Melanin Treatment Effect of Vacuoles-Zinc Oxide Nanoparticles Combined with Ascorbic Acid

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Abstract
Currently, ascorbic acid (AA) is widely used as a skin whitening material, but, AA, an unstable hydrophilic molecule, cannot penetrate the skin easily, due to the hydrophobic character of the stratum corneum. Therefore, we conjugated AA with hydrated zinc oxide—an inorganic matrix with positive surface charge, to improve the stability of AA. The metal-conjugated-ascorbic acid (ZnAA) was then combined with yeast vacuole through the vacuolar membrane proteins that relate to metal transportation to create an enhanced vacuole that contained ZnAA. The characteristics of vacuole with ZnAA (ZnAA_Vac) were next examined by various tests that included X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT–IR), Field emission scanning electron microscopy (FE–SEM), and energy-dispersive X-ray (EDX) analysis. Furthermore, the ability of ZnAA_Vac to degrade melanin was confirmed in both melanoma cell line B16F10, and the artificial human skin MelanoDerm. The results showed that ZnAA_Vac possessed a higher depigmenting effect than the wild-type vacuole or ascorbic acid by reducing 75% of melanin color. Interestingly, ZnAA_Vac was found to be harmless, and did not cause any cytotoxicity to the cells. Overall, ZnAA_Vac is expected to provide a robust, harmless, and effective whitening agent for the skin.

Keywords Nano particles · Ascorbic acid · Metal conjugation · Vacuole · Depigmentation · Human artificial skin

Introduction
Melanin is one of the main factors that regulate the pigmentation in human skin, besides carotenoids and hemoglobin [1]. There are two main types of melanin: pheomelanin (yellow/red soluble polymer) and epidermal melanin (black/brown insoluble polymer) [2, 3], which is the principal melanin synthesized in melanosome inside the melanocytes in the skin [4]. Even though melanin could protect the skin from the effect of DNA photodamage by absorbing and scattering UV rays, which reduces their penetration through the epidermis [5], overproduction of melanin could cause several skin pigmentation disorders, which include melasma, post-inflammatory melanoderma, and dark spot on the skin (freckles) [6]. In recent years, substantial investment has been made in the skin whitening cosmetics industry,

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and despite the impact of Sars-CoV-2, the global market for skin whitening products in 2020 was appraised at around 8.6 billion dollars, and by 2027, may reach 12.3 billion dollars [7]. Nevertheless, skin lightening agents could also harm human health, including inducing high blood pressure, kidney disease [8], dermatoses [9], or more seriously, cancer [10]. Thus, a novel bleaching agent is needed that could degrade melanin without adverse effects on human health.

Ascorbic acid (AA) is a natural water-soluble vitamin (vitamin C) and a nutrient that is necessary for several processes in the human body [11]. First, AA is a potent reducing agent and antioxidant that decreases endothelial permeability, enhances vascular function, and weakens cellular apoptosis in the pathological phase [12]. In addition, as an antioxidant, AA is an essential factor that protects the skin from free radical damage, pollutants, toxins [13], and is involved in a collagen synthesis, which is crucial for curing injury [14]. Interestingly, AA is also a depigmenting factor that can reduce melanin by inhibiting the tyrosinase—an important enzyme involved in the melagenesis process [15]. Most mammals could synthesize AA in the liver, but the non-functional l-gulono-gamma lactone oxidase (GULO) gene means that human can only absorb vitamin C by oral supplementation [11, 16].

In this study, we report a novel potential whitening agent that combines AA and zinc nitrate hexahydrate with yeast vacuoles that we have invented. AA is an unstable hydrophilic molecule, and cannot penetrate the skin easily, due to the hydrophobic character of the stratum corneum [17]. Therefore, the conjugation of AA with other agents, such as hydrated zinc oxide (Zn(NO3)2·6H2O)—an inorganic matrix with positive surface charge, could improve the stability of AA to overcome its limitation in the field of cosmetic [18]. Here, we developed an encapsulating approach of AA from the work of Yang et al. with a modification using yeast vacuoles [18]. Lysosome-like-vacuole in yeast was previously discovered to degrade the melanin [19, 20], and the combination of yeast vacuole and AA could strengthen the original AA to create a powerful whitening agent. In this study, a synthetic material containing ascorbic acid and zinc ion was achieved by a coprecipitation reaction. The characteristics of vacuole with ZnAA (ZnAA_Vac) were next examined by various tests that included X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT–IR), Field emission scanning electron microscopy (FE–SEM), and energy-dispersive X-ray (EDX) analysis. And, the melanin reduction test was conducted with melanoma cell line B16F10 and the three-dimensional human skin model MelanoDerm from MatTek.

