

## Facial Rejuvenation in the Triangle of ROS

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**ABSTRACT:** Recently, we introduced into the conventional catalogue of biological aging a new determinant: ordered interfacial water layers. The discovery of their tunability with skin-tolerated levels of 670 nm light inspired a model, which suggested that the light, by interaction with ordered interfacial water layers in the extracellular matrix, would reverse elastin degeneration. We validated the model in a 10 month self-experiment and arrived at an effective facial rejuvenation program. Importantly, during the experimental phase we avoided extreme oxidative stressors, in particular exposure to extensive ultraviolet and infrared radiation as well as air pollution. Here we report on the adaptation of our model to the extreme oxidative stress levels prevalent in numerous polluted megacities. The results of the extension comprise a new understanding of the protective function of the skin acid mantle, new predictive insight into effects of reactive oxygen species (ROS) on interfacial water layers, and their implication in processes of biological aging, including depletion of follicular stem cell reservoirs and telomere shortening, and led to the design of an accelerated skin rejuvenation method.

Earlier we demonstrated that extended periodic irradiation with intense 670 nm light, generated by light-emitting diodes (LED), significantly reduces facial wrinkle levels.<sup>1</sup> Our previous study was inspired by the results of laboratory experiments performed on model surfaces suggesting that the irradiation of elastin fibers will be instrumental in restoring skin elasticity. Elastin is the protein that provides elasticity to our skin, heart, and arteries. Natively, elastin is hydrophobic but becomes progressively hydrophilic with the physiological changes in the extracellular matrix associated with biological aging. Prerequisite for the functional performance of the elastin fibers is the preservation of a contrast in polarity between their surface and their direct environment. Diminishing contrast promotes dysfunction. The conversion from hydrophobic to hydrophilic is mediated by deposition of an interlayer consisting principally of amino acids, fatty acids, and calcium salts.<sup>2</sup> Native elastin is necessarily coated with a predominantly crystalline interfacial water layer, as predictable from theory<sup>3</sup> and follows from laboratory experiments performed on hydrophobic model surfaces, including polystyrene<sup>4,5</sup> and hydrogenated nanocrystalline and natural diamond.<sup>6–8</sup> The bonding stability of interfacial water molecules on solids depends on their affinity to the solid surface. The affinity is associated with a curvature-dependent asymmetry in charge distribution (for water molecules, the surface charge is maximal at the relatively small hydrogen atoms) – a simple approach to interpret surface polarity behaviors relative to water. Recently, we performed laboratory experiments on nanocrystalline diamond substrates to compare the bonding stability of interfacial water layers on hydrogenated and non-hydrogenated surfaces. We found that the bonding stability was higher on the hydrogenated species.<sup>9</sup> In concert with previous work<sup>6</sup> indicating the polarization of interfacial water molecules by the C–H bond, this result offers an explanation to the origin of the extremely low friction coefficients reported on hydrogenated diamond-like carbon surfaces.<sup>10</sup> The relevance of interfacial water layers increases reciprocally with the scale of measurement. Their explicit consideration in models describing friction at the nanoscale using hydrogenated diamond surfaces<sup>11,12</sup> promises to enhance the predictive capability of the models. In contrast to the lubricative function of interfacial water layers on these hydrophobic species, corresponding water layers masking hydrophilic

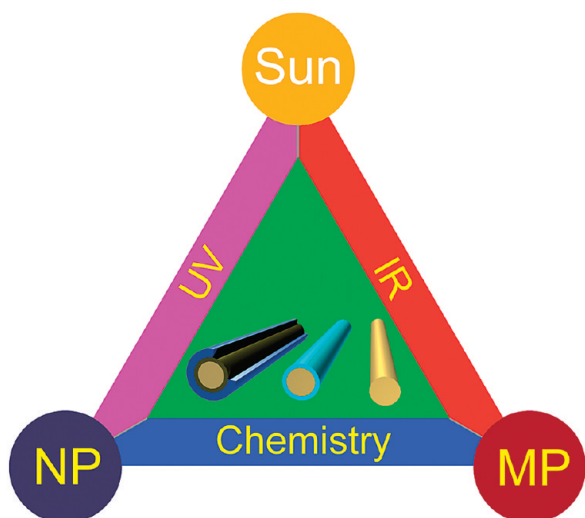
surfaces presented extreme viscosities with glue-like properties, in particular under spatial confinement.<sup>13–15</sup>

