Characterisation and cytotoxicity assay of curcumin nanostructured lipid carrier on HeLa cells

Rabima* and A Oktamauri

Department of Pharmacy, Faculty of Pharmacy, Universitas 17 Agustus 1945, Jakarta, Indonesia.

E-mail: rabima86@gmail.com

Abstract. Low bioavailability is major problem in the use of curcumin. Nanostructured Lipid Carrier (NLC) is an interesting generation of lipid-based nanoparticles, because of its ability to increase the bioavailability of drugs. The objectives of this study were to make a formulation of curcumin in NLC (CRM-NLC) preparations, to determine its characteristic and to examine the cytotoxic effects of CRM-NLC on HeLa cells in vitro. CRM-NLC was made by the method of evaporation and diffusion of solvents in aqueous systems then followed by ultrasonication. Its particle size, polydispersity index, zeta potential were determined by using Particle Size Analyzer. The structure and morphology were observed by using Transmission Electron Microscopy then its cytotoxic activity toward HeLa cells was examined by using the MTT method then Half Maximal Inhibition Concentration (IC50) was determined. CRM-NLC produced in this study had an average particle size of 17.4 nm, a polydispersity index of 0.574, the zeta potential of -63.43 mV, with structure and the morphology of CRM-NLC was round and smooth surface. CRM-NLC IC50 value obtained in this study was 8,872 µg / mL. This study has succeeded in making CRM-NLC preparation with good characteristics and improving curcumin activity on HeLa cells.

1. Introduction
Cervical cancer ranks as the fourth most frequently diagnosed cancer and the fourth leading cause of cancer death in women. It is the most commonly diagnosed cancer in 28 countries and the leading cause of cancer death in 42 countries, the vast majority of which are in Sub-Saharan Africa and South-Eastern Asia [1]. World Health Organisation states that the amount of patient of cervical cancer in Indonesia is one of the highest in the world. The prevalence of cervical cancer in Indonesia is 0.8%, the second highest after breast cancer [2]. Various strategies for treatment of cervical cancer have been carried out including surgical therapy, radiotherapy, and chemotherapy as well as combination of them. Nakano et al., [3] states that radiotherapy is one effective way to treat patients with cervical cancer, but only limited to small tumor because the use of larger doses of radiotherapy can cause damage to normal tissue while chemotherapy can cause adverse side effects such as hair loss, bone marrow suppression, gastrointestinal lesions, neurological dysfunction, cardiac toxicity and resistance [4]. The use of natural materials as alternative medicines is a concern because it is cheaper, abundant, and relatively safer. Curcumin is a phenolic compound that acts as a yellow pigment on the rhizome of the Curcuma plant. Curcumin has been reported to have strong potential as an anticancer in various types of cancer cells including pancreatic cancer, ovarian cancer, breast cancer, colon cancer and cervical cancer [5]-[10]. Previous study reported that Curcuma longa extract had cytotoxicity toward
HeLa cells with an IC50 value of 12.5 µg/ml [11]. Because of curcumin good anticancer effects, the USA National Cancer Institute (NCI) classifies it in the third generation of chemo-preventive drug class. Hence, the fact shows that the bioavailability of curcumin is very low. Curcumin that is given orally less than 10 g is not detected in blood. The low bioavailability is due to its low solubility and rapid metabolism, especially through conjugation of sulfate and glucuronide [12].

NLC become an alternative to increase the bioavailability of curcumin. NLC is the second generation of lipid-based nanoparticle which are introduced to overcome the disadvantages of Solid Lipid Nanoparticle (SLN), namely the limited loading capacity and expulsions of drugs during storage [13]. The other advantages of NLC as a drug carrier are improving drug stability, controlled drug release, and good tolerability [14]. The use of NLC is to deliver anticancer agents via ‘passive targeting’ whereby the particles are accumulated in the tumour area based on the leaky vasculature-enhanced permeability and retention effect has been extensively studied [15]. Thus, NLC have been recently emerged as a multifunctional platform for drug delivery in cancer therapy, several examples for the use of NLCs in delivery and targeting of chemotherapeutic agents were Docetaxel and Paclitaxel-NLC which demonstrated their remarkable ability to boost therapeutic effect of those drugs across a wide variety of malignancies [16]. In this study, we formulated curcumin into NLC using oleic acid as liquid lipid and cholesterol as solid lipid and also evaluated the impact of NLC formulation of curcumin on its cytotoxicity towards HeLa cell line.

