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# Three-channel imaging reveals the comprehensive protein modifications and their impact on skin appearance induced by multiple stimuli

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ABSTRACT

Protein damage repair and prevention are important objectives in skin care industry. Skin protein damage or modifications such as glycation, carbonylation or oxidation, have a significant impact on its function, therefore directly influencing various skin functions or properties including skin appearance. However, there is a lack of comprehensive methods to visualize and assess the protein damage. In this article, we present a three-channel imaging approach to simultaneously visualize and quantitatively evaluate protein oxidation, protein glycation and carbonylation in a full-thickness skin model. We successfully visualized and quantified the impact of the multiple stimuli (ultraviolet radiation A (UVA) and/or methylglyoxal) as well as treatment effect of positive control (vitamins C and E) with this method. Our findings indicate that multiple stimuli exhibit synergistic effects on protein damage. Furthermore, we evaluated a unique combination of skin care ingredients which demonstrated an excellent efficacy in resisting protein damage. Further research revealed that three ingredients of the combination upregulate autophagy in cells, which may contribute to remove damaged proteins and maintain protein quality homeostasis. This method provides a holistic assessment of protein damages and can be employed to evaluate the impact of various stimuli or to assess the efficacy of skin care ingredients in mitigating such damage.



Skin plays a crucial role in overall health and appearance. While the main composition of skin is protein excluding water [1], protein quality significantly impacts skin properties and function, particularly skin appearance, which is a primary concern for many individuals [2]. Alterations in protein due to protein modification or damage can lead to visible changes in skin color like yellowish skin, which is of particular concern among the Asian population [3]. Various cosmetic ingredients have been introduced in skincare products to remain or restore skin health and skin appearance (*e.g.*, even and fair skin tone) through mechanisms such as anti-oxidation, anti-glycation, and anti-carbonylation [3–10], which emphasize the importance of protein integrity in achieving optimal skin health and appearance.

Despite the introduction of all these skin care ingredients, current evaluation methodologies still rely on isolated assessments of individual protein modification [11,12], limiting the overall understanding of their combined effects on skin health and appearance. There is a notable lack of comprehensive method to evaluate various protein modifications simultaneously, in particular, together with carbonylation. This gap in research hinders the ability to assess the collective influence of ingredients on protein modifications. As a result, making holistic comparisons between the effects of different ingredients becomes challenging, restricting the advancement of the skincare industry. A more integrated approach is needed to fully understand how these ingredients work together to improve skin health and appearance, and address skin tone issues effectively.

Currently, the primary method for analyzing overall protein modifications is proteomics. While this sophisticated technique has significantly contributed to skin biomarker identification, it imposes stringent requirements on sample processing and equipment. The highly controlled sample preparation processes, which include protein extraction, purification, and digestion, can lead to sample

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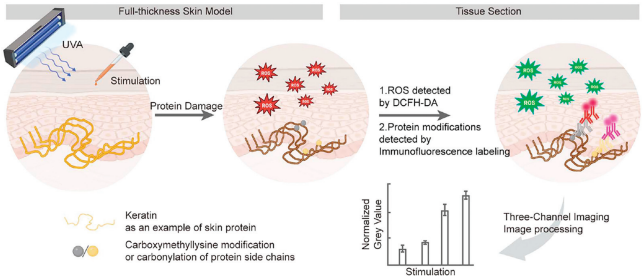


**Scheme 1.** Illustration of stimuli-induced multi-protein damage and the three-channel imaging method for holistic evaluation of protein quality.

loss or degradation. Additionally, the sensitive instruments used in proteomics require skilled personnel to operate, as well as expertise in handling the complex data processing involved. Consequently, these challenges significantly limit the skincare industry's understanding of protein modifications and hinder its development. Among all analytical technologies, fluorescence imaging stands out by offering high sensitivity for detecting low-abundance molecules, providing specificity through the use of targeted fluorophores [13–15], enabling multiplexing for simultaneous detection of multiple targets, and being applicable across various disciplines [13,16,17]. So we turned to fluorescence analysis to address this challenge and developed a method to simultaneously assess the levels of free radicals, glycated and carbonylated proteins in a full-thickness skin model [18] based on fluorescence imaging (Scheme 1). This 3-in-1 method, utilizing confocal fluorescence imaging, allows for the first time the simultaneous detection and evaluation of three types of protein modifications that are of significant concern in the current skin care industry, within the same tissue sample. In this approach, we traced the levels of reactive oxygen species (ROS) in the tissue using the small-molecule fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) [19], identified glycation end-products by employing immunofluorescence technique by detecting carboxymethyllysine (CML) modified proteins [20] and assessed protein carbonylations through their reaction with 2,4-dinitrophenylhydrazine (DNPH) forming dinitrophenyl hydrazone (DNP) and subsequent immunofluorescence labeling with an anti-DNP antibody [21]. Additionally, since the method demonstrated excellent performance in detecting protein modification with treatment of both negative and positive control actives, we further applied the method to evaluate the performance of several skin care ingredients.

