UNIVERSITI PUTRA MALAYSIA

DESIGN AND OPTIMISATION OF FULLERENE-BASED NANOEMULSION SYSTEM FOR NANOCOSMECEUTICAL APPLICATIONS

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in the fulfilment of the requirement for the degree of Doctor of Philosophy

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June 2015

Chairman : Professor Mahiran Basri, PhD
Faculty : Science

Fullerene is gaining interest as an antioxidant in the cosmetic industry but the primary challenges are the incorporation of fullerene due to its hydrophobicity and difficulties in formulating a stable carrier system. Newly developed nanoemulsion system was designed specifically for transdermal application in cosmeceutical applications. Pseudoternary phase diagrams were constructed to serve as platform in building an emulsion system which constituted of palm kernel oil esters (PKOEs), non-ionic surfactant(s), water and fullerene as bioactive ingredient. Non-ionic surfactants applied were polyoxyethylene sorbitan monooleate (Tween 80) and sorbitan monooleate (Span 80) with different mixed surfactant ratios (MSRs). Based on the results, binary surfactant system of Tween 80:Span 80 with MSR of 8:2 performed better in emulsification capability by exhibiting larger monophasic region compared to the usage of single surfactant. The hydrophilic-lipophilic balance value of mixed surfactant unveiled the largest homogeneous and isotropic regions is 12.86.

Compositions selected from pseudoternary phase diagrams were designated as pre-formulation. Excipients contained alongside within the formulation include xanthan gum as rheology modifier, beeswax as emollient and phenonip as anti-microbial agent. Nanoemulsions were prepared by high shear homogeniser, followed by ultrasonic cavitation. The physicochemical behaviours of formulations with various proportions of components were extensively characterised. Essentially, the mean particle size was in the nano-size range of 70-160 nm. It was found that xanthan gum amount with 0.60% (w/w) or higher was able to stabilise the emulsion droplets by forming polymer network with steric stabilising effect.

The effect of composition on nanoemulsion; PKOEs, surfactants and xanthan gum amount on variation of particle size, ζ-potential and viscosity were investigated. Multivariate statistical techniques such as response surface methodology (RSM), Box-Behnken (BBD) and central composite rotatable (CCRD) designs were used to optimise the formulation in acquiring desirable properties of nanoemulsion system. The optimum formulation comprised 12.50% of PKOEs, 7.68% of Tween 80:Span 80 (4:1) and 0.90% of xanthan gum yielded a particle size, ζ-potential and viscosity of 153.6 nm, −53.4 mV and 42.1 Pa s, respectively. Linear relationships were observed in all cases where no interaction occurred between the variables. According to the pareto graphic analysis, surfactant amount gave the largest effect on particle size and ζ-potential whereas viscosity was largely dependent on xanthan gum amount.

Second stage of optimisation was performed to discover the influence of process parameters on the similar responses as investigated earlier. The effect of process types on response variables was complex being dependent on the existence of interaction between the parameters (quadratic polynomial model). The results showed that nanoemulsion prepared under homogenisation rate of 4352 rpm and sonication amplitude of 48% for 97 s would produce particle size, ζ-potential and viscosity of 152.5 nm, −52.6 mV and 44.6 Pa s, respectively. Interestingly, in both optimisation designs, CCRD demonstrated excellent model fitting and estimation of actual values than BBD with lower residual standard error. Nonetheless, both designs predicted similar responses which affirmed one another in terms of reliability to obtain optimum formulation with improved attributes.

Rheological behaviour of nanoemulsion was evaluated using viscometry test. From the rheograms, nanoemulsion exhibited shear thinning (pseudoplastic) behaviour which obeys the power law model. The results from oscillatory strain sweep test showed the wide linear viscoelastic region which directly correlated to high rigidity of the system. The architecture of the nanoemulsion system was analysed using transmission electron microscope to study the morphology. The micrographs showed that the particle size was in agreement with the measured size. In the physical stability and thermal stress test, the optimum formulation was stable under high centrifugal force, storage at room temperature and 45°C for 90 days while maintaining its nano-sized particle and high ζ-potential with texture and consistency being preserved. Likewise, the colloidal system was able to withstand freeze-thaw cycles and having low rate of Ostwald ripening.

