane. The cross-linking of the proteins forms the insoluble structure named “cornified envelope”. Ultimately, a sealed protein envelope of 5-10 nm thickness is formed that later even replaces the initial cellular membrane [38]. This rigid, polygonal envelope weighs around 5-7 % of the total dry mass of a corneocyte [8, 39]: 90 % of this mass is protein, and the rest is lipid. The envelope is even more insoluble and resistant than the keratinous core of the corneocytes. It resists boiling, harsh detergents and chaotropes [8]. Three types of crosslinking account for the stability of the cornified envelope, namely disulphide bonds, $\varepsilon$-(γ-glutamal)lysine isopeptide bonds and bis(γ-glutamyl)polyamine. In normal tissue, the number of crosslinks per 1000 amino acid residues is about six. In the corneocytes, the number of crosslinks is at least twice as high, thus yielding a very stable protein structure [8].

1.2.2. The keratinocyte

Directly below the stratum corneum lies the viable epidermis, composed primarily \textasciitilde 95 % [7] of keratinizing epithelial cells in 10-20 layers. These cells are keratinocytes in various stages of differentiation towards becoming corneocytes. Keratinocytes in the viable epidermis consist of approximately 70 % water by volume, 15 % protein, 5 %

\textsuperscript{13} the process where keratinocytes become terminally differentiated into corneocytes
nucleic acid and 5 % lipid, whereas the corneocyte contains 15 % water, 70 % protein and 15 % lipid [8].

At the stratum basale, the stem cells (or basal keratinocytes) are located. They produce three different classes of keratinocytes [40]. The classes are called holoclone, paraclone and meroclone. The holoclone cells stay in stratum basale to divide as a basal keratinocyte. The paraclone cells, also referred to as transit-amplifying cells, have a short replicate lifespan. They divide no more than 15 times before they terminally differentiate. These cells produce compounds and proteins which are critical to the integrity of the stratum corneum [26]. The keratinocytes in the paraclone class are referred to as the “committed cells”. The committed cells detach from the basement membrane in a process that involves expression of the keratins K1 and K10 (the most abundant keratins in corneocytes). The meroclone class is a transitional stage between the two other classes. The amount of the different clonal types of keratinocytes in the skin are affected by age. Samples of skin from aged individuals hold a higher proportion of the paraclone cell types as opposed to samples of skin from younger individuals. They have a dominant amount of holoclone cells [40]. Thus, skin from young individuals have a larger potential to renew itself compared to skin from older individuals.

The keratinocytes present in the layers above the stratum basale increasingly lose their columnar characteristic and get polygonal shapes (see Figure 3). They begin to flatten as they undergo terminal differentiation, and the diameter changes from 10 µm to 30 µm while their volume decreases.

In the stratum granulosum, dense keratohyalin granules (see Figure 3) are formed in the keratinocytes. These granules are intermediates during the formation of corneocytes and they are composed of profilaggrin, loricrin, and cysteine-rich protein [8, 41-43]. Within the stratum corneum, the granules are joined and occupy the entire volume of the corneocyte. Keratinocytes as well synthesize precursor lipids to the fatty acids found finally in the stratum corneum. The precursor lipids are released into the extracellular space and then degraded there.

1.2.3. Other cell types in the skin

There are three cell types of non-keratinocytes in the epidermis. These are the melanocytes, the Langerhans’ cells (antigen-presenting cell), and the Merkel cells. Another essential cell type found in skin is the fibroblasts that appear in the dermis.

The melanocytes are located at the stratum basale (see Figure 3 and Figure 7). They are unique cells in that they express pigment (melanin). The melanin is then distributed within the skin, including a dendritic targeting that results in providing radiation protective melanin caps to the nuclei of functionally active keratinocytes. With its dendritic extensions, one melanocyte is estimated to have contact to 40 keratinocytes in the stratum spinosum. There are no desmosomal attachments among the melanocytes or between the melanocytes and the neighbouring keratinocytes. However, the melanocytes are linked to the basement membrane with structures similar to hemi-desmosomes.
Through their dendrites, melanocytes transfer melanosomes, i.e. granules consisting of condensed melanin, to keratinocytes. There, the granules transform into melanin caps shielding the nucleus of the keratinocytes. They serve to absorb UV-light and thus reduce UV-induced DNA damage (see 1.4.2) in human epidermis [24]. In addition, melanin facilitates the role of camouflage, heat regulation, and individual variation of facial appearance. Moreover, it directly serves as a scavenger that interacts with reactive oxygen species (ROS, described further in 1.4.2) [7].

Figure 7: Cells of the skin – Melanocytes are found in stratum basale, where they excrete melanin to shield the nucleus of the surrounding keratinocytes. The stratum spinosum contains Langerhans’ cells [44].

The Langerhans’ cells are antigen-presenting cells in the epidermis. They originate from cells in the bone marrow and distributed via the blood stream into the epidermis. There they differentiate into immune-competent cells, staying located in the stratum spinosum (Figure 7). Like melanocytes, Langerhans’ cells do not form desmosomes with neighbouring keratinocytes. When they encounter and process antigens, a migration to a regional lymph node is induced [24].

The Merkel cells are located in the stratum basale. They are part of the sensory organ (see Figure 1: “sensory receptors”) and most abundant in skin regions where sensory perception is most required, as in the fingertips. They are bound to adjacent keratinocytes by desmosomes, and they are connected to a neuron terminal that penetrates the basement membrane [24]. The nerve signals pass via these neuronal terminals across dermis and into nerve fibers that are located in hypodermis.
The fibroblasts are located in the connective tissue of dermis in close contact with the extracellular matrix. With their biochemical apparatus, they give rise to the formation of the fibers in the connective tissue and as well to the formation of the ground substance of the extracellular matrix. The ground substance consists of an aqueous solution of proteoglycans, glycoproteins, and glycosaminoglycans (especially hyaluronic acid) [7]. The ground substance interacts with the fibers produced by the fibroblasts. In addition, it regulates the water-binding capabilities of dermis [24]. Fibroblasts also not only maintain but as well regulate the connective tissue by excreting enzymes\(^\text{14}\) that modulate the degradation of it. With their production of the fibers of the connective tissue, they play an important role in wound healing (described in \textbf{1.3}). The number of fibroblasts is higher in the papillary dermis\(^\text{15}\) than in the deeper dermis. During the lifetime of a person, the size and productivity rate of newly formed fibroblasts in general become smaller. An exception is their hypertrophy\(^\text{16}\) as a response to photodamage. This is related to the biological attempt to compensate damage on the fibers in the connective tissue by enhancing their production in the fibroblasts [24].

\textbf{1.3. Fibers and polymers of the skin}

The connective tissue exists throughout dermis. Connective tissue in general contains fibers in varying amounts, depending on the structural needs i.e. support, connection or separation. There are three types of connective tissue fibers in dermis, all produced by the fibroblasts and composed of long peptide chains. The three types of connective tissue fibers are collagen fibers, reticular fibers, and elastic fibers. They are schematically illustrated in \textbf{Figure 2, 1.1.2}. The fibroblasts also produce hyaluronan. These four constituents of the connective tissue of dermis are described in the following subchapters.

\textbf{1.3.1. Collagen fibers}

Collagen fibers are the most abundant structural component of the connective tissue. They provide high tensile strength to the tissue and they are flexible. Collagen fibers are hierarchically organized tropocollagen assemblies in that they contain bundles of fibrils (\textbf{Figure 8}) made from bundles of microfibrils that finally consist of tropocollagen. The tropocollagen itself is a right-handed triple helix of three collagen polymers in $\alpha$-helical chain conformation.

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\(^{14}\) for example, collagenase and elastase

\(^{15}\) Papillary dermis is the region in \textbf{Figure 1} where the sensory receptors are located.

\(^{16}\) Hypertrophied cells undergo an abnormal non-tumorous growth.
Figure 8: The collagen fiber – The collagen fibers are made up of bundles of fibrils. These are made up of bundles of microfibrils, which consist of bundles of tropocollagen. The tropocollagen itself is a trimer of peptide chain $\alpha$-helices that are intertwined [45].

The collagen $\alpha$-chains vary in size from 600 to 3000 amino acids. Until now, at least 42 different types were identified [26]. From these various collagens, type I is the most abundant collagen type in the skin and makes up more than 90 % of the collagen in the body. Type III collagen provides a supporting scaffold for the cells of blood vessels and various organs. Type IV is the major compound; it serves as a scaffold of the basement membrane. Type VII collagen forms the anchoring fibrils that attach the basement membrane to the extracellular matrix of connective tissue [26].

1.3.2. Reticular fibers

The reticular fibers are composed of type III collagen. They consist of many collagen fibrils, similar to the tropocollagen microfibrils. This gives rise to thinner fibers as compared to those of the thick collagen fibers. The reticular fibers do not form bundles but rather instead form a fine meshwork, illustrated in Figure 2. The enhanced formation of reticular fibers is typical in the initial stages of wound healing and in scar tissue formation. The establishments of reticular fiber networks provides support structures. The tensile strength of healthy skin is established by the collagen fibers. Over time and as the wound healing progresses, the stronger collagen fibers gradually replace some of the reticular fibers [26]. In any case these fibers are produced by the fibroblasts. The fibroblasts are as well responsible for the regulation and for the production of new connective tissue and for the degradation of damaged tissue. Their work is crucial in wound healing, and the integrity of fibroblasts is a signature of healthy skin.
1.3.3. Elastic fibers

The elastic fibers are synthesized in the fibroblasts and then excreted into the connective tissue. They are composed of two structural components: a central core of elastin surrounded by a network of fibrillin microfibrils, illustrated in Figure 9.

![Figure 9: An elastic fiber - the elastin core is surrounded by a network of microfibrils before the elastic fiber is excreted from the fibroblast [46].](image)

The elastin molecule (72 kDa) is a randomly coiled hydrophobic protein. Desmosine units serve to link adjacent elastin molecules (Figure 10) within the fiber core. This way an elastic three-dimensional network is obtained with the ability to recoil to a relaxed state after stretching (see Figure 10, a and b) [26]. The fibrillin shell serves to provide a hydrophilic surface, enabling the immersion of the elastic fiber in the aqueous surroundings of the connective tissue.
The elastic fibers are typically thinner than the collagen fibers. The two types of fibers, elastic fibers and collagen fibers, jointly limit the distencibility (capability to stretch) of the tissue and as well give strength to it.

1.3.4. Hyaluronan

Hyaluronan (hyaluronic acid) was discovered in 1934 by Karl Meyer and John Palmer. They identified the egg white-like substance contains two sugar molecules, one of which is a uronic acid. They proposed the name hyaluronic acid as a combination of hyalos, which in Greek means glass, and uronic acid. Later, in 1986, it was given the term “hyaluronan” as it can be both, an acid and a salt, depending on the physico-chemical surroundings (e.g. solution conditions). It usually is a poly-electrolyte since its carboxyl groups are fully ionized at extracellular pH (pH_{extra} ~7.0) [49, 50].

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17 Uronic acids are a class of sugar acids.

18 The salt sodium hyaluronate forms at physiological pH.

19 The extracellular pH is approximately 7.0 at 37 degrees Celsius. The intracellular pH is lower (~6) due to acid production.
Hyaluronan is a very hydrophilic molecule, especially abundant in loose connective tissue, such as the ground substance of the dermis. Chemically, it is a linear polysaccharide based on a disaccharide group referred to as glycosaminoglycans. Hyaluronan is found in all connective tissues, and it is a functional component in many body fluids of vertebrates (e.g. as lubricant agent in joints).

Among the glycosaminoglycans, hyaluronan represents a peculiar subgroup. It is naturally synthesized by a class of integral membrane proteins\(^{20}\) of the fibroblasts (the so-called hyaluronan synthases [51]). This is different from all other glycosaminoglycans that are synthesized in the Golgi network of cells.

Chemically, hyaluronan is composed of disaccharide repeats of glucuronic acid (the uronic acid) and N-acetylglucosamine [47]. They are linked together through β-1,4 and β-1,3 glycosidic bonds. Under physiological conditions the axial hydrogens form non-polar, relatively hydrophobic patches, while the equatorial groups form more polar, hydrophilic patches. This alternating polarity feature induces the form of an expanded, random coil in solution [47] capable of swelling in water. The coiled structure of hyaluronan can trap approximately 1000 times its weight in water [51].