To validate the method, we first subjected the full-thickness skin model to ultraviolet radiation A (UVA) irradiation, methylglyoxal (MGO), or both, to induce ROS generation or trigger glycation reaction. It is well reported that UVA radiation penetrates deeply into the skin and promotes the generation of ROS, while MGO is a reactive carbonyl compound that can react with proteins through the glycation process, forming advanced glycation end-products (AGEs), altering the structure and function of proteins. Furthermore, the accumulation of AGEs can exacerbate the oxidative stress, resulting in an increase in ROS production [22–25]. Additionally, the protective effect of the combination of vitamin C (VC) and vitamin E (VE) against damage induced by UVA and MGO was also evaluated as a positive control (VC 100 ppm, VE 7 ppm). After stimulation, the full-thickness skin model exhibited visible changes in skin appearance (Fig. 1a), with the tissue treated with both UVA and MGO appearing significantly yellower, while UVA and MGO treatment combined with VC plus VE showed a brighter appearance. L, a*, b* color measurement of these tissues indicated that the skin experienced a decrease of 4.89 in L* value after exposure to oxidative and glycation stimuli (Fig. 1b, L* value; Table S1 (Supporting information): reduction *vs.* blank control: UVA 1.30, MGO 1.55, UVA plus MGO 4.89), while the b* value increased by 4.59 (Fig. 1c, b* value; Table S1: increase *vs.* blank control: UVA 0.02, MGO 0.34, UVA plus MGO 4.59). These changes are beyond the additive effects observed under single-stimulus conditions, suggesting that external stimuli may have synergistic effects on skin damage.

We then detected and visualized protein damages comprehensively using this new 3-in-1 method in the same full-thickness skin samples (Fig. 1d). The three skin layers (stratum corneum, epidermis, and dermis) were clearly observed in the full-thickness skin model sections. Single-channel results from confocal fluorescence imaging demonstrated that both UVA and MGO stimuli significantly increased the levels of ROS within the tissue (Fig. 1d, Fig. 1e top line), while MGO had a more pronounced induction effect on protein glycation and carbonylation (Fig. 1d, Fig. 1e second line and third line). Meanwhile, the reduction effects delivered by VC and VE were also observed in all the three channels (Fig. 1d, far right column). To compare the impact of three types of protein damages holistically, we introduced an indicator "Normalized Grey Value" by summing the grey values of each channel, which enables a comprehensive horizontal comparison of protein modification levels. As shown in Fig. 1f, the tissues treated with UVA and MGO revealed an increase in normalized grey value of 41% and 242%, respectively, compared to the blank control group, while tissues treated with both UVA and MGO exhibited a 338% increase in this value, suggesting a significant synergistic effect of UVA and MGO. In contrast, VC plus VE demonstrated an 11% reduction compared to blank control group.

The Western blot results confirmed above trend, with the levels of CML modifications and carbonylation significantly higher under the combined treatment of UVA and MGO compared to the individual stimulation groups (Fig. 2), while VC and VE group showed a decrease (Fig. S1 in Supporting information). Each individual stimulus could cause multiple types of protein damage, and combination of two stimuli could yield a synergistic damaging effect. This warrants an importance of comprehensive assessment of protein damages and those prevention effect by skin care ingredients.

The establishment of this method aims to provide a rational parameter for a horizontal comparison of various actives or skin care ingredients that act on various pathways. Next, we assessed the effect of aminoguanidine hemisulfate, an investigational drug for the treatment of diabetic nephropathy and well known to inhibit glycation by scavenging reactive carbonyl compounds, such as MGO and other aldehydes that are precursors to glycation. By reacting with these carbonyls, aminoguanidine prevents them from interacting with proteins and forming AGEs [26,27]. We also tested the combination of six skin care ingredients which consists of chemical ingredients and natural extracts (referred as Combo 1, total ingrdients level 11.75 ppm, ingredients and final treatment concentrations: water lily extract 0.49 ppm, lactobionic acid 0.12 ppm, artichoke leaf extract 0.12 ppm, ascorbyl glucoside (AA2G) 0.03 ppm, bisabolol 4.92 ppm, VE acetate 6.07 ppm). We first treated a full-thickness skin model with these two groups along with UVA and MGO stimulation. Compared to the UVA plus MGO treated group, both groups provided a visible brightness enhancement of skin model appearance (Fig. 3a). Objective color measurement in L* value and b* value further confirmed the improvements in brightness and yellowness of the skin (Figs. 3b and c, Table S1).

