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Research Article

Effect of Lipid Composition on Nanostructured Lipid Carrier (NLC) on Ubiquinone Effectiveness as an Anti-aging Cosmetics

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ABSTRACT

The purpose of this research is to determine the optimum composition of solid lipid and liquid lipid in order to increase the penetration and effectiveness of Q10 as antioxidant in anti-aging cosmetics. Solid lipid and liquid lipid used in this study were cetyl palmitate and caprylic, which were combined to four (4) different ratios, namely 10:0; 9:1; 7:3 and 5:5. NLC Q10 in this study was produced by high shear homogenization method at 3400 rpm for 5 cycles and at 24000 for 1 cycle. The fourth formula was evaluated in term of characteristics, penetration and effectiveness. From the pH test , it was known that all formulas met the skin pH range (4.0-6.0). For the particle size test , all formulas (NLC 1 - NLC 4) were in the range from 269.13 to 354.77 nm with NLC 3 (7: 3) had the smallest particle size. The results of viscosity and surface tension test were also consistent with the theory, where the addition of liquid lipid reduced viscosity and surface tension of the system. The entrapment efficiency (EE) demonstrated the EE of NLC 1: 22.24%; NLC 2: 24.71%; NLC 3: 58.21% and NLC 4: 36.94%. The penetration test showed all systems were able to penetrate the dermis layer at the 5th hour. NLC 3 (7:3) had more rapid onset, while the NLC Q10 with the ratio of lipid 9:1, had slower onset of action but can penetrate farther than the other NLC Q10 system. The result of Q10 effectiveness test showed NLC 2 (9:1) has lowest total macrophage (23.33) and very dense collagen observation (score : 4). From this research, it can be concluded that NLC 2 (9:1) had the most optimal lipid composition to increase the penetration and effectiveness of Q10 as an antioxidant in anti-aging cosmetics.

Keywords: Ubiquinone, NLC, lipid composition, antioxidant, anti-aging

INTRODUCTION

Aging is defined as intrinsic inability progressive process of the body to maintain and repair itself to work effectively[1,2]. Aging occur due to excessive UV exposure also called photoaging. To cope with oxidative stress and cell damage due to UV exposure, one of treatment used is antioxidants. Antioxidant that naturally present in body is Isopropyl palmitate, vitamin C and Ubiquinone (Q10)[3]. Ubiquinone (Q10) is one of the antioxidants that can prevent Premature aging caused by chronic UV exposure by inhibiting the formation of ROS, activation of AP-1 and IL-1α[4,5]. Nowadays, Q10 has been developed and used because has many advantages, such as increases the formation of elastin in fibroblasts; increases the expression of type III and IV collagen and lowers the depth of wrinkles around eyes after 6 months of use[5]. However, Q10 also has many weakness, such as very lipophilic, low solubility in water (<1ppb), and had large molecular weight. It causes difficulty for Q10 to penetrate into Stratum Corneum. However, Q10 is unstable and easily degraded when exposed to light. It is necessary to have delivery system that can improve the Q10 stability, to extend the effective time and deliver Q10 to penetrate the stratum corneum (SC) as well as ability to achieve controlled release[6]. One of delivery systems which widely developed is the second generation lipid nanoparticles, called NLC. NLC is developed from SLN by adding liquid lipid into solid lipid. The addition of this liquid lipid will change the crystal lattice structure of solid lipid from ordered into unordered structure, so there will be more space for the active material[7]. This crystal structure change will affect the surface tension, viscosity solubility and stability of active material. Such as, NLC system can improve the stability of antioxidants of tomato extract[8]. The NLC system not only increases the stability of the compound, but also has good skin adhesion and bioavailability. When the particle adheres to the skin surface, it will accumulate to generate packets action effect, which reduces water loss in the skin surface and increases skin hydration in order to protect the skin[14]. From the experiments conducted by Malik et al in 2018, suggested that NLCs could serve as a promising carrier for site specific targeting with better skin retention abilities[17]. One of components that affect the NLC effectiveness as the delivery system of active ingredient is lipid composition[9]. Lipids used in this study is cetyl palmitate (solid lipid) and caprylic (liquid lipids), which are combined into four (4) different ratios. Four NLC Q10 formulas made in this study is the NLC1 (NLC Q10 with ratio of solid lipid: liquid lipid = 10: 0); NLC 2 (NLC Q10 with ratio of solid lipid: liquid lipid = 9: 1); NLC 3 (NLC Q10 with ratio of solid lipid: liquid lipid = 7: 3); NLC 4

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Table 1: Formula of NLC Q10 (%w/w).

Material	Function	Concentration (%)			
		NLC 1	NLC 2	NLC 3	NLC 4
Q10	Active Ingredient	1	1	1	1
Cetyl palmitat	Solid lipid	14	12,6	9,8	7
Caprylic	Liquid Lipid	-	1,4	4,2	7
Tween 80	Surfactant	10	10	10	10
Span 80	Surfactant	10	10	10	10
Propylene Glycol	Co-Surfactant	10	10	10	10
Acetate buffer pH (4.2±0.2) ad	Water phase	100	100	100	100

Table 2: Examination results of viscosity and surface tension from NLC Q10.