Hyaluronan molecules may bear high molecular weight\(^{21}\), they can easily reach 10000 or more disaccharide repeats. Each disaccharide weight amounts to ~400 Daltons, thus the molecular mass of hyaluronan can exceed 4 million Daltons [51], to be compared with the low mass of a glycosaminoglycan monomer of ~ 15-20 kDa. The length of one disaccharide repeat is approximately 1 nm [51]. Thus, stretching a hyaluronan molecule coil of 10000 repeats, results in a length of ~10 μm, comparable to the diameter of a keratinocyte at the basal layer. In coiled solution conformation, the diameter of such a loosely packed hydrated hyaluronan polymer may assume several hundreds of nanometres.

Properties of hyaluronan in the hydrated state are remarkable. Small hydrophilic molecules can freely move within a volume containing hydrated hyaluronan, but macromolecules cannot. Hyaluronan in solution is a viscoelastic material in that its compressibility depends on the presence of a load. The free viscosity of such a solution is 500 000 times that of water but it drops by a factor of 1000 when a flow is applied, e.g. when forced through a thin needle [49]. Hyaluronan is conjectured to play an essential role in relation to physiological lubrication [47], water homeostasis\(^{22}\) [53] and filtering [47].

\(^{20}\) An integral membrane protein is a protein molecule that spans the plasma membrane.

\(^{21}\) The molecular weight of the hyaluronic acid or its salt on average is between 150 000 and 750 000 Daltons 52.  *Generic Solaraze availability.* 1998; Available from: http://www.drugs.com/availability/generic-solaraze.html.

\(^{22}\) maintaining the water equilibrium in the tissue
The normal average concentration of hyaluronan in the human dermis is around 0.2-0.5 mg/g (wet tissue). Overall, in the epidermis, it is around 0.1 mg/g (wet tissue). There is almost no aqueous space in epidermis since the keratinocytes take up most of the space (~95 %, see section 1.2.2). Therefore the actual concentration of hyaluronan in the aqueous volume of the epidermis is much bigger than its overall average suggests, namely 2-4 mg/L of the extracellular substance, and in fact exceeds the estimated concentration of hyaluronan in the big aqueous volume of the dermis, which is up to 0.5 mg/L [10]. Experiments with chemical disruption of the stratum corneum23 gave evidence of an accumulation of hyaluronan in the epidermis as a response to the lesion and as part of the wound healing response. The Hyaluronan concentration was found to increase in the matrix between the basal and the spinous keratinocytes [54]. Accordingly, hyaluronan is a significant participant in the epidermal response to barrier injury. Such injuries are provided by the action of the dermalRollings, and they as well occur in diseases as for example eczema and psoriasis [54].

Hyaluronan24 was shown to assist delivery of drugs to the epidermis in topical treatment of actinic keratosisis25 [10]. The experiment was made with diclofenac in a hyaluronan solution in comparison to the compound being applied to the skin in pure water at equal concentrations. Twice as much diclofenac was located in the epidermis after 24 hours when applied from the hyaluronan solution as compared to the water solution control. A similar effect was found with ibuprofen in a hyaluronan solution. Apparently, hyaluronan promotes the drug penetration across the outer skin barrier with the subsequent formation of a drug reservoir in the epidermis.

Hyaluronan as part of cosmetic treatment may either involve its application by injection of a solution of hyaluronan directly into the dermis or within a serum that is then applied to the skin surface. Aqueous solutions of hyaluronan are prominent choices as dermal fillers. The injection of such a solution into the dermis is inert but it will expand the dermis volume thus smoothing the skin surface and possibly reducing facial lines and wrinkles in the long term [55]. The half-life26 of hyaluronan in tissue ranges from less than one to several days. When introduced into the blood circulation, hyaluronan is removed in liver with a half-life of 2-5 minutes [49]. Application of hyaluronan to the skin within cosmetic products are claimed to moisturize the skin and to restore elasticity of the skin surface, thus achieving an anti-wrinkle effect. However, scientific proof to support this claim seems to be missing during the time of this study. The two sera products in the scope of this project comprise

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23 by acetone application on the skin of hairless mice

24 Hyaluronan is an agent in the gel Solaraze®. The gel contains 3 % diclofenac in 2.5 % hyaluronic acid gel (all wt/wt).

25 the third most common skin complaint in the US, a relatively common skin lesion that results from excessive sun exposure

26 the time it takes for the concentration to drop to half of its initial value
Human skin

two different types of hyaluronan. These, and their possible beneficial effects, are described in 2.1.

1.4. Skin deficiencies

Skin is the most important barrier protecting the human organism against harmful influences from the outside, as for example pathogens or from drying out. Therefore, it is crucial to maintain it healthy, undamaged and functional.

With the melanocytes, skin has its own protective tool against exposure to sun light. Still, their protective capacity against photodamage is limited. Therefore, additional protection of the skin against photodamage is of great concern. Moreover, independent on whether cosmetic or health aspects are in focus, the general health condition of a human widely depends on the status of the skin. It is therefore important to ensure and maintain all vital functions of the skin for as long as possible. The outer appearance of skin aging have been known for centuries, and non-cosmetic inherent effects accompanying skin aging were conjectured ever since. But only during the last fifty years researchers began to systematically explore skin aging in order to reduce or even inhibit its consequences [56].

1.4.1. Aging

Aging is a natural process best defined as increased reduction or even failure of body functions due to non-optimal synchronization of processes, drawback in signal transfer and processing, and reduced transport and process efficiency with a deficiency of supply in nutrition to all tissues. All this results in decreased functionality and reserve capacity in all body organs with an increased likelihood of disease and death. Sometimes aging is regarded as a cancer prevention mechanism by inhibiting unregulated growth of cells, the DNA of which was damaged throughout lifetime [57]. For skin, aging has external appearances like the continued development of wrinkles or a change of complexion that is often considered disadvantageous. To some extent, the perception of these appearances is connected to cultural peculiarities. Still, the external appearances result from internal changes, and they may be signatures of more severe processes that hint to increasing failure of the skin functions, even with the potential of harmful effects that may finally result in death. For example, the breakdown of the barrier function of skin increases the infection probability from bacteria that are regular phenomena in the daily surroundings. Another example is the loss of protection against mechanical damage when the epidermis reproduction fails. Then the healing of small accidental wounds is delayed or even suppressed, and the risk of inflammation up to sepsis 27 is increased. Diabetes patients e.g. especially suffer skin deficiency related wounds of their limbs such that a per se non-dangerous inflammation may propagate to the extent that amputations are needed in order to rescue the individual.

27 illness caused by an infection in a part of the body
Aging of the skin is a complex process depending on lifetime but as well on environmental and cultural factors as well as biological conditions. The latter, if genetic, cannot be modified. However, healthy general nutrition and as well the application of nutrition components aiming at direct tissue uptake by use of skin care products are reasonable choices to aid skin health. Among the cultural factors, the impact of nutrition to the status and well-doing of the whole body is obviously of great importance. In particular, a diet rich in vitamins, especially the vitamins A (retinol), C (ascorbic acid), E (tocopherol), H (biotin), B3 (nicotinic acid) and including polyunsaturated fat, preferentially linoleic acid, is associated with general health but as well especially beneficial in skin care. A healthy diet is as well presumed to slow down skin aging. On the contrary, the consumption of saturated fat and a diet rich in carbohydrates (sugar, alcohol) is associated with destabilizing healthy skin and with an acceleration of skin aging [58].

The unavoidable natural aging occurs in time, even under optimal outer conditions. Phenomenological, aged skin appears thin, dry, pale and translucent, with fine wrinkles and some degree of atony. Histological studies reveal that epidermis is thinner in aged skin as compared to young skin. This is caused by the progressive decrease of the cell division rate in stratum basale. The translucent appearance is a direct consequence of this, with the blood vessels further down in the dermis and even in the hypodermis becoming visible. At the same time, a progressive decrease in melanocyte and Langerhans’ cell density occurs. As a consequence, the natural protection against photodamage is reduced, and the immune defence is hampered. Moreover, there is a loss of extracellular matrix (Figure 11) in the dermis since also the number of fibroblast and as well, their production efficiency decreases. In general, all biochemical processes slows down during aging.

The histological cuts depicted in Figure 11 with schematics illustrating the interpretation of a reduced amount of collagen and of elastin in aged skin as compared to the fiber status within samples of younger skin. The skin samples were selected from places on the body with low probability of sun exposure (i.e. “photoprotected”)29, so that the differences in the connective tissue between the two samples can be attributed to the age difference.

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28 from ancient greek "atonia": slackness

29 protected against UV-radiation
The loss of volume and of elasticity cause the phenomena of flabbiness and the appearance of tiny wrinkles [59].

Among the essential roles of the fibroblasts is the synthesis of enzymes. Examples are collagenase and elastase that both play essential roles for the controlled break down of connective tissue as part of its renewal cycle (see 1.2.3). These enzymes are subsumed under the generic term Matrix Metalloproteinases (MMPs). The MMPs control tissue degradation in general, and also in the dermis. The function of the enzymes is to break down damaged connective tissue such that new and healthy tissue can be build up. This is a vital mechanism in wound healing and tissue repair.

The excretion of MMPs is triggered within the fibroblasts. Undesired triggering may occur after UV-radiation or due to other inflammatory responses. However, the tissue repair is not perfect since it also causes cross-linking of collagen fibers. The external manifestation of this modified collagen matrix is the appearance of age induced wrinkles. The presence of cross-linked collagen in the dermis as well modifies the elastic properties of the skin as a whole. This is sensed (and measured) as a stiffening of the skin.

The biochemical activity of fibroblasts and melanocytes decreases as the body ages. This means the capability for the regeneration and protection of the skin decreases as well. Therefore, it is of high importance to protect the skin against UV-radiation at all ages. Especially skin of older individuals will suffer from this. The skin damage induced by UV-radiation is called photodamage.
1.4.2. Photodamage

Photodamage is due to sun exposure, and it is superimposed on biological aging, possibly resulting in a permanent damage. Photodamage is caused by exposure to high-energy radiation (i.e. UV radiation) that may permanently modify or even destroy molecules and the structures resulting from their aggregation.

UV-radiation is the part of the electromagnetic spectrum. It lies between the x-ray and the visible regions. The composition of the solar radiation is modified by the gases in the atmosphere to yield the solar spectrum at sea level (shown in red in Figure 12). The impact of the different contributions can be harmful or beneficial, depending on the degree of absorption and on the energy of the light.

![Solar Radiation Spectrum](image)

Figure 12: The solar radiation spectrum – The spectrum of radiation outside the atmosphere (yellow) almost follows the theoretical course of black body radiation (the black line). At sea level, the spectrum (represented in red) is modified due to gas absorption in the atmosphere. The UV range can be disintegrated into three contributions (see text) of varying harmfulness [60].

Especially light in the UV-range is known to be damaging, and it does contribute to the phenomena of skin aging [7]. UV radiation is classified into three groups: UV-A (320-
Human skin

400 nm), UV-B (280-320 nm) and UV-C (200-280 nm) (see Figure 12) due to the different effects it may have when incident on matter.

The ozone layer of the atmosphere blocks almost all of the UV-C radiation (see Figure 12) while UV-B and UV-A light reaches the sea level and thus can affect the skin. Most types of glass block UV-B but are transparent for UV-A light. When incident on skin, UV-A light penetrates deep into the dermis. There it may be absorbed and can cause DNA breakage. Consequently, the otherwise healthy and delicately regulated cell biochemistry may break down. Several effects may arise. Among them are a decrease of the production of essential molecules, e.g. the fibers of the connective tissue, and the up-regulation of MMP production followed by an unintended degradation and cross-linking of existing fibers, and the development of undesired cell proliferation, e.g. skin cancer. Moreover, the energy of UV-A light is sufficient to break bonds in the skin fibers, giving rise to loss of skin elasticity and the development of deep furrows. The histological cuts depicted in Figure 11 together with schematics that suggest their interpretation evidence the breakdown of collagen fibers and elastic fibers after 75 years of occasional sun exposure as compared to the findings in young skin, 23 years old.

Based on the microscopy images shown in Figure 11 and Figure 13, the effect of photodamage to skin is even more severe than the lifetime related aging of the skin.
Photodamaged skin exhibits irregular pigmentation and as well deep furrows and fine wrinkles. Melanin pigments are produced as filters in the epidermal cells to naturally protect against photodamage. The occasional development of sunspots upon radiation exposure is due to a large but unevenly spread melanin accumulation in the epidermis. Another natural response to high irradiation is a thickening of the stratum corneum such that the skin gets a thicker dead-cell shield to block the harmful radiation. A clearly harmful effect of photodamage is the decrease in amount of Langerhans’ cells in *stratum spinosum*. This makes the skin less effective in protecting the body against pathogens. Photodamage also results in dermal elastosis. This is the deposition of abnormal amorphous elastic material in the papillary dermis. On a molecular scale, photodamage can induce telomere shortening with the consequence of unsuccessful DNA duplication. This in turn leads to a less effective cell or even a useless one. The photodamage of DNA contributes to the aging phenotype of wrinkles and freckles [56]. An increased probability for the development of skin cancer is another consequence, and a very serious one.