From the three-channel fluorescence imaging, aminoguanidine hemisulfate treatment significantly reduced CML intensity (Fig. 3d, column 2, Table S2 in Supporting information). This finding is consistent with previous reports that aminoguanidine hemisulfate can decrease protein glycation in stimulating conditions. Next, we attempted to prove the hypothesis that suppressing these three types of protein damages by multiple ingredients possessing different

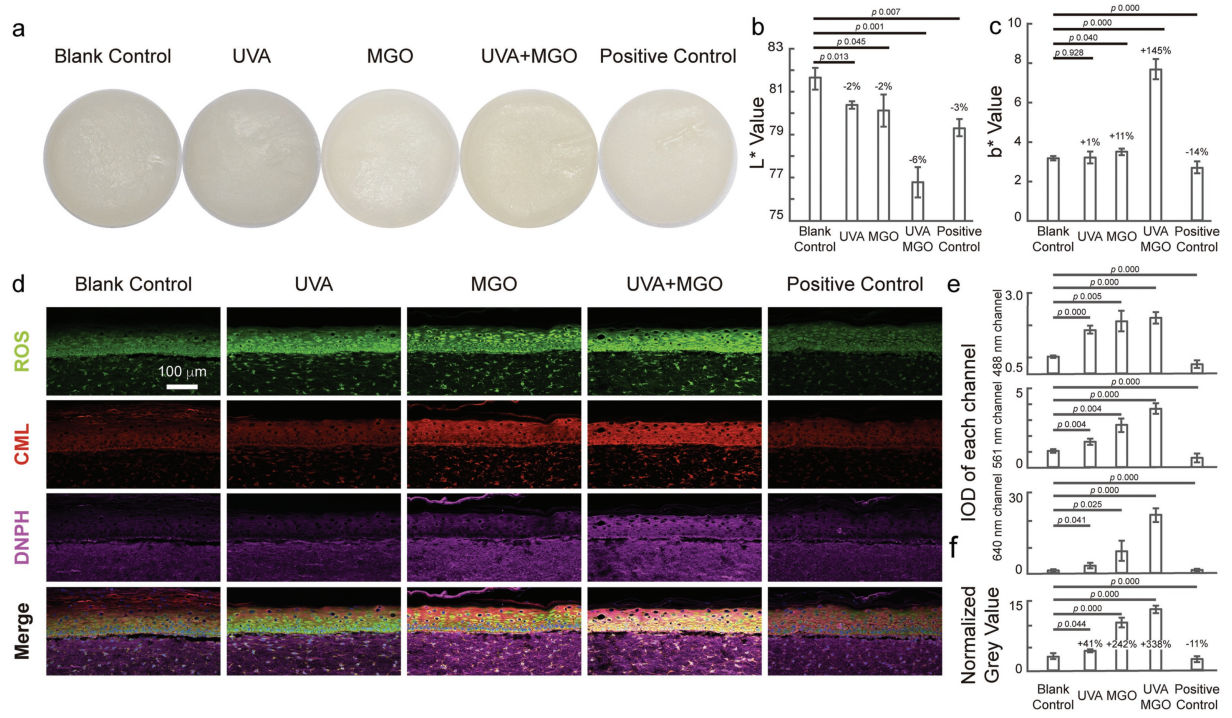


**Fig. 1.** Multi stimuli exhibit a synergy effect on skin appearance and tissue damage status. (a–c) The appearance of full-thickness skin model with the treatment of UV radiation (30 J/cm$^2$), MGO treatment (0.5 mmol/L), UVA plus MGO, and VC 100 ppm plus VE 7 ppm, along with the CIELAB color measurements (L* and b* value). (d) Three-channel fluorescence imaging of the full-thickness skin model slice. Blue channel: ex 405 nm, em 430–470 nm; green channel: ex 488 nm, em 500–540 nm; red channel: ex 561 nm, em 570–620 nm; magenta channel: ex 640 nm, em 650–710 nm. (e) Integrated optical density (IOD) of the three signals. (f) The rational indicator normalized grey value to comprehensively demonstrate the results of comprehensive protein damage. Results are presented as mean ± standard deviation (SD, $n = 3$). Intergroup differences were assessed using two-tailed Student's *t*-test.

