Preparation of L-Glutamine Loaded Liposomes for Drug Delivery to Erythrocytes

Gokce Alp[1] and Yesim Oztas[2,*]

[1]*Department of Chemical Engineering, Faculty of Engineering, Hacettepe University, Ankara, Turkey*

[2]*Department of Medical Biochemistry, Faculty of Medicine, Hacettepe University, Ankara, Turkey*

Abstract: Sickle cell disease (SCD) is a mortal chronic disease caused by a point mutation in the β chain gene in the hemoglobin A (HbA) molecule. Erythrocyte polymerization in SCD is mostly seen as a result of the decrease in the amount of ions and water in the cell, *i.e.* dehydration and deoxygenation of erythrocytes. Deoxygenated and dehydrated erythrocytes become susceptible to clustering, causing clogging of blood vessels which then leads to crisis. Therefore, development of a new treatment method that can effectively prevent deoxygenation of erythrocytes or reduce the oxidative stress of sickle erythrocytes is one of the important issues. The aim of this study is to obtain a new lipid-based drug delivery system that will further be used for decreasing the oxidative stress of sickle erythrocytes. For the purpose, in this study, L-Glutamine (L-Gln) loaded liposomal drug delivery system composed of L-α-Phosphatidylinositol (PI) is prepared. Also, effect of encapsulated amount of L-Gln in liposomes is investigated. Liposomes are prepared via thin-film rehydration method. Characterizations of liposomes are implemented with pH measurements, zeta potential and size measurements. Erythrocytes and liposomes are incubated at 37ºC for 1 and 3 hours. Interactions between the erythrocytes and liposomes are investigated via optical microscopy and hemolysis experiments. The size and zeta potential of unloaded PI liposomes are determined as 89.01 nm with a polydispersity index of 0.438 and -23.4 ± 1.5 mV, respectively. Sizes of L-Gln loaded liposomes are obtained as 126.7, 148.6 and 197.2 nm for 20 mM, 40 mM and 60 mM of L-Gln, respectively. From the optical microscopy images, it is determined that as incubation period of erythrocytes and liposomes are increased, more liposomes are interacted with erythrocytes. Also, as L-Gln amount is increased within the liposomes, it was observed that erythrocytes preserve their morphology.

Overall, with this study, it can be concluded that L-Glutamine loaded liposomes can be used as a new drug delivery platform for erythrocytes. Moreover, the results of this study provide preliminary steps and promising results for design and development of a lipid-based drug carrier system to be used in the treatment of specific erythrocyte-based diseases such as sickle-cell disease.

Keywords: Liposomes, Lipid-based carriers, L-Glutamine, Deoxygenation, Red blood cells, Sickle cell disease.

1. INTRODUCTION

Sickle cell anemia (SCD) is a generally fatal chronic disease that occurs due to a point mutation in the β chain gene in the hemoglobin A (HbA) molecule. Pain, chronic hemolytic anemia, and serious infections are common symptoms in SCD that occurs starting from childhood [1]. In treatment of SCD, primarily efforts are made to prevent complications and reduce the number of sickled cells. Although no initiator can be identified in all cases, fever, dehydration, oxygen deficiency, acidosis and stress are generally considered to be factors that initiate the sickling of erythrocytes [2, 3]. The pathophysiology of SCD is quite complex. As a result of the combination of critical factors such as abnormalities in leukocytes, endothelium, erythrocyte hydration, inflammatory response, coagulation, hemoglobin and erythrocyte membrane, hemolysis and occlusion of small vessels occur and all these cause damage in almost all organ systems [4]. For example, ischemia which occurs due to vascular occlusion causes damage and loss of function in organs such as the brain, kidney, and lungs. On the other hand, frequent transfusions cause iron accumulation in the tissues and organs of these patients, in this respect, the organs are affected and their functions are impaired [5]. It is known that the cell membrane gets oxidative damage due to abnormal iron accumulation in SCD. It has been observed that this situation contributes to erythrocyte dehydration by increasing the loss of potassium and water. Thus, it is thought that use of oral iron chelating agents or antioxidants may also be beneficial for treatment [6, 7].

