A promising protected ascorbic acid-hydroxyapatite nanocomposite as a skin anti-ager: A detailed photo-and thermal stability study

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ABSTRACT

A new ascorbic acid (AA) nanocomposite with low toxicity and high photo and thermal stability is constructed for certain dermatological applications in humans. The presented nanocomposite consists of AA, nano-hydroxyapatite (nHAp) and carboxymethyl cellulose (CMC). The physicochemical properties of such CMC-nHAp-AA nanocomposite were characterized using X-Ray diffractometry (XRD), Energy Dispersive X-ray (EDX) and UV–VIS spectroscopies. The size and morphology of the synthesized nanocomposites were characterized by TEM/SEM techniques. A detailed photo and thermal stability studies were performed to examine the stability of AA in the proposed nanocomposite. The AA content showed great stability against sunlight up to 3 h or more and against heat up to 100 °C, whereas it showed relatively limited stability against laser light up to 10 min depending on the laser type. Cytotoxicity endpoints, evaluating the cell viability and IC50 (50% inhibitory concentration) have been performed for the exposed synthesized nanocomposite. There wasn’t any effect on the cell viability up to 50 μg/mL of CMC-nHAp-AA nanocomposite. Based on IC50 values, it has been found that after 24 h of observation the IC50 of CMC-nHAp-AA nanocomposite was 0.199 μg/mL which depicts high safety profile of the proposed nanocomposite. The produced nanocomposite (CMC-nHAp-AA) is expected to possess great potential in dermatological applications due to its high stability and increased proliferative capacity which lasts longer than AA alone.

1. Introduction

Collagen is a key component of the skin that maintains its firmness and suppleness. But it slowly breaks down over time, depending upon environmental circumstances, genetic factors and lifestyle choices, which cause skin aging. Fibroblasts are skin cells responsible for the production of collagen and replacing damaged fibers with new ones. With age, the skin’s ability to repair itself and building new cellular structures attenuates and finally ends with skin dryness, appearance of fine lines, wrinkles and sagging.

For decades, it has been a common practice throughout the world the use of vitamin C, ascorbic acid (AA), in cosmetics preparation, skincare blends as well as treating hyperpigmentation. AA has a critical role in collagen synthesis and stimulating the proliferation of fibroblasts and, in general, has beneficial effects on human health. It serves as a co-factor of hydroxylase enzyme of proline and lysine which elevates type I and type III procollagen mRNA levels in fibroblasts and stabilizes collagen triple helix. Additionally, AA neutralizes the effects of free radicals generating ubiquitous within our bodies due to overexposure to UV radiation, environmental pollutants and stress.

Thus, AA is considered to be an outstanding anti-ager which has the ability to counteract wrinkles and slow down the aging process. However, the ascorbic acid suffers from numerous drawbacks; mainly light, heat, oxygen, moisture conditions and storage over time affect the stability of AA and makes it prone to damage easily and decomposing into inactive compounds such as 2,3-diketo-l-gulonic acid, oxalic acid, l-threonic acid, l-xylonic acid and l-Lyxonic acid. Therefore, the applications of AA in the fields of cosmetics, dermatologicals, and pharmaceuticals are restricted although of its valuable functions.

A lot of attempts and efforts have been continuously directed towards improving the AA stability through its encapsulation and immobilization or delivering it as a salt. However, most of such trials ended in disappointing results or have been poorly successful. Abbasa et al. found that microencapsulation could improve the stability of AA. The most common encapsulation techniques are liposomes, spray drying, spray chilling and extrusion, which have many applications in food industry. Sheraz et al. performed an attempt in which AA was encapsulated in combination with vitamin E but the outcomes did not have neither the same effects as that of the initial

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compound nor its biological activity. Another attempt reported that ferulic acid may provide increased stability to a solution of AA combined with vitamin E [32]. Noteworthy, yet the above described problem remains unsolved, therefore prevents the reaping of countless benefits of AA which were encouraging enough to merit further attempts and efforts. As a consequence, we suggested a nano-delivery system meeting the requirements for the retention of AA stability and overcoming its drawbacks, besides taking into account the accepted specifications for the cosmetic products in terms of the sensory attributes of the produced formulae (i.e. viscosity, texture, color, odor… etc.), which ensure an optimal outcome for achieving satisfactory results. The proposed nanocomposite, abbreviated by “CMC-nHAp-AA”, consists of three components; a) the active ingredient AA, b) the nanovehicle; nano-hydroxyapatite (nHAp) which is a safe and non-toxic material used to deliver AA to skin cells and enhancing collagen formation, and c) a protective biocompatible coating matrix; carboxymethyl cellulose (CMC) that protects AA against oxidative damage.