Another known effect of UV-A light is the production of reactive oxygen species (ROS)\(^{31}\), e.g. superoxide \(\cdot \text{O}_2^-\). ROS are harmful. They aggressively initiate chemical reactions that do negatively interfere with many molecular species in the body. An increased level of \(\cdot \text{O}_2^-\) can for example inactivate enzymes when the superoxide gets protonated (\(\text{HO}_2^-\)). In the worst case, this can even initiate the apoptotic cascade, causing death of the entire cell. In young skin, the risk of ROS induced photodamage is smaller since the melanocyte activity is higher. They excrete and distribute melanin to protect the keratinocyte nuclei in the epidermis (see Figure 7). The presence of the pigment melanin does not only contribute to skin complexion. Melanin caps in the keratinocytes also act as a natural “sun blocker” by absorbing and scattering UV-radiation. Two types of melanin are present in the skin [61]: The most common is eumelanin that gives rise to brown or black patina (absorption spectrum see Figure 14). The other one is pheomelanin, which is responsible for red tones seen in hair and in freckles.

\(^{31}\) ROS is a term comprising aggressively reactive small molecules like \(\text{O}_2^-\), \(-\text{OH}, \text{H}_2\text{O}_2\), and \(\text{HO}_2^-\)
Figure 14: Absorption spectrum of eumelanin – The absorption of UV and of visible light by two solutions of eumelanin. The two eumelanin types in the solutions are of different average molecular weight. Eumelanin absorbs most of the ultraviolet radiation (<400nm), and the absorption decreases in the visible range (>400 nm) [61].

The dotted line in Figure 14 results from a measurement of an aqueous eumelanin solution with higher average molecular weight of the protein, compared to the full line, which is from a solution of lower molecular weight eumelanin. However, the two absorption curves mostly coincide in the range of UV light and evidence the protective character of eumelanin. Not only does melanin itself absorb a lot of harmful light, it as well can interact directly with ROS and thus work as a scavenger that protects the skin from their harmful effects [7].

Insufficient shielding from UV-B radiation gives rise to the phenomenon of sunburns, an almost immediate effect of overexposure of damaging radiation on the skin. The only known beneficial effect of this radiation is the stimulation of vitamin D synthesis in the epidermis [7]. UV-C radiation is extremely harmful. However, it is of no concern near the sea level, as earth’s atmosphere blocks approximately all of it (see Figure 12).

1.4.3. Selected diseases

There are numerous diseases of the skin. While treatments develop to cure some, even more diseases may arise. One peculiar example is a skin disease discovered in 2009. It appeared in the palms of a 12-year-old girl who had played a computer game (The Sims) for several hours per day during several weeks. As an effect it is called PlayStation palmar hidradenitis [62]. Phenomenological, the disease causes erythema and painful swelling of the palms from neutrophil infiltration of the eccrine sweat glands. Usually, such an appearance is a typical response to infective diseases. In this case, however, it occurred in the absence of any infectious agents and only due to extreme mechanical irritation.

32 abnormal redness of the skin due to capillary congestion
Among the more abundant and representative diseases of the skin only two, namely acne and psoriasis, are going to be explored in more detail in the following since individuals suffering from these are not excluded from the dermaOXY cosmetic treatment.

### 1.4.3.1. Acne

The pathogenesis of acne has not yet been clarified but hypercornification\(^{33}\) of the hair follicle and increased sebum secretion are among the widely accepted phenotypical appearances. The interplay of these two appearances causes the formation of a clog of bacteria (\textit{propionibacterium acnes} (\textit{P acnes})). In the follicle the bacteria start to colonize. They consume triglycerides and release cytokines which in turn induce inflammation in the follicle. As secondary effects, the follicle swells thus forming pimples and the inflammation causes erythema. Finally, an uneven and reddish skin surface is developed. The initial inflammation can easily spread to neighbouring follicles, especially in the absence of antibiotic agents at the skin surface. When volumes filled with bacteria colonies are opened, their contents are released and the infection can spread to nearby regions. Exposure to sunlight has an antibacterial effect. It decreases the activity of the sebaceous glands, thus limits the amount of triglycerides. Extra sun exposure with the aim to treat acne is still not recommended due to the unwanted and harmful effects the radiation induces\(^{[63]}\).

### 1.4.3.2. Psoriasis

Psoriasis is an inflammatory skin disease. It affects about 1-3 % of the population with a typical onset between the ages of 15 and 40 years\(^{[64]}\). The condition has various phenotypes, including psoriasis vulgaris, guttate psoriasis, pustular psoriasis, flexural psoriasis, and erythroderma. The most common type is psoriasis vulgaris also known as plaque psoriasis\(^{[65]}\). This type commonly involves the skin around the elbows, knees and on the scalp. It also affects the scalp and trunk of the body. Flexural psoriasis affects the groin, anal folds and other flexural areas. Guttate psoriasis tend to develop at a young age, often between 15 and 40 years\(^{[64]}\). It is associated with infection, usually streptococcal. The most severe type of psoriasis is the erythroderma. It covers large areas of the body and is associated with fever and loss of nutrients. In extreme cases erythroderma can be life threatening\(^{[65]}\). Psoriasis in general can also induce nail lesions.

Psoriasis is considered a genetic disease that can be triggered by environmental factors. The precise cause of psoriasis is still unknown\(^{[64]}\). The psoriatic skin has two main abnormalities. One of them involves the keratinocytes, while the other involves the immune system. Both of these can induce the other. Keratinocytes produce cytokines\(^{34}\),

---

\(^{33}\) overproduction of epithelial cells lining the follicles

\(^{34}\) Cytokines are small proteins (~5-20 kDa) that are synthesized by various cells such as immune cells, endothelial cells, fibroblasts and keratinocytes.
Human skin

i.e. small proteins important in cell signalling. This production in psoriatic skin leads to keratinocyte hyperproliferation\textsuperscript{35}. It is histologically evident as scales on the skin [64, 65]. The second abnormality, compared to healthy skin, is the increasing amount of immune system cells, such as neutrophils and type T lymphocytes [64]. This inflammatory response may lead to erythema. The psoriatic skin can be associated with itch and even fissures in the skin.

Glycerine has a positive effect on the signalling pathways of proliferating keratinocytes. This makes it useful in aiding psoriatic skin [66]. Also the water soluble vitamin complex of vitamins A, E, and F, present in both dermaOXY sera, are effective in the treatment of dry and irritated skin, such as psoriatic skin [67].

\textsuperscript{35} an abnormally high production of cells
2. The dermaOXY treatment

The dermaOXY method of skin care is the central scope of this project. The treatment of a customer’s skin involves a set of applications, each with the following sequential steps [68].

1. preparation of the skin for the treatment
   The skin is cleansed in order to remove makeup-leftovers and other chemicals, bacteria and loose skin residues.

2. punctuation of the skin by use of the dermalRollings
   The skin is systematically microporated by use of a dermalRollings with 200 µm long needles (see 2.2 for further application details).

3. application of the serum with an oxygen gas stream
   The microporated skin is uniformly covered with a special serum by an airbrush (airbrush, see Figure 15; serum details, see 2.1; instrument details, see 2.3).

4. skin massage by use of an oxygen gas stream
   The serum is massaged into the skin with a 90 % oxygen gas stream at high pressure.

The set consists of six weekly applications that is followed by a break of 3-4 weeks for recovery and then a continuation with roughly one application per month [68].

Figure 15: Serum application – The serum is applied directly onto the skin by the oxygen stream from the specialized tool. The photo was taken at a private demonstration provided by dermaOXY.

For the dermaOXY treatment, two different sera are available: the “SYN” serum, and the “HYALURON” serum. In the first application, the SYN serum is used. In the second
application, the HYALURON serum is used. Then the SYN serum is used again and so on and so forth [68]. In between the applications, an additional home treatment is advised for every-day care, consisting of a combination of the NIGHT, DAY, and EYE crèmes from dermaOXY [68] (these products are not described further in this project).

2.1. The sera

The two sera of the dermaOXY treatment within the scope of this project are the SYN serum and the HYALURON serum. The SYN serum is designed for an instant reduction in wrinkle appearance. It also provides a high nutritional content to the skin [1]. The composition of the HYALURON serum focus on moisture and, as well, nutritional supply. Among its ingredients are essential building blocks for skin recovery and renewal. A full list of ingredients is entered into Appendix A. Selected aspects are addressed below.

Both sera are based on water as a solvent. They contain water-soluble ingredients but as well, compounds that do not or almost not dissolve in water. Therefore, emulsifiers and stabilizers serve to keep all ingredients uniformly distributed within their sera. Examples are the polysorbates, PEG-glyceryl laurate, cethearate, (PEG-20), carbomer.

It is of major concern to prevent the drying out of the skin. The water vapour in the atmosphere never reaches its equilibrium partial pressure. Therefore, water keeps evaporating from all aqueous volumes that are in contact with the atmosphere. Nature has partially met that challenge by providing a thin protective film on the skin surface that prevents, to some extent, the evaporation of water from the thin aqueous interbilayer film in between the corneocytes, and hence limits the TEWL. One role of the sera applied to the skin is the restitution of the natural film, and its stabilisation. Once the serum is applied to the skin, the water in it as well starts to evaporate. This is most undesirable. Instead, the effect aimed for is that the water from the serum transfers to the epidermis. Therefore, each serum contains ingredients chosen with the aim of moisturizing the skin. This is achieved by several moisturizing additives (see Appendix A) and especially by the presence of hyaluronan in the serum. The evaporation from the serum shall be delayed such that water contained in it can even swell the intercellular space within the epidermis and possibly expand the related route to enhance transport through it. The intercellular route contains both lipid bilayers and as well thin water films in between them. Therefore it may serve for the transport of small hydrophilic molecules as e.g. vitamin A and vitamin E, and also for the permeation of hydrophobic compounds as for example squalene, vitamin C and vitamin F (linoleic acid), all of which are ingredients of the sera (see Appendix A).

An example of a moisturizing ingredient, abundant in both sera, is glycerine. Glycerine is a natural three-fold alcohol. It is known to be hygroscopic, meaning that it attracts water [69], for example from a vapour. Cetearyl alcohol is also abundant in the sera. It is a mixture of fatty alcohols. They too bind water, thus increasing the skin hydration. Cetearyl alcohol is known to have an emollient effect on the skin [70]; this is sensed as
a smoothening and softening of the skin. In general, the alcohols in the sera prevent evaporation of the water and thus stabilizes the natural water content within the skin.

Both sera aim at providing the skin with a rich nutritional supplement, meant to support or even restore the natural biochemical processes within the viable epidermis and the dermis. Therefore, both sera contain vitamins, fatty acids, and selected peptides and nutritional cocktails obtained as plant extracts, for example hydrolysed soy protein that is a mixture of peptides, amino acids and mineral salts. Provided these nutritional ingredients from the sera reach the target tissue, viable epidermis or dermis, their supply will enhance a healthy cell metabolism [71].

The ingredients in both sera serve dedicated purposes. Among them are antioxidants. Examples of antioxidants from the sera are vitamin A and E and as well sunflower and carrot oil, and SOD (see Appendix A). Antioxidants are important since their presence inhibits or, at best, suppresses the undesired oxidation of other molecules. The chemical oxidation process, in a cell, causes the formation of free radicals that trigger chain reactions that in turn can result in damage or even death of the cell. Both sera contain a variety of antioxidants. One of them is ubiquinone, also known as coenzyme Q10. Together with vitamin E, ubiquinone is special in that it is a natural lipophilic antioxidant (i.e., it is hydrophobic). Lipophilic antioxidants locate within lipid molecules and protect them against oxidative breakdown. Coenzyme Q10 especially protects the barrier lipids in the skin sebum from oxidative stress. They are known to have a beneficial effect against eczema. And they also promote skin healing and reduce scar tissue formation [72]. Upon aging, the availability of both compounds, vitamin E and as well ubiquinone, is reduced due to a drop in their natural concentration in the body. Photodamage also affects these compounds and causes a dramatic depletion.