Formula	Viscosity	Surface Tension
NLC 1	9775.00	7.61 x 10-3
NLC 2	6808.00	5.66 x 10-3
NLC 3	186.70	4.21 x 10-3
NLC 4	137.50	4.65 x 10-3

Note:
NLC 1: NLC Q10 Formula with Solid Lipid : Liquid Lipid ratio = 10 : 0
NLC 2: NLC Q10 Formula with Solid Lipid : Liquid Lipid ratio = 9 : 1
NLC 3: NLC Q10 Formula with Solid Lipid : Liquid Lipid ratio = 7 : 3
NLC 4: NLC Q10 Formula with Solid Lipid : Liquid Lipid ratio = 5 : 5

(NLC Q10 with ratio of solid lipid: liquid lipid = 5: 5).
The four formulas were tested in terms of characteristics, penetration and in-vivo effectiveness. The physical characteristics test of the study included pH test , particle size, polidispersity index, viscosity, surface tension and particle morphology test . Penetration test was performed by the in-vivo method using Wistar rat skin membrane. After application of 50 mg sample, the skin was then taken on the 3rd, 5th and 7th hour, and then observed using floresense microscope. The effectiveness test of Q10 as antiaging in this study has passed from ethical feasibility test from Airlangga University and included three aspects, namely the amount of macrophages (AM), amount of fibroblasts (AF) and amount of collagen (AK) which counted as qualitative scoring system

METHOD

Instruments

The instruments used in this research were: Differential Scanning Calorymetry (DSC), X-Ray Powder Diffraction (Philips X'Pert, Netherland), Double Beam Spektrofotometer Shimadzu UV-1800, Particle Analyzer Delsa™ Nano Submicron Particle Size, Ultra Turrax IKA® T25 Digital High Shear Homogenizer, Scanning Electron Microscope (SEM) JEOL JSM 840, Magnetic Stirer, Hotplate Dragon Lab MS H-Pro, pH meter Schott Glass Mainz tipe CG 842, viskosimeter Cone and Plate (CPE 41), Fluorescence Microscopy (Olympus FX-1000), Cryostat, sentrifuge (Hettich Rotofix 32), analytical balance of CHYO JP-160, thermometer, complete rat cage (with food and drink), Fixation board, Optilab, LC Optimal camera, broadband ultraviolet B lamp

Table 3: The EE test results of NLC formulas.

Formula	EE average (%) ± SD	KV
NLC 1	22.24 ± 1.50	6.76
NLC 2	24.71 ± 1.77	7.17
NLC 3	58.21 ± 0.09	0.16
NLC 4	36.94 ± 0.46	1.25

MATERIALS

The materials used in this research if did not state otherwise, were in pharmaceutical grade purity. The materials used in this research were Ubiquinone (Q10), Cetyl palmitate, caprylic, Tween 80, Span 80, Propylene Glycol and acetate buffer. Acetate buffer with pH 4.2 ± 0.2 is made of glacial acetic acid and sodium acetate with pro analysis quality.

Preparation Of NLC Q10

NLC Q10 was made by the High Shear Homogenizaton method. The lipid phase and the aqueous phase were made with a high shear homogenizer Ultra-turax with speed of 3400 rpm for 1 minute at temperature (50^0 ± 5ºC) for 3 cycles each. The lipid phase and water phase were then mixed with high shear homogenizer Ultra-turax with speed of 24000 rpm in 3 minutes. Cooling phase then be performed at speed of 500 rpm until reached room temperature.

Physical Charateristic Test of NLC Q10

Physical characterization test included organoleptic, pH, viscosity, particle size and polidipersity index, particle morphology, determination of diffraction pattern and regularity of crystal structure and entrappment efficiency test .

Particle Size and Polydipersity Index Test

The average particle size and particle size distribution test of sample was performed with Delsa ™ Nano Submicron Particle Size Analysis

Particle Morphology Test

particles morphology test was performed using Scanning Electron Microscope (SEM). Magnification used was 5000x.

Determination of Diffraction Pattern and Crystal Structure Regularity:

X-ray diffraction analysis of samples powder was done at room temperature by using X-Ray Powder Diffraction (XRPD)