Hydroxyapatite (HAp) is effectively used in medical and biological applications because it closely resembles bone apatite, exhibits good biocompatibility showing no toxicity compared to other nanoarchitectures used in cosmetics such as titanium dioxide (TiO2) and zinc oxide (ZnO) [33]. HAp as a biomedical material was extensively used as bioactive coating, bone filler, soft tissue repair, and drug delivery system [34–37].

CMC was suggested as a suitable protective coating matrix for AA after running a set of careful comparisons among number of polymers. The reason behind selecting CMC is that, it is non-toxic, odorless and makes the product has a colorless nicely smooth texture without gelling even in the presence of Ca2+ ions [38]. Additionally, CMC has been extensively used for cosmetic purposes such as body creams and lotions because its excellent water retaining capacity which makes it ideally suited to keep skin moisturized and hydrated [39]. Thus, CMC offers an adequate protection against the presumable oxidation and possess supportive effect for cosmetics.

In the current study, we report on the synthesis of new AA-nanocomposite of high stability against light and heat. The cytotoxic effect of the synthesized nanocomposite and the proliferation rate of adult human dermal fibroblasts cells were investigated. The presented nanocomposite provides a skin rejuvenation approach as an anti-aging drug and sheds light on the immense therapeutic potential of AA as anti-free-radicals.

2. Experimental Section

2.1. Materials

All solutions of reacting materials were prepared using deionized water provided by a Milli-Q water purification system. All of glassware was washed with aqua regia solution (HCl, HNO3 = 3:1 (v/v)) and rinsed with Milli-Q water. L-Ascorbic acid (AA), calcium hydroxide (Ca(OH)2), orthophosphoric acid (H3PO4), carboxymethyl cellulose (CMC), sodium hydroxide (NaOH), trypan blue, dimethyl sulfoxide (DMSO), ethanol (Denatured Ethanol) and trypsin solution were purchased from Sigma Aldrich, St. Louis, Missouri, USA and used as received without further purification.

2.2. Preparation of Nano-Hydroxyapatite (nHAp) and CMC-nHAp-AA Nanocomposite

Nano-hydroxyapatite was prepared via modified wet chemical reaction [40] and described as follow: 0.78 wt% Ca(OH)2 and 15 vol% H3PO4 were dissolved in highly transparent solution of CMC which was produced by dissolving 0.25 g of CMC in 50 mL of ultrapure water separately. 0.44 g of AA was added to the calcium hydroxide solution. The pH of each solution was adjusted to 11 by using NaOH solution. H2PO4/CMC solution was added in dropwise regime to vigorously stirred Ca(OH)2/AA/CMC solution at room temperature for about 1 h producing a transparent clear solution that was aged at room temperature for 24 h. In an attempt to separate the CMC stabilized AA/nHAp particles, solvent/non-solvent method [41] was applied using acetone as a non-solvent. Through this method, excess CMC, AA, aceton and other reagents were removed and CMC stabilized AA/nHAp particles was precipitated. The produced precipitate was collected by centrifugation at 3000 rpm for 15 min. The process of centrifugation and redispersion was repeated three times. The purified CMC stabilized AA/nHAp composite was dispersed finally in ultrapure water and stored in dark conditions for further characterization, study and applications.

2.3. Stability Study

The purpose of stability study was to provide evidence on the behavior of the proposed nanocomposites under the influence of light and temperature, and also to establish recommended storage conditions for the produced nanocomposite in case of various proposed applications.

Four samples were prepared to examine the stability of AA and to determine the optimal conditions for retaining its biological functionality. Sample ‘A’ was AA solution (0.0008 g of AA dissolved in 500 mL ultrapure water), sample ‘B’ was a mixture of AA and CMC (0.0008 g AA and 0.25 g of CMC dissolved in 400 mL of ultrapure water), sample ‘C’ was nHAp-AA (prepared using the same procedure of Section 2.2 in absence of CMC), sample ‘D’ was CMC-nHAp-AA composite (as prepared in Section 2.2). Dilution series was performed till we obtained the concentration appropriate for absorption measurements.

2.3.1. Photostability

2.3.1.1. Stability Against Light. The above mentioned samples A, B, C and D were irradiated by sunlight (in the summer- in Egypt at temperature 35–40 °C). The samples were exposed to sunlight for 0, 5, 10, 20, 40, 50, 80 and 170 min. After exposure to these conditions, the stability of AA in the four samples was evaluated by tracking its absorption spectrum using UV-visible spectrophotometer (T80+, PG instruments, U.K.) in the range 200–400 nm.