In the safety evaluation test, nanoemulsion showed no cytotoxicity effect on fibroblast cell (3T3) up to 48 hours. Results also showed that nanoemulsion was non-irritant with Human Irritancy Equivalent (HIE) scores of 0.36. *In vivo* biophysical attributes of skin studies showed that the skin hydration increased without any increase in transepidermal water loss up to 28 days of the treatment period. However, with this finding, the skin hydration increased more progressively on application of fullerene-laden formulation compared to placebo (without fullerene). Collagen content was increased significantly which lead to improved water binding capacity. No visible skin reactions caused by dermal irritation, contact sensitisation or rash were experienced by the subjects during the treatment. This work concluded that a stable fullerene nanoemulsion fits for cosmetic was successfully developed and showed potential collagen regeneration in human skin.

Abstrak tesis dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

REKABENTUK DAN PENGOPTIMUMAN FULERENA BERASASKAN SISTEM NANOEMULSI BAGI KEGUNAAN NANOKOSMESEUTIKAL

Oleh

NGAN CHENG LOONG

Jun 2015

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Fulerena semakin mendapat perhatian sebagai antioksidan dalam industri kosmetik tetapi cabaran utama adalah penggabungan fulerena kerana kehidrofobikannya dan kesukaran untuk memformulasikan sebuah sistem pembawa yang stabil. Sistem nanoemulsi baru telah dicipta khusus untuk aplikasi transdermal sebagai formulasi kosmeseutikal. Gambar rajah tiga fasa telah dibina sebagai platfom dalam pembinaan sistem emulsi yang terdiri daripada ester minyak isirong sawit (PKOEs), surfaktan bukan ion, air dan fulerena sebagai bahan bioaktif. Surfaktan bukan ion yang digunakan adalah polioksietilena sorbitan monooleat (Tween 80) dan sorbitan monooleat (Span 80) pada nisbah campuran surfaktan (MSRs) yang berbeza. Hasil keputusan menujukkan sistem binari surfaktan bagi nisbah Tween 80:Span 80 dengan nilai MSR 8:2 mempunyai keupayaan pengemulsian yang lebih baik dengan menghasilkan kawasan monofasik yang lebih besar berbanding dengan penggunaan surfaktan tunggal. Nilai keseimbangan hidrofilik-lipofilik bagi campuran surfaktan yang menghasilkan kawasan homogen dan isotropik terbesar adalah 12.86.

Komposisi yang dipilih daripada gambar rajah tiga fasa kemudian digunakan sebagai pra-formulasi. Bahan-bahan lain yang terkandung dalam formulasi termasuk gam xantan sebagai pengubahsuai reologi, lilin lebah sebagai emolien dan phenonip sebagai agen anti-mikrob. Nanoemulsi telah disediakan dengan menggunakan homogeniser ricih tinggi dan diikuti oleh peronggaan ultrasonik. Ciri-ciri fizikokimia bagi formulasi dengan pelbagai bahagian terhadap komposisi telah ditentukan secara mendalam. Secara umumnya, purata saiz zarah adalah dalam julat 70-160 nm dalam saiz nano. Gam xantan sebanyak 0.60% (w/w) atau lebih didapati mumpunyai kebolehan untuk menstabilkan titisan emulsi dengan pembentukan jaringan polimer terhadap kesan pengstabilan sterik.

