European Journal of Pharmaceutical Sciences 70 (2015) 22–28

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Skin intervention of fullerene-integrated nanoemulsion in structural and collagen regeneration against skin aging

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ARTICLE INFO

Article history:
Received 4 October 2014
Received in revised form 4 December 2014
Accepted 13 January 2015
Available online 22 January 2015

Keywords:
Fullerene
Nanoemulsion
Oxidative stress
Skin aging
Sonography

ABSTRACT

Despite the fact that intrinsic oxidative stress is inevitable, the extrinsic factor such as ultraviolet radiation enhances reactive oxygen species (ROS) generation resulting in premature skin aging. Nanoemulsion was loaded with fullerene, a strong free radical scavenger, and its efficacy to provide protection and regenerative effect against ROS-induced collagen breakdown in human skin was studied. Stable fullerene nanoemulsions were formulated using high shear homogenization and ultrasonic dispersion technique. An open trial was conducted using fullerene nanoemulsion on skin twice a day for 28 days. The mean collagen score significantly increased ($P < 0.05$) from 36.53 ± 4.39 to 48.69 ± 5.46 with 33.29% increment at the end of the treatment. Biophysical characteristics of skin revealed that skin hydration was increased significantly ($P < 0.05$) from 40.91 ± 7.01 to 58.55 ± 6.08 corneometric units (43.12% increment) and the water was able to contain within the stratum corneum without any increased in transepidermal water loss. In the *in vitro* safety evaluation, fullerene nanoemulsion showed no acute toxicity on 3T3 fibroblast cell line for 48 h and no indication of potential dermal irritation. Hence, the fullerene nanoemulsion may assist in protecting collagen from breakdown with cosmeceutical benefit.

1. Introduction

Skin aging is stimulated by reactive oxygen species (ROS) which linked firmly to intracellular and extracellular oxidative stress (Masaki, 2010). Oxidative stress is associated with excessive ROS generation where the biological system's ability fails to neutralize the reactive intermediates resulting in imbalance within the body system (Palmer and Kitchin, 2010). ROS formed in cells include superoxide anion ($\cdot O_2^-$), hydroxyl free radicals ($\cdot OH$), lipid peroxyl radicals ($LOO\cdot$), hydrogen peroxide, and singlet oxygen under specific conditions (Floyd et al., 2011; Masaki, 2010). Intrinsic oxidative stress refers to the natural aging process of degenerative effects within the human body system due to the genetic factor and hormonal changes (Farage et al., 2008; Pinnell, 2003). Extrinsic oxidative damage can be traced to environmental factors such as sunlight, smoking and other pollutants, an unbalanced diet, lack of exercise, stress, and illnesses (Farage et al., 2008; Pinnell, 2003). The synergistic effect of both factors cause functional deficit through structural and molecular degradation which is often characterized by wrinkles formation, atypical pigmentation, laxity, and loss of tension/elasticity (Krutmann, 2011).

Age-related skin alterations are naturally genetically-governed by the influence of intrinsic factors which are inevitable over the human lifespan. Nevertheless, premature skin aging could happen promptly due to extrinsic factors such as exposure of ultraviolet (UV) radiation. ROS liberated would trigger the activation of nitrogen-activated protein kinases (e.g. c-jun and c-fos) which then induce the formation of activator protein-1 (AP-1). Subsequently, AP-1 activates the matrix metalloproteinases (MMPs) which stimulate the production of collagenase, gelatinase, and stromelysin (Zouboulis and Makrantonaki, 2011). Eventually, MMPs would cleave the collagens which cause the breakdown of triple helix structure into fine fragments and further undergoes denaturation immediately into gelatinous peptides. Enhanced collagen degradation is mediated by AP-1 activation and inhibition of transforming growth factor (TGF)-β signaling (Rittié and Fisher, 2002).

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http://dx.doi.org/10.1016/j.ejps.2015.01.006
0928-0987/

Skin often exposed to UV radiation and ROS were generated within the epidermis and dermis layer which resulted in skin aging. Antioxidant produced in human body might not be sufficient to balance up the excessive radical generated. External surplus intake of antioxidant was necessitated to counteract the damaging effects in the cells. Fullerene is an effective free radical scavenger with higher performance than natural antioxidant through multiple addition by forming radical adduct (Wang et al., 1999). Structurally, it made up of 60 carbon atoms but also in the other form. It is characterized as a hydrophobic compound in nature but found to be biocompatible with fatty acid esters (Cataldo, 2008). Likewise, fullerene served as sacrificial antioxidant to lipid autoxidation while still remained active as antioxidant after undergoing the first stage of oxidation (Cataldo, 2010). This makes fullerene suitable to be delivered transdermally which targets directly to the source of radical development.

A biocompatible oil-based carrier for fullerene which enhances the skin bioavailability is required. Nano-sized colloidal delivery system has drawn a lot of attention for transdermal application mainly for its direct application. Nanoemulsions, particularly oil-in-water (O/W) emulsion, consists of oil phase which serve as the carrier for the bioactive is an attractive technique for enhancing hydrophobic active compounds' solubility. Nanoemulsion is a non-equilibrium system which features nanometric droplet size in the range of 20–200 nm (Solans et al., 2005). Nanoemulsions can be prepared either by low energy, high energy or combination of both emulsification methods. However, high shear homogenization and ultrasonic dispersion are better choice over the high pressure homogenization due to being rapid in nature and relatively lower cost in production.