2.3.1.2. Stability Against Laser. The samples were irradiated at different wavelengths such as 405 nm, 488 nm, 532 nm and HeNe laser (532.8 nm) for different durations (0 min, 2 min, 4 min, 6 min, 8 min, and 10 min) and the stability of AA was evaluated by means of spectrophotometry.

2.3.2. Thermal Stability

The thermal stability of the four stated samples was evaluated in the temperature range of 30 to 100 °C. The stability of AA in the heat-treated samples was examined by UV-visible spectrophotometer in the range 200–400 nm.

2.4. In Vitro Study

2.4.1. Maintenance of Cell Culture

Adult human dermal fibroblast cells (AHDF) (Lonza, Basel Switzerland) were cultivated in 75-cm2 cell culture flasks using D-MEM (Gibco, Thermo Fisher Scientific Inc. USA) supplemented with 10% fetal bovine serum (FBS) as culture medium. Cell line was maintained as the following; the growth media were discarded from the cell culture flasks and the cell layer was washed gently by PBS (Ca2- and Mg2- free). The cell monolayer was washed by 5 mL trypsin (pre-warmed to 37 °C in water bath). Trypsin was decanted and the cell culture flasks were incubated at 37 °C. The flasks were microscopically examined to check detachment of cells, when the cells became rounded up and partially detached, the side of the flask was lightly tapped to dislodge the remaining cells from the flask. Fresh growth media were added to
the detached cells. Cells were resuspended in growth medium to the desired concentration according to cell count. Cell suspension was cultured and incubated at 37 °C until cells reached confluence again.

2.4.2. Cell Counting

For determination of growth rates, cells were counted accurately using the hemocytometer. The surface of hemocytometer slide and coverslip were cleaned with 70% alcohol.

2.4.3. Preparation of Cell Suspension

Double fold dilution of the original cell suspension was prepared by adding 0.5 mL of undiluted cell suspension to 0.5 mL of 0.4% trypan blue dye. A sterile pipette was used to transfer cell suspension to hemocytometer counting chamber then left to settle for a few minutes before beginning to count. All viable (unstained) cells in the 8 squares of 2 hemocytometer chambers were counted omitting cells lying on the upper line and left line of each chamber. The volume of each chamber = 0.1 mm³ (1.0 × 1.0 × 0.1). The mean count of the cells in each chamber was calculated. While, the total number of cells in the suspension was calculated using the following formula: \[ N = \frac{m \times t_b \times V}{10^4} \] where, \( N \) = number of cells in the cell suspension, \( m \) = mean of cell count per 0.1 mm³, \( t_b \) = correction of the trypan blue dilution (2 in double fold dilution with trypan blue), \( V \) = volume of the original cell suspension in mL, \( 10^4 \) = conversion factor for counting chamber volume and \( N \) (number of cells per mL) = \( N_1/V \). The new suspension (with new concentration \( N_2 \) and new volume \( V_2 \)) could be calculated by simple dilution law.

2.4.4. Cryopreservation of Cell Lines

a) Freezing

Cell suspension was prepared by trypsinization. The cell suspension was centrifuged 5 min at 300 to 350 × g (~1500 rpm), at room temperature. The supernatant was discarded and the cell pellet was adjusted to a concentration of 5–10 × 10⁶ cells/mL in preservation medium containing 10% dimethyl sulfoxide (DMSO) and 10% serum. Each 1 mL of the resuspended cells was dispensed in a cryotube clearly labeled with cell type, passage number, cell concentration and date of preservation. Cryotubes were Placed in cryobath for 30 min then kept frozen overnight at −70 °C. Cryotubes were transferred to a liquid nitrogen storage freezer (−196 °C).

b) Thawing

The cryotube was removed from liquid nitrogen freezer and immediately placed into a 37 °C water bath, then continuously agitated until medium is thawed. When the medium was completely thawed, the tube was wiped with alcohol to reduce bacterial load. The cell suspension in the cryotube was transferred to a culture flask containing growth medium (added very slowly, drop by drop as the viability of the thawed cells was severely affected if the cells were added rapidly). The culture flask was incubated overnight at 37 °C. The growth medium was carefully decanted (to remove DMSO) and replaced with fresh growth medium. The flask was then incubated till confluence. Thawed cells were tested concerning viability and sterility.