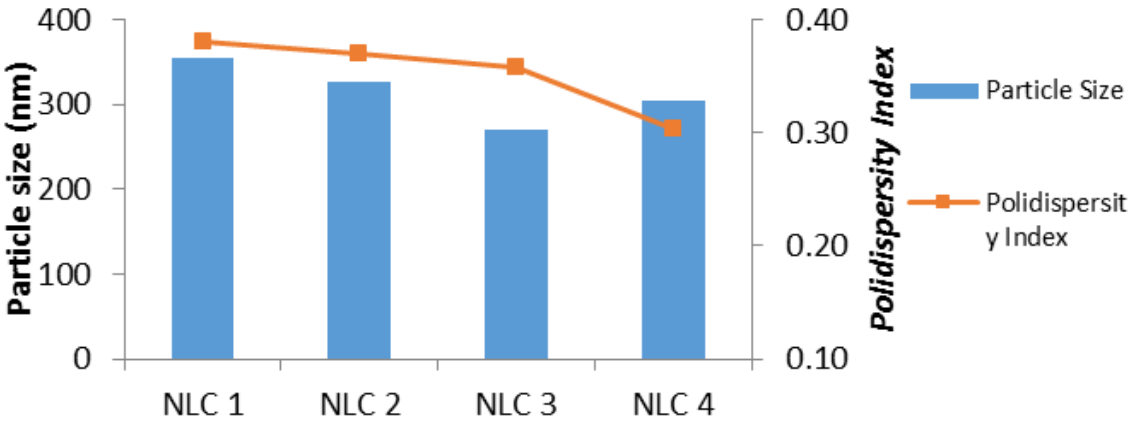

Figure 1: Graph of particle size and polidispersity index(PI) of NLC Q10.

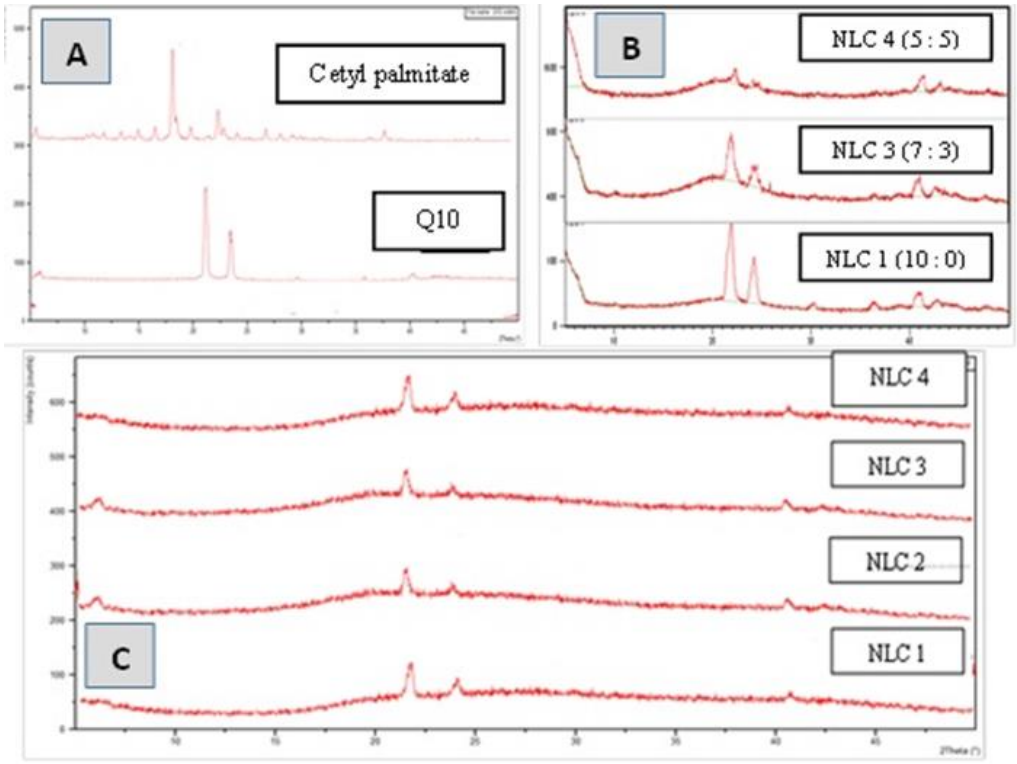

Figure 2: Diffraction Patterns and Regularity of Crystal Structure: A : Single material, Q10 dan cetyl palmitate (at 0-5000 intensity); B : lipid mixture of solid and liquid lipid at various lipid compositions (at 0-1600 intensity) ; C : NLC Q10 on various lipid compositions (at 0-600 intensity).

Entrappment Efficiency Test

Entrappment efficiency eveluation was performed using centrifugation method at 1500 rpm for 15 minutes. % EE was calculated from the initial concentration of Q10 subtracted with the untrapped Q10 content divided by initial content of Q10

$$EP\ (\%) = [(Ct-Cf)/Ct] \times 100\%$$

Note :

Ct : Initial concentration of Q10 in NLC

Cf : Untrapped concentration of Q10

In-vivo Penetration Test

The research object used in this penetration tests in NLC system was Wistar rats what met the inclusion criteria (healthy; 8-10 weeks age; weight of 100-250 grams) and did not meet the exclusion criteria (there is concomitant skin diseases and prevent bleeding in mouse skin). The sample size used was 36 rats, which consisted of 12 test groups in the observations of 3, 5 and 7 hours. Rats is anesthetized with ketamine, and then the abdomen hairs were cleaned using mechanical hair clipper. 50 mg test sample which have been given fluorescent dye (rhodamine) then applied to the rat skin. At each observation hour (3^{rd}, 5^{th} and 7^{th} hour) the skin was cut using frozen microtome in 5µm thickness. Skin sample was then observed with microscope fluorescent at 70 times magnification.