Both sera contain two varieties of hyaluronan (described in 1.3.4), differing in the number of monomers in the polymer. One variety exhibits a relatively low molecular weight average (10-50 kDa) and the other one has an average molecular weight of 2000 kDa. Transport across the stratum corneum (see 1.1.5) is increasingly inefficient the bigger the molecule. This rule out the direct permeation of the large hyaluronan. Moreover, hyaluronan is highly hydrophilic. Its permeation across a tissue barrier requires an aqueous path. The aqueous film of the intercellular route (see 1.1.5.2) may serve as a transport route for the low molecular weight variety provided the aqueous gaps between the lipid layers are wide enough. Therefore, the diffusive transport using the intercellular route will be more efficient if the aqueous film of this route is widened by swelling through water uptake. Here the high molecular weight variety is useful. It cannot enter into dermis across the stratum corneum and therefore it will constitute to the formation of a wet film on top of stratum corneum. With its hygroscopic property, it is preventing water loss from the epidermis and thus supporting the existing intercellular route for transport of other (smaller) molecules. Moreover, water from the serum is available for redistribution in between the intercellular lipid film, thus widening the existing aqueous path. This way, the passage of small hydrophilic molecules (see p. 38) and possibly even the entry of the
small molecular weight variety of hyaluronan is supported. In addition to the natural routes, the presence of micropores through the epidermis supports the direct translocation of both types of hyaluronan across the *stratum corneum* and into the dermis (depending on the needle length, here 200 µm).

The low molecular weight hyaluronan, when entered into the dermis via the micropores, shall serve to partially compensate for the insufficient amount of the naturally occurring compound. As the skin ages, the production in the fibroblasts decays leading to a deficiency in the natural hyaluronan. The low molecular weight hyaluronan functions as a filler and binds water molecules thus keeping the dermis hydrated.

The high molecular weight variety of hyaluronan does poorly translocate through the skin. Instead, its major role is to support the formation of a protective film at the skin surface, and to stabilize it there. This hyaluronan variety also has a high binding capacity for water. Therefore, it reduces TEWL, and doing so, it keeps the aqueous part of the intercellular route moist and open and slows down the closing of micropores. Its impact on transport across the skin barrier is indirect: small water-soluble molecules can easily move within the liquid film and diffuse into the intercellular space. Small hydrophobic molecules are transported through the film by use of detergent carriers and can as well use the intercellular route by diffusing through the lipoidic bilayers within it.

During the treatment, the SYN serum is applied first. It provides an instant wrinkle-reducing effect that comes from the presence of the dipeptide diaminobutyroyl benzylamide diacetate, also known as SYN®-AKE. SYN®-AKE is a synthetic non-toxic tripeptide derivative of a snake venom. It was developed based on the results from venom research. Similar to its toxic natural counterpart, the molecule is a receptor antagonist of a muscular receptor, i.e. it blocks or dampens signals transmitted to the receptor. Thereby it causes relaxation of the facial muscles. The molecule has thus a direct impact on the mimic wrinkles that are a specific attribute of every individual. A wrinkle reduction can be quantified by measuring skin roughness. For example, the average skin roughness per individual was reduced by up to 37 %, as measured in forehead and in crow's-feet on 45 volunteers that applied SYN®-AKE twice a day during 28 days [73].

Though many ingredients are present in both sera, the relative amount varies. Sorted lists of the ingredients of the HYALURON and SYN sera are found in Appendix B and Appendix C, respectively. There, the ingredient with the highest mass percent is listed first. The HYALURON and SYN sera are similar in ingredients. However, the HYALURON serum contains more nutritional compounds than the SYN serum. One example is the highly purified extract from the microalgae *Dunaliella*, which is found only in the HYALURON serum. This extract is said to protect and enhance the activity of the mitochondria, thus reviving the skin cells by supplying more energy [74] and enhancing the productivity of the fibroblasts. Another example of a highly nutritional ingredient only found in the HYALURON serum is the Macadamia Ternifolia Seed Oil.
2.2. The dermalRollings

The dermalRollings device (Figure 16) consists of a hand-held barrel with 192 conical microneedles [68]. The needles are made of stainless steel, and the handle and the barrel are made of the thermoplastic acrylonitrile butadiene styrene (ABS). In the dermaOXY skin care approach, the conical microneedles on the dermalRollings barrel are 200 μm long. The needles are arranged in a regular array on the barrel, and the barrel can turn around a central axis. There is one needle per 6.54 mm$^2$, i.e. 0.15 needles per mm$^2$ [68].

When the barrel is rolled over the skin surface as part of the dermaOXY application, the needles are used to puncture the upper layers of the epidermis and thus locally break the barrier function of the *stratum corneum* and hence the whole epidermis. The barrier recovery takes between 3 and 15 hours if the skin is clean and healthy. Even though the formation of the micropores and their presence are not painful, the pores constitute open wounds and proper precaution must be taken to avoid infections.

The beneficial effect of the dermalRollings microporation treatment is the potentially enhanced transport of sera ingredients into the deeper regions of the skin (see 2.1). This means the sera are not only targeting the outer skin surface. They also contain nutritive and protective serum ingredients to be effective in the deeper regions of the skin. They are transferred much more effectively into the dermis through the micropores than by passive diffusion through the narrow intercellular route. Once translocated from the skin surface into the dermis, antioxidants, nutritional compounds and even hyaluronan serve to improve or even reconstitute the diverse functionalities there.

![Figure 16: A dermalRollings - The dermalRollings is a dermaOXY trademark. It is equipped with rows of microneedles that puncture the epidermis when rolled across the skin [1]. The needles are made by stainless steel and the rest is ABS plastic [68].](image)

The beneficial transport breaks down as soon as the pores close as an effect of the normal healing response to the artificial wounding. To trigger the initiation of the barrier recovery response it requires air (see 1.1.5.3) and a related TEWL. Preventing air access to the micropores (for example by clogging them) hampers the increase of the TEWL and
The dermaOXY treatment

the onset of the wound healing. This prolongs the duration of the enhanced transport period up to 120 hours [31].

During application, the dermalRollings is rolled back and forth six times [68] in one direction over the facial skin. The procedure is repeated at three more directions, each at an additional angle of 45° to the previous rolling direction. This results in a systematic formation of a regular array of micropores across the facial skin. With the thorough poration of the epidermis, the main barrier of the skin is disturbed such that chemicals like several ingredients of the sera can bypass the *stratum corneum* and move directly to the viable epidermis. Depending on the poration depth, they can even translocate directly to the superficial dermis from where direct uptake or further diffusion of drugs can happen. Additionally, the movement of the dermalRollings massages the skin. This causes slight erythema. The poration wounds, though tiny, initiate an inflammation response. Both effects together result in an increased flow in the blood vessels of the dermis. Thereby improving the oxygen supply and nutrition flow from the blood stream to the dermis, enhancing the biochemical activity of the fibroblasts (and their mitochondria) as well as the keratinocyte proliferation. As a result, new material to the extracellular matrix is established, and the age dependant reduction of keratinocyte production is partially suspended.

### 2.3. The oxygen treatment

The Yuyue 7F-5 Oxygen Concentrator provides a gas stream enriched in oxygen (90 %) at a flow of 5 L/min, at a maximum pressure of 45 kPa, to be used in the dermaOXY treatment. At a lower flow of 1-3 L/min the concentration may even get as high as 94 % pure oxygen. The oxygen stream drives an airbrush device to apply and distribute the serum under clean and controlled conditions. The serum is first dispensed onto the skin surface of a client and then massaged into skin.

Pure oxygen has been used for medical purposes for centuries; the first recorded treatment with pure oxygen was performed in 1662 [75]. Because of previous reports on oxygen toxicity, it was not fully approved until 1937, where hyperbaric oxygen therapy (HBOT) was put into practice as a treatment for decompression sickness [76]. Oxygen recently has been found to play an important role in skin rejuvenation and treatment of photo damaged skin [77]. Ongoing research seeks to reveal the responsible mechanisms for the effectiveness of the use of pure oxygen and so far the evidence relies on photography before and after treatment of the skin.

UV-B radiation causes angiogenesis\(^\text{36}\), i.e. an increased formation of blood vessels in the viable layers of epidermis, seen as a dense network of tiny red lines just beneath the skin surface. Angiogenesis is a product of a skin self defence mechanism. When the skin suffers from hypoxia (low levels of oxygen) the response is an increased amount of the so called hypoxia inducible factor (HIF-1) protein [77]. HIF-1 is a protein that up-regulates

\(^{36}\) Angiogenesis is the formation of new blood vessels from pre-existing ones.
the transcription of the vascular endothelial growth factor (VEGF) which then in turn promotes angiogenesis to compensate for the hypoxic condition [77]. Returning to normal oxygen levels, the HIF-1α subunits degrade. Most probably, hyperoxia (high levels of oxygen) condition increases the degradation [77].

Hyperoxic conditions in the tissue is in general able to decrease blood flow. This slows down active angiogenesis in the skin, which decreases the influx of neutrophils (immune system cells) to a sun damaged area. Neutrophils have been shown to express a few MMPs, and MMP-1 seems to be co-localized with neutrophil elastase after sun exposure [78, 79]. MMPs are involved in remodelling the extracellular matrix of dermis and have important roles in angiogenesis, degradation of collagen and many glycoproteins [77, 80, 81]. Investigation of fibroblast cultures have shown that MMP-1, -2, -3 and -9 increase in amount after UV-radiation an further research is needed to establish a correlation between hyperoxic conditions and MMPs on a molecular level in order to explain in more detail the HBOT’s role in wrinkle reduction. Some MMPs are created by the fibroblasts to remove damaged connective tissue, such it can be replaced by healthy tissue. Ongoing studies are exploring MMPs and HIF-1 as the main causes in wrinkle formation [77].

The exact mechanisms of how HBOT applied on the skin can reduce wrinkle formation and photoaging are yet to be understood [77]. Research has claimed positive HBOT effects in reduction of wrinkles, increasing skin elasticity and stimulating collagen production [82-84]. However, scientific evidences for the claims are still waiting to be provided [77].

2.4. The photon treatment

The dermaOXY photon treatment uses light from LED’s. The use of light in cosmetic treatments of skin is called photorejuvenation. The first clinical article on photorejuvenation as a cosmetic and aesthetic procedure was published in 2000 [85, 86].

The process referred to as photorejuvenation is the aesthetic treatment of fine wrinkles, photodamaged skin, herpes and scars by electromagnetic radiation in the visible and infra-red range [87]. The visible range extends from the wavelength of 380-780 nm (Figure 17).

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37 light emitting diodes

38 Electromagnetic radiation is a phenomenon of energy behaving like waves and particles (photons) as it progresses. Better known as light in common speech. However, visible light is only in the range of approximately 380-780 nm in wavelength.
Electromagnetic photorejuvenating treatment of skin, provided the proper wavelength is chosen, was shown to up-regulate the collagen production in human fibroblast cell cultures [87], diminish rosacea\(^{39}\) symptoms, smoothen fine wrinkles and reduce pore size [89], and counteract UV-induced erythema. Apart from the cosmetic treatment that is aiming at an improvement of the aesthetic appearance of skin, electromagnetic treatment is also applied for medical purposes. For example, anti-inflammatory effects were reported [90]. Moreover, target specific variations of phototherapy are used to accelerate healing after skin peeling and some elective surgeries and to treat acne, vitiligo, rosacea and even skin cancers [63].

The dermaOXY skin care system includes the application of electromagnetic waves. The instrument used is the dermaSEMITA proHZ by MedicTinedic ApS. It provides low intensity light\(^{40}\) in five different types of specific LED irradiation settings: red light of 620-625 nm, blue light of 465-475 nm, yellow light of 588-595 nm, and mixtures of red and blue and of red and yellow light. The irradiation exposure time pattern can be varied from permanent exposure to periodic on/off exposure in the range from 0-190 Hz [91].

The specific phototherapeutic applications recommended by dermaOXY are entered into Table 2.

Each irradiation application extends over 10-35 minutes, with a recommended pulse frequency of 10 Hz. Blue and red light frequencies are recommended to 50 and 190 Hz, respectively [68].

\(^{39}\) Rosacea is a chronic inflammation in the skin resulting in erythema.

\(^{40}\) The blue light treatment reaches a maximum power of 60 mW.
Table 2: LED applications – The table shows what type of light is used to get which type of effect in the skin.

