RESEARCH ARTICLE

# Preparation and *In-vitro* Hematological Evaluation of Chlorambucil Loaded Lipid Nanoparticles

Ameer S. Sahib,[1*] Shaimaa N. A. Alhammid,[2] M. Akrami,[3] Hussein A. Muhammed[4]

[*1]*Ministry of Health and Environment, Babylon Health Directorate, Babylon, Iraq*
[2]*Department of Pharmaceutics, College of Pharmacy, University of Baghdad, Baghdad, Iraq.*
[3]*Department of Pharmaceutical Biomaterials and Medical Biomaterials Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.*
[4]*Department of pharmaceutics, College of Pharmacy, University of Al Qadisiya, Al Diwaniya, Iraq.*

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## ABSTRACT

Chlorambucil (CBL) is an orally administered anticancer drug currently utilized in the therapy of lymphomas, breast, and ovarian carcinomas. CBL was a candidate to be loaded inside lipid nanocarrier for parenteral administration to eliminate its serious adverse effects. Six formulas were prepared using the homogenization-ultrasonication method. Distearoyl phosphatidylethanolamine (DSPE) and soybean oil were the best lipids selected for CBL solubility and incorporation inside the nanoparticles. Poloxamer F68 ratio (1:2) relative to the total amount of lipids used and soy lecithin to surfactant ratio of 1:2 were the best ratios to get the best results of zeta potential (ZP), particle size (PS), and polydispersity index (PDI). Formula F5 was the best selected one with 85 ± 8 nm PS, 0.26 ± 0.01 PDI, and -35 ± 1.3 mV ZP. These results reveal an acceptable size for parenteral administration and good surface potential stability for colloidal dispersion. Drug entrapment efficiency (88.7 ± 3.7) and loading capacity (4.25 ± 0.03) were high concerning CBL solubility in the lipids. Particle morphology was studied by transmission electron microscopes (TEM), and appeared the spherical shape of the particle. Some erythrocyte hemolysis and high protein corona (34.59%) with plasma protein led to particle size enlargement to about 327 nm and decreased absolute ZP to about -28.5 mV.

**Keywords:** Chlorambucil, DSPE, Hemolysis, Protein Corona.

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## INTRODUCTION

According to global cancer statistics, an estimated more than 268 thousand new cancer cases with 41 thousand cancer-related deaths of breast cancer and more than 83 thousand new cancer cases with approximately 21 thousand cancer-related deaths of lymphoma occurred in 2018.[1] While chemotherapy has dominated the treatment of malignant tumors, multidrug resistance (MDR) is the leading cause of chemotherapy failure.[2] CBL is a bifunctional nitrogen mustard (Figure 1); it is used as an antineoplastic therapy for lymphocytic leukemia treatment and useful for solid tumors treatments such as breast and ovarian carcenomas.[3] Administration of CBL, like other anticancer drugs, causes highly toxic adverse effects, like anemia, reduce immune system functionality, and bone marrow suppression, due to the relatively nonselective killing of some of the rapidly dividing tissues in the host like epithelium villa and bone marrow cells.[4]

Nowadays, the drug delivery systems have highly interested in chemotherapy, which specifically aims at tumor drug delivery, thus reducing the toxicity and enhancing the efficacy of antitumor agents. Liposomes, lipid emulsions, solid lipid nanoparticles, and nanostructured lipid carriers (NLC) were developed to get a specific targeting way for the tumor site.[5] NLC is composed of liquid and solid lipids derived from natural sources. Because of its character of

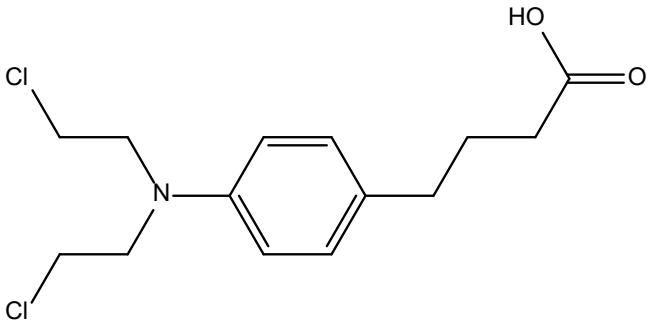


**Figure 1:** Chemical structure of chlorambucil

*Author for Correspondence: bassamalsafee@utq.edu.iq*

the non-ordered core structure of the lipid particles, NLC is highly characterized for the accommodation of various hydrophobic drugs, including paclitaxel, doxorubicin, and chlorambucil.[6,7]

This study aimed for parenteral NLC investigation as a CBL loaded nanocarrier. CBL-NLC was prepared for parenteral purposes, characterized for particle size, zeta potential, drug encapsulation efficiency, hemolysis study, and protein corona study.