Anti-aging In Vivo Activity Test

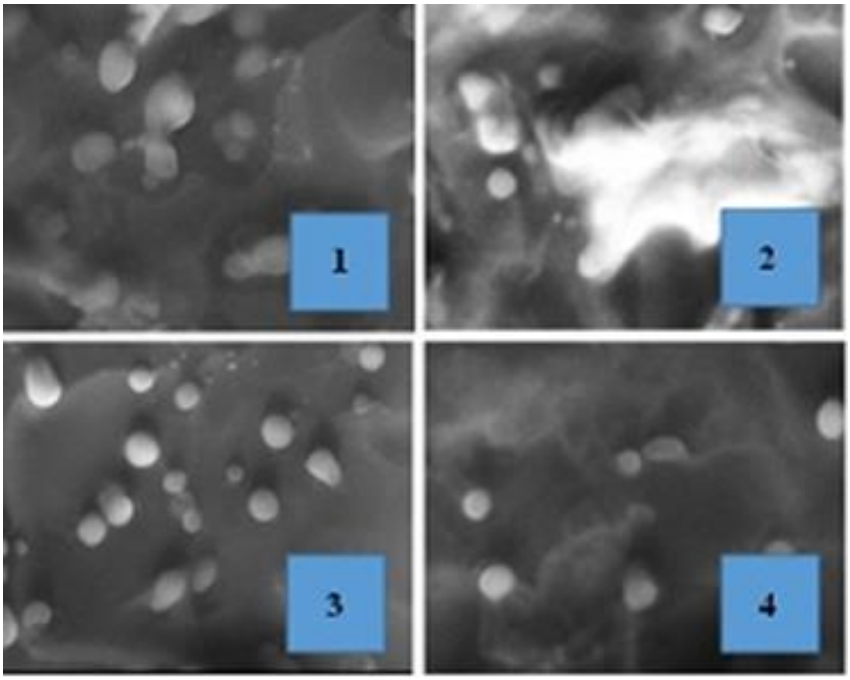

Figure 3**:** NLC Q10 particle morphologies at various lipid compositions.

The research object used in this anti-aging effectiveness test has the same citeria with object which was used in penetration test. The sample size used was 24 rats, which divided into six test groups, four groups of test samples and two control groups. Rat back were shaved exposed to UV light at dose of 840 mJ / cm^2. Exposure was three times a day for 5 days until total dose reached 840 mJ / cm^2. Four (4) different test samples were applied two times for each treatment, which was 20 minutes before exposure (to allow time for topicals absorption into the skin) and 4 hours after exposure (ROS formation started 4 hours after exposure). On day 5, the skin was taken and then analyzed by optical microscopy to obtain total amount of macrophages, fibroblast and collagen.

RESULTS AND DISCUSSION

Organoleptic Examination

The result of the organoleptic test showed that all formulas have yellow color, the distinctive smell of acetate buffer and semisolid consistency. NLC 3 and NLC 4 have the less semisolid consistency because of the more liquid lipid addition on both formulas. However, blends with liquid lipid >10% exhibited miscibility issues. This could be attributed to the disruption of ordered arrangement of solid lipid and expulsion of oil from the lipid matrix at higher liquid lipid concentration[17,18]

pH Examination

From the pH examination results at various lipid compositions can be seen that pH of all NLC samples have fulfilled skin pH range of 4.00 to 6.00.

Viscosity and Surface Tension Examination

Results of viscosity and surface tension tests from all NLC formulas were shown in the following table.

From Table 2, it can be seen that with the increasing concentration of the liquid lipid, the system viscosity and system surface tension reduced. The addition of liquid lipid reduced the system surface tension, where it also reduced the system viscosity[10].

Particle Size and polidispersity index Examination

The examination results of all NLC formulas can be seen in figure 1From the graph it can be seen that size of all NLC Q10 formulas were below 400 nm, The particle size was slightly increased by encapsulation of molecules (Q10) compared to the drug-free NLCs[24]. Polidispersity index of NLC Q10 can also be seen below 0,4. This result indicating a monodisperse, narrow and homogeneous size distribution because PI was below 0.3[19,24]. In summary, by adding liquid lipid concentration, the particle size and system PI were smaller, which means that the formed system become more homogenous [9,10]. Based on the ANOVA statistical analysis resultas, system particle size examination of NLC Q10 obtained p-value (sig) lower than 0.05. It showed significant difference of particle size between all NLC formulas at various lipid compositions. Whereas anova statistical analysis results of polidispersity index of NLC Q10 system obtained that p-value (sig) was greater than 0.05. This showed that no significant differences of polidispersity index between NLC formula at various lipid compositions.

Crystallinity Test

The diffraction patterns and regularity test results of crystal structure over angles range 2θ 5^0-50^0 can be seen in the ffigure.