2. Material and methods

2.1. Material

Oleic acid, Tween 80, ethanol and acetone (Merck), cholesterol (Sigma-Aldrich). HeLa cells line obtained from LAPTIAB (Indonesia), 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, St. Louis, MO, USA), RPMI Medium, sera, and antibiotics for cell culture purchased from Sigma-Aldrich (US).

2.2. Methods

2.2.1. Fabrication and characterisation of CRM-NLC. Fabrication and characterisation of CRM-NLC was prepared using emulsion solvent diffusion and evaporation followed by ultrasonication according to a previously published method [16]. Firstly, the oil phase was prepared by mixing 60 mg of cholesterol (Merck) and oleic acid (15%, Merck) which was then dissolved into a mixture of acetone (3 mL) and ethanol (3 mL) at 60°C. The oil phase was then added with curcumin (Merck) 5% according to drug/lipid weight ratio to form homogenous oil-curcumin phase. In the second step, a 60 ml aquades was added to 1% tween 80 at 60°C to form an aqueous phase. The oil-curcumin phase was then dispersed in an aqueous phase with a magnetic stirrer for 5 minutes at a speed of 800 rpm. Then, the mixture was ultrasonicated for 2 minutes at a speed of 800 rpm by using ultraturrax homogenizer (IKA Works, Inc., Wilmington) and 40% amplitude to produce an oil in water CRM-NLC. After homogenization was completed, the mixture was cooled at room temperature with a magnetic stirrer at 800 rpm speed for one hour. CRM-NLC was characterized using Delsa™Nano C (Beckman Coulter, Inc.) to analyze the particle size, and transmission electron microscope (TEM) (JEOL JEM-1400) to analyze the shape and its morphological structured.

2.2.2. Cell culture. Cell culture of HeLa was purchased from culture collection of LAPTIAB, Agency for The Assessment and Application of Technology (Indonesia) and grew at 37°C in a humidified atmosphere of 5% CO2 in RPMI medium mixed with 10% fetal bovine serum, 1 0000 U/L penicillin, and 10 mg/L streptomycin. For the experiments, the cells were detached by trypsin treatment, seeded at a density cells/mL into 96-well culture plate, and incubated for 24 h at 37°C in 5% CO2 and 100% humidity before cell viability test.
2.2.3. Cytotoxicity assay of CRM-NLC. Cytotoxicity assay of CRM-NLC was done using the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, St. Louis, MO, USA) assay. This was performed to evaluate cell viability treated by curcumin and CRM-NLC according to a published method [15] with minor modification. HeLa cells were grown in 96-well plates, treated with curcumin (31.25-1000 µg/mL) and CRM-NLC (4.7-151 µg/mL) and incubated for 48 h. Then, 10 µL MTT (5 mg/mL) added to each well and incubated at 37°C for four hours. Finally, HeLa cells were lysed with DMSO, and the absorbance was measured using a microplate reader at 570 nm. The experiment was repeated at three times. The viability cells and IC₅₀ values were determined.

3. Result and discussion

3.1. Fabrication and characterisation of CRM-NLC

Fabrication of CRM-NLC consisted of three main stages, which were pre-emulsions, homogenisation and ultrasonication. The combining techniques had several advantages such as the preparation of nanoparticles obtained having smaller particle sizes, simple and effective equipment for laboratory scale [17]. In addition, several previous studies have proven successful using the incorporation of this method in producing NLC [18][20]. Pre-emulsion was made by mixing the oil-currucmin phase with the water phase at 60°C. The oil-curcumin phase was in a liquid state when it was dispersed into the water phase so that it was dispersed in the form of small droplets in the aqueous phase which is stabilized by emulsifiers [21]. This mixing of oil-curcumin and water phases forms pre-emulsions which were then homogenized. It was aimed to unite the oil-curcumin phase with the water phase until it was homogeneous and breaking down large particles into smaller ones. Homogenisation results were cooled into room temperature so that the droplets of oil-curcumin phase dispersed in the aqueous phase could crystallized as soon as possible with small particle sizes before the droplets clumped back into larger droplets to obtain a homogeneous emulsion. The CRM-NLC was then ultrasonicated to help reduce particle size [18]. In the ultrasonication process, ultrasonic waves are produced which cause high speed liquid flow by ultrasonic cavitation so that the particles collided with each other very quickly. This caused damage to the van der Waals force and even the main bond in the particle of CRM-NLC.