## MATERIALS AND METHODS

### Materials

Chlorambucil (CBL), olive oil, castor oil, and soybean oil were purchased from Sigma-Aldrich (Taufkirchen, Germany), distearoyl phosphatidyl ethanolamine (DSPE) was purchased from Xi'an Ruixi Biological Technology (Xi'an, China), glyceryl monostearate (GMS) was purchased from Hangzhou J&H Chemical Co. (China). Poloxamer 188 (Lutrol F 68) was supplied by BASF (Ludwigshafen, Germany), soybean lecithin for parenteral use was purchased from Beiya Corp. (Tieling, China). All other chemicals and reagents purchased from the commercial source were obtained from Sigma-Aldrich (Taufkirchen, Germany).

### Solubility Study of Chlorambucil (Selection of lipids)

The solubility of CBL in different liquid oils was detected through following the methods of Higuchi and Connors, where saturated chlorambucil solutions in castor oil, olive oil, and soybean oil were prepared by the addition of an excess CBL amount to 1-mL of oil, then, on the shaking water bath, shaking reciprocally at 25 ± 0.50C for 48 hours after mixing by using vortex for 10 minutes. Centrifugation for 15 minutes at 10,000 rpm, then dilute the supernatant with methanol and spectrophotometrically assayed at 258 nm and using a linear equation to calculate the concentration of CBL.[8]

Naked eyes were used under normal light to visualize the clear melted lipid with solubilized CBL to study CBL solubility in solid lipids, where CBL was added to specific quantities of selected lipids (stearic acid, compritol888 ATO, GMS, and DSPE) which heated in 5 mL glass vials to about 3°C above its melting point in a controlled temperature water bath.[9] Room temperature must be constant, where any change in temperature may affect the results.

### Preparation of CBL-loaded Nanocarrier

Chlorambucil-loaded nanostructured lipid carrier (CBL-NLC) was formulated using the high-pressure homogenization and ultrasonication method.[10] Calculated amounts of CBL, soybean oil, and DSPE, were dispensed into a glass beaker after using a suitable organic solvent. After evaporation of the organic solvent when using a hot plate magnetic stirrer at 75°C and 900 rpm. An aqueous phase at the same temperature consisting of surfactant (poloxamer F68) with or without co-surfactant (soya lecithin) was dropped onto the melted lipid film resulted in the organic phase. After the coarse emulsion formation, it was homogenized with an Ultra-Turrax Digital Homogenizer (IKA T25 basic, IKA Werke GmbH, and Co., Germany) at 12000 rpm for 5 minutes. Finally, the high-energy ultrasonication (Misonix ultrasonic liquid processor S-4000, Hielscher, GmbH, Germany) was used for 10 minutes (on-off 4-1 sec) to get the formulation in nanosized form. Temperature controlling of the sample was accomplished by keeping it in an ice bath.

Finally, the volume was adjusted with water to the desired limit, passed through 0.45-nm MF-Millipore Membrane Filter (Merck, Darmstadt, Germany) to remove any contamination resulting from homogenization ultrasonication methods, and then kept in a clean and sealed vial. The blank formula was prepared similarly but without chlorambucil. The composition of different formulations was shown in Table 1.