Interestingly, yeast vacuole is the primary site for zinc sequestration, and has two vacuolar zinc transporters Zrt3 and Zrc1, which are known to be involved in zinc’s transportation vacuole [21–23]. Therefore, zinc ion in the synthesis product would drive the conjugation zinc ion-ascorbic acid (ZnAA) to the vacuole, which technique could be used to carry drug [24, 25]. In summary, we have created a novel bleaching agent that could reduce melanin using vitamin C, zinc ion, and yeast vacuoles. The nanoparticles were nontoxic, stable and suitable for human skin treatment.

Materials and Methods

Cell Culture and Materials

B16F10 (KCLB 80080) cells were grown at 37 °C under 5% CO2. The cells were cultured in RPMI 1640 medium adding 10% NCS (Newborn calf serum, Cat No: 16010-159, GIBCO), 5 mM glutamine (Cat No: 56-85-9, Sigma Aldrich), 25 mM HEPES (Cat No: 7365-45-9, Sigma Aldrich) and 1% penicillin–streptomycin (Cat No: 15140-122, ThermoFisher). The media was exchanged every conducive day, and we treated ZnAA as the cells were grown at about 70%.

Saccharomyces cerevisiae s2805 (ATCC 208280) was kindly provided by the Korea Research Institute of Bioscience and Biotechnology (KRIIBB). S. cerevisiae was grown to mid-log phase in YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 30 °C in a shaking incubator at 180 rpm.

Encapsulation of Ascorbic Acid by Co Precipitation with Zinc Nitrate Hexahydrate (Zn(NO3)2·6H2O)

0.05 M zinc nitrate hexahydrate (Zn(NO3)2·6H2O, Cat No: 10196-18-6, Sigma Aldrich) was dissolved in distilled water (DW), then 0.1 M l-ascorbic acid (AA, Cat No: 50-81-7, Sigma Aldrich) was added in metal solution. The pH of the reaction solution was calibrated to pH 6.7 by adding NaOH with forceful stirring. To reduce the disintegration of AA and air contamination during the synthesis, nitrogen gas was flowed continuously through the reaction solvent. A white suspension appeared, and was kept at room temperature for 12 h, before being filtered and washed by DW. Finally, a vitamin C-hydrated zinc oxide hybrid was prepared, and called ZnAA [18, 26]. Figure 1 shows the synthetic process.

The characteristics of ZnAA were analyzed multi-purpose high performance X-ray diffractometer (XRD, X’PERT-PRO Powder, Malvern Pananalytical, England) operating at 40 kV and 30 mA with Cu–Kα radiation (λ = 1.5404) for the fixed samples on a microscope slide. Furthermore, ZnAA was characterized by Fourier transform infrared spectroscopy (FT–IR, Perkin Elmer Spectrum GX, USA). Each FT–IR spectrum was collected at a resolution of 2 cm−1 from (4000 to 600) cm−1.
**Vacuole Isolation from *S. cerevisiae***

*Saccharomyces cerevisiae* was cultured in YPD medium at 30 °C for 24 h. Cells were harvested and mixed with 0.1 M Tris-SO₄ buffer (100 mM Tris-SO₄ (pH 9.4) and 10 mM dithiothreitol (DTT)). This mixture was incubated at 30 °C (90 rpm) for 10 min, followed by centrifugation at 3000 rpm for 5 min. The supernatant was discarded, and the pellet was mixed with lyticase for 1 h to make the cell membrane flexible. After that, the mixture was centrifuged at 3000 rpm for 5 min, and the pellet was washed twice with Sorbitol K⁺ phosphate buffer. For the next step, breaking buffer (20 mM Tris–Cl (pH 7.4), 0.6 mM Sorbitol, and 1 mM phenyl methane sulfonyl fluoride (PMSF)) were mixed with the pellet, followed by ultra-sonication at 40 W for 30 min (20 s on/10 s off pulse) on ice. The mixture was centrifuged at 3000 rpm for 10 min, and the supernatant was discarded. The pellet was remixed with breaking buffer, followed by a second ultra-sonication for 20 min (10 s on/10 s off pulse). After centrifugation at 500×g for 5 min, the supernatant was collected. The remaining pellet was subjected to a second centrifugation at 20,000×g for 30 min, and the pellet containing vacuoles from cells was collected [27].