This study has succeeded in formulating CRM-NLC (Figure 1a). Cholesterol was used in this study because based on previous research reports, cholesterol-enriched nanoemulsion could increase LDL receptors after being injected into the blood [16], [26]. The use of tween 80 as a surfactant because it had a Hydrophilic Lipophilic Balance (HLB) value of around 15 and it is also a suitable surfactant used in the manufacture of oil-in-water emulsions. Tween 80 was safe and non-toxic surfactant, and it was compatible with materials used in the CRM-NLC carrier system [22]. Tween 80 has been used successfully in previous studies as a surfactant in the production of NLC [18], [23], [24]. Tween 80 was a surfactant that gives the best characteristics in curcumin nanoemulsion [25], [26]. One of the parameters of successfulness of CRM-NLC preparation was indicated by the absence of phase separation and creaming [26]. This research has succeeded in producing CRM-NLC according to these parameters (Figure 1a). The NLC-curcumin produced did not have a precipitate, was a clear liquid, yellow in color and homogenous. Sari et al., [27] had formulated curcumin nanoemulsion with several concentrations of active substances, which produced nanoemulsion in the form of clear yellow liquid. This clear liquid showed good solubility and well dispersed particles in nanoemulsion.

The particle size of nanoparticles was closely related to their absorption in the body. The small particle size will increase the surface area which causes high solubility making it easier for the particle to be absorbed into the body, increasing bioavailability thereby increasing the effectiveness of treatment [28]. Particle size determination was done using the principle of dynamic light scattering Particle Size Analyzer (PSA). The obtained average particle size was 17.4 nm. Particle size distribution can be seen in (Figure 1b). The size was in accordance with the particle size range for NLC, which according to Rosli et al., [29], NLC had a particle size of 10-100 nm. The particle size was also suitable for cancer treatment, where particles with a size of less than 50 nm provide the best
efficacy in preventing tumor growth \textit{in vivo} [30]. The particle size obtained in this study was smaller than the results of previous studies. Madane & Mahajan [28] and Behbahani \textit{et al.}, [31] obtained CRM-NLC particle sizes of 146, 8 nm and 220 nm, respectively.

\textbf{Figure 1}. CRM-NLC produced (a) and characterization of particle size diameter (b), a selected TEM image (c), and zeta potential (d).

This was probably due to the fact that in this study CRM-NLC was prepared using a combination technique to obtain nanoparticles in smaller sizes. Particle size was also influenced using surfactants and liquid lipid components in the NLC system. The used of tween 80 as a surfactant produced smaller particle sizes of NLC compared to other types of surfactants [24]. In addition, lower surface tension and viscosity of lipid matrices containing oleic acid contribute to the formation of NLC with smaller particle sizes [32]. This result was in line with Swidan \textit{et al.}, [33] which stated that NLCs with oleic acid as liquid lipids have smaller particle sizes compared to the use of Capryol 90 because oleic acid has a higher viscosity.

Polydispersity index described the level of uniformity in a system, where the smaller the polydispersity index value, the distribution of particles in a mono-dispersion system was more uniform [34]. The polydispersity index value obtained was 0.574. The value $<0.7$ of polydispersity index was said to have a mono-dispersion particle distribution. The mono dispersion particle system shows a distribution of particle sizes that tends to be narrow and indicates a stable nanoparticle system because of the fewer particles that form aggregates. Zeta potential described the charge of particles contained on the surface of a colloidal system, and in addition it is used to predict physical stability in the long storage [22]. The zeta potential obtained was -63.43 mV, where a colloidal dispersion system with a zeta potential of $\pm 30$ mV was considered to be a stable formulation. High negative or positive values
of zeta potential will cause nanoparticles to repel each other and to prevent aggregation tendencies [29]. Therefore, it can be said that CRM-NLC had a good dispersion system stability. This is influenced by the presence of Tween 80 as surfactant. This surfactant stabilized CRM-NLC by forming a layer around the surface of the CRM-NLC thereby reducing the electrostatic repulsion between the particles, increasing the physical stability of the CRM-NLC and preventing aggregation.

