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The Protective Role of Natural Melanin Nanoparticles Under UV-C Exposure

Doğal Melanin Nanoparçacıklarının UV-C Maruziyeti Altındaki Koruyucu Rolü

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Abstract

Ultraviolet-C (UV-C) irradiation cause serious cellular and molecular tissue damage or ocular problems when it directly exposed to living organisms without filtering. Melanin is an organic UV absorbing biomaterial, that has no cytotoxicity or any harmful effects on human health. This study focused on UV-blocking ability of natural melanin nanoparticles (MNP) against the damaging effects of UV-C irradiation on the gram-negative bacteria *Escherichia Coli* (*E. coli*). Experiments showed that existence of MNPs enhanced cell viability of *E. coli* bacteria when exposed to different application time of UV-C irradiation.

Keywords: Melanin nanoparticles; UV-C irradiation; Nanomaterial; UV protecting agents

Öz

Ultraviyole-C ışıma filtre uygulanmaksızın doğrudan yaşayan organizmalara maruz bırakıldığında ciddi hücresel ve moleküler doku hasarına yol açmakta ve oküler rahatsızlıklara sebep olmaktadır. UV soğurma özelliğine sahip ve organik bir biyomalzeme olan melanin, herhangi bir toksik özellik içermemekte ve insan sağlığına olumsuz etkisi bulunmamaktadır. Bu çalışma doğal melanin nanoparçacıklarının (MNP) UV engelleyici özelliğini kullanarak, UV-C ışımasının gram negatif *Escherichia Coli (E. coli)* bakterisi üzerindeki zarar verici etkisinin azaltılmasına odaklanmıştır. Yapılan deneyler ortamda MNP'lerin varlığının farklı UV-C ışıma sürelerine maruz kalan *E. coli* bakterilerinin hayatta kalmalarını arttırıcı etkisi olduğunu göstermiştir.

Anahtar Kelimeler: Melanin nanoparçacıkları; UV-C ışıma; Nanomalzeme; UV koruyucu ajanları

I. INTRODUCTION

Nowadays, the need in effective UV-shielding agent has increased in construction, paints, coating, packaging, cosmetic and biomedical industries [1]. Recent advances in the development of new nanoscale and multifunctional materials like nanoparticles, nanowires, nanotubes and/or thin films with the help of nanotechnology have brought a new dimension to UV-shielding technology. To date, significant progress has been made in the production of photo-stable nanoparticles like ZnO, TiO_2 [2-5]. However, with the changing world standards, researchers have started to seek for new sustainable, biocompatible, and biodegradable nanoparticles produced with environmentally friendly techniques and their adaptation in applications instead of nanoparticles produced by wet chemistry, which are harmful to the environment and cause high amount of solvent waste.

Melanin, produced in melanosomes, is a well-known pigment with free radical scavenging ability, antioxidant activity, electronic-ionic hybrid conductivity, metal-ion chelation, redox activities, and electrical stability, most importantly their photoprotective ability by absorbing the light in the visible and UV regions of the electromagnetic spectrum [6-11]. It is found in many living organisms such as bacteria, fungi, plants, catfish, and cuttlefish ink. Among these, melanin nanoparticle can easily be extracted from cuttlefish ink with a simple centrifugation and washing process without the need for performing extraction or purification steps using harsh chemicals [12]. Natural melanin nanoparticles (MNPs) have been started to take place of metallic and metal oxide nanoparticles as a "green and sustainable" alternative [13-15]. Herein, natural melanin nanoparticles with excellent biocompatibility and biodegradability were prepared via centrifugation from cuttlefish ink. We attempted to extract

nanoparticles with sustainable and low-cost way, which has potential application as UV-shielding agent. Then, these nanoparticles were incubated with a commensal gram-negative bacterium, *E. coli* [16,17]. Protection efficacy of MNPs were analyzed after UV-C irradiation with different exposure durations.

II. MATERIALS AND METHOD

Preparation steps for experiments delineated in Figure 1 starts with design of UV-C light source, extraction of adequate amount of melanin nanoparticles stock and bacterial cultivation of *Escherichia Coli (E. coli)* on TSA. A set of experiments were applied for varied groups incorporating control group, MNPs solution and the mixture of bacterial solution and MNPs solution. Following that cell viability was observed and analyzed for each experimental group under different UV-C exposure time.