**Liquid Chromatography–Mass Spectrometry (LC–MS)**

*Saccharomyces cerevisiae* was exposed individually to AA and ZnAA for 24 h. After exposing samples to the cells, the remaining AA and ZnAA in the medium would be determined. The analysis used column C18 (150 mm × 4.6 mm) which was maintained at 20 °C. The LC–MS data were collected, and processed by MassHunter software (Agilent).

**Field Emission Scanning Electron Microscope (FE–SEM) with Energy-Dispersive X-ray (EDX) Spectroscopy**

The morphology of ZnAA was observed by Field emission scanning electron microscopy (FE–SEM) (Carl Zeiss SUPRA, Germany). Samples were softened with DW, and were then prepared using the freeze-drying process [28]. While taking the image of ZnAA, electron was immediately analyzed by EDX spectroscopy.

**Melanin Degradation Test**

After being exposed to various samples, the melanoma cell line B16F10 was washed twice with Phosphate-buffered saline (PBS); after that, it was dissolved in NaOH with 10% DMSO, and heated at 80 °C for 1 h. The amount of melanin was determined by spectrophotometry at the absorbance of 400 nm [20].

**Skin Whitening Test with Human Skin Model MelanoDerm**

MelanoDerm (MatTek Corp., Ashland, NA) was used for skin whitening test as a viable three-dimensional human skin that includes normal melanocytes and keratinocytes. The cells are derived from African–American (MEL-B), Asian (MEL-A), or white (MEL-C) donors. MEL-B tissues were used in this study and were maintained in the NMM-113 medium. The MelanoDerm tissues were fed daily with 5 mL of fresh NMM-113 and various vacuole concentrations or ZnAA_Vac for 12 days [29].
Measurement of Melanin Content

Melanin content was measured as described in the previous work [29]. The skin tissue was taken out of the culture dish after 12 days, then moved in an Eppendorf tube and homogenized for 1 min in lysis buffer (20 mM Tris–Cl pH 7.4, 1 mM EDTA pH 8.8, 1 mM EGTA pH 8.5, 1% v/v Triton X-100). After homogenization, 20 μL protease K (5 mg/mL) was supplied, and the cells incubated at 45 °C overnight. The next day, 20 μL protease K was added for 4 h. Next, 50 μL 0.5 M sodium carbonate and 10 μL 30% H₂O₂ were added, and incubated at 80 °C for 30 min. After that, 100 μL of a 2 chloroform: 1 MトOH mixture was added, followed by centrifugation at 10,000×g for 10 min. The sample supernatant was used to determine melanin content by spectrophotometry at the absorbance of 405 nm.

Cell Viability

The cell viability test for MelanoDerm was described previously [30]. The tissues were washed twice by DPBS, before adding 300 μL MTT solution. All tissue samples were covered to protect from light and incubated 37 °C for 3 h. After being cleaned by sterilized gauze, the tissue samples were moved to a new plate, and then isopropanol and 0.04 M HCl were added. At the next step, the samples were stirred at 120 rpm without air in the anaerobic bag at RT for 2 h. Finally, the solutions were moved to an Eppendorf tube, and measured by ELISA plate reader on 96-well plates at the absorbance of 570 nm.

Data Analysis

All data were obtained from three independent samples, run simultaneously for error analysis, and the results are reported with the standard deviation. The data were analyzed using Sigma Plot (SPS, Chicago, IL. USA), and a p-value < 0.05 was considered significant.

Results

Characterizations of the Synthetic ZnAA

The properties of the synthesis product between ascorbic acid and zinc nitrate hexahydrate were analyzed by X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FT–IR).

Figure 1 presents the encapsulating process of AA with zinc ion, while Fig. 2 provides the XRD patterns that show the crystalline structure through the specific diffraction peaks of ZnO (peaks (002), (110), and (201) observed at 2θ = (34.1, 58.8, and 69.2)°, respectively), as reported previously [31–35].