From figure 2 (B), it can be seen that with the increasing concentration of liquid lipid, the diffraction intensity was decreasing. This is due to the more liquid lipid addition the more changes of internal crystalline structure, where it able to increase drug loading[11,12]. From figure 2 (C), it can be seen that the absorption peaks of cetyl palmitate in NLC system was still visible, although the intensity was not as high as in single material diffraction. This showed that Q10 were completely dissolved and encapsulated in the lipid

Result of penetration depth test at 3rd hour

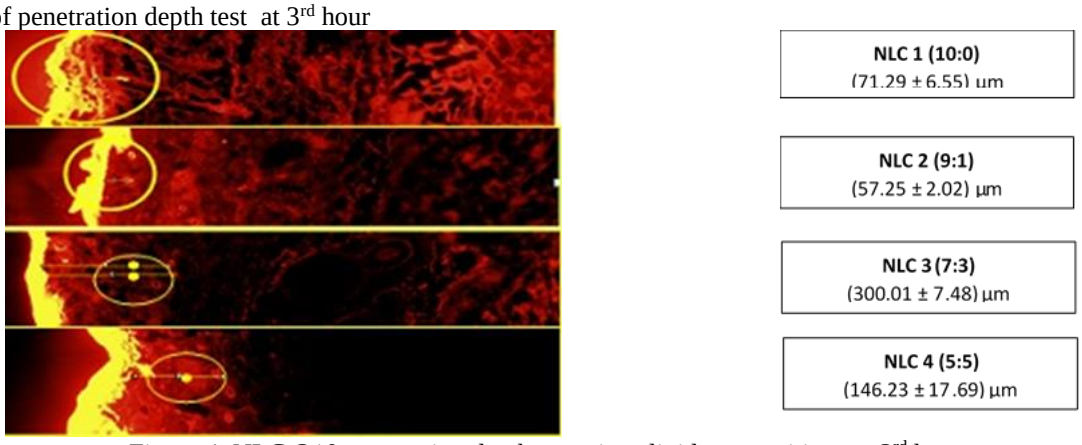

Figure 4: NLC Q10 penetration depth at various lipid compositions at 3rd hour

Result of penetration depth test at 5th hour

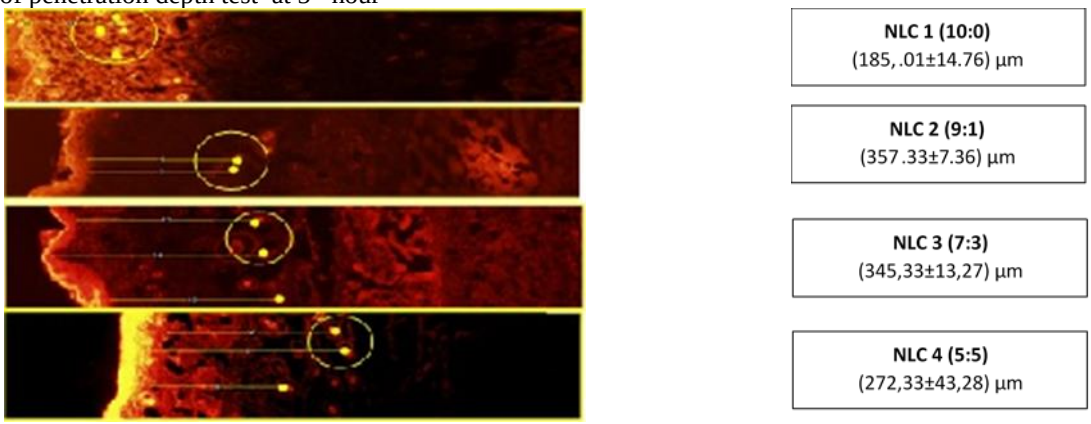

Figure 5: NLC Q10 penetration depth at various lipid compositions at 5th hour.

Result of penetration depth test at 7th hour

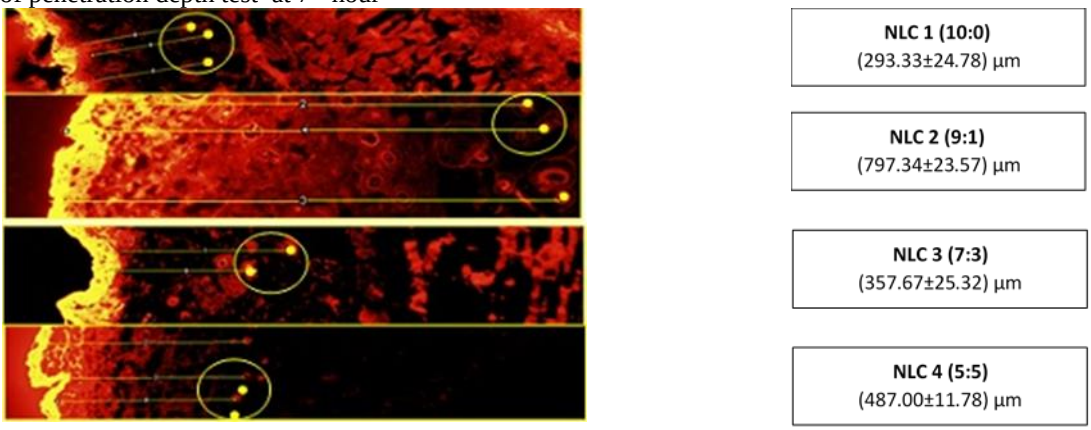

Figure 6: NLC Q10 penetration depth at various lipid compositions at 7th hour.

matrix therefore the properties of the pure Q10 structure could not be observed[15,16]. From those figures can be seen that the appearance of diffraction angle between NLC systems with different lipid compositions showed no significant difference.