The primary target of the LED treatment<sup>1</sup> was a gradual liberation of matured elastin fibers from glue-like water layers in the extracellular microenvironment, where we exploited the finding that 670 nm light increased the fluidity of interfacial water layers masking hydrophilic surfaces.<sup>5</sup> The process of liberation, re-establishment of the native surface polarity, and eventually restoration of the functionality of the elastin fibers received support from the simultaneous activation of cellular metabolic processes in the dermis. The activation of cellular metabolic processes by light (laser or LED) is routinely exploited in clinical practice to facilitate the uptake of properly administered chemical substances by the skin, for instance, to accelerate the healing of complicated wounds, complementary to the actual light effect. The cooperative interplay between the physicochemical and biological effect of light is based on ample evidence obtained individually for each part, by us and other groups, in laboratory experiments and clinical studies, respectively.

Clearly, the biological relevance of the order of interfacial water layers is not limited to nanoscale processes in the extracellular matrix. The order is believed to play a key role in modulating a variety of bidirectional flow processes in the cell, for instance, in nuclear pores, where water and water-soluble molecules are selectively transported in and out of the nucleus across the nuclear envelope. Correlations between metabolic flow processes across the cell membrane and traffic of cargo across the nuclear membrane, with water layers lining the channels' (pores') entrance and/or wall, represent an unexplored field, specifically with regard to the order of the interfacial water involved, and importantly, the impact of the environment on the order of the interfacial water, for instance, oxidative stress and chemical alterations related to climate change and air pollution – a highly attractive multidisciplinary arena. Therefore, the focus on the facial skin – a major target of environment-provoked oxidative stress – is not a coincidence.

**Triangle of ROS.** Natural ultraviolet and infrared radiation and air pollution, including but not limited to hydrophilized carbon particles,<sup>16</sup> with sizes ranging from a few nanometers to several micrometers, cause independently significant structural damage to the exposed skin – stratum corneum, viable cells, and extracellular matrix – partly by contributing to an increase of ROS levels. The total damage caused by the interplay of these components appears, however, to exceed that of their sequential

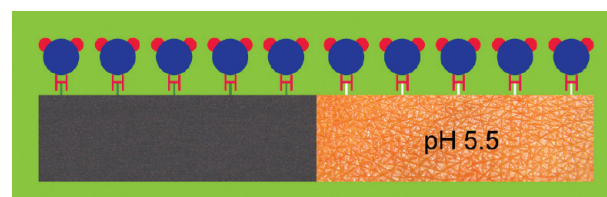
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**Figure 1.** Hydrophilized airborne pollution particles – nanoparticles (NP) and microparticles (MP) – can block the pores of the skin, thereby inhibiting skin respiration. The sun, emitting ultraviolet (UV), visible, and infrared (IR) radiation, is the principal factor in skin elastin degeneration.<sup>17</sup> The term chemistry stands for airborne substances involved in converting the polarity of surfaces. By acting as antennas, and in combination with suitable wavelengths of light, nanoparticles on the epidermis might be involved in processes of radiative energy transfer.<sup>18</sup> In a worst case scenario (megacity with intense air pollution) lasting exposure to the interplay of these effects accentuates the physiological changes involved in the degeneration of elastin, thereby accelerating wrinkling. The inset in the center of the triangle of ROS summarizes the physicochemical aspects of elastin maturation.<sup>1</sup> Native hydrophobic elastin without (right), and with crystalline water layer (middle), and matured hydrophilic elastin with glue-like water layer (left).

sum. For instance, by blocking skin pores, air pollution particles will enhance the impact of infrared radiation on the denaturation of proteins. It is of paramount importance in this context to realize that the action spectrum of ROS is not limited to biological effects: Prior to inducing biological changes, the predominantly negatively charged ROS will affect the order of interfacial water layers, both on the epidermis and deep in the dermis. Figure 1 is a visual synopsis of the natural oxidative stress factors involved in ROS generation, establishing the triangle of ROS. Their interplay can have dramatic consequences for the exposed part of our skin. In a worst case scenario (megacity with intense air pollution) the damage exceeds protein denaturation (photoaging) induced by ultraviolet radiation. In the following, we show that the triangle of ROS is useful for modeling the interplay between different oxidative stressors and their synergistic impact on biological aging. As all the assumptions entering the triangle of ROS (Figure 1) and derived from the interplay of its components with elements of the biosystem are of a qualitative character (do not depend on the assumption of special sets of numerical data) we are justified in expecting, if the whole way of considering is coherent, accurate model predictions.