Figure 3 displays the characteristics of ZnAA by FT–IR and FE–SEM. Figure 3a shows that there were strong absorption bands in the ascorbic acid sample at (1686 and 1773) cm⁻¹, which reflect the C=C and C=O stretching vibrations, respectively, while several bands from (1000 to 1500) cm⁻¹ are related to C–O–C, CH₂ scissoring, and other C–H modes, as reported previously [36, 37]. On the other hand, Fig. 3b depicts the FT–IR spectra of ZnAA in which a vibration band appears at 503 cm⁻¹, indicating the formation of ZnO. Additionally, the peak at 3452 cm⁻¹ shows O–H stretch, while peaks at (1397 and 1496) cm⁻¹ are due to C–O–H bending vibrations. These FT–IR spectra results are similar to previous studies that analyzed the characteristics of ZnAA or ZnO [32, 33, 38, 39].

Morphology of ZnAA with EDX Analysis

Figure 3C shows the morphology of ZnAA particle taken by FE–SEM, in which ZnAA is formed as a very tiny particle at (19–30) nm. Furthermore, Table 1 describes the ZnAA surface components using energy-dispersive X-ray (EDX) spectroscopy, where zinc ion is presented in the synthesis product, while not in the AA sample.

Vacuolar Targeting of ZnAA in Saccharomyces cerevisiae

Saccharomyces cerevisiae was exposed individually to AA and ZnAA for 24 h. After exposing samples to the cells, to verify the existence of ZnAA in vacuole, the remaining
ascorbic acid and ZnAA were measured by LC–MS. Table 2 shows that no ZnAA remained in the yeast cytosol and culture media, while (71 and 6.45) % AA remained in the media and cytosol, respectively. On the other hand, the 99.9% of ZnAA found in the vacuole affirmed that ZnAA was targeted to the vacuole as expected, while only 22.55% ascorbic acid was delivered to the vacuole. This phenomenon confirmed the role of zinc ion in the vacuolar targeting approach.

Table 1 The EDX data of ZnAA with ascorbic acid on the surface

| Element | Wt % | AA | ZnAA |
|---------|------|----|------|
| C       | 46.02| 3.43|      |
| O       | 53.98| 20.5|      |
| Zn      | 0    | 76.07|      |

Table 2 The LC–MS analysis data of AA and ZnAA after exposure to S. cerevisiae

|                      | AA (%) | ZnAA (%) |
|----------------------|--------|----------|
| Initial value        | 100    | 100      |
| The ratio in media   | 71     | N.A      |
| The ratio in cytosol | 6.45   | N.A      |
| Inflow ratio in vacuole| 22.55 | 99.9     |

N.A. no absorbance; AA ascorbic acid; ZnAA zinc ion-conjugated-ascorbic acid

Melanin Reduction in Melanocytes by ZnAA-Vacuole

In this experiment, the vacuole that contained ZnAA was used to treat melanocytes for the melanin reduction test, and called ZnAA_Vac. ZnAA_Vac were isolated from S. cerevisiae, and 5% (wt/vol) vacuoles were exposed to melanocyte for 24 h. “Wild-type” vacuole was used to compare with the activity of ZnAA_Vac, while 2% ascorbic acid was the positive control, due to its depigmenting effect [40, 41].
The result in Fig. 4 shows no distinction in the melanin-reducing effect between 2% ascorbic acid, vacuole, and vacuole that possessed 100 ppm ZnAA, which degraded approximately 24% of melanin. Meanwhile, vacuole with 1000 ppm ZnAA degraded around 75.5% melanin; thus, 1000 ppm ZnAA provided a higher reducing ability. In fact, ZnAA without vacuole also showed a high effect of degrading melanin; however, it also caused high cytotoxicity that killed many cells (Fig. 4a).

The depigmentation efficiency of ZnAA_Vac depicted in Fig. 5 was observed at day 0 and day 12 of the treatment. Figure 5a presents the effect of several vacuole’s concentrations from wild-type yeast on the artificial human skin after 12 h of treatment, while Fig. 5b shows the depigmenting effect of ZnAA_Vac. After treatment with (5 and 10)% vacuole or all samples exposed to ZnAA_Vac, the color reduction data were analyzed using Sigma Plot, and a p-value<0.05 was considered significant. ZnAA_Vac 100 ppm, ZnAA_Vac 500 ppm, ZnAA_Vac 1000 ppm: vacuoles that contained ZnAA at (100, 500, and 1000) ppm, respectively.
is clearly evident to the naked eye. Nevertheless, to precisely assess the depigmenting effect, melanin contents were measured after 12 days of treatment.