Entrappment Efficiency (EE)

The test results of NLC formulas can be seen in Table 3. Based on the ANOVA statistical analysis results, EE of NLC Q10 system obtained p-value (sig) lowers than 0.05. It showed that there was significant difference of EE between all NLC formulas at various lipid compositions, whereas NLC 4 has the best EE than any other systens.

Particle Morphology Test

The Test result of NLC Q10 particle morphology can be seen in figure 3.

From particle morphology test in Figure 3 can be seen that the SLN Q10 (NLC 1) has less spherical shape compared with other NLC. NLC 2, 3 and 4 did not show significant morphological differences with same spherical shape with smaller size than NLC 1, but it can be seen that the NLC 3 shape (Formula NLC Q10 with the ratio of lipid solid:

Penetration depth comparison of NLC preparation.
Below is the depth of penetration comparison from various NLC Q10 systems with various different lipid composition.

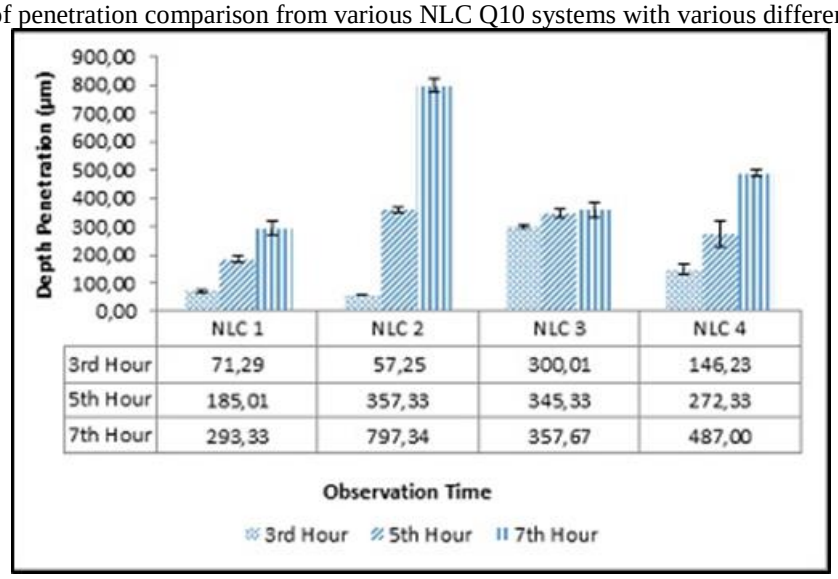

Figure 7: Penetration depth comparison of NLC Q10 with various lipid compositions at each observation hour

Qualitative intensity comparison of NLCQ10 penetration with various lipid compositions at each observation hour

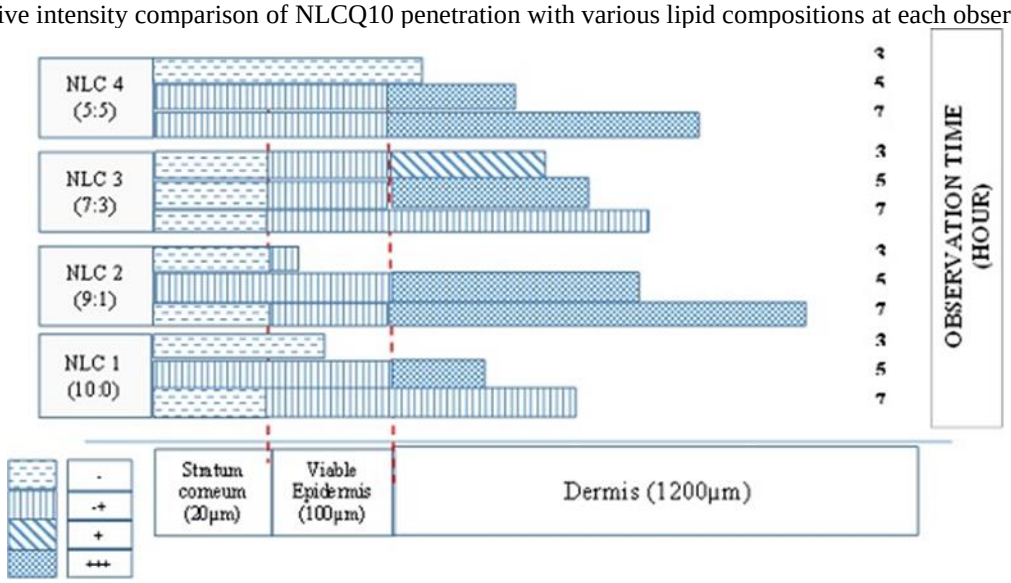

Figure 8: Qualitative intensity comparison of NLCQ10 penetration with various lipid compositions at each observation hour.

Table 4: Macrophages and fibroblasts amount from NLC Q10 observations at various lipid compositions.