Kesan kandungan PKOEs, surfaktan dan gam xantan terhadap perubahan saiz zarah, keupayaan ζ dan kelikatan telah disiasat. Teknik statistik multivariat seperti kaedah gerak balas permukaan, rekabentuk Box-Behnken (BBD) dan komposit pusat berputar (CCRD) telah digunakan untuk mengoptimumkan formulasi dalam mencapai sistem nanoemulsi yang diingini. Formulasi optimum terdiri daripada 12.50% PKOEs, 7.68% Tween 80:Span 80 (4:1) dan 0.90% gam xantan telah menghasilkan saiz zarah, keupayaan ζ dan kelikatan sebanyak 153.6 nm, -53,4 mV, dan 42.1 Pa s masing-masing. Hubungan linear telah dapat diperhatikan dalam semua kes di mana tidak ada saling tindakan berlaku antara pembolehubah-pembolehubah. Menurut analisis pareto grafik, jumlah surfaktan telah memberi kesan yang terbesar kepada saiz zarah dan keupayaan ζ manakala kelikatan adalah sangat bergantung kepada kandungan gam xantan.

Pengoptimuman peringkat kedua dilakukan untuk mengetahui kesan bagi proses parameter terhadap gerak balas yang telah disiasat sebelum ini. Kesan bagi jenis proses pada pembolehubah respon adalah kompleks bergantung kepada kewujudan saling tindakan antara parameter (model polinomial kuadratik). Hasil kajian menunjukkan bahawa nanoemulsi yang disediakan pada kadar homogenisasi 4352 rpm dan sonikasi amplitud 48% selama 97 s akan menghasilkan saiz zarah, keupayaan ζ dan kelikatan 152.5 nm, -52,6 mV dan 44.6 Pa s masing-masing. Dalam kedua-dua rekabentuk pengoptimuman, CCRD menunjukkan model yang lebih baik bersesuaian dengan anggaran nilai sebenar berbanding BBD dengan ralat piawai residu yang lebih rendah. Walau bagaimanapun, kedua-dua rekabentuk telah diramalkan mempunyai gerak balas yang sama yang disahkan di antara satu sama lain dari segi reliabiliti untuk mendapatkan formulasi yang optimum dengan sifat-sifat yang telah ditingkatkan.

Ciri reologi nanoemulsi telah ditentukan menggunakan ujian kelikatan. Daripada reogram, nanoemulsi mempamerkan sifat ricih penipisan (pseudoplastik) yang mematuhi model 'power law'. Hasil daripada ujian ketegangan ayunan menunjukkan rantau linear viskoelastik yang luas yang berkadar terus dengan sistem ketegaran yang tinggi. Struktur sistem nanoemulsi telah dianalisis dengan menggunakan mikroskop elektron transmisi bagi mengkaji morfologinya. Mikrograf menunjukkan bahawa saiz zarah adalah bersesuaian dengan saiz yang diukur. Dalam ujian kestabilan fizikal dan tekanan haba, formulasi optimum didapati stabil di bawah daya empar tinggi pada suhu bilik dan 45℃ selama 90 hari di samping mengekalkan zarah bersaiz nano dan keupayaan ζ yang tinggi dengan tekstur dan konsistensi yang dapat dikekalkan. Sistem koloid mampu menahan kitaran beku-cair dan mempunyai kadar 'Ostwald ripening' yang rendah.

Dalam ujian penilaian keselamatan, nanoemulsi tidak memberi kesan sitotoksiti pada sel fibroblast (3T3) sehingga 48 jam. Keputusan juga menunjukkan bahawa nanoemulsi adalah tidak merengsa dengan skor 'Human Irritancy Equivalent' (HIE) pada 0.36. Kajian *in vivo* sifat biofizikal kulit menunjukkan bahawa penghidratan kulit meningkat tanpa sebarang kenaikan kehilangan transepidermal air sehingga 28 hari dalam tempoh rawatan. Walau bagaimanapun, penghidratan kulit meningkat dengan lebih progresif dalam penggunaan fulerena berbanding dengan plasebo (tanpa fulerena). Kandungan kolagen telah meningkat dengan ketara yang membawa kepada kapasiti pengikatan air yang lebih baik. Sepanjang tempoh rawatan, kesan tindak balas pada kulit yang berpunca daripada kerengsaan kulit sensitif terhadap sentuhan atau ruam yang dialami oleh subjek tidak kelihatan. Kajian ini merumuskan bahawa nanoemulsi fulerena yang stabil dan sesuai untuk kosmetik telah berjaya dicipta serta menunjukkan potensi pertumbuhan semula kolagen dalam kulit manusia.