**Skin Acid Mantle.** The outermost layer of healthy skin is hydrophobic<sup>19</sup> with a slightly acidic pH between 4 and 5.5,<sup>20</sup> presenting a positive charge in this milieu, which changes to negative at neutral pH.<sup>21</sup> Whereas the acid mantle of the skin has been described in the literature for more than 100 years, its provenance and intrinsic functions are intensively discussed. New research<sup>22</sup> ascribes to it, however, two vital protective functions: epidermal permeability barrier and antimicrobial barrier. Interestingly, these functions are consistent with those of ordered interfacial water layers on hydrophobic surfaces in general, and because of the anticipated excess protons at the acid mantle, on



**Figure 2.** Hydrogen atoms on hydrogenated diamond inducing crystalline interfacial water layers by polarizing water molecules (left), and hydronium ions (pointing with the lone pair side toward the hydrophobic region)<sup>3</sup> polarizing water molecules at the top layer of the skin (right).

healthy skin in particular.<sup>3</sup> Whereas on hydrogenated diamond the bond and polarization of the first layer of interfacial water molecules is determined by the C–H bond (in agreement with experiments),<sup>6,9</sup> it is reasonable to assume on healthy skin a similar ordering, induced by the interfacial layer of excess protons.<sup>3</sup> Figure 2 illustrates the principle and compares the organization of water molecules on hydrogenated diamond with that on healthy skin. Lacking the stabilizing C–H bond, the ordering on skin will be less pronounced (more statistical) than on hydrogenated diamond. However, as long as the skin maintains its acidic pH, its outermost layer will be coated by a nanoscopic, predominantly ordered water layer, which, as shown for a variety of hydrophobic surfaces, will instantly be depleted when exposed to visible light of the intensity of the solar radiation.<sup>23,24</sup> In contrast, on hydrophilic surfaces the same light intensities only increase the fluidity of the nanoscopic water layers, however, without depleting them. This observation is crucial for understanding the role of the acidic pH of our skin. Considering that the largest fraction of the airborne pollution particles is hydrophilic,<sup>16</sup> it becomes clear that by accentuating the glue-like nature of the interfacial water, a neutral or alkaline pH of the skin (converting the top skin layer practically to hydrophilic) would reinforce the adhesivity of the particles eventually landing on the skin. Because of the specific size range, such particles have the capacity to effectively block the pores of the skin (both hair follicles and sweat pores). At higher concentrations, they may completely set off the thermoregulatory function of the skin. By casting shadows on the skin, the particles are instrumental in preserving the glue-like character of the interfacial water layers established between them and the skin. This mechanism is not only valid for pollution particles originating from anthropogenic sources – it holds also for volcanic soot, sand, organic debris, pollen, bacteria, viruses, and in general all kinds of pathogens. Probably, the immobilizing potential of the shadow increases with particle size. Obviously, by keeping its pH acidic, our skin is perfectly protected both against overheating by potentially pore-blocking particles and against infections caused by the attachment of microorganisms. Presumably, we are facing here an extraordinarily efficient evolutionary adaptation. In summary, the biological relevance of ordered interfacial water layers is not restricted to the functionality of the elastin – in humans they also play a role in skin defense. By comparison, the skin of cats and dogs, whose skin is more or less protected by fur, presents pH values close to neutral.

**Accelerated Facial Rejuvenation.** From the aforementioned scenario, it becomes evident that prolonged bombardment with ROS, as for instance, stimulated by a persisting interplay of the oxidative stressors represented in Figure 1, will convert hydrophobic native elastin to hydrophilic, and/or accentuate the hydrophilic character of matured elastin, thereby transiently restricting the elastic function of the elastin fibers, encouraging their immobilization. The transition is mediated by changes in the order of the interfacial water layers enveloping the elastin fibers. Surprisingly, the generation of ROS in the dermis is not only triggered by the left (UV) and right (IR) side of the triangle of



**Figure 3.** Representative photographs show wrinkles subsequent to 2 months of extreme oxidative stress (left) and after 3 months of daily LED treatment (right): initially 2 months of LED only, followed by 1 month of green tea assisted LED. The change resulting from 1 month of combinational treatment (less pronounced wrinkle levels, shorter wrinkle valleys, and juvenile complexion) was previously realized in 10 consecutive months.<sup>28</sup>