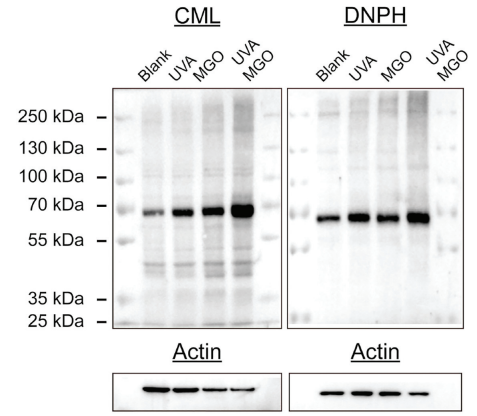


**Fig. 2.** Synergistic effects of stimulation on protein damage modifications. Western blot analysis of protein CML and carbonylation modifications in the epidermal layer of the full-thickness skin model in response to UVA, MGO, and UVA plus MGO.

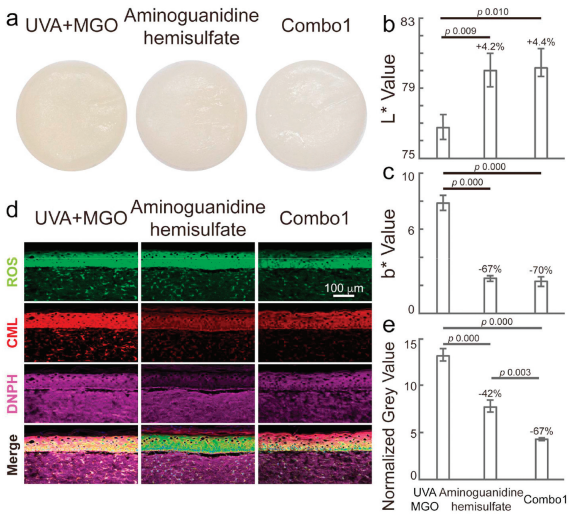


**Fig. 3.** The protective effects of two groups of active ingredients/combinations against protein damage. (a–c) The appearance of the full-thickness skin models after exposure to UVA and MGO stimulation with the treatment of two active groups, aminoguanidine hemisulfate (3 mmol/L) and Combo 1, along with the CIELAB color measurements (L* and b* value). (d) Three-channel fluorescence imaging of the full-thickness skin model slice. Blue channel: ex 405 nm, em 430–470 nm; green channel: ex 488 nm, em 500–540 nm; red channel: ex 561 nm, em 570–620 nm; magenta channel: ex 640 nm, em 650–710 nm. (e) The indicator "Normalized Grey Value" shows the overall ability of three test groups to resist damages including oxidation, glycation and carbonylation. Results are presented as mean ± SD ($n = 3$). Intergroup differences were assessed using two-tailed Student's *t*-test.

mechanisms is effective to suppress protein damages holistically. As Combo 1 designed, artichoke leaf extract, AA2G, and vitamin E acetate are well known for their antioxidant properties [2,28–31], water lily extract and lactobionic acid both have clearance benefits from two different angles; bisabolol is reported to be an anti-inflammatory ingredient [2,32,33]. Experimental results supported this hypothesis; single-channel imaging revealed that, compared to the negative control group (UVA and MGO combination), this Combo 1 group significantly reduced levels of ROS, CML modifications, and carbonylation modifications (Fig. 3d, column 3, Table S2), even stronger than aminoguanidine hemisulfate.

While a detailed analysis of each channel showed that different ingredients possess unique advantages in specific pathways, protein modifications are complex processes influenced by multiple factors, thus targeting one particular pathway can also improve the other two types of protein modifications simultaneously.

