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A bioinspired, photostable UV-filter that protects mammalian cells against UV-induced cellular damage

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While commercially available suncare products are effective at absorbing ultraviolet (UV)-light, recent studies indicate systemic toxicities associated with many traditional chemical and physical UV-filters. We demonstrate the application of xanthommatin, a biochrome present in arthropods and cephalopods, as an alternative chemical UV-filter that is cytocompatible while maintaining its photostability and photoprotective properties.

The skin is a complex, multi-layered organ comprising biomolecules such as DNA, proteins, 7-dehydrocholesterol, melanin, hemoglobin, and urocanic acid that have the ability to absorb a wide range of solar radiation (290-1440 nm).[1-4] Solar ultraviolet (UV) (290-400 nm) alone can generate enough energy to transition many of these biomolecules from their stable states to reactive, excited states. As a result, these changes can trigger a cascade of biochemical reactions,[2, 4-9] including the overproduction of reactive oxygen species (ROS),[10, 11] DNA mutations,[12] and the activation of inflammatory signalling pathways.[4, 13-17] UV-overexposure has also been linked to photoinduced aging via the absorption of UV-A (321-400 nm) photons by DNA, resulting in photosensitization and the generation of ROS and nitrogen species (RNS).[18] These events can initiate the activation of matrix metalloproteinases (MMPs),[1, 19] triggering the breakdown of collagen fibers, and ultimately causing the formation of coarse wrinkles.[20-22] In addition, these reactive species have been associated with swelling in the epidermis, depletion of Langerhans cells, and microvascular injury throughout the skin.[3, 23] Thus, there has been a great effort to mitigate these negative effects through the application of topical interventions, such as sunscreens.

Typical commercial sunscreens contain a combination of chemical and/or physical UV-filters.[24] Small molecules that absorb UV-radiation are known as chemical UV-filters; these compounds are generally conjugated systems that can readily absorb incoming photons. More specifically, chemical UV-filters are composed of one or more benzene rings and/or carbonyls that allow electron delocalization resulting in high molar absorptivity in the UV-A and UV-B (290-320 nm) regions. Out of the 16 U.S. Food and Drug Administration (FDA) approved UV-filters, avobenzone is the only chemical filter that provides long range UV-A protection (λ_{max} 360 nm).[25] Unlike the chemical filters, physical UV-filters such as titanium dioxide and zinc oxide particles are designed to block, scatter, and reflect UV-light to protect the skin.[26] Regardless of the type, both components have been reported to pose a threat to human health.[27-29] For instance, many chemical UV-filters in commercially available sunscreens can quickly absorb into the skin and interact with epidermal cells such as keratinocytes and Langheran cells.[30-32] Additionally, upon exposure to solar radiation, some compounds degrade into reactive by-products that have been implicated as contributors to reproductive and developmental toxicities in animals.[27, 33-35] More recently, commonly used UV-filters such as avobenzone, oxybenzone, octocrylene, and ecamsule have been detected in the bloodstream at concentrations beyond FDA standards, prompting their revaluation as human-safe materials by several regulatory agencies.[36] Furthermore, early iterations of physical UV-filters resulted in thick, white coatings that were aesthetically undesirable,[37] leading manufacturers to develop micro- and nano-sized particles that create more transparent films. However, reducing particle size increased skin penetration, contributing to local and systemic toxicities.[18, 26] Therefore, there is a need for new broad-spectrum filters that can minimize the negative effects of solar radiation while being safe to humans.

Bio-derived materials are great alternatives to commercially available UV-filters due to their innate cytocompatibility, structural complexity, photostability, and high molar absorptivity.[38-41] One example is the mycosporine-like amino acids (MAAs) isolated from marine cyanobacteria and algae, which have been explored as UV-filters due to their broad spectrum absorbance and photostability.[42-44] While these unconventional materials, and others like it, show great promise as sunscreens in formulations, their commercialization has seen limited success due to the continued dependence on the biological source coupled with historically low extraction yields.[45]

In this report, we explore the application and utility of an unsuspecting material, xanthommatin (Xa), as an alternative UV-filter. Both natural and synthetic forms of Xa provide a broad absorption and scattering profile that spans UV through short-wave infrared regions (λ_{max} = 360 and 480 nm).[46, 47] Given these features,

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we hypothesize that Xa could be repurposed and packaged as an alternative UV-filter. The rationale is that the photostability innate to Xa can mitigate UV-induced cellular damage by absorbing and dissipating a broad spectrum of solar radiation. In this study, we investigated the cytocompatibility and bioactivity of soluble Xa and Xa-based coatings *in vitro* as a broad-spectrum UV-filter. An added, unexpected feature is that Xa also behaves as a free radical scavenger, suggesting its utility in both suncare protection and as a potential therapeutic agent.