<table>
<thead>
<tr>
<th>light type</th>
<th>effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>red (620-625 nm)</td>
<td>phenotypic antiaging effects</td>
</tr>
<tr>
<td></td>
<td>reduction of erythema</td>
</tr>
<tr>
<td></td>
<td>enhancement of collagen production</td>
</tr>
<tr>
<td></td>
<td>smoothening of skin</td>
</tr>
<tr>
<td>blue (465-475 nm)</td>
<td>acne reduction</td>
</tr>
<tr>
<td></td>
<td>anti-inflammatory effect</td>
</tr>
<tr>
<td>yellow (588-595 nm)</td>
<td>photorejuvenation</td>
</tr>
<tr>
<td></td>
<td>activation of lymph and blood circulation</td>
</tr>
<tr>
<td>red and blue</td>
<td>acne reduction</td>
</tr>
<tr>
<td></td>
<td>reduction of skin greasiness</td>
</tr>
<tr>
<td></td>
<td>antibacterial effect</td>
</tr>
<tr>
<td>red and yellow</td>
<td>reduction of fine wrinkles</td>
</tr>
<tr>
<td></td>
<td>increase of fibroblast activity</td>
</tr>
</tbody>
</table>

The method of low-level light therapy (LLLT) was introduced in the 1960s [63]. By that time, lasers were used as light sources during treatment of photodamaged skin, and patients had to accept a significant risk of harmful side effects. A long post-treatment recovery period was needed because of ablative damage to the superficial layers of the skin. Alternative light sources became available with the invention of LEDs. Their use is much less expensive since the light sources are cheaper. Moreover, safety concerns are suspended, and there is no demand for trained personnel. LED treatments are non-ablative\textsuperscript{42}. Therefore, short or no recovery periods are needed [63, 92].

\textsuperscript{41} in the terms of normal wound healing, i.e. a couple of weeks

\textsuperscript{42} Ablative laser treatments etch the affected tissue, creating easy access to the dermis.
Healthy effects of LLLT are obtained when a sequential exposure to blue and then to red light is applied. P acne bacteria synthesise and store a large amount of porphyrins. These become chemically active when exposed to visible, especially blue light. This blue light exposure causes the formation of free radicals and ROS that in turn cause membrane damage in the bacteria themselves. The example illustrates how a normally harmful effect can be partially exploited in a useful way, serving as an antibacterial tool. Subsequently, red light is applied in acne treatments, since it reduces inflammatory responses and in general has beneficial healing effects [63].

Infrared light (IR) and near infrared light (NIR) have beneficial effects in photorejuvenation too. However, so far they are not included in the current dermaOXY treatment. The mechanisms associated with the cellular photo-bio-stimulation are not yet fully understood. Further research is needed to document the exact beneficial effects of each range of wavelength. However, the absorption of red and NIR light by mitochondrial chromophores (in particular cytochrome c oxidase) is thought to enhance cellular processes e.g. to activate stem cells and boost tissue repair and healing [63].

The beneficial impact of IR is also currently used to reduce UV-induced erythema. According to one theory [93] the exposure to IR light is claimed to prepare cells such that their UV-induced damage is reduced. The model conjectures that exposure to IR-light affects the mitochondrial apoptotic pathway in a positive way. The argument is linked to the daily illumination cycle and due to the related varying spectral composition of sun light. The intensity of IR radiation at dawn is high compared to the intensity of UV-radiation. During that period, the IR light is supposed to prepare the skin such that it is capable to resist the harmful UV-radiation later when the spectral composition of sun light at sea level exhibits an increasing amount of UV-radiation [63]. This is a quality gained through evolution. Research results also claim a noticeable reduction in UV-induced erythema when skin is exposed by LED light illumination within hours after UV-exposure. Both observations indicate a general beneficial effect gained from IR exposure [89].

IR has an up-regulating effect on the collagen production in skin. Laser doses of 0.099-0.522 Jcm\(^{-2}\) at 904 nm wavelength showed to have the most significant, positive effects on fibroblast function and to induce a maximum collagen production [87]. Yellow and IR light was found to reduce MMPs. Especially MMP-1 was reduced after exposure to 590 nm and 870 nm wavelength light with a flux of 0.1 Jcm\(^{-2}\)[89]. The impact of MMPs is explained in 2.3.
3. The analytical methods

The dermaOXY method is a cosmetic routine that consists of a set of tools and procedures that are jointly or subsequently applied in order to improve skin quality and revert aging effects. The overall outcome must be quantified such that the positive impact of the treatment can be evidenced and, at best, quantified. The DermaLab® Combo test system (distributed by Cortex Technology ApS, Hadsund, Denmark) offers a standardized tool set to cover this need [94].

The test system comprises a probe for high resolution 20 MHz ultrasound skin imaging, a conductance probe for quantifying the hydration level (water content) of the skin, a suction cup probe for skin elasticity measurements, and a colorimeter probe for skin colour assessment. These four methods are described and evaluated in the following. Furthermore, the instrument provides systems for assessing the TEWL and the skin sebum translucency characteristics. In addition, it comprises a video scope for acquiring direct images of the skin. However, these three probes are not comprised within the scope of this project [94].

3.1. Ultrasound

Every sound wave is a propagating density wave. The audible range of humans roughly covers frequencies from 16 Hz to 16 kHz [95]. The frequencies above this range establish the region of the so-called ultrasound while the lower frequencies constitute the infrasound range. The speed $v$ of the wave depends on the density $\rho$ of the material and on its uniform compressibility modulus.

An ultrasound wave is generated e.g. by use of a piezoelectric crystal and then emitted to the surroundings of the crystal. Piezoelectric materials contract and expand when exposed to electrical potential differences. Vice versa, when deformed by compression or expansion, an electrical polarisation is generated that can be measured as an electrical signal. This way, a single-element device can be constructed to be used both for the excitation and as well for the detection of an ultrasound wave. In such a device, the piezo crystal is mounted in between two electrodes (see Figure 18). The electrodes serve to apply an oscillating voltage, also known as alternating current in pulse mode. The crystal is electrically excited to mechanically oscillate with the same frequency as the voltage, far into the MHz range. The probe consists of a linear array of piezoelectric crystals that create a homogeneous wave density within the medium. The array direction is parallel to the skin surface, and the crystals are excited one after the other. Data are collected from each crystal separately and merged to create an entire image of the skin within a fraction of a second [95].
The analytical methods

The acoustic wave is then coupled into any other material that is in contact with the surface of the crystal. Single-element transducers are used in pulsed-echo mode. Each wave pulse is followed by a period of no wave emission. This means, the piezo-electric transducer first emits a short pulse of a high frequency wave into the object of interest and then is used to measure the reflected wave (the "echo") during the emission pause (see Figure 18, bottom).

The application of ultrasound in medicine results in the acquisition of images that depict the different response of tissue to an incident ultrasound wave. The Cortex DermaScan C Ultrasonic System is a B-scan type single-element ultrasound imaging system with a hand-held probe for 2D-scanning (see Figure 18, top). It exploits the reflection (the “echo”) that results when a wave is reflected from an interface in between two materials of different density and compressibility. The ultrasound wave is directly coupled into the tissue by use of a contact gel. The purpose of the gel is to exclude the presence of air bubbles and hence artificial boundaries in between the probe and the tissue to be studied.
The ultrasound method in the scope of this project uses a focused (linear) sound wave of 20 MHz; it has a 60 x 200 µm resolution and up to a 3.4 mm penetration in skin [94]. The scan length is 17 mm with a footprint of 11 mm such that the width of the image corresponds to 11 mm in reality [94].

The speed of sound in a medium depends on its acoustic impedance which is specific for all materials and their organisation. For example, the speed of sound in soft tissue (see Table 3) is similar to the speed of sound in water and in blood. In air it is much slower because the density of molecules in a gas is low compared to a liquid, and a gas is highly compressible. The density of atoms in a metal is large compared to a liquid, which is evident from high speed of sound through it.

Table 3: The speed of sound v in various materials. All values are measured at 37 °C [95].

<table>
<thead>
<tr>
<th></th>
<th>air</th>
<th>fat</th>
<th>water</th>
<th>soft tissue</th>
<th>blood</th>
<th>metal</th>
</tr>
</thead>
<tbody>
<tr>
<td>v (m/s)</td>
<td>330</td>
<td>1460</td>
<td>1524</td>
<td>1540</td>
<td>1570</td>
<td>&gt;3000</td>
</tr>
</tbody>
</table>

The different values of ultra sound speed in air and in soft tissue require that a gel be applied at the target skin area such that reflection artefacts are avoided at the air-skin interface (see Table 3). An image of the target area skin is produced from the ultra sound echo data as illustrated in Figure 19.
Figure 19: Principle of B-scan sonographic image acquisition from skin. Locally resolved amplitude data (A-scan data) are converted into brightness or false colour presentations (B-scan data) [98].

When an electrical impulse is applied across the piezoelectric material, a sound wave is propagating into the sample. The wave amplitude is not changed if there is no absorption. The biggest cause of absorption of the wave is the conversion from mechanical energy from the sound wave to heat within the tissue. When the wave reaches an interface that separates two media of different acoustic impedance some portion of it is transmitted and some portion is reflected. The reflected part propagates back through the sample as an echo of the initial wave until it finally compresses the piezoelectric crystal and produces an electrical signal there (see Figure 18, bottom). The time $\Delta t$ for the echo to come back through the sample medium with the speed of sound $v$ is a measure of the distance $x$ to the reflecting interface. The distance is calculated by: $x = c \Delta t / 2$. This is known as the pulse-echo principle [95]. At each interface the propagating wave is split into a reflected wave and into a transmitted one. The transmitted wave passes through the new medium until it is again reflected and transmitted at the next interface or finally completely absorbed.

Accordingly, bright reflections are obtained from all sharp boundaries between different materials. Inhomogeneous materials like the dermis with polymer structures extending loosely packed through the dermal matrix will result in striated or grainy image appearance caused from fiber aggregates immersed within their aqueous bulk solution.
As a result of the various reflections from within the skin tissue, the DermaLab® Combo ultrasound device generates a 2D B-image (B for brightness, see Figure 19).

### 3.2. Hydration

The DermaLab® Combo tool set comes with the DermaLab USB Hydration pin probe. It consists of eight pins (electrodes) and a power supply. A potential difference $V$ is applied across the pins and an ampere meter monitors the current $I$ that flows through the surface of e.g. stratum corneum. The output is the conductance $G$ measured in the SI unit Siemens (S), which is the reciprocal of resistance $R$ measured in ohm $Ω$ [94]. The electrodes are used pairwise to measure the conductance $G$ between them (full range 0 to ~10000 µS, at ~5 % accuracy). The pairwise use of the electrodes results in eight independent measurements. The resulting final value is an average of these. At constant given probe diameter $A$, the conductance measured in a medium of length $l$ can be converted into its conductivity $κ$. For example, conductivity values of drinking water range between 0.005-0.05 S/m depending on the salt contents, whereas the value for seawater is 5 S/m [99]. Wood consists mainly of cellulose, somehow similar to hyaluronic acid. The conductivity of wood is between $10^{-3}$ S/m and $10^{-16}$ S/m (at 20 °C) depending on whether it is moist or dry, respectively [100]. A film of hyaluronan with no water present, has a conductivity of $10^{-13}$ S/m [101] which corresponds to about the value of dry wood compared to moist wood. The conductivity of a serum is mostly reflecting its high contents of water and dissolved ions therein and therefore the conductivity is higher than that of hyaluronic acid alone.

### 3.3. Elasticity

For elasticity measurements, the DermaLab® Combo device set comes with a suction cup probe (radius: $r = 5$ mm). The principle is to measure the response of the skin to suction. Physically, an extension is induced when stress is applied in the form of strain to the surface of skin by reduced pressure (“vacuum”). In the device used, a pump connected to the probe generates a pressure difference $ΔP$ of 0-75 kPa relative to the atmospheric pressure $p_a$ [100]. The cup probe is adhered to the skin by a double adhesive sticker [94]. The sticker seal serves to apply a force to a selected region of the skin. The force $F$ can roughly be estimated from the effective suction area of the probe $A$ and the applied pressure difference $ΔP = p_a - p_s$ [46]. The force $F$ is a “suction” force as the pressure

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$S = \Omega^{-1}$

$G = I/V$

$κ = G \cdot l/A$

$F = A/Δp$, where $A = \pi r^2$
$p_i$ in the probe is less than the atmospheric pressure $p_a$ (~100 kPa at sea level [102]). This way the skin is stretched by the force $F$.