2.4.5. Cytotoxicity of Human Dermal Fibroblast Cell Line Using Trypan Blue Exclusion Assay

MTT assay is a sensitive, quantitative and reliable colorimetric method that measures viability of cells. The assay is based on the ability of mitochondrial lactate dehydrogenase enzymes (LDH) in living cells to convert the water soluble substrate 3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyl tetrazolium bromide (MTT) into a dark blue formazan which is water insoluble. A solubilization solution (dimethyl sulfoxide) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring it using spectrophotometer at a wavelength usually between 500 and 600 nm. Cytotoxic effect of test materials (A, B and C) were evaluated using AHDF cells for 24 h post-treatment using MTT assay, where test samples were sterile filtrated using 0.22 μm syringe filter. Double fold dilutions were prepared by adding equal volumes of the prepared materials to fresh medium. IC50 inducing concentration was calculated using Master Plex 2010 program. Tissue culture flasks 25 cm² surface area were pre-cultured then treated with the safe concentration and cell proliferation was monitored throughout 1, 3 and 7 days. The cells were counted using trypan blue exclusion assay. Cell count was recorded and plotted against duration of exposure.

2.5. Instrumentations

2.5.1. TEM Analysis of CMC-nHAp-AA Nanocomposites

Transmission electron microscopy (TEM) images were performed using a JEOL 200 CX (Akishima, Tokyo, Japan) at 200 kV. A droplet of each sample was placed on a copper grid and allowed to dry before being examined in the transmission electron microscope. The TEM images were analyzed using the Image-Pro Plus and Gatan Digital Micrograph program (Yubinbango103-0027 Nihonbashi, Chuo-ku, Tokyo, Japan).

2.5.2. FTIR Analysis

The FTIR investigations were carried out with a Scimitar Series FTS 2000 Digilab spectrophotometer (Marlborough, MA, USA) in the range of middle infrared of 4000–400 cm⁻¹. 0.0007 g sample was pressed with 0.2 g of KBr for IR spectroscopy Uvasol® purchased from Merck, Germany. The number of scans was 16 and a resolution of 4 cm⁻¹ characterized these measurements.

2.5.3. Powder XRD

Powder X-ray diffraction (XRD) patterns were recorded with a PANalytical: XPert PRO diffractometer using Cu Ka radiation source for investigation of the crystalline structure and phase transition.

2.5.4. UV–Vis Spectroscopic Analysis

The stability of CMC-nHAp-AA nanocomposite was monitored by UV–Vis spectrophotometer (T80 +, PG instruments, U.K.).

2.5.5. Laser Systems

(a) CW-DPSS laser [Changshun New Industries Optoelectronics Tech Co, Ltd. (China)] of 40 mW power at 405 nm wavelength.
(b) A green laser light (532 nm) from a Diode Pumped Solid State (DPSS) laser [LSR-PS-II] with an average output power of 1 mW.
(c) A (CW) argon ion laser (American laser company USA – ARJ – MED. INC, class 3b) delivering laser light of 4.8 mW power at 488 nm wavelength.

2.5.6. Equipment used for cell culture

(a) CO₂ incubator (Jouan - France)
(b) Cooling centrifuge (Jouan GR 412 - France)
(c) Vertical laminar air flow (Nunclon - USA)
(d) Cell culture inverted microscope (Hund - Germany)
(e) ELISA plate reader (Dy Dynatech medical products - England)
(f) Hemocytometer (New power - Germany)

3. Results and Discussion

3.1. Physicochemical Characterization of CMC-nHAp-AA Nanocomposite

The XRD pattern of nHAp in CMC-nHAp-AA nanocomposite was recorded and plotted against duration of exposure. The absorbance of this colored solution can be quantified by measuring it using spectrophotometer at a wavelength usually between 500 and 600 nm. Cytotoxic effect of test materials (A, B and C) were evaluated using AHDF cells for 24 h post-treatment using MTT assay, where test samples were sterile filtrated using 0.22 μm syringe filter. Double fold dilutions were prepared by adding equal volumes of the prepared materials to fresh medium. IC50 inducing concentration was calculated using Master Plex 2010 program. Tissue culture flasks 25 cm² surface area were pre-cultured then treated with the safe concentration and cell proliferation was monitored throughout 1, 3 and 7 days. The cells were counted using trypan blue exclusion assay. Cell count was recorded and plotted against duration of exposure.
The d-spacing values were at 6.7939 Å, 3.49094 Å, 3.37912 Å, and correspond to the anorthic system with space group P1 with the monetite phase of HAp (JCPDS 04-009-4184), and crystallized in the anorthic system with space group P-1 [42]. The XRD pattern of the as-prepared HAp has been depicted via JCPDS standard HAp card [43]. The d-spacing values were at 6.7939 Å, 3.49094 Å, 3.37912 Å, 3.13205 Å, 2.96167 Å, 2.76253 Å, 2.72226 Å, 2.50056 Å, 2.25391 Å, 1.91568 Å, 1.85095 Å and 1.72465 Å assigned to the Miller's indices of reflection plane are (010), (T21), (020), (T11), (T12), (T30), (T02), (T22), (030), (T32), (322) and (312) respectively. The small-angle peaks indicate the porous structure of the material [44]. This characteristic porous nature, allows nHAp to absorb, carry, and subsequently release components of the composite. In other words it can act as a miniature sponge carrying the components and delivering them to target cells. XRD analysis provides a detailed knowledge of the produced particles, as it gives structural information regarding mean grain size and microstrain of the produced nHAp by Scherrer equation. Additionally, the particle size, the fraction crystallinity Xc and specific surface area of nHAp were determined (Supplementary Table 1). The obtained data suggested that, the average crystallite size of nHAp produced by calcium hydroxide and phosphoric acid, ranges between 50 and 78 nm.