#### *Evaluation of Particle Size, Polydispersity Index, and Zeta Potential*

Mean PS, PDI, and ZP of all the formulations were determined by photon correlation spectroscopy (PCS) using a particle size analyzer (Horiba SZ-100, Horiba Instruments Incorporation, Kyoto, Japan). Before the particle analysis, each sample of the formulations was diluted with deionized water, then the mean particle size and PDI were detected. At the same time, for ZP measurement, formulations were diluted with deionized water and put in a special cell (electrophoretic cell) of the Zetasizer.[11]

### Morphology Characterization

Transmission electron microscopy (TEM) was used for particle morphology. The formulation samples dropped on a mesh copper grid, and 1% (w/v) of phosphotungstic acid at room temperature was used for negatively staining the sample. The filter paper was utilized for excess liquid drain-off. Then, the dry

**Table 1:** Composition of CBL-loaded nanocarrier formulations

| *Formula code* | *CBL (mg)* | *DSPE (mg)* | *Soybean oil (mg)* | *Soya lecithin (mg)* | *Poloxamer F68 (mg)* |
|---|---|---|---|---|---|
| F1 | 1 | 15 | 5 | — | 25 |
| F2 | 1 | 15 | 5 | — | 20 |
| F3 | 1 | 15 | 5 | — | 10 |
| F4 | 1 | 15 | 5 | — | 6.66 |
| F5 | 1 | 15 | 5 | 5 | 10 |
| F6 | 1 | 15 | 5 | 10 | 10 |

film of the sample on the grid was put on the TEM sample holder and scanned with a transmission electron microscope device (Philips EM208S, Netherland) at different magnifications.[12]

**Drug Entrapment Efficiency and Loading Capacity Evaluation**

The drug loading capacity and entrapment efficiency of the CBL-NLC formulations were identified by ultrafiltration.[13] In the outer chamber of the Amicon Ultra Centrifugal Filter 15 mL tube (MWCO 10,000 Da; Sigma-Aldrich, Darmstadt, Germany) the sample was placed and centrifuged at 3500 rpm for 15 minutes. The nanoformulation and encapsulated drug remained in the outer chamber, while the aqueous phase and un-entrapped drug moved into the sample recovery chamber through the filter.

The concentration of CBL in the aqueous phase was estimated using UV-visible spectroscopy after dilution of 200 uL sample with methanol. Calculating drug entrapment efficiency and loading capacity by using:

$$\text{Entrapment Efficiency EE (\%)} = [(W_{initial} - W_{obtained})/W_{initial}] \times 100$$

$$\text{Loading capacity LC (\%)} = [(W_{initial} - W_{obtained})/W_{lipid+loaded\ drug}] \times 100$$

Where $W_{initial}$ is the initial amount of the drug in the formulation, $W_{obtained}$ is the measured amount from the formulation's aqueous media, and $W_{lipid+loaded\ drug}$ is the total amount of the lipids used and loaded drug.

**Hematological Study**

*In-vitro Hemolysis Test*

**Erythrocyte Separation Technique**

A fresh blood sample (10 mL) was obtained from a healthy donor by venipuncture and collected into a blood collecting vial containing ethylene diamine tetra-acetic acid (EDTA) in a ratio of 1.5 mg/mL (1.5 mg EDTA for each 1-mL fresh blood sample). The erythrocytes were immediately separated by centrifugation at 2000×g/4°C for 5 minutes and washed three times with PBS (pH 7.4). Collected erythrocytes (precipitated in the bottom of the centrifuge tube) were resuspended in 9 mL PBS (pH 7.4); this considered erythrocyte's stock media suspension for the study.[14] The plasma (supernatant) was transferred to labeled tubes and stored at -80°C until used (for protein corona study).

**Table 2:** Compositions of positive control, negative control, and formulation samples for hemolysis test

| *Components* | | *Triton X-100 2% (µL)* | *PBS pH7.4 (µL)* | |
|---|---|---|---|---|
| Code | *Erythrocyte suspension (µL)* | | | *Formulation (µL)* |
| *+ve control* | 200 | 800 | --- | --- |
| *-ve control* | 200 | --- | 800 | --- |
| *Sample* | 200 | --- | --- | 800 |

**Process of Hemolysis Test**

Immediately after that, 800 µL of 2% Triton X-100 (20% Triton X-100 and 80% deionized water) was added to 200 µl of media as a positive control reference (100% hemolytic).[15] Negative control reference (assumed to be non-hemolytic) prepared by addition of 800 µL of PBS (pH 7.4) to 200 µL media. Finally, the selected formulation (DLF) was prepared in different concentration ratios (1-9 to 9-1 ratio) with PBS (pH 7.4) dilution. The hemolysis study samples for the positive control, negative control, and nanoformulations were prepared by addition of 800 µL of each concentration to 200 µL of the media (Table 2).