Currently, the only treatment for SCD is bone marrow transplantation, which usually requires a lymphocyte antigen conjugate family member donor. As a result of this treatment, disease-free survival is 85%, 7% transplant-related death and 9% tissue incompatibility [8]. However, the insufficient number of suitable bone marrow donors and the risk of death due to transplantation limit the widespread use of bone marrow transplantation. On the other hand, therapeutic approaches are still in progress such as gene therapy or gene editing. Most treatment modalities are

*Address correspondence to this author at the Department of Medical Biochemistry, Faculty of Medicine, Hacettepe University, Ankara, Turkey;
Tel: +90 3123051652; E-mail: yoztas@hacettepe.edu.tr

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symptomatic, and although medicine is improving day by day, people with this disease still die because of sudden infections, stroke, acute chest syndromes, or acute splenic sequestration crises [9]. In summary, preventing cell deoxygenation and dehydration is a critical step in the case of SCD and this can be implemented via drug carriers.

Nowadays, drug delivery to the targeted point is successfully provided owing to developments in nanomedicine and nanotechnology and recent advances in smart drug delivery systems. Although different approaches exist, as stated in the literature, there is a need for a new drug delivery system towards SCD that can function more effectively and which will be an alternative to the methods currently used for this purpose.

Within this context, the drug delivery system planned to be used in this study is liposomes. Liposomes are lipid-based structures, composed of the lipids that are generally naturally found in human body. In recent years, for drug delivery, liposomes are frequently preferred over other systems due to their advantageous properties [10-14]. Generally, liposomes are formed from one or more phospholipids combined at different mole ratios. As liposomes are lipid-based carriers, they are generally composed of biocompatible and biodegradable lipids [15]. They are easy to formulate and also their surfaces can be modified easily for targeting. In addition, they are known to be tolerated well in the systemic circulation. They possess the advantage of having hydrophilic and hydrophobic drug molecules in their structure at the same time owing to their amphiphilic structure [16]. Owing to all these advantages and many others, liposomes can be considered as important candidates to be used in drug delivery to erythrocytes if the liposome charge and composition is optimized carefully [17].

There are studies in the literature on drug delivery to sickle erythrocytes using a liposomal drug delivery system. For example, in a study in which phenylalanine and tryptophan active substances were applied to erythrocytes with sickle cell anemia using a liposomal delivery system, it was reported that sickling was reduced as a result of the interaction of liposomes with erythrocytes [18]. In another study, it was determined that phenylalanine or tryptophan active substances do not have an adverse effect on the metabolism and functions of erythrocytes, and it was suggested that these substances can be used in the treatment of SCD using a liposomal carrier [19]. There is also a study investigating the effects of vanillin, which is known for its antioxidant properties, on erythrocytes by loading in multilayered liposomes [20]. However, still an effective treatment system is yet to be developed. Also, combined delivery of different active substances to a target cell simultaneously with a single carrier such as liposomes is another approach. Each component can provide different advantages and their simultaneous delivery to the cell can make the treatment much more effective.

The active substance planned to be included in the liposome structure is L-Glutamine. L-Gln is known as the most important metabolic nutrient that stimulates the proliferation of enterocytes and leukocytes and maintains their mucosal integrity [21,22]. It is the most abundant amino acid in the bloodstream. Most importantly, L-Gln is the precursor molecule of glutathione (GSH), an effective antioxidant agent. For example, when kidneys undergo oxidative stress, it is known that the limiting factor for GSH synthesis is the presence of L-Glutamine [23]. In recent years, the use of L-Glutamine has been approved by the Food and Drug Administration (FDA) to reduce acute pain in SCD patients [24]. Moreover, in a recent study, which evaluates the viability of neutrophils, it was concluded that compared to control group, a greater viability was observed when glutamine encapsulated liposomes were applied to neutrophils [25].

In the light of these findings, in this study, it is planned to obtain a drug delivery system by encapsulating this hydrophilic active substance within the inner segments of the liposomes. To our knowledge, L-Gln administration to erythrocytes via a liposomal carrier has not been previously studied in the literature.

As a first step of development of smart multifunctioning liposomal drug carriers, the aim of this study is to prepare L-Gln loaded PI liposomes and to investigate their interaction with erythrocytes. Therefore, preliminary results will be obtained towards decrement and elimination of deoxidation of erythrocytes, which further causes erythrocytes to sickle.

2. MATERIALS AND METHODS

2.1. Materials

L-α-Phosphatidylinositol from soybean (PI), L-Glutamine (Gln≥ 99%), sodium chloride (NaCl), sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$), disodium hydrogen phosphate dodecahydrate

(NaH2PO4.12H2O), chloroform (GC grade), methanol (HPLC grade) were purchased from Sigma-Aldrich and used without further purification. The water used in all experiments was ultrapure water with a resistivity of 18.3 MΩ-cm (Millipore, USA).