**Fig. 2** represents FTIR patterns of nHAp and nHAp-AA produced using calcium hydroxide and phosphoric acid after washing with acetone and in absence CMC. The spectrum possess an (OH)⁻¹ group in the region of 3169–3538 cm⁻¹. The broadening of the peak of (OH)⁻¹ group indicates the presence of hydrogen bonding between hydroxyapatite and carbonyl of ascorbic acid (AA) [45]. The small band comes out at 2352 cm⁻¹ probably assigned to the gaseous CO₂ which indicates the slight contamination of samples by atmospheric CO₂ [46].

The stretching peak at 1640 cm⁻¹ is probably due to the presence of AA carbonyl group [47]. The CH deformation and bending of AA appear at 1433 and 1133 cm⁻¹. The band at 1062 cm⁻¹ belongs to C–O of AA and its C–C peaks were observed at 871, 789 cm⁻¹. The phosphate groups (PO₄)³⁻ of hydroxyapatite appear at 575, 527, 1046 and 1092 cm⁻¹ [44,48]. The FTIR results, further confirm the nanoconjugation between nHAp and AA.

The morphology of the prepared samples (nHAp, nHAp-AA and CMC-nHAp-AA) was investigated by TEM measurements (Fig. 3a, b & c). The micrographs revealed that the produced particles have spherical shape displaying a relatively uniform morphology. The porous microstructure was due to high content of aggregated small crystals (Fig. 3a). Presence of AA (Fig. 3b) resulted in a meshwork with cohesion between the crystals. In Fig. 3c, the TEM micrograph depicted the precipitation of nHAp aggregated in CMC matrix. TEM analysis showed a relative uniform distribution of nHAp with self-assembled and aggregates of uniform size and morphology. Due to the very fine size of precipitated particles present in the aggregates, they could not be individually resolved.

SEM micrograph of nHAp-AA synthesized from calcium hydroxide and phosphoric acid after washing with acetone is shown in Fig. 3d. As can be seen from the micrograph, the particles are highly agglomerated with roughness of size around 10 nm. The nanoparticles were clustered into large agglomerates of irregular shapes and distributions. The SEM micrograph also shows bright patches distributed randomly in all the particles. These patches are suggested to be nano-pores. The irregular shapes of the particle may be attributed to washing with acetone.

**Fig. 2.** FTIR spectra of (a) nHAp and (b) nHAp-AA produced using calcium hydroxide and phosphoric acid.

**3.2. Stability Study**

In an attempt to examine the stability of AA in different matrix conditions, four samples were irradiated by sunlight (at temperature 35–40 °C in the summer - in Egypt) for 0, 5, 10, 20, 40, 50, 80 and 170 min. The four samples (“A” (AA solution), “B” (mixture of AA and CMC), “C” (nHAp-AA), “D” (CMC-nHAp-AA)) were investigated by UV–Vis spectrophotometry in the range 200–400 nm.

As clearly shown in Table 1 and Fig. 4(A–D), there are changes in the absorption maxima of AAA in the four samples (A, B, C and D). In sample A which represents AA solution, the absorption peak was at 260 and in sample B (mixture of AA and CMC) the peak wavelengths tended to be slightly shifted toward the long wavelength region (bathochromic shift less than 10 nm). In samples C and D, the absorption peaks were shifted to shorter wavelengths due to the influence of the functional groups on the conjugated systems, which caused the absorption peaks to appear at longer wavelengths than the peak wavelength of AA (sample A). This kind of shift in the absorption spectrum of AA can be explained in terms of the blocking effect of CMC as well as the conjugation effect to the surface of HAp through the hydroxyl groups that surround AA molecules similar to the pH effect [49,50]. These effects and interactions resulted in changing the dielectric medium of AA which consequently shift its absorption maximum to shorter wavelengths (hypsochromic effect).
3.2.1 Stability Against Light