ROS (Figure 1), but apparently also by the visible part of the spectrum. *In vitro* studies indicated a dependence of the ROS effect on three light parameters: wavelength, intensity, and dose. As a general tendency, smaller doses generated ROS levels that were beneficial for cellular processes, whereas higher doses generated levels with potentials reaching from the inhibition of cellular functions to bactericidal effects.<sup>25</sup> To compensate for a possibly extensive ROS generation by the intense LED light and subsequent inhibition of cellular processes, we included into our facial rejuvenation program a powerful ROS scavenger: epigallocatechin gallate (EGCG) extracted from green tea.<sup>26</sup> Orally administered EGCG is known to compensate for environment-induced oxidative stress.<sup>27</sup> Topical effects are ROS compensation and extension of the survival rate of the cells involved in the incorporation and transport of extracellular metabolic waste from the basal membrane across the epidermis to the top skin layer.

It is instructive to recapitulate: Between November 2007 and September 2008 one of us irradiated the skin around the corner of the eyes with intense LED light (WARP 10, Quantum Devices, Inc. WI): Central wavelength 670 nm, integral intensity  $728 \text{ W m}^{-2}$  and dermal dose  $4 \times 10^4 \text{ J m}^{-2}$ . The resulting change in wrinkle levels for 10 consecutive months of daily irradiation was communicated earlier.<sup>28</sup> The second phase of the facial rejuvenation program started December 2008. First, we continued the previously established protocol. The difference to the previous phase was residence in megacities: in China (one month) and Africa, including Cairo (one month), directly prior to phase two of the experiment. This exposed the skin to massive environmental stresses, including high levels of ultraviolet, and infrared radiation causal for heat stress reinforced by its interplay with extreme particulate matter concentrations in the air. Figure 3 (left) shows the condition of the facial skin in Africa, one day before the end of the journey. Evidently, two months of extreme sun and air pollution were sufficient to neutralize the success of 10 months of light treatment. Phase two of our facial rejuvenation program started upon return from Africa and persisted for a further two months without a visible change in wrinkle levels. Here we modified the routine and introduced topical application of green tea (3 g of dry leaf mass per 250 mL of water, brewing temperature  $100 \text{ }^\circ\text{C}$ , cooling time 30 min), applied onto the skin around the corner of the eyes 20 min before irradiating the wrinkled zones according to the protocol. The temporal coordination between use of ROS scavenger and light, and the coupled functional complementarity between the biological and physicochemical

processes in the skin offers an explanation to the accelerated rejuvenation of the facial skin displayed in Figure 3 (right). In our study, we exploited the protective effect of EGCG. However, it is clear that the combination polyphenolic component of green tea and red light is not the only possible one. An arsenal of powerful ROS scavengers can be found in the literature.

**Depletion of Follicular Stem Cell Reservoirs and Telomere Shortening.** The new understanding emerging from a systematic analysis of the implication of ordered interfacial water layers in conserving or converting the polarity of biological surfaces is not limited to the function of elastin fibers. It applies likewise to the design of practical strategies allowing us to prevent and reverse topical skin deteriorations related to anomalous ROS levels provoked by the environment. This opens the door to a multitude of novel biomedical and cosmetic applications related to biological aging, for instance, skin rejuvenation formulas compatible with pulsed light, promising to improve their transepidermal penetration. As a general recommendation, our model suggests to use for long-lasting topical cosmetic applications exclusively hydrophobic formulas. Hydrophilic formulas, for instance, nutritive creams, are beneficial when applied for shorter periods, but are preferably to be avoided for long-lasting protective-topical applications. This is, however, not the end of the list of biological implications, which are potentially controlled by the order of interfacial water layers. *In vitro* work showed that the graying of human hair is caused by  $\text{H}_2\text{O}_2$ -mediated oxidative stress,<sup>29</sup> the process of oxidation involving the entire hair follicle – a uniquely protected compartment and coincidentally a prominent stem cell niche.<sup>30,31</sup> Therefore, follicular stem cells are probably susceptible to the same bursts of ROS, which are responsible for the graying of hair. Noticeable, follicular stem cells reside continuously in a virtually hydrophobic milieu, proximal to the sebaceous gland producing and containing the hydrophobic sebum.<sup>32</sup> One may speculate that prolonged ROS bombardment in the narrow follicular space might not only turn the hair gray but also, by transiently changing the order and pH of the interfacial water layers separating and enveloping stem cell colonies, change the nature of their niche, thereby successively depleting the reservoirs. The stem cell hypothesis proposed by us receives support from studies showing that a change in intracellular/extracellular pH dramatically affected the lifespan and differentiation of stem cells.<sup>33</sup> The speculative quality of this assumption might be relaxed by considering that this is exactly what one normally encounters in the course of biological aging.