For the measurement, the cup is tightly attached to the skin and the reduced pressure (“vacuum”) is applied. The net suction force needed in order to lift the skin in the centre of the probe by a predetermined amount (range of 0-1.5 mm) is measured and then used to calculate Young’s modulus\(^47\). Young’s modulus $E$ describes the linear Hookean response (“elastic”) to the deformation. The device is used to determine yet another parameter. When the suction force is released at once the time $t_R$ needed for the skin to relax to the original state is measured. In viscoelastic matter, material may be temporarily redistributed during a deformation. This results in a characteristic relaxation time $t_R$. The skin exhibits both elastic and as well viscoelastic properties. The elasticity tool of the DermaLab® Combo device set quantifies the viscoelastic properties of skin by the use of a parameter $V_e$\(^48\) that is calculated by a combination of Young’s modulus $E$ and the relaxation time $t_R$ [103].

The skin is elevated in response to the suction force in the cup, and the deformation is then electronically measured and translated into Young’s modulus. In healthy skin on a young individual, i.e. in the absence of wrinkles, the response to a low deformation force is linearly proportional to the applied force and a complete restoration of the skin appearance happens after the measurement. The deformation upon a suction force can then be interpreted as the joint response of all materials that determine skin elasticity.

The situation is different in the presence of wrinkles. Wrinkles may roughly contribute in at least two different ways, depending on whether they are surface folds or permanent ridges.

Surface folds result from excess surface skin when the underlying tissue is partially collapsed, as seen in skin samples from old individuals (see Figure 11 and Figure 13). Upon suction, these wrinkles are easily unfolded already at low suction pressure (i.e. still within the range of low deformation forces). The probe area $A$ of the suction cup is then not the same as the actual surface area of skin below it. The unfolding of surface wrinkles is similar to inflating a hot air balloon and does not change the surface area. It occurs simultaneously to the elastic deformation presented above. Both effects, wrinkle unfolding and the elasticity of the skin as a whole, can only be separated by measurements that allow a systematic variation of the suction force with an instantaneous measurement of the elevation height. The different contributions to viscoelasticity from elastic relaxation and from wrinkle re-folding can only be distinguished when the time course of the relaxation is tracked in detail.

\[^{47}\text{Young’s modulus is a measure of the stiffness of an elastic material.}\]

\[^{48}\ V_e = E/t_R\]
The number and as well the depth and the total area consumption of surface wrinkles may in part be compensated by restoration of the dermis volume upon the treatment with the dermaOXY method.

Different from that, permanent wrinkles cannot be flattened at low pressure but are embossed to the individual skin appearance since they are related to lasting modifications in the skin. Their development during lifetime is related to individual history and attitudes. They reside partially from wound healing (scars) or are partially due to individual mimic and a related local imprinting effect. Elastically, the impact of permanent wrinkles appears as an increase of the Young modulus within the range of normal elasticity, at low force. At high suction force, such wrinkles may be the site of skin rupture.

Modifications of skin elasticity happen during the lifetime due to natural development (maturation, “aging”), due to environmental conditions (artificial exposure to radiation including excessive sun light, to dissolving or degenerating chemicals) and also due to individual habits like smoking and diet ingredients. A change in skin viscoelasticity was also reported for women undergoing menopause [104]. Smokers may have to face an inhibition in the production of collagen, an increase in the production of MMPs and even elastosis\(^\text{49}\) [105].

### 3.4. Colorimetry

The DermaLab® Combo tool set comes with a DSM II ColorMeter. The target area for probing skin color is 7 mm in diameter. The device consists of a Colour sensor with 64 active elements, thus allowing for spatially resolved characterisation of skin colour perception. The resolution of fine details is 0.75 mm x 0.75 mm. Illumination is provided by white light LED’s. The colorimeter allows a quantified measure of erythema and other causes of skin color variations, e.g. the local presence of melanin. The method is based on the light absorption characteristics of the skin and applies colorimetry to quantify skin color in a reproducible way [94].

The human skin color perception is primarily due to the presence of chromophores [106] such as melanin, carotene (synthesized by plants but present in the mammalian adipocyte tissue through dietary entry), haemoglobin from the blood circuit and bilirubin (a yellow breakdown product of the heme catabolism\(^\text{50}\), excreted in bile and urine – elevated levels indicate certain diseases like hepatitis).

Colorimetry functions as an objective measure of perceived skin colour, making use of the CIELab colour space (CIE: quantification system of colour perception by the Commission Internationale de l'Éclairage) [107]. This is a tri-stimulus colour system where any colour is described by three parameters: \(L^*\) representing luminosity on a 0 (black) to

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\(^{49}\) Elastosis is the accumulation of abnormal elastic tissue.

\(^{50}\) red blood cells undergoing a hemolysis (breaking apart) process
The analytical methods

100 (white) scale, $a^*$ ranging from magenta (+$a^*$) to green (-$a^*$) and $b^*$ ranging from yellow (+$b^*$) to blue (-$b^*$) [106]. Erythema, such as the common UV-induced erythema, is correlated with the extend of redness in the skin [106].

All kind of erythema can be quantified by colorimetry, for example the erythema caused by the dermalRollings induced skin-healing response and the reduction of UV-induced erythema by LED irradiation (see 2.4) [89].
4. Evaluation of the dermaOXY treatment

The following chapter contains additional considerable aspects for future treatments. First the cosmetic dermaOXY treatment is addressed and thereafter the analytical methods.

4.1. The treatment

The diverse components of the dermaOXY treatment are addressed sequentially, with occasional cross-correlation below. In general, it is important to maintain a clean environment throughout the treatment.

4.1.1. The sera

Both sera are based on water and contain a variety of ingredients aiming at supporting and improving skin health, recovery potential and cosmetic appearance. The challenge is to provide the target volume of skin with ingredients across the stratum corneum into the viable tissue.

The skin surface is directly accessed. The sera aim at moisturizing it by prolonging the half-life of water using hyaluronan as a biogeneous hygroscopic water reservoir.

The viable epidermis and dermis are more difficult to target. The transfer of serum ingredients from the surface into deeper parts of the skin is supported by methods to widen the intercellular route and by providing a poration route (see 1.1.5). The latter is done invasively; thus, the serum must contain antibacterial ingredients and any kind of contamination of the serum must be avoided. The extent of transport of the diverse sera ingredients across the epidermis into the dermis is unknown. As a rule of thumb, the transfer probability from the skin surface where the sera are applied into the deeper parts of the skin is higher the smaller these molecules to be translocated are. Larger molecules and especially the small hyaluronan variety may possibly permeate into the dermis mostly via the poration route. The extent of transfer is as well unknown for smaller molecules. This is most essential since the production and thus the amount of some natural compounds in the skin such as ubiquinone decreases with age and as well after UV-radiation. Therefore, it is important to supply the skin with these nutritional and protective ingredients. Detailed studies should be applied to confirm the effectiveness of the intended routes.

4.1.2. The dermalRollings

The use of the dermalRollings induces an artificial inflammation response in the skin. This enhances the blood supply thus as well the healing response of the skin. As part of it, the fibroblasts produce new fibers that fill the dermal volume. This in turn enhances skin elasticity and distencibility. The method temporarily opens pores that allows for direct transepidermal transfer of serum ingredients. Therefore, the method also holds infection risks. Preferentially, sterile conditions should be assured. At least, the skin must be cleansed before the treatment begins and kept clean until a serum is applied, i.e. the
cleansed skin must not be touched. New, sterile or at least sterilized dermalRollings should be used for each treatment.

The use of a dermalRollings is not advised in treatments of acne, as the needles can pick up bacteria from infected skin areas and then transport them to healthy skin areas and thus spread the infection. Treatment of pre-damaged skin should in general be handled under careful consideration from case to case.

In the diabetes society, a needle-free drug delivery technology has been around since the 1850s. However, the first research in the area began in the 1930s leading to the first patent on the technology in 1936-38. The theory is to propel the drug into the skin by a small jet and some uses nitrogen gas to achieve this. The liquid needle can penetrate drugs into the subcutaneous space [108]. One such devise is the Medi-Jector VISION®, which is claimed to create a micro thin drug jet that penetrates into the fatty tissue of hypodermis. Other studies list ultrasound and thermal regulation as well as jet injection as great methods of increasing transepidermal drug delivery [30]. These options can possibly be implemented to enhance transepidermal transport of chemical agents in cases where the dermalRollings are not advised, e.g. when treating skin with acne.

4.1.3. The oxygen treatment

Filtering the gas stream is important. This provides the vulnerable skin with the cleanest environment possible such that dirt and pathogenic cells are absent.

The pressure from the gas stream massages the skin. This increases the blood flow to the underlying tissue. Most probably, the gas stream does not provide oxygen to this tissue where it is highly needed by the cells. However, the oxygen supply form the blood stream increases due to the massage. As the blood flow increases, the supply of oxygen, nutrients and waste removal in target areas of the skin increase. This is highly needed in successful renewal of the cells, fibers, polymers and as well the basal keratinocytes in epidermis.

The stream does not distribute bacteria, if filtered properly. Therefore, the gas stream is preferred rather than mechanical massaging. Distribution of bacteria might be the case when massaging with hands or other solid tools.

4.1.4. The photon treatment

The aesthetic treatment of skin with light holds large potential. It is non-invasive, cheap and does not require specialized personnel. The selection of specific wavelengths of light may be used to target specific issues (see 2.4). The photon treatment is powerful, especially in the case of diseased and damaged skin, e.g. acne. It is a promising supplement to the standard dermaOXY treatment and can be used in connection with it. As an example, the photon treatment can be used to initiate the collagen production by activating the fibroblast mitochondria before the dermaOXY treatment is begun. As well, the healing effects of light irradiation (see 2.4) can be implemented after the application of serum to microporated skin.
4.2. Analytical methods

The analytical methods provided by the DermaLab® Combo test system are adequate assessment tools to track changes in the skin state before and after the treatment and as well to track long time effects. The available toolset is non-invasive and therefore directly applicable in vivo. The quantification measures and their detailed interpretation though are delicate. They do require more detailed studies and proper calibration, even the introduction of calibration systems.

The details of the methods are described in chapter 3. These techniques are most probably reliable tools to monitor and to qualify, to some extent, the impact of the cosmetic treatment during a series of applications for an individual. Since the frequency of the treatments requires waiting times of weeks, the probes will be applied to several test persons during that period. Again, this requires proper calibration procedures such that the results from measurements on a specific test person at different stages of the treatment can be linked properly and turned into comparable qualification. Only then can the changes in the skin status of that individual, i.e. the desired skin improvement, substantiated by a relative assessment before and after each application, be reasonably tracked throughout the extended treatment period. For a higher degree of significance, it is though necessary to establish a considerate protocol for an assessment sequence.

Additional knowledge may be gathered by in vitro methods but this of course is impossible for the in situ quantification of a treatment success since it requires skin sample extraction.

In general, all in vitro methods involve surgery extracted human skin samples or extracted skin from animal models or purely artificial skin models. Studies on such systems permit to test diverse assumptions; the results may possibly be used to refine the treatment as is. In the scope of this project and addressing cosmetic aspects rather than medical ones such studies could e.g. address the extent and effect of the skin punctuation in view of skin morphology, roughness, hydration, and transport of essential species within the dermaOXY sera like the two types of hyaluronan.

The use of in vitro methods to study and confirm basic assumptions and general processes of the treatment applied, is reasonable under certain conditions. First, an essential question or problem is identified and then addressed thoroughly within a well-defined protocol of skin pre-treatment (i.e. a preserving sample preparation). Combined with a proper experimental plan for studying the sample by choice with an adequate method, under controlled conditions and in a defined way makes it possible to gain significant quantitative information. For example, the presence of water before and after poration and its assumed intrusion into the deeper regions of skin after the artificially

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51 An initial study with 3-5 selected individuals under defined conditions and throughout a fixed treatment sequence (6 initial weeks plus 3-4 subsequent months) is possibly a good starting point to identify typical appearances, correlations, and open questions.
applied lesions can be studied by Raman spectroscopy. The surface of skin can be assessed by roughness measurements using tapping sensors, its morphology can be depicted by electron microscopy. On the other hand, selected components of interest in the sera may be marked or even replaced by fluorescent probes such that the distribution of oily and aqueous parts in the sera is made visible, or the transfer of labelled molecules, e.g. hyaluronan, from skin surface into its deeper regions is depicted. Time dependent measurements will then allow to quantify the transport of the labelled ingredient. In the following, some selected options for potential in vitro approaches are presented. If applicable, the tentative outcome from their application will be compared to the results that can be gathered using the analytical methods provided by the DermaLab® Combo.