At 37°C, incubations were carried out with gentle shaking for 2 hours of all the samples and controls. The samples were centrifuged, after incubation, at 8000×g for 5 minutes. The UV-absorbance of the supernatant was measured at 541 nm to determine the percentage of cells undergoing hemolysis.[16] Hemolysis induced with Triton X-100 was taken as 100% as a positive control, while that induced by PBS (pH7.4) was taken as a negative control. The following equation was used for the calculation of the percent of hemolysis:

$$\%Hemolysis\frac{(Abs\ sample) - (Abs - ve)}{(Abs + ve) - (Abs - ve)} \times 100$$

Where Abs sample, Abs -ve, and Abs +ve were UV-absorbance for sample, negative control, and positive control, respectively.[17]

*Protein Corona and SDS-PAGE Test*

Protein corona (PC) is defined as the array of proteins that become attached to the surface of the nanoparticles (NPs). Its composition is highly variable and depends on many factors, including composition, size, and NP surface charge.[18] In sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), the mixture of protein is separated depending on molecular weights after an electrical field exposure. The different proteins travel through a polyacrylamide gel and are according to their size separated due to their different electrophoretic mobilities. After the migration, the proteins can be stained using Coomassie brilliant blue stain and analyzed by ImageJ software program.[19]

For NP-PC complex formation, the selected formulation DLF in PBS (pH7.4) was incubated in 90% v/v plasma (prepared from hemolysis study) for one hour at 37°C/70 rpm in a shaking water bath, then centrifuged for 30 minutes at 13000 rpm/15°C. Remove supernatant (Gently remove the supernatant without disturbing the pellet to prevents going back into dispersion), and wash by adding 0.5 mL PBS to pellets and recentrifuged 20 minutes/13000 rpm/15°C, repeat washing step three times. Finally, redisperse the pellets (from the bottom of the centrifuge tube) with 100 µl PBS[20] proteins bind rapidly on their surface resulting in a formation of a so-called 'Protein Corona'. These proteins are strongly attached to the NP surface and provide a new biological identity which is crucial for the reaction at the nano-biointerface. The structure and composition of the protein corona is greatly

determined by the physico-chemical properties of the NP and the characteristics of the biological environment. The overall objective of this study was to characterize the role of NP size/surface curvature and PEGylation on the formation of the protein corona. Therefore, we prepared NP in a size of 100 and 200 nm using the biodegradable polymers poly (DL-lactide-co-glycolide. The NP-PC complexes should be visible as a pellet at the bottom of the tube.

For the SDS-PAGE technique, took 10 µL from the pellets of NP-PC complex prepared previously with 10 µL of Laemmli buffer and heated at 95°C for 5 minutes in a boiling water bath for proteins denaturation. Subsequently, a polyacrylamide gel of 10% was prepared, and the samples were applied to the gel. The SDS-PAGE was accomplished at a constant voltage of 100 V for 3 hours or until the run has traveled down to 1-cm from the bottom edge of the gel. The resulting gel was fixed (79% water, 1% orthophosphoric acid, 20% methanol), stained with a colloidal Coomassie Brilliant Blue G-250 solution overnight, and destained in methanol: water (1:3, v/v).[21] Finally, the Image J software program (ij version 1.52v) was used after scanning the gel for quantitative analysis.

## RESULTS AND DISCUSSION

### CBL Solubility in solid and Liquid Lipids

For the formulation of NLC, the CBL solubility in different lipids is a character of the lipid nanoparticles' encapsulation efficiency. It was concluded that high entrapment efficiency of the final formulas resulted from the high lipid solubility of the drug.[22] To study the lipid solubility of CBL, a range of liquid lipids (soybean oil, olive oil, and castor oil) and solid lipids (stearic acid, compritol 888 ATO, GMS, and DSPE) was selected. For solid lipids with a melting point above that of room temperature, solubility must be determined in the lipid melt. However, this method is not precisely guaranteed because the heat used for lipid melting may also affect CBL (melting point 69°C), and this may interfere with lipid solubility, so these results are considered approximately and not exactly.

Figure 2 shows the percent of CBL solubilized in a specific amount of lipid. DSPE and soybean oil had the highest potential to solubilize CBL and have been used for NLC formulation.