The effect of ROS on the order and pH of interfacial water layers might also affect processes in the nucleus. Whereas the mechanism and control of cargo migration through nuclear pore complexes is still poorly understood, it is clear that prolonged ROS bombardments could transiently accentuate hydrophilic aspects of the nuclear pore complex, thereby disturbing the selective transport of vital cargo in and out of the nucleus. Likewise, macromolecules representing the cargo could transiently change their surface polarity. The result is likely a temporary nutritive deprivation in the nucleus. Possibly, interfacial water layers and interfacial pH play a central role in processes of stress-mediated telomere shortening – one key factor in cellular aging. Telomere shortening can be induced by various causes, including both external and internal stressors,<sup>34</sup> and has already been linked to an overproduction of ROS by mitochondria.<sup>35</sup> Chromosomes are known to attach to the nuclear matrix via telomeres.<sup>36</sup> Polarity contrasts between nuclear matrix and telomere segments seem to serve the telomeres as anchors, which protect chromosomes from unfavorable interactions.<sup>37</sup> It is now clear that minimal changes in polarity can maximally affect space-selective anchoring and repulsion processes between telomere and nuclear matrix. In a mechanical picture, telomere shortening is favored by the coincidence of glue-like water layers (penetrating into the telomere-nuclear matrix interface and/or masking the relevant contact zone) and dynamic detachment and separation processes, for instance, cell divisions.

Characteristic for both compartments – the contact space separating the epithelial cells forming the follicular lining from the enclosed hair shaft and nuclear volume – is their microscopic dimension. From the dimension of the compartments, it is plausible that minimal prolonged ROS generation will create pH gradients and giant fluctuations in interfacial pH. Within the nucleus, for instance, minimal amounts of ROS are expected to considerably change the pH of interfacial water layers and to affect pre-existent polarity contrasts between telomere portions and nuclear matrix. By their tendency to penetrate into interfaces and by masking surfaces, interfacial water layers play presumably a critical role in both the transfer of information (order and/or pH) and initiation of connection–disconnection processes (pH modulated switch). For the hair follicle the gradients in interfacial pH and their propagation will be inescapably experienced by the follicular stem cells. Therefore, treatment of the hair with improper pH could, by diffusional intrusion into the follicular space, affect the lifespan of follicular stem cells. These perspectives, including the possibility of the use of interfacial pH as a sensitive marker for ROS, may motivate competent groups in efforts to improve techniques to measure interfacial pH.

**Conclusion.** A comprehensive consideration of the dependence of biological functions on the structural property of interfacial water layers, which may present a perfect crystalline order on hydrophobic surfaces and extremely high viscosities of hydrophilic surfaces, enriches our insight into basic processes implicated in biological aging, in particular under the accelerating impact of ROS. Further exploration of ROS-related changes in interfacial water layers (interfacial pH) with focus on the link between stress and hydrophilicity may inspire unforeseen routes to retard aging, in the skin and deep in the body. Besides this preventive prospect, there is increasing evidence that the quality of light employed in our rejuvenation study is effective in accelerating stem cell proliferation in vitro. With this possibility, our study might provide robust strategies not only to protect stem cells in their niche but also to identify stimuli allowing us to use light to fill up partially depleted stem cell reservoirs in vivo, thereby to retard and reverse biological aging. Finally, we wish to put forward a strategy to possibly minimize the impact of oxidative stress on telomere shortening. If there is indeed a stress-mediated build-up of a glue-like interfacial water layer,

which contributes to the shortening of telomeres, then it must be possible to switch off the glue effect by irradiation with moderate levels of visible light. This expectation is derived from previous laboratory experiments showing that the light increased the fluidity of interfacial water layers on hydrophilic surfaces – equivalent to reducing their glue-like character.

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