Figure 5c shows that 2% ascorbic acid exhibited a high depigmenting ability by decreasing around 33% of melanin, while the vacuole’s maximum effect was approximately 14% (vacuole (5 and 10)). Regarding ZnAA_Vac, vacuole that contained 100 ppm ZnAA did not show a high depigmentation effect; however, the melanin degradation increased along with the augmentation of ZnAA dose. More precisely, ZnAA_Vac with 500 ppm ZnAA reduced 31% of melanin, while ZnAA_Vac with 1000 ppm ZnAA provided a superb ability by degrading 75% melanin after 12 days. These results were identical with the melanin reduction test in melanocytes, in which the ZnAA_Vac 1000 ppm had degraded 75.5% melanin (Fig. 4).

Discussion

In this study, we combine AA with yeast vacuoles that we have invented, and to improve skin penetration when used as a cosmetic, we conjugated AA with hydrated zinc oxide (Zn(NO3)2·6H2O). Through the X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FT–IR) analysis, it was confirmed that the synthetic ZnAA (zinc ion-ascorbic acid) is consistent with the previously reported properties of ZnAA or ZnO [27, 28, 34, 35]. The formation of ZnO from the chemical precipitation of zinc nitrate hexahydrate, sodium hydroxide, and ascorbic acid was commonly investigated before in which vitamin C had occupied an essential role in ZnO crystal formation [32, 33, 42]. The formation of ZnO can also be confirmed through the reduction of carbonic ion in EDX analysis. To combine ZnAA with yeast vacuoles by internalization into the vacuole, we incubated yeast with ZnAA. As mentioned before, the vacuole is the primary site for zinc sequestration; therefore, the two vacuolar zinc transporters Zrt3 and Zrc1; thus, the conjugation ZnAA is expected to agglomerate at the vacuole [21–23]. Recently, considering the hazardous nature of the chemical synthesis of nano-particles, the current investigation is focusing on the green synthesis using eco-friendly biological extracts [43–46].

The melanin reduction test of ZnAA_Vac, the vacuole that contained ZnAA, was conducted with melanoma cell line B16F10 and the three-dimensional human skin model MelanoDerm from MatTek. The highest depigmentation effect of ZnAA_Vac in both melanoma cell lines and artificial human skin cells demonstrated our precise approach when conjugating ascorbic acid, Zn ion, and vacuole. After being conjugated with the vacuole, ZnAA became an efficient and harmless whitening agent, while individual vacuole or ascorbic acid displayed a lower effect, and ZnAA without vacuole caused high cytotoxicity that killed a lot of cells. Therefore, the encapsulation approach of ZnAA with vacuoles could provide a safer and more robust agent for melanin degradation. According to recent studies, the use of biological compounds for the synthesis of inorganic nanoparticles has shown promising results such as cost-effective and environmentally friendly compared to the conventional nanoparticles synthesis pathway [47–50]. Previously, it was reported that a combination of tyrosine, zinc and vitamin C has been shown to increase the bioavailability of vitamin C 20-times vis-a-vis using just vitamin C [51]. Like these researches, many studies using ZnO to improve the biocompatibility and stability of AA have been reported. However, in this paper, the whitening efficacy of these complex nanoparticles was verified using melanocytes and artificial skin tissue for the first time, therefore it includes novel and meaningful results can be used in the pharmaceutical and cosmetic fields.

In this study, vacuole as a carrier to protect and enhance the activity of metal-conjugated-ascorbic acid was reported for the first time. Ascorbic acid synthesized with zinc ion was transported to vacuole through the vacuolar membrane protein, and then the vacuole that contained ZnAA was isolated, which we called ZnAA_Vac. ZnAA_Vac was found to be safe for the cell without cytotoxicity, and provided a considerable effect to degrade the melanin content in both melanocyte and artificial human skin cell.

Conclusions

In this study, vacuole as a carrier to protect and enhance the activity of metal-conjugated-ascorbic acid was reported for the first time. Ascorbic acid synthesized with zinc ion was transported to vacuole through the vacuolar membrane protein, and then the vacuole that contained ZnAA was isolated, which we called ZnAA_Vac. ZnAA_Vac was found to be safe for the cell without cytotoxicity, and provided a considerable effect to degrade the melanin content in both melanocyte and artificial human skin cells. The discovery of ZnAA_Vac could open up a new way to develop a state-of-the-art whitening agent that effectively reduce melanin, without damage to the skin, but in order to be commercialized, additional clinical and in vivo experiments must be conducted, and research on materialization is also required.

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