2.2. Methods

2.2.1. Preparation and Characterization of L-Gln-Loaded Liposomes

Liposomes are prepared via well-known thin film hydration method [26]. Briefly, 0.01 g PI was dissolved in the organic solvent composed of 3:1 chloroform:methanol (v/v) to be in final concentration of 1000 ppm under magnetic stirring. It is waited overnight for removal of solvent and thus formation of thin lipid film. Afterwards, lipid film was hydrated with 10 ml of phosphate buffered saline solution (PBS, pH 7.4) under magnetic stirring at 65ºC for 60 min. Hydrated suspension was then subjected to ultrasonic homogenizer (Bandelin, Sonopuls) at 96% power, 5 cycles for 10 minutes and liposomes were obtained.

For the preparation of L-Gln-loaded liposomes, L-Gln was dissolved in PBS prior to hydration of lipid film to be in different final concentrations of 20 mM, 40 mM and 60 mM. Then aqueous phase was added to the dried lipid phase and the same procedure was applied for the formation of liposomes.

Size and zeta potential values of the liposomes were characterized by using Zeta-sizer (Malvern, Zetasizer Nano). Stabilities of the liposomes were also tested by implementing the same measurements both for unloaded and L-Gln-loaded liposomes after 10 days.

2.2.2. Investigations of Interactions between the Liposomes and Erythrocytes

Separation of erythrocytes from whole blood. Heparinized whole blood from a young healthy individual (with a hemoglobin value of 12.2 g/dl and mean corpuscular volume of 81.2 fL) was centrifuged at 1400 g for 10 min. Resulting cell pellet was washed three times with PBS. Then, the pellet was suspended at %30 hematocrit.

Interactions between erythrocytes and liposomes. Erythrocytes and unloaded liposomes were incubated at 37ºC in shaker for different erythrocyte:liposome ratios (v/v) such as 1:1, 1:2, 1:4 and 1:6 and for different time periods such as 1 and 3 hours. Then, 10 µl of suspension from each sample was transferred to microscope slide for further investigation with optical microscopy (Nikon, Eclipse E200). Prior to visualization, samples were covered with another slide to prevent oxygenation. For L-Gln loaded liposomes, erythrocytes were interacted with liposomal suspensions containing different L-Gln amount with erythrocyte:liposome ratio of 1:1 (v/v) at 37ºC in shaker for 3 hours. After the interaction, 10 µl of suspension from each sample was transferred to microscope slide and fixed in methanol and stained with Wright-Giemsa to investigate the samples via optical microscopy.

3. RESULTS AND DISCUSSION

3.1. Preparation and Characterization of Liposomes

The first step of this study to obtain stable liposomes composed of PI. Figure **1a** presents the rehydrated lipid suspension, whereas Figure **1b** shows the suspension after sonication. The clear and transparent suspension indicates the successful formation of liposomes [27]. The formation of liposomes is also supported via size measurements presented at Table **1**. The sizes of the agglomerates before sonication are determined to be in 613.5 nm with a Polydispersity index (PdI) of 0.615. Therefore, it can be deduced that prior to sonication, these lipid agglomerates are considerably large in size. After sonication, the sizes are reduced and suspension became homogeneous, as expected. Sizes of the liposomes are determined as 86.9 nm of diameter with lower PdI of 0.438. Decrease in PdI value is an indication of formation of homogeneous structures within the suspension [28]. In addition, formation of liposomes with diameters smaller than 100 nm makes these drug delivery vehicles advantageous for delivery of therapeutics [29]. The zeta potential values of

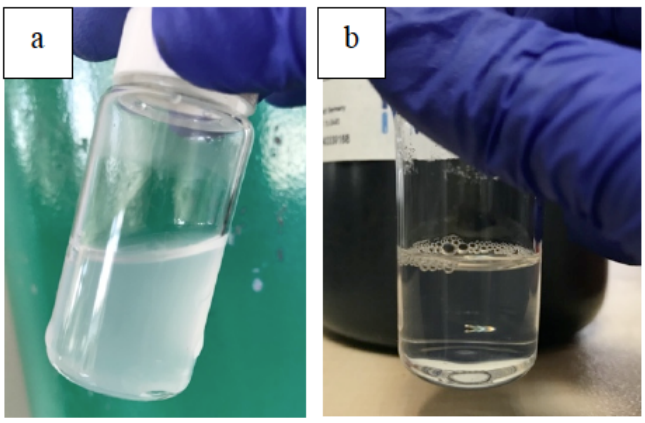

Figure 1: (**a**) Lipid suspension prior to sonication, (**b**) liposome suspension.