Both soybean oil and olive oil were revealed approximate solubility and, at the same time, higher than castor oil solubility, although all of them consist of three C18 chains. This confusion may be attributed to castor oil's hydrophilicity property due to hydroxyl groups attached to the three chains.[23] On the other hand, due to the difference in the degree of unsaturation between the other two oils, soybean oil's solubility appeared almost higher than olive oil.[24] The DSPE consists of two $C_{18}$ chains without unsaturation; theoretically, it must be much more CBL solubilizer than stearic acid (consists of one $C_{18}$ unsaturated chain); instead, there is a slight difference between their solubilization effect. This difference may be attributed to the polar poles in DSPE (phosphate and amine groups), for that both were got an approximate and an acceptable drug solubility character.[25] Compritol ATO888 was revealed a low solubility character comparatively to DSPE and stearic acid, although it consists of three long $C_{22}$ chains. The general ranking order of drug solubility in lipids was cleared that the long-chain lipids appeared lower solubility character than smaller ones, according to Alskar L. et al., with the high voids result from these long chains, especially when there is no unsaturation.[26] The highest propensity for solubilizing CBL was closed to DSPE and soybean oil, used for nanocarrier formulation.

### Characterization of CBL Nanocarrier

Particle size acts as the fundamental index for evaluating nanoparticles, which plays an essential role in selecting the best ratio and type of the lipid, surfactant, and co-surfactant. Table 3 shows the mean particle size of NLC formulations in the range of 73 nm–132 nm with polydispersity index (PDI) varies from 0.23–0.46 and ZP -35mV to -38mV. The particle size changed significantly upon manipulating the ratio of the surfactant to the lipid. Specifically, the ratio of 1:2 (surfactant to lipid ratio) was considered the best for poloxamer F68 to get better size distribution and surface potential; these results.[27] In detail, the particle size of higher surfactant concentration in F1 formulation may be due to the adsorption of the surfactant on the particle surface that results from the intrinsic thermodynamic instability of the nanoparticle system.[28] Simultaneously, there is little increase in particle size with the decreasing of poloxamer ratio (F2–F3) and much more increasing in particle size after more ratio decreasing (F4).

Polydispersity index values of all prepared formulations were considered in an acceptable range (<0.5).[29] In general, there is an inverse relationship between the particle size and surfactant amount with PDI values. Almost increasing size (decreasing surfactant amount) correlated with the better PDI results, Kipriye Z reached the same outcomes.[30] For F3, the particle size is 117 nm with the best PDI value of 0.23 compared with F1 and F2, hence considered the best formula for other studies.

The amplitude of the zeta potential exhibits the stability of the emulsified solution. ZP can be determined by many parameters, such as particle surface charge density, solution counter ion concentration, temperature, andsolvent polarity.[31] As shown in Table 3, all the prepared formulations' zeta potential values were in the acceptable range, in-between -35 mV and -38 mV. The particle size, PDI, and zeta potential