As a phenoxazone-based biochrome abundant in arthropods and cephalopods, Xa contains a variety of functional groups such as carbonyls, amines, hydroxyl groups and unsaturated regions (**Figure 1**). Given these features, we tested whether Xa could be used as a new UV-filter, where we began by evaluating the photoprotective properties of soluble Xa. We measured the UV-absorption of Xa (0.03-1.00 mM) and observed a broad profile that increased in intensity as a function of concentration (**Figure 1A, Supplementary Figure 1**). Next, we integrated the spectral absorbance curve from 290 to 400 nm to identify the effective critical wavelength. According to the FDA, UV-filters can be considered "broad-spectrum" if they have a critical wavelength of 370 nm.[25] We observed that Xa has a critical wavelength of 385 nm, indicating it can be considered as a broad-spectrum UV-filter. From the absorption profiles in **Figure 1A**, we calculated the sun protection factor (SPF) of Xa at varying concentrations (see supplementary information for details). We measured a concentration dependent SPF that ranged from 1 ± 0. to 18 ± 1 (**Figure 1B**), suggesting that Xa is a tunable UV-filter. The SPF values generated by Xa were also comparable to other commercially available UV-filters within this specified range (S**upplementary Figure 1**).

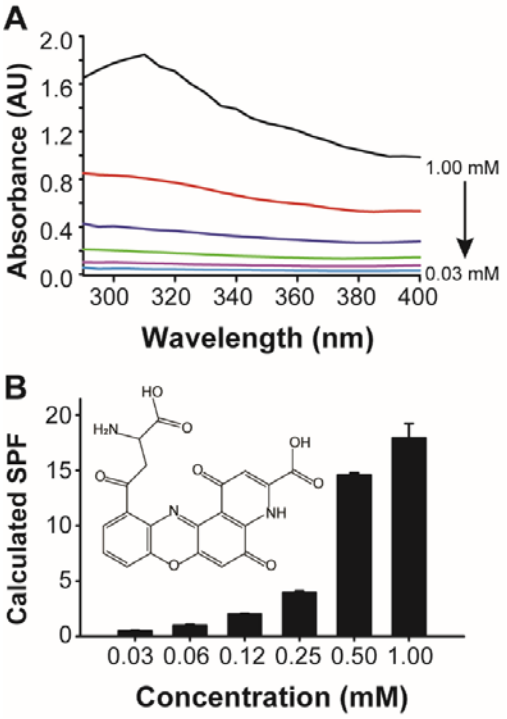

Figure 1. Tunable optical properties of Xa. A) Absorption spectra of Xa (0.03 – 1.00 mM) in the UV-region. Solutions tested in PBS, pH 7.4. **B)** The concentration dependent SPF of Xa as calculated from the Sayre derived Mansur equation. Values represent mean ± SD (n= 3). Inset is the non-ionized chemical structure of Xa.

Given its UV-filtering capability, we next characterized the photostability of Xa both with and without application of solar irradiation. To test this, thin films were first constructed by varying Xa concentration from 0.30 to 5.00 mg/cm^2 on UV-transmitting PDMS substrates (**Figure 2A**). Next, these films were analysed using a standard plate reader, where the PDMS films were used as the plate cover over a standard 96-well plate. From the measured absorption profiles, SPF values of Xa films were calculated, exhibiting a range from 3 ± 0 to 20 ± 2 (**Supplementary Figure 2**). Like the solution-based experiments, Xa films exhibited SPF values that increased based on the concentration used during fabrication.