Table 1: Zeta Potential and Size Measurements of Lipid Suspensions before and after Sonication

Suspensions	Zeta Potential (mV)	Size (nm)	PdI
Before sonication	-21.3 ± 1.4	613.5	0.615
After sonication	-23.4 ± 1.5	86.85	0.438

suspensions before and after the sonication are obtained to be around -22 mV. As it is known, PI is an anionic lipid. Therefore, measured zeta potential values are found to be consistent with the literature [30]. For both suspensions, the only material with in the solution is PI, so, even though sizes of the agglomerates had changed after the sonication, variation at the zeta potential values would be an unexpected result.

L-Gln Loaded Liposomes. L-Gln loaded liposomes with different L-Gln amounts (*e.g*. 20 mM, 40 mM, 60 mM) are also prepared following successful formation of unloaded liposomes composed by PI. After obtaining transparent liposomal suspensions (Figure **2**), same characterizations are utilized for them as described previously. In addition, stabilities of the liposomes are assessed by employing the same measurements 10 days after formation of liposomes (Table **2** in Figure **2**).

As presented in Table **2**, independent from L-Gln concentration, zeta potential values of drug loaded liposomes do not significantly differ from one another. Also, when these values are compared with zeta potential values of unloaded liposomes, it can be seen that after drug encapsulation, zeta potential values of liposomes had not changed at all. Since L-Gln is a positively charged hydrophilic molecule, negative zeta potential values can be considered as a direct indication of high encapsulation efficiency of the liposomes. 10 days after formation of liposomes, zeta potential values had been nearly the same with the results of day 1. This supports the fact that liposomes remain stable.

Moreover, when the size values of unloaded and L-Gln loaded liposomes are compared, the increment at the sizes of drug loaded liposomes demonstrates the

Table 2: Zeta Potential and Size values of L-Gln Loaded Liposomes

Liposome Samples	Zeta Potential (mV)		Size (nm)	
	Day 1	Day 10	Day 1	Day 10
A (20 mM L-Gln)	-21.4± 1.5	-20.2± 1.2	126.7±12.1	118.3±9.3
B (40 mM L-Gln)	-23.3± 1.6	-22.9± 1.4	148.6±10.7	139.7±14.2
C (60 mM L-Gln)	-22.8± 1.3	-21.6± 1.8	197.2±8.3	190.4±10.5

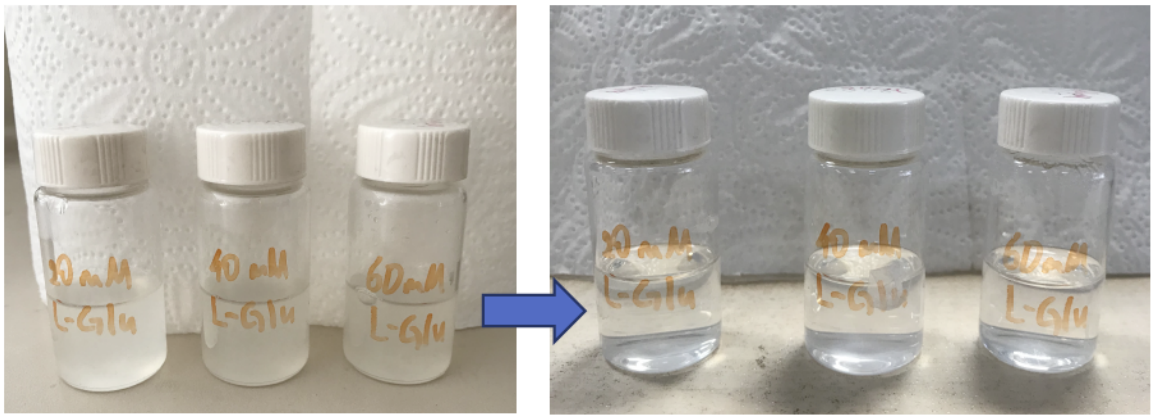

Figure 2: L-Gln loaded liposomes prior and after ultrasonication. Table **2** shows zeta potential and size values of liposomes at day 1 and day 10.

successful encapsulation of drug. Diameters of L-Gln loaded liposomes increases with increasing glutamine amount. This result is consistent with our expectations. Again, size values after 10 days had not changed significantly, which is a good indication of stability of liposomes.