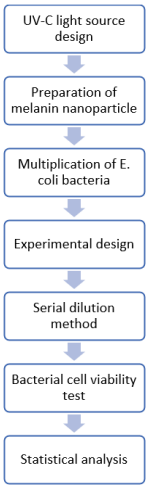

Figure 1. Block diagram of the experimental steps

a) Personal Protective Equipment

The ISO 15858 Standard shares the necessary safety information for humans and other living organisms to use devices incorporating UV-C. According to the ISO 15858 – UV-C Devices – Safety Information Standard [18], UV-C irradiation may result in serious cutaneous or ocular damage on human body when it directly contacts with skin or eye without filtering. Unprotected and unfiltered exposure to UV-C increases the risk of developing skin cancer arisen from DNA and RNA damage [19]. Furthermore, long-term eye exposure to UV-C may lead to partial and permanent blindness [18]. Regarding the ISO 15858 standard, it is extremely important to use the necessary personal protective equipment which consist of EN 166 (personal eye protection) and EN 170 (UV filters) approved goggles, a non-porous glove with irradiation protection up to 400 nm of wavelength, clothing covering exposed skin known not to be transparent to UV-C penetration [18] as shown in Figure 2. Moreover, warning signs should be placed for environmental protection. During this study, all personal and environmental protection were applied in accordance with ISO 15858 standard directives.

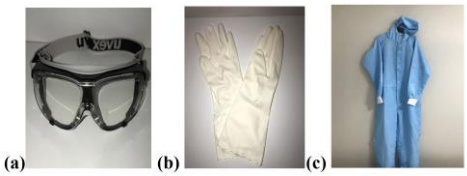

Figure 2. Personal protective equipment provided for use in the project. (a) An EN 170 (UV filters) approved goggle. (b) non-porous glove. (c) UV protective coverall.

b) UV-C LED Light Source Design

Light emitting diode (LED) based ultraviolet (UV) lamps became a popular instrument in many industries, since they have mercury-free nature, low cost, small size and longer lifetime compared to traditional UV lamps [20]. Therefore, surface mounted device (SMD) 3535 UV-C LEDs were supplied from Secol Elektronik as UV-C sources that are in the wavelength range of 270-280 nm. As demonstrated in Figure 3, application-specific 3X3 UV-C LED array matrix was designed for a 24-well plate after soldering process and was fixed on the aluminum plate to prevent excessive heating during use. The designed light source was operated in 49 V and the current passing through one LED was about 0.28 A.

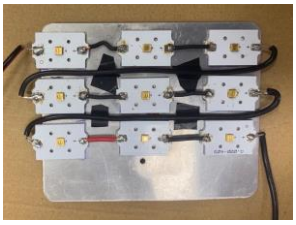

Figure 3. The designed 3X3 matrix UV-C light source.

c) Extraction of Natural Melanin Nanoparticles

In this study, natural melanin nanoparticles extracted from commercially available cuttlefish (*Sepia officinalis*) ink according to a method described by Jakubiak et al., indicated in Figure 5 [21]. Firstly, the cuttlefish ink (Ekol Food Products) was diluted five times with distilled water and adequate amount of melanin stock solution was obtained. The resulting melanin stock was re-diluted five times and centrifuged at 10000 rpm for 20 minutes. The supernatant was removed. Deionized water (DIW) was added onto the pellet to remove salt and impurities and solution mixed with a vortex device for 1 minute. This washing and centrifugation process was repeated 5 times. At the end of process, the pellet was left in the oven to dry for 1-2 days at 80°C.