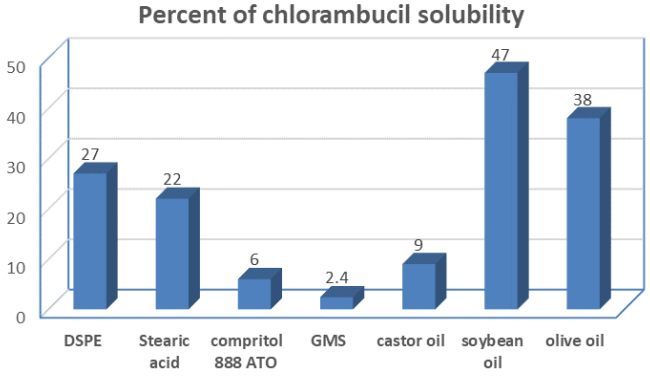


**Figure 2:** CBL solubility in different solid and liquid lipids

**Table 3:** Mean particle size, zeta potential, PDI, EE, and LC of the formulated CBL nanocarrier

| *Formula code* | *Particle size (nm)* | *PDI* | *Zeta potential* | *EE%* | *LC%* |
|---|---|---|---|---|---|
| F1 | 125 ± 18 | 0.46 ± 0.02 | -37 ± 2.4 | 87.3 ± 2.4 | 4.18 ± 0.02 |
| F2 | 109 ± 14 | 0.44 ± 0.04 | -35 ± 1.7 | 86.9 ± 1.4 | 4.16 ± 0.03 |
| F3 | 117 ± 7 | 0.23 ± 0.01 | -36 ± 1.2 | 89.4 ± 0.8 | 4.28 ± 0.08 |
| F4 | 132 ± 20 | 0.31 ± 0.02 | -38 ± 2.1 | 90.1 ± 4.2 | 4.31 ± 0.1 |
| F5 | 85 ± 8 | 0.26 ± 0.01 | -35 ± 1.3 | 88.7 ± 3.7 | 4.25 ± 0.03 |
| F6 | 73 ± 11 | 0.57 ± 0.03 | -33 ± 1.2 | 89.9 ± 0.7 | 4.30 ± 0.8 |

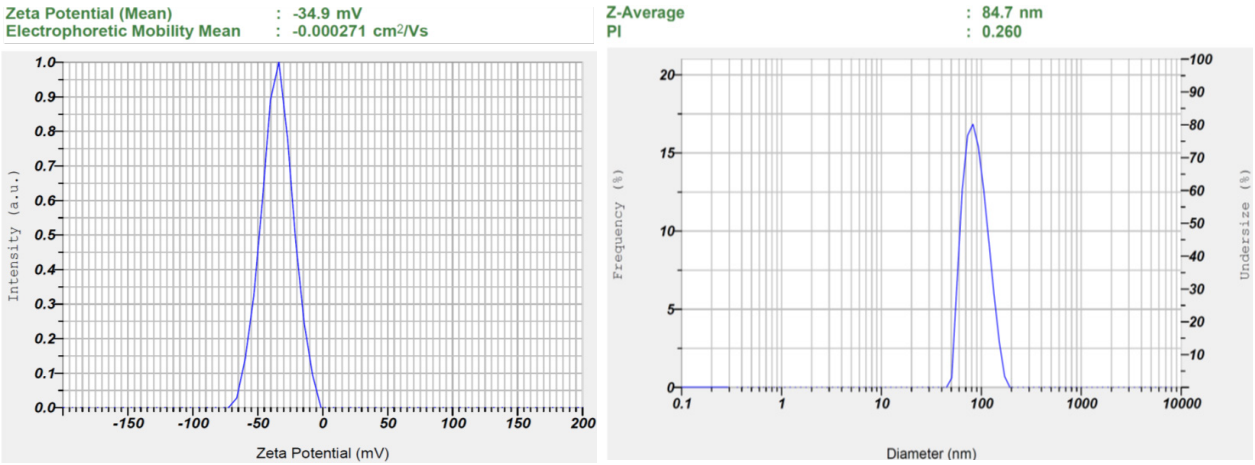


**Figure 3:** Particle size, PDI, and zeta potential of the optimum formula F5, measured by HORIBA particle analyzer

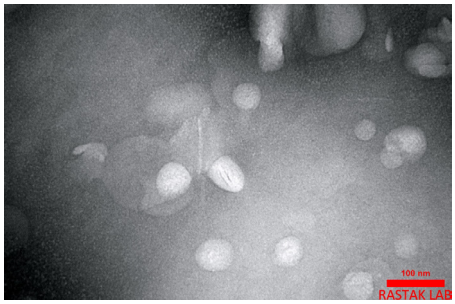


**Figure 4:** Morphology of F5 observed by transmission electron microscope

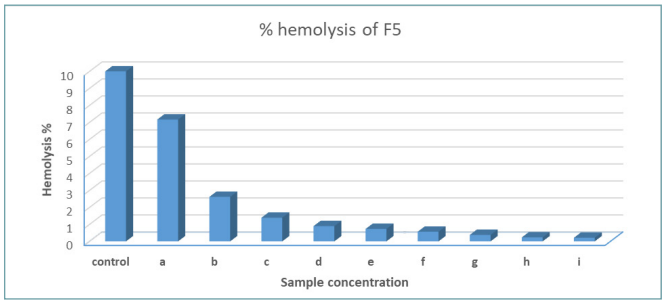


**Figure 5:** Hemolysis percentage of different concentrations of F5 and Triton X-100 as positive control

results of an optimum formula (F5), measured by the HORIBA analyzer,are illustrated in Figure 3. Moreover, drug entrapment efficiency and loading capacity appeared at optimum scale, confirming the high drug solubility in solid and liquid lipids used in this study and the high hydrophobicity of the CBL. All the formulations loaded drugs in the range of 86.9–90.1% EE.