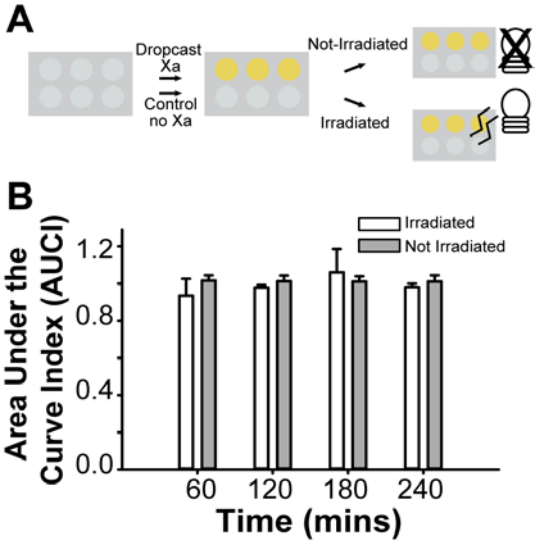

Figure 2. **Xa as photostable coatings.** A) An illustration of Xa-based coatings and controls prepared on imprinted PDMS substrates. Photostability was tested both with and without application of solar radiation. **B)** Solar simulated light (820 W/m^2) was applied to the coatings for 240 mins, and the AUCI was calculated at various time points. All irradiated samples were compared to non-irradiated samples. Values represent mean ± SD (n= 3).

Because Xa contains several auxochrome functional groups (e.g., hydroxyl, amino, aldehyde, carboxylic acid) that are known to enhance the photostability of UV-absorbing molecules,[48] we postulated that Xa would remain stable when subjected to solar radiation. To test this hypothesis, we monitored the photostability of Xa-coated PDMS films under solar simulated light. In this study, films comprising of 5.00 mg/cm^2 Xa were exposed to 820 w/m^2 total solar radiation. This is equivalent to a 4-hour global irradiance at solar noon on a clear day in spring in Phoenix, AZ. Absorption spectra were then measured every 30 minutes, and the area under the curve index (AUCI) was calculated for each condition. As shown in **Figure 2B**, the AUCI for Xa-coated PDMS films remained > 0.90 over the course of the 4-hour experiment which was similar to our solar radiation-free, control films. This set of data suggests that Xa is photostable within the window of maximum activity of topical sunscreens.[49]

As a first step in assessing its cytocompatibility, we performed a proof-of-concept study where Xa was incubated in a 2D cell culture model with murine fibroblasts (NIH 3T3). As fibroblasts are the primary cell type in the dermis, looking into their interactions with Xa *in vitro* will provide valuable insight into Xa as a safe material. To test Xa's cytocompatibility, cells were exposed to the concentrations used to define the SPF range (0.03-1.00 mM) for a 24-hour incubation period. As shown in **Figure 3** and **Supplementary Figure 3**, cell viability was found to be 94% ± 6% and 96 ± 5% as quantified by fixable dead and AlamarBlue assays, respectively. These data indicate that Xa treatment does not alter mammalian cell behavior,

with viability similar to the control group (Xa-free incubated cells) (**Figure 3A**). To further assess the impact of Xa treatment on fibroblasts, we looked at cell morphology and proliferation using confocal microscopy. Following a 24-hour incubation, Xa treatment at 1.00 mM did not alter cell morphology, as the elongated and spindle-shaped cell characteristics were comparable to untreated cells (**Figure 3B**). Additionally, cell proliferation as well as cell number and confluency in both treated and untreated conditions were comparable. These results suggest that Xa is cytocompatible at UV-protective concentrations.

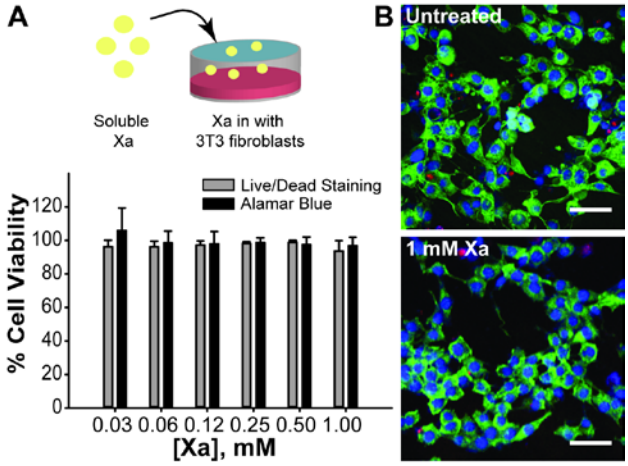

Figure 3. Cytotoxicity of Xa monitored *in vitro*. A) The cytotoxicity of Xa was monitored by the AlamarBlue and fixable dead cell staining assays. In both assays, the cell viability was measured after 24 hours incubation of 0.03 to 1.00 mM Xa with fibroblasts. The cell viability was determined to be statistical similar between the two assays except for the 0.03 mM condition ($p < 0.05$). Data represents mean ± SD from both assays (N=9 per concentration). **B)** Confocal microscopy images of cells cultured for 24 hours in 0mM (untreated) and 1.00 mM Xa. Here, cell nuclei were stained with DAPI (blue), dead cells with far-red staining (red), and actin with AlexaFluor 488-phalloidin (green). Confocal images are representative of n =9 samples per condition. Scale bar = 50 µm.