Figure 4. The extraction steps of natural melanin nanoparticles.

d) Dynamic light scattering analysis

The size distribution of melanin nanoparticles before and after UV-C exposure for 10 minutes was measured by a Zetasizer (Nano ZS, Malvern, Worcestershire, UK).

e) Bacterial Strain

Escherichia coli ATCC (American Type Culture Collection) 25922 strain was used in this study. Bacteria were streaked diluted from frozen stocks stored at -80°C. The single colony of the bacterial strain was added on Tryptic Soy Agar (TSA) via streaking and incubated at 37°C overnight. After an overnight incubation period, a single colony was selected and removed from TSA to inoculate in 5 mL Tryptic Soy Broth (TSB) and cultured overnight at 37°C on an orbital shaker (180 rpm). The bacterial suspension was centrifuged and the supernatant TSB was removed. The pellet (bacteria) was dissolved in phosphate-buffered saline (PBS) to obtain units / mL (CFU / mL) forming approximately 10^8 for the bacterial strain.

f) Installation of the UV-C Irradiation Set

In the UV-C irradiation system, the light sources are positioned perpendicularly to the plate. The distance between the plate surface and the light source was kept constant at 1 cm. Irradiation from each LED was exposed to single well of a 24 well plate and only 9 wells of the plates were irradiated by the UV-C LED light source which has 9 UV-C LEDs in total (Figure 5). The application time for bacterial inactivation were selected in different time intervals including 10 seconds, 1 minute, 5 minutes, 15 minutes, and 30 minutes.

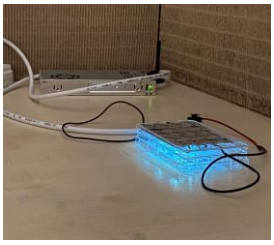

Figure 5. UV-C LED light source application

g) Experimental Design

In this study, the effects of the combined use of MNPs and UV irradiation on bacteria (*E. coli*) were investigated with 4 different groups as follows;

• Control Group: Bacterial solution is neither treated with nanoparticles nor UV-C irradiation.

• Melanin Group: Bacterial solution is only incubated with melanin nanoparticles.

• UV Group: Bacterial solution is only exposed to UV-C irradiation.

• UV+Melanin Group: Bacteria solution is both incubated with melanin solution and exposed to UV-C irradiation.

Melanin solution in 100µg/ml concentration was prepared and sonicated overnight using bath-type sonicator before the experiments. Each group was replicated with 3 samples on 9-well plates. For Melanin and UV + melanin group applications, 250 µl bacterial solution and 250 µl MNP solution of various concentrations were mixed in a well of 24-well plate and incubated for 4 hours. Then UV irradiation was applied for 10 seconds, 1 minute, 5 minutes, 15 minutes and 30 minutes for UV and UV + melanin group. A different set of experiments was prepared for each parameter change.

h) Colony Counting Method via Serial Dilution for Bacterial Cell Viability Analysis

To detect the number of bacteria that remained alive after each application, serial dilution method was used. Since there are too many bacteria to count in the original sample, the dilution process is carried out gradually. The dilution factor of this solution is 1/10 to calculate the number of bacteria in the wells. 20 µl of the treated sample were taken and combined with 180 µl of PBS in one well. Then 20 µl was taken from this well and combined with 180 µl PBS in the other well. This process was repeated gradually until the desired dilution was reached. 100 µl of diluted solutions were poured onto TSA containing petri dishes and incubated at 37°C overnight. At the end of this process, bacteria became visible as shown in Figure 6. After counting the number of bacteria on the agar, it was multiplied by the dilution factor and the number of viable bacteria was determined in each original sample at the end of the applications.

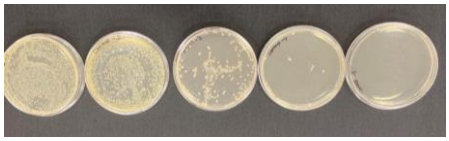

Figure 6. Bacterial colony counting after serial dilution.

i) Data and Statistical Analysis

Student t-test was used to compare whether there was a statistically significant difference between the control and experimental groups, and when this value was less than 0.05, the results were considered as significant.

III. RESULTS

a) Dynamic size distribution characterization

Dynamic light scattering analysis points out that the average size of melanin nanoparticles was found 248 nm with a standard deviation of 3 nm. After UV-C exposure, the size of melanin nanoparticles was slightly decreased to 213 ± 7 nm.

b) Cellular Protection at 100 µg/mL Melanin Concentration

The cellular protection effects of 100 µg/ml melanin concentration on *E. coli* under UV-C irradiation at different times were investigated. In general, all cells in both the UV-C group and the UV-C + 100 µg/mL melanin concentration groups were inactivated under 15 and 30 minutes of UV-C exposure. For this reason, it is not possible to comment on the properties of melanin nanoparticles at the irradiation times of 15 and 30 minutes. When 5 minutes and 1 minute of UV-C irradiation was applied to the experimental groups, the decrease in cell viability was 99.9% in bacterial cell count in UV Group as the same as preceding UV-C exposure durations. On the other hand, the cell viability-enhancing effect of melanin nanoparticles was observed at both 5 minutes and 1 minute UV-C irradiation in UV+Melanin Group (Figure 7-8).