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I certify that a Thesis Examination Committee has met on 24 June 2015 to conduct the final examination of Ngan Cheng Loong on his thesis entitled "Design and Optimisation of Fullerene-Based Nanoemulsion System for Nanocosmeceutical Applications" in accordance with the Universities and University College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

3D	three dimensional
BBD	Box-Behnken design
c.u.	corneometric unit
CCRD	central composite rotatable design
CIR	Cosmetic Ingredient Review
DMEM	Dulbecco's Modified Eagle Medium
FDA	Food and Drug Administration
HLB	hydrophilic-lipophilic balance
LCFS	lipid formulation classification system
LVR	linear viscoelastic region
MSRs	mixed surfactant ratios
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
O/W	oil-in-water
OD	optical density
PKOEs	palm kernel oil esters
ROS	reactive oxygen species
rpm	revolutions per minute
RSE	residual standard error
RSM	response surface methodology
TEM	transmission electron microscopy
TEWL	transepidermal water loss
UV	ultraviolet
W/O	water-in-oil
w/w	weight per weight

CHAPTER 1

INTRODUCTION

Background of Study

So long as the desire to seek ultimate beauty, eternalise youthful appearance and revitalise skin wellness is instilled in oneself, demands for new effective and innovative skincare products in the market will continue to soar. Since then, the term "cosmeceutical" is often used in the cosmetic industry which has been classified as cosmetic products which yield therapeutic or medicinal benefits (Choi and Berson, 2006). Similarly to cosmetics, cosmeceuticals are applied topically but vary in which they contain bioactive compounds that can influence the biological function of skin and deliver nutrients to enhance skin health (Brandt *et al.*, 2011).

Cosmeceutical products show promising prospect which is highlighted by the substantial market growth in the coming years. According to the insight report, it forecasted the global cosmeceutical market, which is dominated by top European countries (United Kingdom, Germany, France, Italy and Spain) with 65% of the worldwide market, followed by United States and Japan, will reach US $42.4 billion by 2018 (GBI Research, 2013). Even so, massive cosmeceutical products are not strictly regulated and often times, consumers are deceived by the fascinating claims in the product information which is not scientifically proven or backed by rigorous clinical studies (Brandt *et al.*, 2011). As a matter of fact, efficacy of a cosmeceutical product is largely dependent on the bioavailability of bioactive compounds used in the formulations.

In order to improve the bioavailability on human skin, increase in penetration of bioactive compounds into human skin is undoubtedly necessary. Manufacturers of cosmetic products have recently shown increasing preference for multifunctional products in which different active agents can be incorporated to improve bioavailability and decrease side effect or toxicity due to lower active agent content. Recent advanced in nanotechnology and engineering proves to be effective in surging innovative formulations and product solutions. Novel strategies for skin delivery systems in cosmeceutical include vesicular systems, particulate systems, fibrous matrices and emulsion systems (Golubovic-Liakopoulos *et al.*, 2011).

A new generation hybrid of nanotechnology and emulsion technology is able to manipulate the delivery of bioactive compounds in achieving the best outcome of the treatment. Nanoemulsions are able to ensure these terms. Various bioactive compounds incorporated in nanoemulsions for application in the treatment for rheumatoid arthritis (Salim *et al.*, 2012) and medicinal research for inflammatory disorder (Peng *et al.*, 2010), have shown enhanced penetration of drugs. Nanoemulsions have also been developed for waterinsoluble active ingredients such as tocotrienol (Han *et al.*, 2011) and kojic acid dipalmitate (Al-Edresi and Baie, 2010) as cosmetic delivery system. Eventually, this comes down to the competency of the bioactive compounds and their purported biological activity.