Next, the photoprotective activity of Xa-based coatings was evaluated at a fixed concentration representing SPF = 19 ± 4 (**Supplementary Figure 4**). In this study, cells were irradiated for 30 minutes with 73 W/m^2 UVR, representing 11 MED for skin type III (see supporting information for details) in the presence of Xa-containing and Xa-free PDMS films. Upon irradiation, we measured the concentration of pyrimidine (6-4) pyrimidone photoproducts (6-4PP), a biomarker of UV-induced DNA damage by ELISA.[6, 7] As expected, the Xa-based coatings substantially reduced UV-induced cell damage, where we observed a 46 ± 20% reduction of 6-4PP when compared to unprotected cells (**Figure 4A**). Altogether, these results indicated that Xa provided broad-spectrum protection against the harmful effects of UV-light in our experimental setup.

Due to its unique structural complexity, photostability, and photoprotective properties, we explored whether Xa had additional features to protect mammalian cells. To that end, we investigated Xa's antioxidant activity *in vitro* using the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) assay, where we compared its antioxidant activity with vitamin C (ascorbic acid), a commonly used antioxidant.[11, 50-52] Based on our findings, the half maximal effective concentration (EC_{50}) of Xa was found to be 1.00 mM. In comparison, ascorbic acid's EC_{50} was found to be 0.13 mM as shown in **Figure 4B**. Although the antioxidant capacity of Xa is not as strong as ascorbic acid, it is significantly higher than avobenzone and oxybenzone, the two main commercially available sun blockers (**Supplementary Figure 5**).

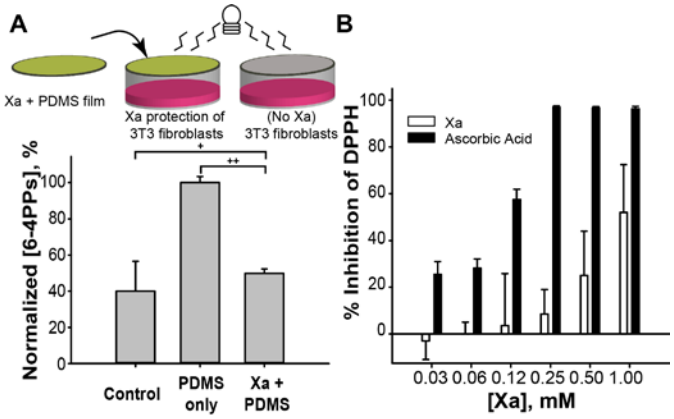

Figure 4. Bio-activity of Xa monitored *in vitro.* A) The bio-activity of Xa as an UV-protectant was evaluated by ELISA. The percent of normalized [6-4PPs] corresponds to the relative concentration of 6-4PPs in samples covered with PDMS substrates (uncoated) compared to Xa-coated PDMS substrates and control (not irradiated) samples. Data was analysed by Student's two tail t-test where + denotes statistical similarity, p=0.453, and ++ denotes statistically significant difference, p =0.003. Values represent mean ± SD (n= 6). **B)** The inhibitory effect of DPPH by a commercial antioxidant ascorbic acid was compared to Xa at concentrations ranging from 0.03-1.00 mM. Values represent mean and SD (n= 3).

In this study, we found that Xa is a potent UV-absorbing compound. As a material, Xa is photostable and provides broad-spectrum UV-R protection over multiple hours. When tested *in vitro* with fibroblasts, Xa is safe and cytocompatible. Finally, with the added, unexpected antioxidant activity, we believe Xa could potentially be applied to prevent oxidative skin damage in future formulations. Still, additional work is required to investigate absorption/skin permeation, dermal and mucosal membrane irritation, skin sensitization, and chronic genetic-, photo-, and geno-toxicities in animal models similar to previous reports.[32, 53] For now, our findings support the application of biomaterials like Xa and its derivatives in the design of next generation suncare products.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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