The absorption of AA in Fig. 5(A–D) shows different behavior in the four samples depending on the period of exposure to sunlight as well as the nature of the host materials (nHAp, CMC). The photodegradation of AA in sample A (Fig. 5A), increased with increasing the exposure to light, whereas it has been slowed in sample D (Fig. 5D). This can be explained in terms of the presence of CMC and HAp. CMC acted as a protective coat and helped in the slower degradation of AA. On the other hand, HAp acted as a substrate and hold material which conjugated to AA and lead to protection of the AA [25]. In one way or another, HAp contributed to improve the stability of AA against light. The photodegradation of sample B (Fig. 5B) and C (Fig. 5C) proceeded rapidly and showed broadening until the peaks almost vanished/bleached over time. The absorption peak at 260 nm is well defined as being specific to the AA [25]. In sample A, the initial absorbance at \( \lambda_{\text{max}} = 265 \) nm was determined as 1.188 then decreased to be 0.303 after 170 min. Interestingly, when comparing the absorption of AA in samples A and D we found that, the initial absorbance value for sample A at \( \lambda_{\text{max}} = 245 \) nm was 1.009 then decreased to 0.249 after 170 min, whereas in sample D the absorbance at the same wavelength was initially 2.898 and after 170 min it decreased to 2.319. Based on these findings, light caused photolysis of AA which resulted in a rapid decrease in absorbance as in case of sample A. In case of sample D, the AA was not degraded easily by irradiation with sunlight, which confirmed its higher photo-stability due to the coating effect of CMC and conjugation to HAp. On the other hand, the AA in the other samples did not exhibit noticeable stability after sunlight exposure because of missing the effective protection articles [24,25,27,28]. Thus, we can conclude that photodegradation of AA could be prevented and controlled by adding a protective polymer as CMC and conjugation to a

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample composition</th>
<th>Absorption maximum (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Ascorbic acid (AA)</td>
<td>265</td>
</tr>
<tr>
<td>B</td>
<td>Mixture of AA and carboxymethyl cellulose gum (CMC)</td>
<td>267</td>
</tr>
<tr>
<td>C</td>
<td>nHAp-AA composite</td>
<td>245</td>
</tr>
<tr>
<td>D</td>
<td>CMC-nHAp-AA composite</td>
<td>240</td>
</tr>
</tbody>
</table>

Fig. 4. The absorption peaks of AA solution (A), mixture of AA and CMC (B), nHAp-AA (C), and CMC-nHAp-AA nanocomposites (D).

Fig. 3. TEM micrographs of (a) nHAp, (b) nHAp-AA and (c) CMC-nHAp-AA nanocomposites; and SEM micrograph of (d) nHAp-AA nanocomposite.
biocompatible substrate like HAp. Such information would be helpful for establishing appropriate storage protocols to minimize the degradation of the produced AA-nanocomposite.

Based on the results and discussion presented in the current section (irradiation by sunlight) as well as the information collected from absorbance decay of AA (Fig. 6(A–D)), we can conclude that, CMC-nHAp-AA composite offers high stability against sunlight compared to the other synthesized composites. This achievement explains the purpose of this study, as it was being directed to produce a stable AA composite, keeping all the benefits of AA and avoiding its poor stability problems. This high stability against sunlight may make CMC-nHAp-AA a promising candidate as a skin anti-aging drug. In comparison to other studies aiming to stabilize the AA by encapsulation [25], micro-encapsulation form [31] and others [25,27,28], it has been found that CMC-nHAp-AA is the most promising AA composites in terms of stability, compatibility, feasibility and safety which makes it an efficient candidate for dermatological cosmetics.

3.2.2. Stability Against Lasers

For the first time, different types of lasers at different wavelengths were applied to examine the stability of AA in the produced composites; (samples A (AA solution), B (mixture of AA and CMC), C (nHAp-AA) and D (CMC-nHAp-AA)). The four samples were irradiated by HeNe laser (632.8 nm), diode laser (405 nm), Argon ion laser (488 nm) and Nd:YAG laser at the second-harmonic wavelength (532 nm).

The four samples were irradiated with the different laser sources for different time durations (0 to 10 min). Sample D which represents (CMC-nHAp-AA nanocomposite) showed a relatively higher stability than the other samples (A, B and C) against different laser sources (Fig. 7(D1–D4)). This clearly indicates the role of CMC and nHAp in stability retention of AA in the proposed composite.

Fig. 8(D1–D4) shows the absorbance decay rate of sample D against different lasers. Samples A, B and C were irradiated with the same laser sources and the AA content showed very low stability in most cases (Supplementary Figs. 2(A1–A4), 4(B1–B4) and 6(C1–C4)).

Supplementary Figs. 3(A1–A4), 5(B1–B2) and 7(C1–C4) showed the absorbance decay rate of samples A, B and C using different lasers. Surprisingly, in some cases of presence of nHAp or CMC, AA showed some stability with laser irradiation and this explains clearly the role of nHAp and CMC as host and coating agents for AA, respectively.