4.2.1. Skin morphology

A tool that directly measures skin roughness in terms of height variation per area can quantify the evaluation of skin roughness and wrinkle state.

The presence of wrinkles or scars is in fact not assessed by any of the in vivo analytical methods applied so far. This could be an easy and direct accomplishment, even available for in vivo use [109]. Examples comprise tapping devices that mechanically probe and record height differences by moving a tiny stylus systematically across a selected skin area (see Figure 20) and non-contact optical devices (see Figure 21) that base on the analysis of pattern modulations (e.g. PRIMOS – phase shift rapid in vivo measurement of skin [110].)

Figure 20: In vivo method to assess the presence and character of skin surface (wrinkles, scars, etc.). Schematic of roughness assessment by a mechanical scanning profilometer. Lateral resolution is limited by stylus shape and size [111].
Evaluation of the dermaOXY treatment

Often, these methods are indirectly applied to replica from skin taken as silica imprints [112, 113]. Depth resolution from optical and as well from mechanical profilometry extend into the micrometer range for a test area of several mm² (see Figure 22).

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Surface area, mm²</th>
<th>Picture points, n</th>
<th>Depth resolution, μm</th>
<th>Time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantimet 970</td>
<td>100</td>
<td>300,000</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Standard IA</td>
<td>50</td>
<td>180,000</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Mechanical profilometer</td>
<td>25</td>
<td>62,500</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>Optical (laser) profilometer</td>
<td>50</td>
<td>250,000</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Confocal microscope</td>
<td>1</td>
<td>250,000</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

Figure 22: Compilation of roughness assessment methods [111].

The use of use skin replica allows to apply advanced *in vitro* methods like AFM (atomic force microscopy) and SEM (scanning electron microscopy). The AFM is optimal for investigating very detailed height variations within a small area of no more than several μm². This is not adequate for the study of scars, skin wounds, and wrinkles. Here, the scanning electron microscopy provides a better tool since the variation of the magnification allows for zoom-in series of images from a well-defined area and at increasing resolution. Especially the range of μm is easily accessible. Therefore, the SEM is most adequate for systematic studies on skin to explore the details of the skin punctuation by application of the dermalRollings.

*In vitro* samples to be studied by SEM are covered by a thin metallic film of a few nanometers that represents the replica of the skin. The thin film can be applied directly onto properly prepared samples taken from surgically extracted skin. Alternatively, silica
replica can be taken as a first step from individuals during their treatment (before and after the application of the dermalRollings) and later be further processed by addition of the thin film onto the silica template.

The use of silica replica is suggestive if long-term studies on an individual (or a group of individuals) are desired. Such an extended study is though time consuming.

The use of replica from extracted skin allows to simulate the poration treatment with the purpose of e.g. testing the type of punctuations obtained on test skin by several rolling methods. Parallel samples may be prepared and compared. Moreover, the punctuation result may be correlated with results from confocal microscopy (see 4.2.4.1) studies about the filling of the pores when water or sera are applied to the porated skin.

Before inserted into the SEM, all samples must be fully dried by standard laboratory procedures such as critical point drying such that the vacuum in the electron microscope is preserved. The metallic film prevents accumulation of electrons at the surface.

4.2.2. Complexion

The colorimeter that is available within the DermaLab® Combo test system is an appropriate tool for assessing skin complexion, and its change. As for all methods in the test set, proper calibration procedures must be implemented before comparative studies can be performed such that modifications upon the treatment of an individual can be quantified throughout a series of applications.

4.2.3. Skin hydration

The term “hydration” and its measurement by surface conductivity mostly involves the properties of a thin aqueous film at the skin surface. The contribution of the ions to the electrical properties of skin depends on their concentration in the aqueous solvent. This is then taken as an indirect measure of the amount of water in this film. This base on the assumption that the type and amount of the dissolved salt ions is known. Therefore, it bears a big insecurity since salt on the skin varies from individual to individual and is depending on their response to external conditions as season and ambient room conditions in general. However, relative changes may be tracked for an individual if it is possible to set up a defined protocol for the conditions of the measurement.

In general, it is tricky to measure water contents. This is in part due to the fact that water is not only located as a thin film at the skin surface but as well to a small amount within the aqueous space between the lipid layers in the stratum corneum and to a much higher extent within the extracellular matrix of the dermis. Another aspect is the sampling volume. An electric field applied to the skin surface or injected deeper into skin by use of flat or spiky electrodes will drive a responding current. Flat electrodes will mostly reduce the current response to the surface film whilst spiky electrodes will collect a more complex response due to the complex conducting volume. This is not easy to interpret.
Raman spectroscopy was recently suggested as a tool for both *in vivo* and *in vitro* quantification of skin composition, including water contents [114-116]. The method involves the molecular dynamics in the system, namely the vibrational modes within a molecule. The higher mass of deuterium (D) as compared to hydrogen (H) gives rise to a related shift in the optical Raman spectrum. After proper calibration, the method can be applied to quantify water amounts and, to some extent, water state, within the probing volume.

Another direct method exploring dynamics in aqueous system is dielectric spectroscopy. This method exploits the dipole properties of water by measuring their relaxation in an external electric field. The use of the method for the quantification of water amounts is limited; its potential is though unique for exploring the presence of water bound to interfaces like in capillaries and other narrow cavities.

More direct methods to measure water contents in the skin are not at hand, at least not *in vivo*.

All the above methods can be applied as well for the *in vitro* case. However, the removal of a sample from the body by default provides smaller volumes to be studied. Small samples can be mounted into instrumentation that is not compatible with full body experiments. Moreover, the sample can be further processed: its thickness can be cut to the smallest significant volume, and chemical labels may be introduced into it.

Especially, optical transmission microscopy methods require thin samples of no more than maximally 100 μm in thickness, depending on the extent of material present in the sample that is capable of absorbing light. In thin samples, the presence of water can for example be directly depicted by Raman confocal microscopy on extracted skin samples [114, 115]. Using deuterated water (D₂O) instead of normal water (H₂O) allows to distinguish the OH-vibrations from biomolecules from the OD-vibrations of the immersing water. 3D-false color images can then be established and show the distribution of water in skin. Especially pores and their filling depth can be directly assessed.

Normal confocal microscopy can be applied after injection of fluorescent dyes. Spinning wheel confocal microscopy allows to reduce the time for the acquisition of a simple image such that kinetic processes in the range of milliseconds can be tracked.

Regarding water contents, all aqueous contents could be labeled by a fluorophore that dissolves in water. For example, if the water in a serum is labeled by such a dye, the spinning wheel technique will be able to report on the permeation of the dye from the thin serum film at the skin surface into the aqueous compartments of the skin, and especially into microneedle induced pores. Alternatively, the injection of an aqueous label into the dermis and the subsequent monitoring of its presence will tell about the change of water

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52 Hydrogen is composed of one proton, which is chemically written \( ^1 \text{H} \). Deuterium is an isotope of Hydrogen and has one neutron besides the proton of hydrogen. Chemically, deuterium is specified \( ^2 \text{H} \).
contents there, be it by transport into or across the epidermis, or by collapse of the dermis due to water extraction. Optimally, such a dye has the same size as water has. This is inherent in the Raman technique since $\text{D}_2\text{O}$ is about the same size as $\text{H}_2\text{O}$ but not possible for fluorophores as their size is always bigger.

Drying out of the skin, i.e. the loss of water from skin, is a completely different matter. The evaporation from skin is assessed as the trans-epidermal water loss (TEWL). This is a valid quantity for the net water loss acquired during a given time and across a defined area of surface. The method for measuring this [117], is straightforward and available implemented into the DermaLab® Combo test system. The measurement of the TEWL can though not be used directly to correlate the water loss with the skin permeability for water [118]. Still, the quantification of the TEWL will report on the evaporation from unprotected skin and can directly be compared with the evaporation status measured from skin protected by a thin film of serum. Systematic in vivo measurements over time and with varying contents of the high molecular weight hyaluronan will for example allow to assess the assumption that the presence of this polymer does indeed stabilize the water contents of the skin by reducing the evaporation from it.

4.2.4. Tissue status
A series of layers constituted from thin film of a serum on top of the epidermis (mostly stratum corneum) and the dermis represent a three layered compartment of physically different properties regarding density, elasticity, permeability for water and other molecules, and molecular contents. Tissue morphology and as well elasticity are assessed by two tools implemented into the DermaLab® Combo in vivo test system.

4.2.4.1. Composition of the skin layers
The ultrasound feature in the test toolset uses the ultrasound B-scan method to report on the depth and the linear extension of acoustic inhomogeneities (e.g. fiber volumes in the dermis) as a profile view of the facial skin. The ultrasound profiles obtained reveal a qualitative picture about density variations within the upper layers of the skin. The epidermis and the dermis can be clearly distinguished. Since contrast is based on modifications in mass density the method might be used, after proper calibration, to monitor changes in the thickness of the dermis, and its density. This way, it might be possible to quantify the status of the fibers in the dermis for an individual under a sequence of treatments. Calibration samples should be well-known materials that exhibit similar sound response as the samples to be studied and that therefore can be used as reference standards [119] before each new measurement. Such standard samples might phantom samples that mimic test samples [120], or consist of a tailor made test set (e.g. a high volume of pure water (for the no reflection case), an air-metal sample (for the total reflection case), and layered samples that contain for example metal sheets in a resin) or commercially available test objects.
Optical imaging techniques basing on specific labelling of selected molecules or volumes constitute more direct methods for depicting morphology. Usually, they involve in vitro samples.

For example, extracted skin samples can be studied in a confocal microscopy for the presence of fluorescently labeled molecules. Such molecules are usually either hydrophobic or hydrophilic. Hydrophilic dyes were already discussed above regarding their potential to mark accessible water volumes.

4.2.4.2. Elasticity
Skin elasticity is readily assessed by use of the related DermaLab® Combo tool. The system reports on the net elasticity of the skin region studied. Contributions to the elasticity of the skin from different components cannot be separated.

4.2.5. Transport
Hydrophobic dyes were successfully used to identify and explore the lipid pathways in stratum corneum [121]. If applied into a serum, the migration of the labeled compounds from the serum film into the lipoid parts of the skin will show up directly in confocal images and report on the transfer of these molecules of interest. Time dependent studies using spinning wheel microscopy could thus be used to track the rate of transfer of labeled hyaluronan into the upper skin layers.

The same applies for transport through aqueous pathways. Here, a water soluble dye must be chosen. The direct transport of water can only be measured by use of labeled water, i.e. using deuterated or radioactive isotopes of hydrogen (D or T)\(^{53}\). All other dyes are by default bigger than water molecules. Still, this can as well yield significant results since the choice of dyes of varying size may serve to get an idea about dimension constraints through aqueous pathways.

Samples of extracted skin without and with micropores can be studied in the confocal microscope to gain information about pore presence, pore density and pore profile, and as well about the filling of a pore after application of a skin serum.

Another aspect is the general transport of molecules into the deeper part of skin from a serum that is applied to the skin surface. Information can be gained when selected molecule types in the serum are in part replaced by their fluorescent counterpart. This might especially be interesting for the case of the small hyaluronan that is meant to target the dermis and partially replace the lack of natural fibers there as a “filler”.

\(^{53}\) T is an abbreviation for tritium, chemically specified as \(^{3}\)H.
5. Conclusion

The *dermaOXY skin assay* constitutes an advanced type of skin care for cosmetic purposes. It does so by combining mechanical skin pre-treatment with the subsequent administration of sera in a series of application sessions, to be possibly supported by light treatment. The cocktail of ingredients in the sera is delicately balanced. It aims at shielding the skin surface from harmful external effects like UV-light, dehydration and even provides, to some extent, mechanical protection. Simultaneously, essential nutritional ingredients to the skin are provided from the sera. They aim at enhancing the natural processes in the deeper part of the skin (dermis). This is supported by the mechanical poration technique that does initiate an increased metabolism with a subsequent natural reconstitution of essential skin components as a response to the minute skin lesions. At the same time, the pores represent a partial breakdown of the natural barrier function of the stratum corneum. This provide an additional route into the dermis, to be used by larger molecules in the sera that are else how excluded from transport from the skin surface into the dermis. The risk of water loss whilst the porated skin is open for evaporation is largely reduced by the presence of high molecular weight and effectively water binding semi-natural polymers, the large hyaluronan, within the sera. This hyaluronan not only provides a soft protective shield at the skin surface but it as well minimizes water loss from within the skin by stabilizing the water contents in the serum film.