3.2. Investigations of Interactions between the Liposomes and Erythrocytes

Interactions of erythrocytes with unloaded liposomes. To observe if liposomes can interact with erythrocytes, and to understand the effect of amount of liposomes and incubation time on erythrocyte morphology; erythrocytes are incubated with liposomes at different v/v ratios at 37ºC for 2 different incubation periods of 1 and 3 hours. Sample codes are corresponding v/v ratios with incubation times are given in Table **3**.

The optical microscope images are given in Figure **3** for the samples described at the Table **3**. As it can be seen from the images, for all samples, 1 hour of incubation period had not been adequate for liposomes to interact with erythrocytes. However, after 3 hours of incubation, it is observed that liposomes had maintained to adsorb to membranes of erythrocytes and interact with them for all samples.

From Figure **3**, it can be seen that for all samples, erythrocytes had preserved their morphology in terms of their size and shapes. It can be seen that even after 3 hours of incubation, erythrocytes had not been deformed. Among all samples, best results are obtained with sample A-3. For this sample, it is obtained that liposomes had been able to reach to nearly all cells and integrated within the membranes of erythrocytes. Therefore, it is decided to continue to study with erythrocyte:liposome ratio of 1:1 and 3 hours of incubation period.

Interactions of erythrocytes with L-Gln loaded liposomes. The interactions between the L-Gln loaded liposomes and erythrocytes have been implemented via incubating the components at 37ºC for 3 hours. The optical microscopy images of corresponding samples are given in Figure **4**. From the figure it can be seen clearly that liposomes were successfully interacted with red blood cells. In addition, presence of L-Gln loaded liposomes has contributed to erythrocytes to preserve their morphology. Images of erythrocytes without the presence of liposomes in PBS, indicate that cells had started to corrupt. However, for the samples, in which L-Gln loaded liposomes are present, erythrocytes maintain their size, shape and structure and did not disrupt. Even though they have been preserved in the same conditions (same medium and temperature), adsorption of L-Gln loaded liposomes to the membranes of erythrocytes had maintained the stability of cells. Liposomes that integrated to the erythrocyte structure can be seen more clearer in the samples that have been stained with Wright's stain. The dark areas belong to liposomes and it is observed that independent of L-Gln amount, liposomes had been able to adsorb to cells. In fact, this is a key finding for this study. We propose that presence of lipids support the erythrocyte membrane to retain its integrity. Moreover, after adsorbing and interacting with erythrocyte membranes, we propose that presence of L-Gln also provides erythrocytes to preserve its morphology. Therefore, we believe that after further investigations, this system may carry an important potential to be used in drug delivery to erythrocytes in

Table 3: Sample Codes for Different Erythrocyte:Liposome Ratios and Incubation Times

Sample Code	Erythrocyte:Liposome Ratio (v/v)	Incubation Time
A-1	1:1	1
A-3	1:1	3
B-1	1:2	1
B-3	1:2	3
C-1	1:4	1
C-3	1:4	3
D-1	1:6	1
D-3	1:6	3