The zeta potential obtained was negatively charged (Figure 1d). This of course was greatly influenced by the components of the CRM-NLC itself. The type of lipid used, had a significant effect on zeta potential. Oleic acid has a carboxyl group with a very strong negative charge when ionized. This result was in line with the results obtained by Swidan et al., [33] who also used oleic acid as a component of liquid lipids in the NLC formulation. In addition, it was also likely due to the hydroxyl groups in cholesterol and Tween 80 which can be partially negative if ionized in line with the results obtained by Zardini et al., [36] who used Tween 80 as a surfactant in the NLC-Lycopene formulation as well as research done by Emami et al., [16] using cholesterol, oleic acid and Tween 80 in the NLC-Paclitaxel formulation. Morphological examination of CRM-NLC particles was carried out using TEM JEOL JEM 1400 at an operating voltage of 200 kV with magnification of 100000 times. The structure and morphology of the CRM-NLC seen globular (spherical) with a smooth surface and had no aggregation of particles as can be seen in (Figure 1c). These results were in accordance with the study of Sriarumtias et al., [37] which stated that nanoparticles with lipids as carriers, were round in shape with a smooth surface, whatever type of lipid is used [36]. This indicated a good homogeneity and unity of NLC-Curcumin so that a regular release of curcumin trapped in CRM-NLC can be obtained [33].

3.2. Cytotoxicity assay of CRM-NLC

![Figure 2](image_url)

**Figure 2.** Cell viability assay for curcumin (A) and CRM-NLC (B), and IC<sub>50</sub> values (C) of curcumin and CRM-NLC on HeLa cell line. Control, untreated cells. P < 0.001 indicated significant difference from curcumin standard.

The Half Maximal Inhibitory Concentration IC<sub>50</sub> of curcumin and CRM-NLC obtained in this study were 24.062 and 8.872 µg / ml, respectively (Figure 2). According to the National Cancer Institute, a compound said to have an active anticancer activity when the value of IC<sub>50</sub> is <30 pg / ml [38]. IC<sub>50</sub> of CRM-NLC obtained in this study were smaller than IC<sub>50</sub> of curcumin in previous studies but the value obtained is larger than the results of the study of Wang et al., [39] who tested the cytotoxicity of CRM-NLC against lung cancer cells with IC<sub>50</sub> of 5.66 µg/mL. Some previous studies also showed a
tendency to decrease the $IC_{50}$ of active substances formulated into NLC preparations. Therefore, the results obtained were indicating that CRM-NLC was more toxic compared to standard curcumin. It could also be seen from the results of Hela cell image using microscope *inverted* after being given standard curcumin treatment and CRM-NLC, it was seen that Hela cells with CRM-NLC treatment appeared to have more deaths than with standard curcumin treatment (Figure 3).

This was due to the ability of NLC to attach and to pass through cell membranes which depended on their physicochemical characteristics in terms of size, composition and surface charge. CRM-NLC with a small size is likely to be very easy to pass through the cell membrane. In addition, based on previous research reports, cholesterol-enriched nanoemulsions could increase LDL receptors after being injected into the blood. Most cancers shown an increased regulation of LDL receptors, therefore, cholesterol-enriched nanoparticles might be used as an antineoplastic drug carrier system to cancer cells. This is in line with several research results that show that cholesterol-enriched microemulsions and nanoemulsions could internalize drugs such as carmustine, etoposide and paclitaxel into cultured neoplastic cells. So it was thought that the cholesterol-enriched CRM-NLC formulation combining passive and active targeted drug delivery systems. Passive targeted delivery through increased permeation and retention (EPR) effects due to its small particle size. Whereas the active targeted delivery of CRM-NLC is carried and internalized to cancer cells through the *endocytosis pathway* of the LDL receptor. Thus, the amount of curcumin released into cancer cells could increase [16, 39].

The mechanism of curcumin inhibition of HeLa cells was not known with certainty. However, research shown that curcumin play a role in regulating the activity of the NF-κB and Akt pathways on HeLa cells which triggered the process of apoptosis and inhibits cell proliferation [7, 9]. Another possible mechanism was to inhibit telomerase activity. Cervical cancer cells infected with HPV are known to express E6 oncogene. E6 protein stimulated the activity of the telomerase enzyme. Curcumin could inhibit telomerase activity in human neuroblastoma cells in line [4] and telomerase activity in brain tumor cells [41]. This was also supported by *docking* simulation results which showing that curcumin had an interaction with good stability toward the 12-lipoxygenase enzyme which played a role in the inflammatory process [42, 43].

![Figure 3. HeLa cells without treatments (a) and HeLa cells after treatment with CRM-NLC (b).](image)

4. **Conclusion**

The results obtained in this study indicated that curcumin could be formulated as an NLC preparation with good characteristics and potential to be developed as an effective drug delivery system because it improved cytotoxicity of curcumin on HeLa Cells.
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