3.2.3. Thermal Stability

To continue the investigations of the AA stability in different composites, its thermal stability has been also studied in details. Thermal decomposition of AA was followed up by raising its temperature in steps of 10 °C/min from room temperature up to 100 °C. The thermal stability of AA-composites was determined from the data recorded by UV–Vis spectrophotometer in the range 200–400 nm. When the four samples (A, B, C and D) were heat treated, there was sequential decrease occurring in the stability (Fig. 9(A–D)). During thermal treatments, sample D showed the highest thermal stability; as it was nearly stable until around 50 °C while the other samples (A, B and C) were almost decomposed at the same temperature. Sample D had eventual decline at the highest temperatures tested for extended periods. Fig. 10(A–D) shows the absorbance decay rate of samples (A–D) at different temperatures. There was a slight decrease in the absorbance value over heat. Thus sample D showed the lowest absorbance decay rate (Fig. 10D) and its absorbance value was 1.988 at room temperature and decreased slightly to reach 1.395 after the sample's temperature was raised to around 100 °C. Whereas in sample A the absorbance was...
1.021 at room temperature and became 0.051 at around 100 °C.

It has been found that AA entirely enzymatically oxidized to Dehydroascorbic acid (DHAA) during processing of crushed vegetable products (juices and purees) at temperatures below 70 °C [51]. Other study showed that AA in aqueous solution degrades completely in 4 h at ambient conditions, whereas paracetamol remains stable under the same conditions for about 11 days. However, relative humidity under the test conditions does not affect significantly the stability of individual components, and their mixtures [52]. In comparison, our findings fully support the main hypothesis of this work which is; the produced CMC-nHAp-AA nanocomposite is a promising approach toward achieving relatively thermal stable composite up to 100 °C.

From photo- and thermal stability studies of AA in different hosting environment, we can conclude that the best recommended composite for AA to keep its viability is CMC-nHAp-AA nanocomposite. Such kind of composite is introduced in the present work to be skin anti-aging drug.

3.3. Cell Culture

3.3.1. Cytotoxic Effects of CMC-nHAp-AA Nanocomposite on Human Skin Cells (HFB4 Cell Line)

Cell viability and cytotoxicity are commonly used to monitor the response of cells in culture after treatment with test materials by observing the number of the cells survived for different period of time after the test materials were added to the wells. To analyze the effects of CMC-nHAp-AA nanocomposite on cell viability, human skin cells (HFB4 cell line) were treated with varying concentrations of CMC-nHAp-AA nanocomposite for 48 h. As shown in Supplementary Fig. 8 and Supplementary Table 3, CMC-nHAp-AA nanocomposite did not affect the cell viability up to 50 μg/mL. However, concentrations higher than 100 μg/mL led to a significant reduction in cell viability. Thus, CMC-nHAp-AA nanocomposite was used at concentrations up to 100 μg/mL to evaluate its effect on cell proliferation. As a result, there was a concentration dependent increase in cytotoxicity at high doses, i.e. 100 μg/mL, so care should be taken when administrating the nanocomposite in high doses.

3.3.2. Cytotoxic Effects of CMC-nHAp-AA Nanocomposite on Normal Human Dermal Fibroblasts Cells - Adult (NHDF-Adult)

Another detailed in vitro cytotoxic study was performed on normal human dermal fibroblasts cells-adult (NHDF-Adult). In this study, three compounds were prepared; sample “A” represent AA solution, sample “C” represent nHAp-AA and sample “D” represent CMC-nHAp-AA. The sample “B” which represents a physical mixture of AA and CMC is a voided from this study, as we going to examine the synergetic effect of the real synthesized composites (clearly shown from TEM study in Fig. 3a, b & c). The effects of the three samples were examined on (NHDF-Adult) by comparing IC50 (50% inhibitory concentration) values as clearly shown in Fig. 11A, C and D. Based on IC50 values, it was found that after 24 h of observation the IC50 of sample “A” was 1.581 μg/mL and sample “C” was 0.445 μg/mL, whereas in case of sample “D” it was around 0.199 μg/mL. In other words, sample “D” seemed to have a higher IC50 value, compared to compound “A” and “C”. It is worth to highlight that, CMC-nHAp-AA nanocomposite has IC50 value higher than AA solution which means that it has high safety profile. The low IC50 value of nHAp-AA could be due to the absence of CMC which acts as a coating matrix preventing AA oxidation. From this...
discussion, CMC-nHAp-AA nanocomposite exhibited reduced cytotoxicity on (NHDF-Adult) compared to other samples. This reduced cytotoxicity effect may be attributed to the synergic effect for the presence of other constituents of the composite.