In this present study, the developed fullerene nanoemulsions were applied transdermally to discover the effects in regulating the human skin structure and amelioration of collagen within the skin. Safety evaluation of fullerene nanoemulsions was conducted based on cytotoxicity test of fibroblast cell line using MTT cell viability assay and dermal irritation test by Irritection® Assay System. Protective effects of fullerene were determined by using high resolution ultrasound imaging to monitor the structure and assess the collagen content of the skin over a period of 28 days of application. Further extension of this research involved biophysical measurements focus on skin hydration and transepidermal water loss.

2. Materials and method

2.1. Materials

Fullerene, C_{60} (purity: 99.5%), xanthan gum from *Xanthomonas campestris*, white beeswax, polyoxyethylene sorbitan monooleate (T80), and sorbitan monooleate (S80) were purchased from Sigma–Aldrich (St. Louis, USA). Palm kernel oil esters (PKOEs) were synthesized directly in our laboratory via enzymatic transesterification of palm kernel oil and oleyl alcohol using Lipozyme RM IM as the catalyst (Keng et al., 2009). Preservative, phenonip was obtained from Bramble Berry (Bellingham, USA). Deionized water was purified using Milli-Q water system (Billerica, USA). All ingredients were used as received without further purification.

2.2. Fullerene nanoemulsions formation

Nanoemulsions formation was carried out using a method based on high energy emulsification technique developed previously (Ngan et al., 2014a,b). Oil and aqueous phases were separately preheated up to 75 ± 1 °C (Table 1). Fullerene nanoemulsion was prepared by adding the aqueous phase dropwise into the oil phase and homogenizing the mixture at 4350 rpm using a high shear homogenizer (PT3100, Kinematica AG, Switzerland) at room temperature (25 ± 2 °C) for 15 min. The premixed emulsions were further subjected to ultrasonication by 24 kHz ultrasonic tip processor (UP400S, Hielscher Ultrasonics GmbH, Germany) with a maximum power output of 400 W. The titanium probe tip of 14 mm diameter and 100 mm length was submerged into the emulsions while being sonicated at 50% amplitude for 100 s (alternating on–off every 1 s). Samples were immersed in ice bath to mitigate the ultrasound thermal effect. Blank nanoemulsion composed the same content of PKOEs as fullerene nanoemulsion was used as the placebo.

Table 1
Composition of the developed fullerene nanoemulsion.

Compounds	Nanoemulsion compositions (%, w/w)			
	FNE1	FNE2	FNE3	BNE
Oil phase				
Palm kernel oil esters	12.5	12.5	12.5	12.5
Span 80	1.54	1.54	1.54	1.54
Phenonip	0.7	0.7	0.7	0.7
Beeswax	1	1	1	1
Fullerene	0.001	0.005	0.010	–
Aqueous phase				
Tween 80	6.16	6.16	6.16	6.16
Xanthan gum	0.9	0.9	0.9	0.9
Water, deionized (q.s.)	100	100	100	100

2.3. Characterization of nanoemulsions

2.3.1. Particle size determination

Nanoemulsion particle sizes were measured by dynamic light scattering technique using a particle size analyzer (Zetasizer Nano ZS90, Malvern Instruments, UK) equipped with an argon laser (λ = 488 nm). The measurement was performed with an angle of 173° at 25 °C with each measurement being average of 16 runs, each of 10 s duration. All samples were diluted (1:200) with deionized water to avoid multiple scattering effects before filling into the zeta disposable cells. Diluted samples were filled into the capillary cell using 3 mL syringe to avoid the presence of air bubbles before inserting into the module. Samples were measured after 2 min equilibration at 25 °C and the results were reported as the average five measurements. For data analysis, each sample was analyzed in triplicate after 24 h of sample preparation. The particle size of the nanoemulsions was also was monitored for 1, 7, 15, 30, and 90 days to detect the variation of size over time.

2.3.2. Nanoemulsion stability study

Macroscopic analysis by visual observation of the fullerene nanoemulsions was performed in order to observe any macroscopic instability such as creaming, flocculation, coalescence, and phase inversion including color changes. Centrifugation and thermal accelerated stability studies were conducted. In the centrifugation test, 10 mL of samples were taken immediately after preparation and centrifuged at 4000 rpm for 20 min (Hermle Labor Technik, Germany) at 25.0 ± 0.5 °C. For thermostability study, vials containing 10 mL of sample were stored in an incubator (DK-S1020, DAIKI Sciences Co. Ltd, Korea) at 45 °C and also at room temperature (25.0 ± 0.5 °C) for 90 days. In addition, freeze–thaw cycles stability study was carried out by allowing the samples to freeze in refrigerator at 5 °C for 24 h and left to thaw at room temperature for another 24 h. These steps were repeated for three cycles for 6 days.

2.3.3. Transmission electron spectroscopy (TEM)

Fullerene nanoemulsion was characterized by high resolution TEM (H-7100, Hitachi, Japan) with operating system at 200 kV to