The treatment especially involves the compensation of the undesired appearances caused by skin aging, be it environmental (excessive exposure to sun light or long-time stays in dry atmosphere) or natural (due to illness induced or natural aging). It does so by slowing down the aging processes in the skin. This is achieved by triggering blood circulation, by providing nutrition and anti-oxidants, by partially replacing skin fibers and stabilizing water contents. The treatments aim at a combination of supporting natural functions with compensating methods. This all-together result in an improvement of complexion and a reduction of skin elasticity loss due to aging. Skin collapse with the appearance of wrinkles is partially counteracted and may even be suspended to some extent.
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73. Pentapharm, *SYN-AKE*.
88. *Wavelength spectrum of light*.
90. ***INVALID CITATION ***
91. dermaOXY, *dermaSEMITA proHZ user manual*. 
96. Piezoelectric transducer.
97. *echoing ultrasound wave*.
103. *DermaLab® elasticity*.


Appendix A

The ingredients in the table are not sorted. Lists of ingredients sorted by mass percentage are found in Appendix B and Appendix C of the HYALURON and SYN serum, respectively.

Table A: Ingredients of the SYN and HYALURON sera, and intended properties [68].

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>SYN Serum</th>
<th>HYALURON Serum</th>
<th>Function and effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolyzed Hyaluronic acid</td>
<td>X</td>
<td>X</td>
<td>Sodium Hyaluronate – den høj molekylære Hyaluron, der binder store mængder fugt, danner et beskyttende lag, så elasticitet og blødhed bliver i huden.</td>
</tr>
<tr>
<td>Sodium Hyaluronate</td>
<td>X</td>
<td>X</td>
<td>Vitamin C, kraftig antioxidant og anti-ageing, Beskytter mod frie radikaler, DNA &amp; UV stråling. Øger elasticitet og collagen produktionen.</td>
</tr>
<tr>
<td>Magnesium Ascorbyl Phosohate</td>
<td>X</td>
<td>X</td>
<td>C vitamin ester dannet ud fra askorbinsyre og palmitin syre. For at skabe en fedtopløselig form af C-vitamin.</td>
</tr>
<tr>
<td>Ascorbyl Palmitate</td>
<td>X</td>
<td>X</td>
<td>Vand bindinge effekt, der hjælper andre fugtigivere. Indeholder jern, zink, magnesium, kobber, silicium. Forebyggende mod nedbrydning af collagen, samt har en opstrammende effekt.</td>
</tr>
<tr>
<td>Algea Extract</td>
<td></td>
<td>X</td>
<td>Peptide der stimulerer collagen niveau &amp; reducerer rynker.</td>
</tr>
<tr>
<td>Palmooyl Tripeptide-5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DermaOXY, Menov og Klösgen</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Appendix A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<p>| <strong>Palmitoyl Oligopeptide</strong> | X | X | Peptide der stimulerer cellefornyelse og reducerer dybe rynker. |
| <strong>Palmitoyl Tetrapeptide-7</strong> | X | X | Peptide der reducerer tab af fugt, poser og mørke rander under øjet samt øger hudens elasticitet. |
| <strong>Squalene</strong> | X | X | Naturlig til stede i huden; fungerer der effektivt som &quot;transportør&quot; for vitaminer og andre aktive ingredienser. Modvirker tørhed og øger fleksabiliteten, virker også som en naturlig fugtgiver. |
| <strong>Lonicera Caprifolium Flower Extract</strong> | X | X | Ekstrakt af kaprifolium. Virker anti-bakteriel og blødgørende, |
| <strong>Macadamia Ternifolia Seed Oil</strong> | X | X | Vitaminer og essentielle olier. Beskytter celle membranen, og tilfører konsistens til cremerne i form af at ændre viskositeten. |
| <strong>Helianthus Annuus Seed oil</strong> | X | X | Binder fugt. Rig på vitaminer og antioxidanter. |
| <strong>Dipeptide Diaminobutyroyl Benzylamide Diacetate</strong> | X | Peptide med speciel aminosyre sammensætning, der får musklerne til at slappe af, hvilket resulterer i øjeblikkelig rynke reduktion. |
| <strong>Superoxide dismutase</strong> | X | Kraftig antioxidant der beskytter mod de frie radikalers skadelige virkning. |
| <strong>Hydrolyzed soy protein</strong> | X | X | Særlig aktiv kompleks af peptider, aminosyrer og mineraliske salter der booster illoptagelsen og stimulerer celle fornyelsen ved at efterlignes udmærkede og stimulerer cellernes egen metabolisme. |
| <strong>Daucus Carota Sativa Root Extract</strong> | X | X | Rig på vitamin E, der virker blødgørende og fuglighedsberigende. Indhold af carotenoider modvirker dannelse af frie radikaler og virker beskyttende på hudens celler. Modvirker oxidation af hudens lipidlag. |</p>
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Daucus Carota Sativa seed oil</strong></td>
<td>Højt indhold af vitamin E og antioxidanter.</td>
</tr>
<tr>
<td>(Gulerodsoolie)</td>
<td>Fremmer hudens celle regenerering og stimulerer produktionen af sebum ved tør hud. Virker blødgorende og øger hudens flexibilitet.</td>
</tr>
<tr>
<td><strong>Beta-Carotene</strong></td>
<td>Aktivt stof I gulerod. Modvirker oxidation af hudens øverste lipidlag og fremmer hudens immunforsvar.</td>
</tr>
<tr>
<td><strong>Cetyl/Cetearyl Alcohol</strong></td>
<td>Blødgorende agent.</td>
</tr>
<tr>
<td><strong>Ubiquinone</strong></td>
<td>Coenzym Q10, der beskytter mod omgivelsernes iltning af hudens øverste lipidlag. Har sammen med vitamin E en synergistisk effekt, der medvirker til at genopbygge hudens lipidbarriere.</td>
</tr>
<tr>
<td><strong>Tocopherol</strong></td>
<td>Vitamin E, antioxidant.</td>
</tr>
<tr>
<td><strong>Linoleic Acid</strong></td>
<td>Vitamin E - antioxidant, der modvirker dannelsen af frie radikaler. Fungerer som naturlig fugtbinder i de øverste hudlag.</td>
</tr>
<tr>
<td><strong>Retinyl Palmitate</strong></td>
<td>Kombination af retinol og palmitinsyre; antioxidant og cellefornyende egenskaber.</td>
</tr>
<tr>
<td><strong>Caprylic/capric triglyceride</strong></td>
<td>Fugtgivende og Anti-inflammatoriske egenskaber.</td>
</tr>
<tr>
<td><strong>butylene glycol</strong></td>
<td>Blødgorende egenskaber og fugtighedsgiver (moisturizer)</td>
</tr>
<tr>
<td><strong>Octyldodecanol</strong></td>
<td>Blødgorer og udglatter.</td>
</tr>
<tr>
<td><strong>Bisabolol</strong></td>
<td>Fugtgiver. Reducerer tør og skællet hud.</td>
</tr>
<tr>
<td><strong>Carbomer</strong></td>
<td>Stabilisator</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>-----</td>
</tr>
<tr>
<td><strong>Polysorbate 20</strong></td>
<td>X</td>
</tr>
<tr>
<td><strong>Polysorbate 65</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Ceteareth-25</strong></td>
<td>X</td>
</tr>
<tr>
<td><strong>PEG-20 glyceryl laurate</strong></td>
<td>X</td>
</tr>
<tr>
<td><strong>Sorbic acid</strong></td>
<td>X</td>
</tr>
<tr>
<td><strong>Citric Acid</strong></td>
<td>X</td>
</tr>
<tr>
<td><strong>Ethylhexyl-Glycerin</strong></td>
<td>X</td>
</tr>
<tr>
<td><strong>Phenoxyethanol</strong></td>
<td>X</td>
</tr>
<tr>
<td><strong>Diethylhexyl Syringylidene-Malonate</strong></td>
<td>X</td>
</tr>
<tr>
<td><strong>Potassium Sorbate</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Sodium Benzoate</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Glycerin</strong></td>
<td>X</td>
</tr>
<tr>
<td><strong>Perfume</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Ingen Parabenes</strong></td>
<td>X</td>
</tr>
</tbody>
</table>
Appendix B

The ingredients of the dermaOXY HYALURON serum (585-7-5-1) are listed with the most abundant ingredient first [68]. The ingredients are sorted by mass percentage, and the one with the highest is listed first, as illustrated by the blue diagram.

<table>
<thead>
<tr>
<th>Ingredient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqua</td>
</tr>
<tr>
<td>Glycerin</td>
</tr>
<tr>
<td>Hydrolyzed Soy Protein</td>
</tr>
<tr>
<td>Macadamia Ternifolia Seed Oil</td>
</tr>
<tr>
<td>Cetearyl Alcohol</td>
</tr>
<tr>
<td>Squalane</td>
</tr>
<tr>
<td>Polysorbate 20</td>
</tr>
<tr>
<td>Algae Extract</td>
</tr>
<tr>
<td>PEG-20 Glyceryl laurate</td>
</tr>
<tr>
<td>Hydrolyzed Hyaluronic acid</td>
</tr>
<tr>
<td>Magnesium Ascorbyl Phosphate</td>
</tr>
<tr>
<td>Butylen Glycol</td>
</tr>
<tr>
<td>Lonicera Caprifolium Flower Extract</td>
</tr>
<tr>
<td>Lonicera Japonica Flower Extract</td>
</tr>
<tr>
<td>Helianthus Annuus Seed Oil</td>
</tr>
<tr>
<td>Tocopheryl Acetate (Vit. E)</td>
</tr>
<tr>
<td>Tocopherol</td>
</tr>
<tr>
<td>Sodium hyaluronate</td>
</tr>
<tr>
<td>Palmitoyl Tripeptide-5</td>
</tr>
<tr>
<td>Palmitoyl Oligopeptide</td>
</tr>
<tr>
<td>Palmitoyl Tetrapeptide-7</td>
</tr>
<tr>
<td>Bisabolol</td>
</tr>
<tr>
<td>Ubiquinone</td>
</tr>
<tr>
<td>Daucus Carota Sativa Root Extract</td>
</tr>
<tr>
<td>Daucus Carota Sativa Seed oil</td>
</tr>
<tr>
<td>Linoleic Acid</td>
</tr>
<tr>
<td>Retinyl palmitate</td>
</tr>
<tr>
<td>Beta-carotene</td>
</tr>
<tr>
<td>Ascorbyl palmitate</td>
</tr>
<tr>
<td>Phenoxyethanol</td>
</tr>
<tr>
<td>Ethylhexylglycerin</td>
</tr>
<tr>
<td>Carbomer</td>
</tr>
<tr>
<td>Citric acid</td>
</tr>
<tr>
<td>Sorbic acid</td>
</tr>
<tr>
<td>Sodium benzoate</td>
</tr>
<tr>
<td>Potassium sorbate</td>
</tr>
<tr>
<td>Diethylhexyl Syringylidenemalonate</td>
</tr>
<tr>
<td>Caprylic/capric triglyceride</td>
</tr>
</tbody>
</table>
Appendix C

The ingredients of the dermaOXY SYN serum (591-7) are listed with the most abundant ingredient first [68]. The ingredients are sorted by mass percentage, and the one with the highest is listed first, as illustrated by the blue diagram.

Aqua
Hydrolyzed Soy Protein
Cetearyl Alcohol
Glycerin
Squalane
Ceteareth-25
Polysorbate 20
PEG-20 Glyceryl laurate
Hydrolyzed Hyaluronic acid
Squalane
Butylen Glycol
Magnesium Ascorbyl Phosphate
Octyldodecanol
Lonicera Caprifolium Flower Extract
Lonicera Japonica Flower Extract
Tocopheryl Acetate (Vit. E)
Helianthus Annuus Seed Oil
Superoxide Dismutase
Bisabolol
Dipeptide Diaminobutyroyl Benzylamide Diacetate
Sodium Hyaluronate
Palmitoyl Oligopeptide
Palmitoyl Tetrapeptide-7
Carbomer
Daucus Carota Sativa Root Extract
Daucus Carota Sativa Seed oil
Tocopherol
Linoleic Acid
Ubiquinone
Retinyl palmitate
Beta-carotene
Ascorbyl palmitate
Phenoxyethanol
Ethylhexyglycerin
Sorbic Acid
Citric Acid
Diethylhexyl Syringlyidenemalonate
Caprylic/capric triglyceride