### Transmission Electron Microscopy Examination

TEM images in Figure 4 showed that F5 formula with particle size below 100 nm is almost spherical in shape, which is nearest to the particle size obtained by DLS analysis or a little smaller. As particle diameter was calculated using TEM and dynamic light scattering techniques, slight variations in particle size were observed. Particles seem smaller in size when measured by TEM comparing with the larger diameter revealed by DLS. In the DLS technique, NLCs are detected in an aqueous state, and lipid nanoparticles are extremely hydrated, and their diameters are hydrated and typically bigger than their actual sizes; hence, DLS measures a hydrodynamic diameter; in contrast, TEM has a drying step before measurement, and it reveals the exact size and shape of the particle.[32]

### Hematological Study

*In vitro Hemolysis Test*

The *in-vitro* hemolysis test is mainly used to assess the nanoparticles' results on red blood cell integrity and estimate the nanoformulation's optimal use for parenteral administration. This assessment was performed by estimating the concentration of hemoglobin released after damaging the RBCs, where oxygenated hemoglobin (oxyhemoglobin) can be detected and measured spectrophotometrically.[33] As shown in Figure 5, aftertesting different dilutions of F5 formulation, almost all the formulations revealed acceptable hemolysis percentages. Hemolysis percentage below 5% is considered

within the acceptable limit for intravenous administration and medicinal application.[34] Exceptfor its stock solution (without dilution),when lipid concentration is equal to 0.5 mg/mL, thehemolysis percentage was appeared to be more than 7%.The image of the test is illustrated in Figure 6.

As the phospholipids and emulsifiers used in this project, such as soybean lecithin, are known to induce hemolysis, direct interaction betweennanoparticles and RBCs is predicted to have cell-damaging effects. The role of the phosphatidylcholine group of lecithin allows for nanoparticle penetration into the erythrocytes,[35] explaining the hemolysis effect of the DLF formulation.

*Protein Corona and SDS-PAGE Test*

Bands of electrophoresis gel, Figure 7, wererevealed the high protein corona related with F5 formula, which approximately reaches that of albumin, albumin used as a marker in this test. The band's location elucidated in the gel reflects albumin's position, meaning the main protein responsible for corona construction is albumin, representing about 92% of F5 formula-related PC. Figure 8 shows the intensity and percentage of the electrophoresis gel bands for plasma, albumin, and F5 formula using the Image J software program.

Figure 9 shows the results of particle size, PDI, and zeta potential, directly after PC formation for the F5 formula. It

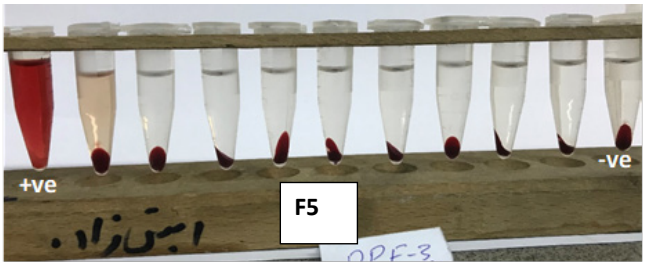


**Figure 6:** Hemolysis image of the F5 dilutions with positive control (left) and negative control (right), showing the low hemolysis at higher concentration