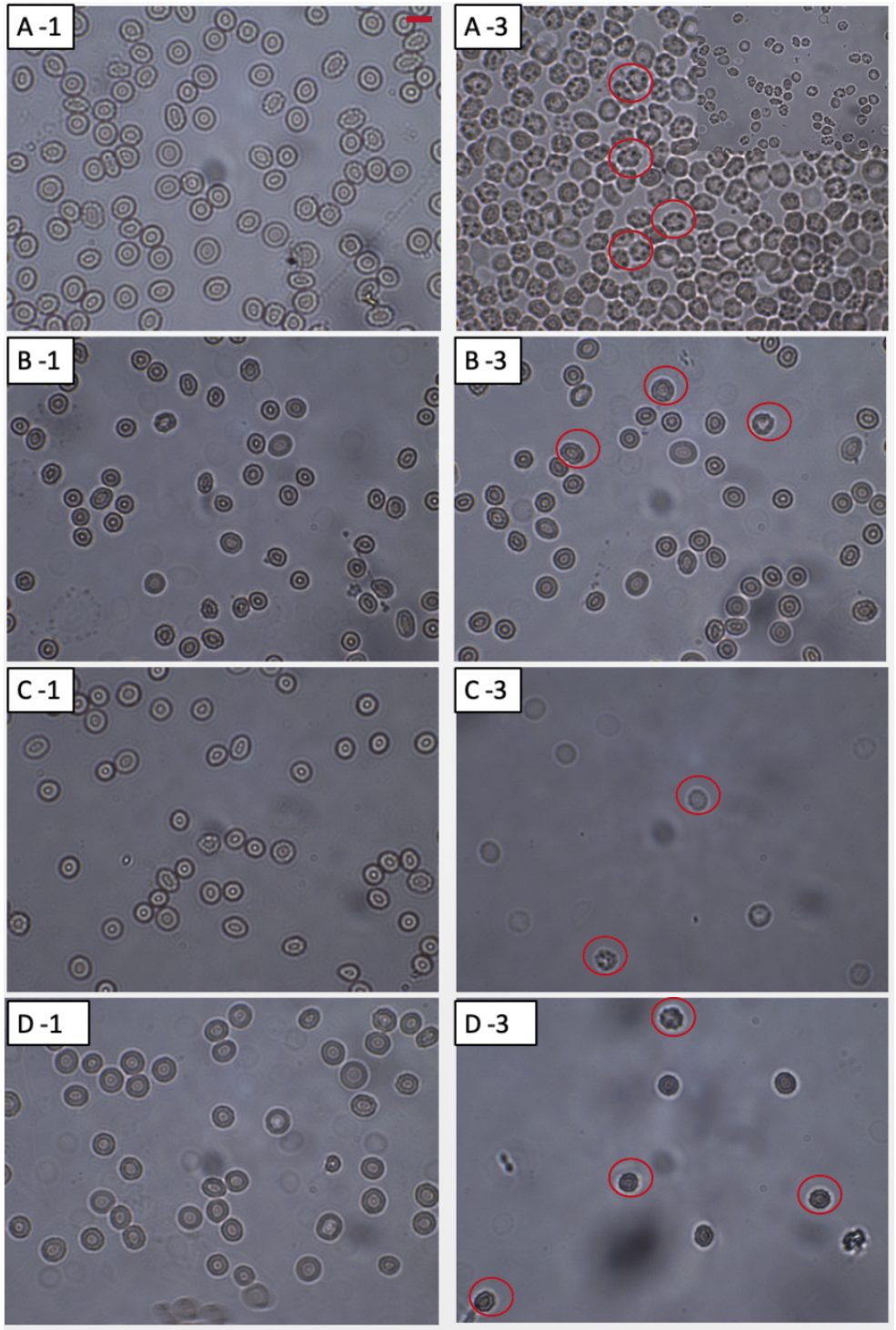

Figure 3: Optical microscopy images of the samples coded A, B, C, and D after interaction with erythrocytes for 1 hour (**A-1**, **B-1, C-1** and **D-1**) and 3 hours (**A-3**, **B-3**, **C-3** and **D-3**). Magnification of the images is 100x. Scale bar at picture A-1 represent 0.01 mm.

case of different diseases, particularly for treatment of sickle cell disease.

4. CONCLUSION

Successful drug delivery to healthy erythrocytes is a first step that should be accomplished to continue with further studies involving sickled erythrocytes. For this purpose, biodegradable and biocompatible lipid-based drug delivery systems are good candidates to study with. Within this context, in this study, PI liposomes loaded with L-Glutamine are prepared and characterized. Afterwards, their interactions with healthy erythrocytes are investigated using optical