Formula	Total Macrophages	Total Fibroblast
NLC 1	46.00 ± 6.48	13.33 ± 4.03
NLC 2	23.33 ± 2.05	19.33 ± 3.09
NLC 3	25.67 ± 8.34	22.67 ± 2.87
NLC 4	25.00 ± 4.97	No appearance of fibroblast – PMN cell (±52)

liquid lipid = 7: 3) was the most spherical particle compared to other formulas. They reported that morphologies and particle sizes were greatly influenced by oil concentration. They showed that increasing oil up to 30% produce spherical particles with smooth surfaces and small sizes[21]. Moreover, the micrograph also revealed the agglomeration of nanoparticles which might be due to the lipid nature of the carrier and the drying process during sample preparation prior to SEM analysis[22,23]. In summary, with the increasing concentration of liquid lipid, the formed particles become more spherical with smoother surfaces[10]

Penetration Test Results of Q10 at Rat Skin membranes

Test results of penetration depth from NLC Q10 at various composition as follows:

From Figure 7 and 8 above can be seen that at 3rd hour, NLC 3 and NLC 4 showed deeper penetration than other NLC systems. At the 5th hour, all NLC systems showed significant increase in penetration depth. At the 7th hour, all systems still showed increase in penetration depth, but

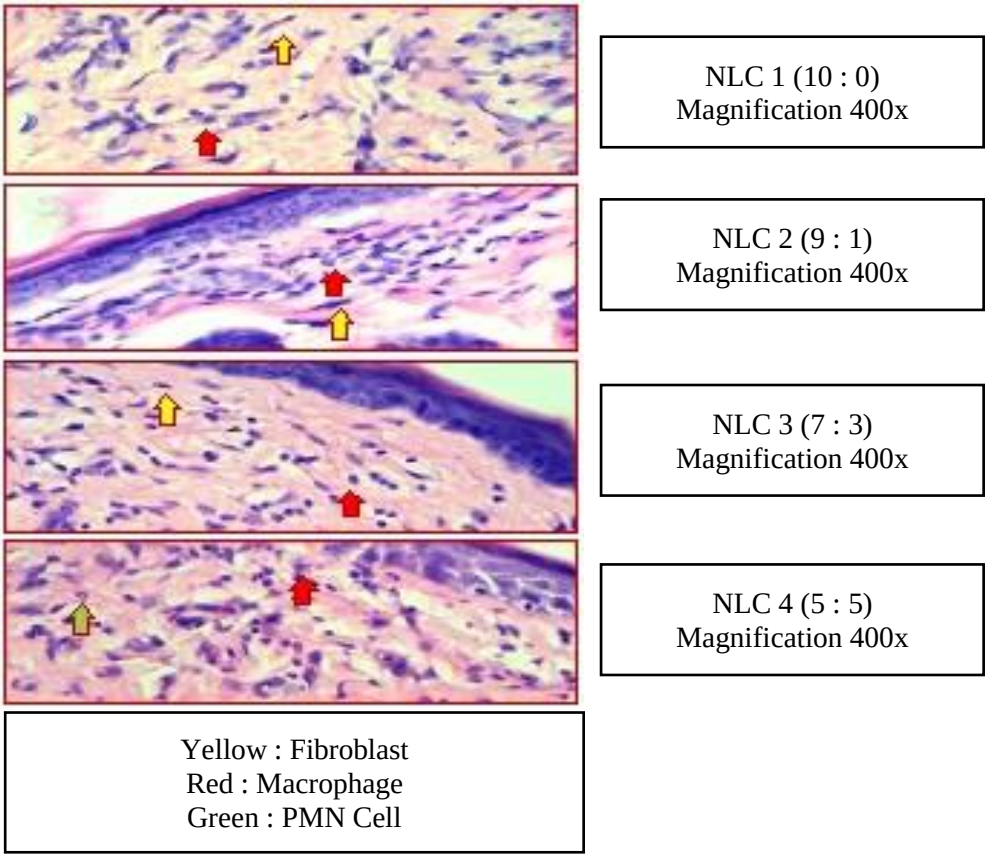

Figure 9: Comparison of macrophage and fibroblast amount after 5 days treatment.

Table 6: Scoring amount of collagen density from effectiveness observation of NLC Q10 preparation at various lipid compositions.

NLC System	Scoring
NLC 1	1
NLC 2	4
NLC 3	2
NLC 4	0

the most optimal penetration depth was NLC 2, the NLC Q10 system with lipid composition ratio of 9: 1. In summary, the NLC-based systems led to deeper skin penetration of both lipophilic model drugs[19]. Slower drug release profile of NLCs may increase the drug effect and durability on active site[20].

Effectiveness Test of NLC Q10 Preparation

Observations results of total macrophages, total fibroblasts and total collagen amount qualitatively

Observation of Total Macrophage and fibroblast

Functionally, the macrophages actively remove dead and damaged cells, bacteria and cellular debris from the body. Macrophage amount showed higher damaged / dead cells that exist on the skin, in this study, which is caused by UV exposure [13]. Otherwise, fibroblasts amount indicated that cell repair initiated by the use of NLC with various lipid compositions after being exposed to UV. From Table 4 it can be seen that after 5 days treatment, NLC 2 showed macrophages amount were lower than other NLC systems, while NLC 3 showed higher fibroblast amount than other NLC systems. From this observation, it can be concluded that NLC 2 and NLC 3 could provide better cellular repair after being exposed.