### 3.3.3. Cell Proliferation

The proliferation assay allows determining the number of growing cells in the absence or presence of certain materials. Collagen is produced in the fibroblasts of the human dermis, thus raising fibroblast proliferation rate can increase collagen production [4]. Adult human dermal fibroblasts were treated with 3 samples ("A" which represents AA solution, "C" nHAp-AA composite and “D” which represents CMC-nHAp-AA composite). It was observed that, there was a substantially higher level of proliferation of the cells treated with sample “D” (CMC-nHAp-AA) compared with the other samples after 7 days of growth. After day 3, the enhanced response was barely observable in sample “A” (which represents AA solution) and declined in sample C (Fig. 12).

Regarding to the action of CMC-nHAp-AA nanocomposite in a biological system, it is well known that the presence of HAp content enhances the interface activity and support cell growth [53]. Additionally, ascorbic acid (AA) promotes cell proliferation by activation of extracellular signal-regulated-kinase (ERK)-controlled pathway along with its role in enhancing cell immunity as reported by Vetter et al. [54]. The absence of the polymer (CMC) which serves as a protector or an isolator from direct contact with oxygen, heat, and moist conditions keeping AA from oxidation may be a reason for the reduced cell proliferation in response to sample C (nHAp-AA). Thus, the presence of CMC in sample “D” provides enhancement of the bioavailability of the stated composite by increasing the solubility [55]. This means that HAp-AA nanocomposite increases cell growth through prolonged bioavailability and activation of cell signal transduction.

These findings prove that, there is a positive proliferation response in term of cell growth through prolonged bioavailability and activation of cell signal transduction to sample “D” with longer duration than sample “A” and sample “C”. This is attributed to the synergic effect for the presence of the three constituents of the composite and confirms the observations collected from photo- and thermal stability studies.

The microscope images of the fibroblasts before and after treating with samples A, C and D are shown in Supplementary Fig. 9.

### 4. Conclusion

In the present study a new ascorbic acid (AA) nanocomposite with low toxicity and high photo- and thermal stability has been constructed for dermatologic cosmetic applications. Various AA-nanocomposites with nHAp and/or CMC were synthesized via wet chemistry using calcium hydroxide and orthophosphoric acid as precursors. This reaction produced a clear colorless solution with water as a major by-product which meets requirements of the proposed approach. The produced composite has been examined by UV–VIS, XRD, FTIR, EDX-TEM and SEM techniques.

Results of stability testing of AA nanocomposites have been also reviewed, which indicated that the proposed CMC-nHAp-AA composite is the most stable one against heat and light. Consequently, the current study was extended to evaluate the toxicity and the cell proliferation rate of the most stable AA-composite (CMC-nHAp-AA) together with the
Fig. 11. The half maximal inhibitory concentration (IC50) determination of samples A (AA solution), C (nHAp-AA) and sample D (CMC-nHAp-AA) against adult human dermal fibroblasts cell line.

Fig. 12. Comparative evaluation of adult human dermal fibroblasts cell line under the effect of three samples (“A” represents AA solution, “C” represents nHAp-AA and “D” represents CMC-nHAp-AA) relatively to time compared to non-treated control.
less stable AA-composites. In cell culture tests, adult human dermal fibroblasts have been exposed to three types of AA-nanocomposites (ascorbic acid (AA) solution, CMC-nHAp-AA and nHAp-AA nano-composites). The results revealed that the most stable composite (CMC-nHAp-AA) significantly increased cell attachment/proliferation and promoted the production of collagen compared with the other composites (AA and nHAp-AA) which coincides with the data collected from photo- and thermal stability studies. So, it can be concluded that the proposed composite has the highest rate of cell proliferation. Thus it might hold great potential as biomaterial enhancer for cell proliferation rate and collagen production in dermatologic applications.

Finally, from these findings it is expected that the produced CMC-nHAp-AA nanocomposite is a promising paradigm in cosmetics and skin rejuvenation applications giving a vital rationale for restoring skins’ elasticity and moisture. Another important feature of CMC-nHAp-AA composite is that it can also be easily designed to fit in various cosmetic purposes due to the capability of having its properties tailored by changing one of a series of variables. Some of these variables are the type of the active ingredients, concentration, size and shape of the nano-carrier thus it could act as a dermal filler or for anti-aging of skin.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jphotobiol.2017.07.004.

References

[4] C. Bagole, D. Ray, S. Bernstein, S. Feldon, T. Smith, More than structural cells, purposes due to the capability of having its properties tailored by changing one of a series of variables. Some of these variables are the type of the active ingredients, concentration, size and shape of the nano-carrier thus it could act as a dermal filler or for anti-aging of skin.