Fullerene, being the third carbon allotrope, has been explored and deployed dynamically in the cosmeceutical industry on account of its excellent antioxidant activity (Lens, 2009). Fullerene and its derivatives are proven far better than α-tocopherol in term of antioxidant activity (Takada *et al.*, 2006; Wang *et al.*, 1999). Application of fullerenes in cosmeceutical has always been a challenge for the researchers due to their immiscibility in the water (Cataldo, 2008). Hence, some have been formulated where fullerenes are dissolved in squalane (Kato *et al.*, 2010a) and encapsulation of fullerenes in polyvinylpyrolidone (Xiao *et al.*, 2007) for cosmetic applications. However, there is limited information on the development of stable nanoemulsion systems for pristine fullerene.

Surfactants play an important role in acquiring a stable emulsion without any occurrence of sedimentation or flocculation over time. Ionic surfactants are not suitable for human use because they are easily affected by pH and change in ionic strength. Nevertheless, nanoemulsions with low surfactant concentration in the formulation could reduce any adverse toxicological and dermatological effects to human body (Han *et al.*, 2011). For high skin-compatibility cosmetic purposes, non-ionic surfactants are being used, namely Tween 80 and Span 80 with non-toxic and biocompatible (Azeem *et al.*, 2009), and vegetable oil, palm kernel oil esters (PKOEs) which show non-irritancy and increase in skin hydration (Keng *et al.*, 2009), would be suitable candidates in cosmetic formulations for skin rejuvenation.

In this study, palm-based colloidal systems were developed from the pseudoternary phase diagrams. Palm kernel oil esters are specialty esters which were chosen to be added into the formulation due to their excellent moisturising effect and wetting behaviour. Apart from its goodness and pure cosmeceutical benefit richness, development of palm-based products in Malaysia also serve as an initiative to promote market expansion of palm oil globally. Tween 80 and Span 80 were selected due to their high degree of compatibility with other ingredients, good chemical stability and low toxicity. Xanthan gum and beeswax are natural adjuvants added as rheological modifier and moisturiser, respectively to enhance the skin benefits and properties of the formulation. For longer shelf life purpose, a very low amount of phenonip was added as preservative. Formulations were developed after the suitable compositions were determined by using high-energy emulsification method to produce nanoemulsion systems. The potential of the nanoemulsion formulations were then evaluated on its safety and clinical trial on human skin.

Research Problems

Fullerene is well established for its antioxidant activity in various fields especially in cosmeceutical industries. Human skin has the water content of 10−20%, while more than 40% of bioactive materials used in the pharmaceutical and cosmetic industries are poorly water soluble (Tal-Figiel and Figiel, 2008), thus causes failure in delivery of bioactive compounds into human skin. Unfortunately, fullerene is hydrophobic in nature. Surface modification of fullerene has been done to increase its solubility in water but original activity of fullerene will be affected. Hence, a novel biocompatible delivery system is required to deliver fullerene to the human skin.

Like any other colloidal systems, nanoemulsion is prone to numerous types of physical instabilities. In fact, the greatest challenge is to maintain the particle size in nanometer range while remain physically stable for a period of time. The right compositions of each component and techniques in the preparation of formulation would be crucial to acquire such sophisticated colloidal system. This process is tedious and time-consuming to find the best formulation if the formulator was to consider the effect of each component individually. Besides, the potential of fullerene in collagen regeneration is still unknown.

Research Objectives

The specific objectives of this work are:

(i) To develop biocompatible palm kernel-based nanoemulsion systems incorporated with fullerene for transdermal applications.
(ii) To characterise the physicochemical, rheological properties and stability of the newly developed nanoemulsions.
(iii) To conduct optimisation of the formulation composition and process of the developed nanoemulsions.
(iv) To evaluate the safety of nanoemulsions and their biophysical attributes of human skin.

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