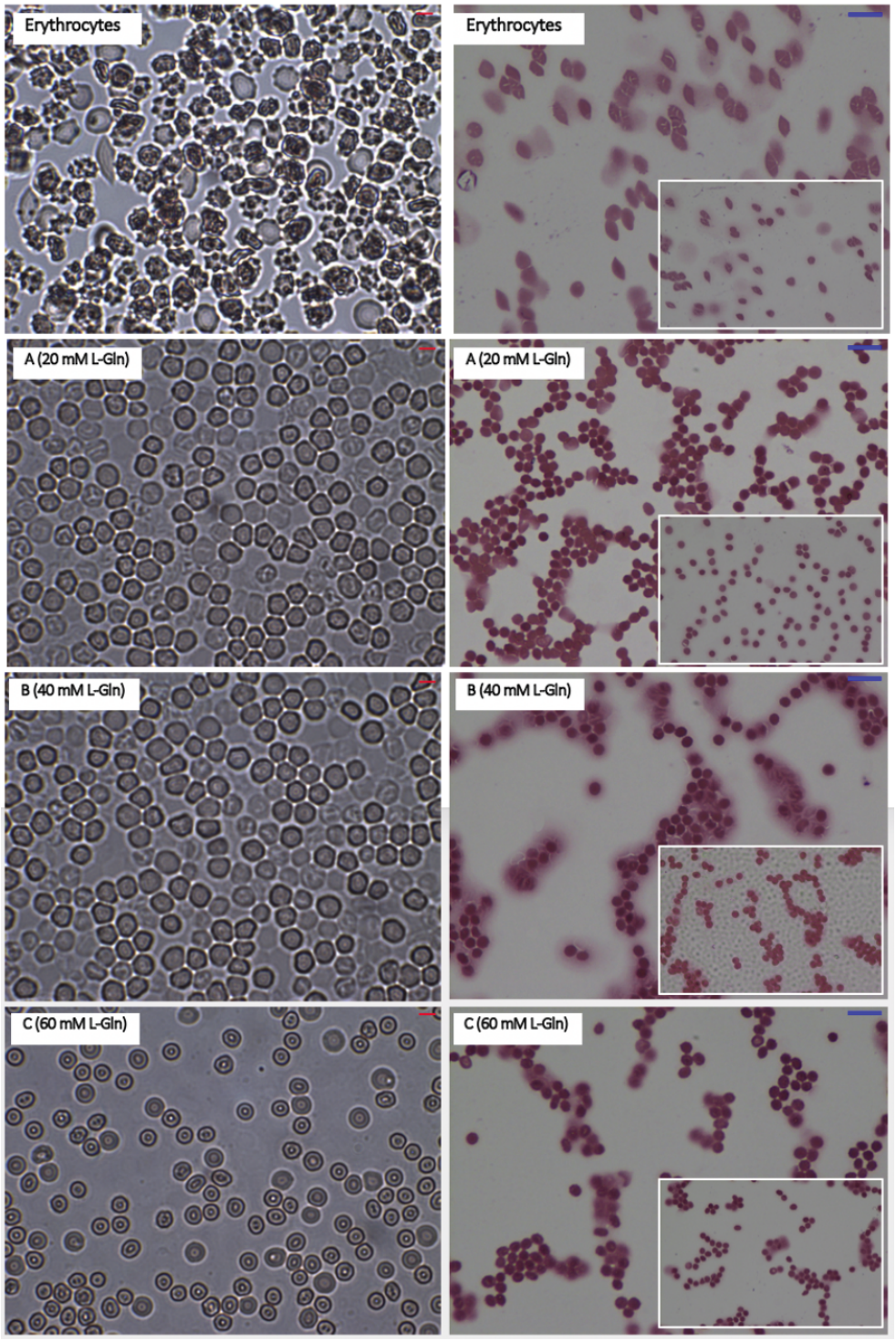

Figure 4: Optical microscope images for non-interacted erythrocytes, and erythrocytes that interacted with L-Gln loaded liposomes which contain different amounts of L-Gln: 20 mM (**A**), 40 mM (**B**) and 60 mM (**C**). Magnification of images at the first column (black & white) is 100x. Magnification of images at the 2nd column is 40x. Scale bars represent 0.01 mm.

microscope. It was concluded that after 3 hours of incubation of erythrocytes with liposomes, interaction between them could be more pronounced. Also, as the amount of encapsulated L-Gln was increased, the morphology of erythrocytes had been preserved. This can be considered as a key finding to continue with further development and optimization of this delivery system. To conclude, we propose that L-Glutamine loaded liposomes can be used as a new drug delivery platform for erythrocytes. Moreover, the results of this study provide preliminary steps for the design and development of a lipid-based drug carrier system.

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ETHICAL APPROVAL

This article contains experiments conducted with healthy erythrocytes isolated from whole blood samples provided from volunteers. Ethical approval of this study is provided by Non-interventional Clinical Researches Ethics Board of Hacettepe University with the issue number of GO 19/1005.

CONFLICTS OF INTEREST

Nothing to disclose. The authors declare no conflicts of interest.

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