Statistical analysis from both macrophages and fibrobast amount showed that p-value (sig) was less than 0.05. This showed there were significant differences in macrophage and fibroblast amount which were formed after treatment.

Collagen amount observation

Collagen amount was determined based on hispatological parameters score in the calculation of several field of view at 400x magnification as seen in table below

The observation results of collagen density from NLC preparation at various lipid compositions is:

From table 6, it can be seen that after 5 days treatment, NLC 2 showed high scoring of collagen density qualitatively compared to other systems. Initial formation of collagen fibers showed sustained improvement after the formation of fibroblast cells. It can be concluded that the NLC 2 showed better cell repair compared to other systems. The initial rapid release of Q10-loaded NLCs may be caused by the enrichment of Q10 in the outer surface of the NLCs that immediately diffuses into the release medium. The later sustained release could be attributed to the degradation and erosion of the inner lipid matrix where the drug could be molecularly dispersed or dissolved[24] From the EE value, it can be seen that NLC 2 had low value but it gave good effectiveness. This was because the smaller EE value, the more active ingredient in the outer system and not in entrapped condition. It allows active ingredient to penetrate deeper and gave better

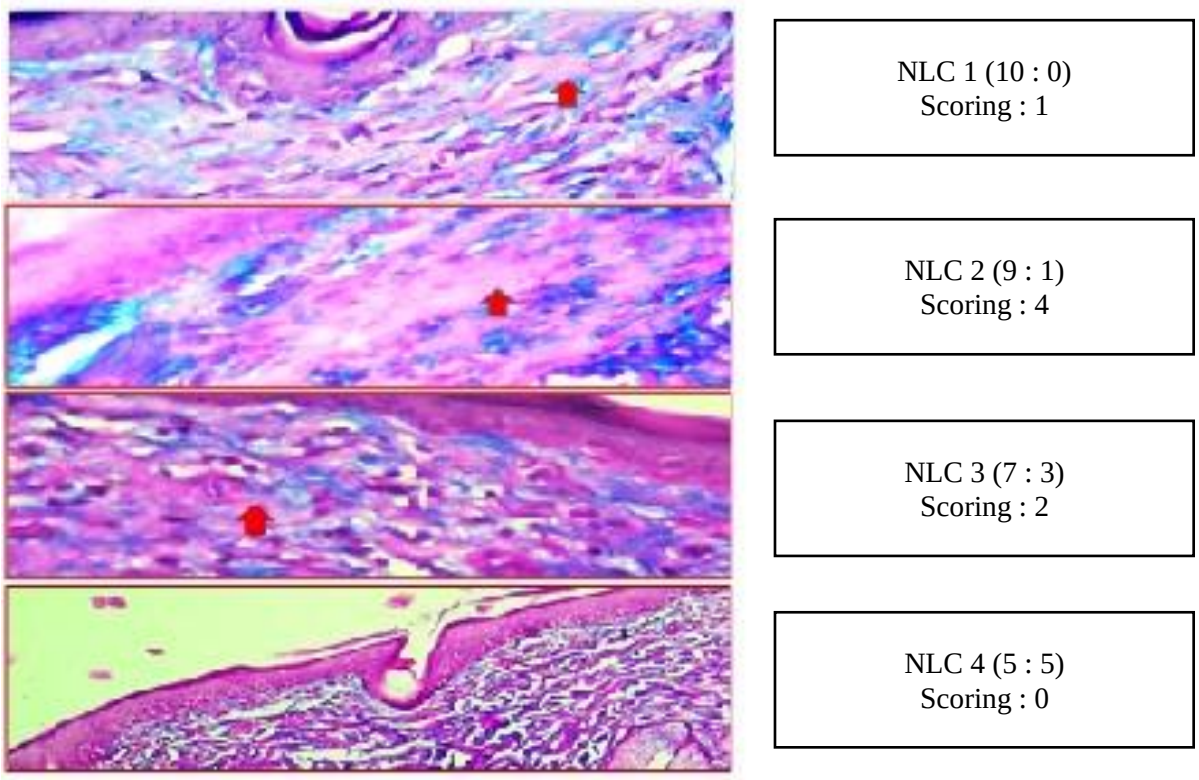

Figure 10: Collagen density comparison after 5 days treatment.

Table 5: Histopatological scoring parameters for collagen density.

Scoring	Parameters
0	There was no collagen appearance
+1	The collagen density was low (approximately 10% per field of view)
+2	The collagen density was moderate (10 s / d 50% per field of view)
+3	The density of collagen is dense (50 s / d 90% per field of view)
+4	The density of collagen is very dense (90 s / d 100% per field of view)

effect. Further more, the entrapped active ingredient served as reservoir that can maintain drug levels.

CONCLUSION

From this research can be concluded that lipid composition affected the NLC characteristics, Q10 penetration and effectiveness of Q10 as antioxidant, NLC 2 (9: 1) was the most optimal lipid composition to increase the penetration and Q10 effectiveness as antioxidant in anti-aging cosmetics.

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