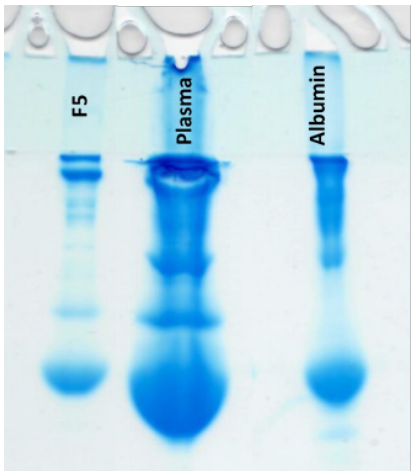


**Figure 7:** Electrophoresis gel representing bands of F5, plasma, and albumin for protien corona test

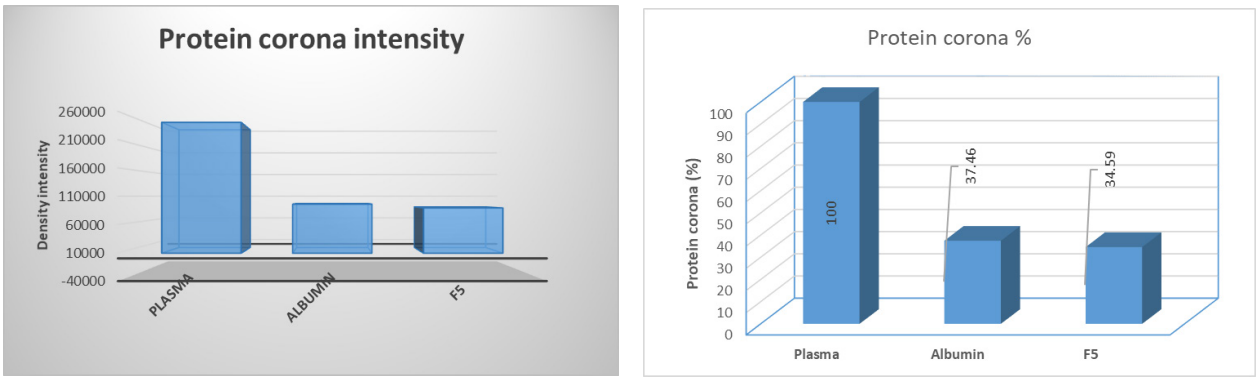


**Figure 8:** Issntensity of protien corona density (left) and percentage (right) histogram, derived from electrophoresis gel after using ImageJ software program

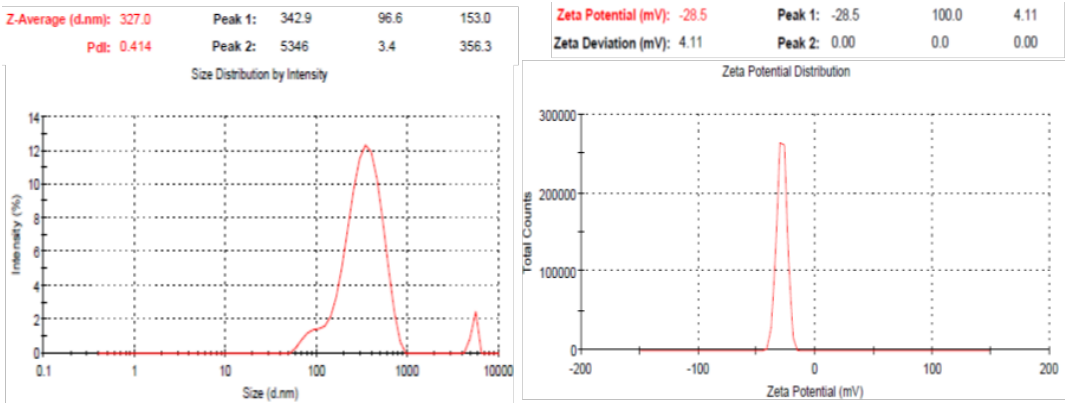


**Figure 9:** Particle size, PDI, and zeta potential of the F5 formula at the end of the PC test

was observed that the mean hydrodynamic size isabout 327 nm with PDI 0.414 and -28 mV zeta potential. Size increment analysis explains and confirms the difference in plasma protein binding recorded in the SDS-PAGE test. This rise in particle size and reduction in absolute zeta potential, seen after plasma incubation, correlates to the coating the surface of nanoparticles with positive or neutral charged proteins, confirming that albumin is the main protein that contributes to this analysis.[36].

## CONCLUSION

CBL lipid nanocarrier was successfully prepared using the homogenization-ultrasonication method. Nanocarrier characterizations were revealed an acceptable particle size and zeta potential for parenteral use. These results got after using poloxamer F68 as a surfactant and soy lecithin as a co-surfactant. Moreover, the high solubility of CBL in DSPE and soybean oil was led to the high EE. There were some erythrocyte hemssssolysis and high protein corona formation indicating the formula has to be modulated. PEGylation surface grafting could be a good choice to solve this problem.

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