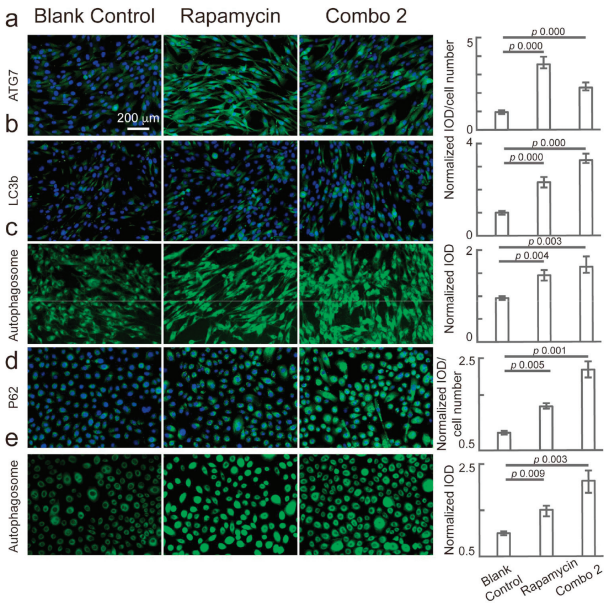


**Fig. 4.** The active ingredients Combo 2 upregulated the expression of key proteins in the autophagy pathway and the intercellular autophagosome amount in fibroblasts and keratinocytes. (a–c) Imaging and fluorescence intensity of ATG7, LC3b and autophagosome in fibroblasts. (d, e) Imaging and fluorescence intensity of P62 and autophysome in keratinocytes. The concentration of rapamycin is 50 nmol/L. The ingredinets composition and concentration is water lily extract 994 ppm, lactobionic acid 251 ppm, artichoke leaf extract 248 ppm. Results are presented as mean ± SD ($n = 3$). Intergroup differences were assessed using two-tailed Student's *t*-test.

This was also observed in Western blot results (Fig. S1). Therefore, it is essential to comprehensively assess the effects of ingredients on protein damages. The new index "Normalized Grey Value", as described previously, allows a horizontal evaluation of different ingredients or ingredient combinations regarding their capacity to prevent protein damage. In Fig. 3e, aminoguanidine hemisulfate showed a 42% reduction of total protein damage modification, while the Combo group demonstrated a 69% reduction. This is the first time that there is a method allowing for a comprehensive evaluation of the effectiveness of various ingredients or their combinations in reducing protein damages, which holds great promise for further application.

With the help of this method, the holistic reduction efficacy of Combo 1 in decreasing oxidative free radical levels, protein glycation, and protein carbonylation in skin tissue was observed, from a prospective of healthy protein protection. To address skin protein damage, the removal of damaged proteins is equally important. Previous studies indicated that the metabolic clearance of mis-modified proteins in cells and tissues is crucial for maintaining skin protein homeostasis [34] and autophagy plays a critical role in regulating the accumulation of damaged proteins, including misfolded and incorrectly modified ones [2]. For instance, water lily extract was reported to remove formed CML by stimulating autophagy in keratinocytes [32]. Therefore, we further investigated the clearance efficacy of Combo 1 by assessing the autophagy status of living cell treated with a combination of water lily extract, lactobionic acid, and artichoke leaf extract (referred to as Combo 2). We evaluated the effects of Combo 2 on the regulation of ATG7, LC3b and P62, which are well recognized autophagy markers and responsible for the elongation of autophagosomal membrane and their further maturation into autolysosomes [35–38], respectively.

The expression levels of ATG7 and LC3b in fibroblasts, and P62 in keratinocytes were visualized by immunofluorescence imaging, after the cells were stimulated by rapamycin (50 nmol/L, positive control of autophagy activation) [38] or the Combo 2 (Fig. 4). Results show that fibroblasts treated with the Combo 2 exhibited a significant upregulation of ATG7 and LC3b expression (Figs. 4a and b, Table S3 in Supporting information). Moreover, the expression levels of LC3b and the number of autophagosomes were even higher than those in the rapamycin-induced autophagy group (Figs. 4b and c, Table S3). Similar upregulation of P62 and an increase in autophysome amount were also observed in keratinocytes (Figs. 4d and e, Table S3).

These results demonstrate the effectiveness of Combo 2 as part of Combo 1 in promoting cellular autophagy, clearing damaged proteins, and maintaining protein quality homeostasis. This provides us an additional insight into the potential mechanisms of ingredients to enhance brightening appearance of skin under the stress by both resisting and removing protein damage.

Overall, we have developed a comprehensive method to assess the effects of various protein modifications together with the changes in skin appearance induced by multiple stimuli based on fluorescence imaging and analysis. By leveraging this new method, we demonstrated the protective effects of various ingredients against protein damage. The method would become a useful tool to assess the holistic impact of various stimuli and protective effects of ingredients on protein homeostasis and skin appearance in a comprehensive manner. Moreover, this work thoroughly demonstrates the application of fluorescence imaging and fluorescence analysis techniques in addressing one of the most challenging industrial problems. It will significantly elevate the application of fluorescence imaging as a sensitive, convenient, and rapid analytical technology in a broader industrial context.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## CRediT authorship contribution statement

**Wenjuan Liu:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Shanshan Zhang:** Writing – review & editing, Funding acquisition, Data curation, Conceptualization. **Yu Wang:** Writing – review & editing, Data curation, Conceptualization. **Bin Fang:** Supervision, Conceptualization. **Weirui Wang:** Conceptualization. **Shujing Song:** Data curation. **Tomohiro Hakozaki:** Writing – review & editing, Supervision, Conceptualization.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2025.111182.

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