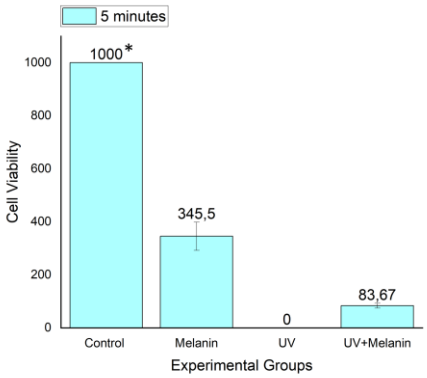

Figure 7. Effect of MNPs on mean cell viability of *E. coli* bacteria under UV-C irradiation for 5 min.
(*cell viability of control group was outnumbered; therefore, an assumption was applied.)

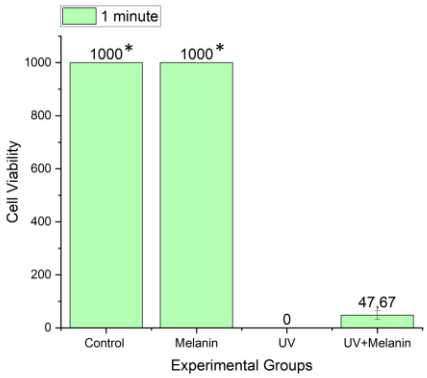

Figure 8. Effect of MNPs on mean cell viability of *E. coli* bacteria under UV-C irradiation for 1 min.
(*cell viability of control and melanin group were outnumbered; therefore, an assumption was applied.)

As a result of 10 seconds of UV-C irradiation applied to the UV groups, the decrease in cell viability was significantly decreased. However, an increment in bacterial cell viability was observed for UV+Melanin group compared to UV group (Figure 9).

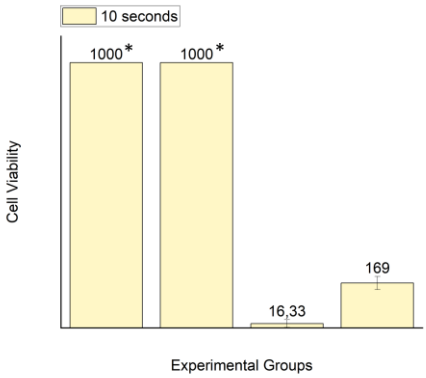

Figure 9. Effect of MNPs on mean cell viability of *E. coli* bacteria under UV-C irradiation for 10 sec.
(*cell viability of control and melanin group were outnumbered; therefore, an assumption was applied.)

IV. DISCUSSION

In the experiments using UV-C LED light source, 99.9% bacterial photoinactivation was observed in the experimental groups that were exposed to UV-C irradiation on *E. coli* bacteria. However, when UV-C irradiation was applied to the experimental groups in which, bacteria were incubated with MNP, an increase in cell viability was observed compared to the UV-C experimental group alone. In other words, MNPs exhibited the protective feature of microorganisms in the irradiation with UV-C LED. The set of the experiments for observing the bacterial photoinactivation of microorganisms using the combination of UV-C light source and melanin is insufficient. Nevertheless, we clearly observed the protective effect of melanin nanoparticles on bacteria after UV-C exposure and its efficacy increased by decreasing the irradiation time. As a future perspective, we aim to continue this study by changing the parameters of not only the exposure time, but also the concentrations of the melanin nanoparticles to determine the exact ranges that exhibit protective effect. Besides, we will continue to study the protective effect of these nanoparticles on healthy human cells such as fibroblasts or keratinocytes which are the primary cells exposed to UV-irradiation in daily life and have crucial roles in the skin against UV-C light.

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