



# RGD-Modified Nano-Liposomes Encapsulated Eptifibatide with Proper Hemocompatibility and Cytotoxicity Effect

Hassan Bardania<sup>1</sup>, Seyed Abbas Shojaosadati<sup>2\*</sup>, Farzad Kobarfard<sup>3</sup>, Dina Morshedi<sup>4</sup>, Farhang Aliakbari<sup>4</sup>, Mohammad Taher Tahoori<sup>5</sup>, Elahe Roshani<sup>6</sup>

<sup>1</sup> Cellular and Molecular Research Center, Yasuj University of Medical Sciences, Yasuj, Iran

<sup>2</sup> Biotechnology Group, Department of Chemical Engineering, Tarbiat Modares University, Tehran, Iran

<sup>3</sup> Department of Medicinal Chemistry, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran

<sup>4</sup> Industrial and Environmental Biotechnology, National Inst. of Genetic Engineering and Biotechnology, Tehran, Iran

<sup>5</sup> Department of Immunology, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

<sup>6</sup> Department of Nanobiotechnology, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

\* **Corresponding author:** Seyed Abbas Shojaosadati, Biotechnology Group, Department of Chemical Engineering, Tarbiat Modares University, Tehran, Iran. E-mail: shoja\_sa@modares.ac.ir

## Abstract

**Background:** Eptifibatide (Integrilin®) is a hepta-peptide drug which specifically prevents the aggregation of activated platelets. The peptide drugs are encapsulated into nanoliposomes in order to decreasing their side effects and improving their half-life and bioavailability.

**Objectives:** In this study, the *in vitro* cytotoxicity and hemocompatibility of RGD-modified nano-liposomes (RGD-MNL) encapsulated a highly potent antiplatelet drug (eptifibatide) was investigated.

**Material and Methods:** RGD-MNL encapsulated eptifibatide was prepared using lipid film hydration and freeze/thawing method. The morphology and size distribution (about 90 nm) of RGD-MNL were characterized using transmission electron microscopy (TEM). The *in-vitro* cytotoxicity of nano-liposomes was examined using the MTT, LDH release and reactive oxygen species (ROS) generation assays. The effect of RGD-MNL on red blood cells (RBC) was investigated using hemolysis and LDH release assays.

**Results:** The results revealed that RGD-MNL had no significant cytotoxic effect on HeLa and HUVEC cell lines, and also no ROS generation increase in the cells. In addition, the adverse effect of RGD-MNL on LDH release and membrane integrity of RBC was not observed.

**Conclusions:** In conclusion, the recommended RGD-MNL formulations have not any significant cytotoxicity on normal cells or RBC and have potential for protecting and enhancing the activity of antiplatelet drugs.

**Keywords:** Liposomes; Cytotoxicity; Materials Testing; Eptifibatide

## 1. Background

Atherosclerosis as a cardiovascular disease is a multifactorial disease and various efficient drugs such as eptifibatide was developed for treatment of thrombosis (1). Eptifibatide (Integrilin®) is a highly potent peptide drug that selectively inhibits aggregation of activated platelets (2, 3). Nevertheless, it has side effects and short half-life due to accumulation in non-targeted tissues and elimination by renal filtration (2). Therefore, nanocarriers such as liposomes with the ability to target specific tissues or cell types can improve the function of antithrombotic agents like eptifibatide (4, 5). Among various nanocarriers, liposomes are the most commonly used drug delivery system for the delivery of thrombolytic drugs (6, 7). While larger than their nanocounterparts, liposomes have many advantages

such as high biocompatibility, biodegradable, low immunogenicity. In addition, liposomes could efficiently encapsulate and protect drugs and improve selectivity and effectively lengthen the half-lives of drugs (8, 9). The surface of nanoliposomes can be easily modified with specific ligand for targeting a specific part of a disease such as cardiovascular diseases (10, 11). Atherosclerosis plaques have various important parts such as platelets that can be used for targeting drug delivery. Platelets play a vital role in initiate thrombus formation (12). RGD (Arg-Gly-Asp) motif is a ligand derived from fibrinogen that has high affinity for platelets (13-15). In several studies, the surface of liposomes was modified by RGD ligand and used for the targeted delivery of thrombolytic agents (13, 14, 16-18).

In our previous study, eptifibatide was successfully encapsulated into RGD-modified nanoliposomes and its applicability was tested (19, 20).

## 2. Objectives

The main objective of this study was the evaluation of cytotoxicity and hemocompatibility of RGD-modified liposomes encapsulated eptifibatide. The size and morphology of prepared liposomes were characterized utilizing transmission electron microscopy (TEM). *In vitro* cytotoxicity of nanoliposomes was investigated using MTT, lactate dehydrogenase (LDH) leakage and reactive oxygen species (ROS) assays. Furthermore, the hemocompatibility of liposomal samples was evaluated with a special focus on hemolytic activity and membrane integrity.

## 3. Material and Methods

### 3.1. Materials

1, 2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) was obtained from Avanti Polar Lipids (USA), and 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), cholesterol from Sigma Aldrich (USA). Cancer HeLa cell line and normal human umbilical vein endothelial (HUVEC) cells was obtained from Pasteur Institute (Tehran, Iran). Eptifibatide was provided by the School of Pharmacy, Shahid Beheshti University of Medical Science (Tehran, Iran). The cell culture medium (DMEM) and antibiotics (penicillin, streptomycin) were purchased from GibcoBRL (Life Technologies, Paisley, Scotland) and fetal bovine serum (FBS) was from Biosera (England). And other salts and solvents were obtained from Merck (Germany). All materials were used without any further purification. Dipalmitoyl-GRGDSPA was synthesized as reported in our previous study (19).

### 3.2. Liposome Preparation and Characterization

DSPC/cholesterol/dipalmitoyl-GRGDSPA (7/3/1 mM respectively) compounds were dissolved with chloroform in a 10 mL round-bottomed flask. The solvent was removed by rotary evaporator under low vacuum to form thin lipid films. Typically, the lipid films were hydrated at 65 °C with 2 mL Tris buffer (50 mM; pH 7.4). 2 mL of the prepared drug-free liposomes was vortexed and mixed with eptifibatide solution (3.65 μmol in deionized water) subjected to seven freeze/thawing cycles (5 min at -196°C and 5 min at 65°C with 150 rpm shaking). The newly formed multi-lamellar vesicles (MLVs) were passed 10 times through an extruder (Avanti polar) containing two stacked 100 nm polycarbonate filters. The size and lamellarity of prepared nanoliposomes analyzed by transmission electron microscopy (TEM) (Philips CM30, Netherlands). Prior TEM analysis, the sample was

prepared by placing on carbon coated copper grids (400 mesh, Agar Scientific, UK).

### 3.3. *In vitro* Cytotoxicity Assays

#### 3.3.1. MTT Assay

Cytotoxicity of RGD-MNL encapsulated eptifibatide was evaluated based on the MTT assay (21). Cancerous cell line (HeLa cells) were seeded in a 96-well flat-bottomed microplate ( $4 \times 10^3$  cells per well) and incubated for 24 h in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37 °C. RGD-MNL encapsulated eptifibatide, drug-free RGD-MNL and unmodified nanoliposomes (UNL) encapsulated eptifibatide each with various concentration (50, 100 and 200 μg/ml) were added into the wells and incubated for 24 h. The cells were also incubated in the presence of free eptifibatide at the concentrations equivalent to the eptifibatide content of the nanoliposomes. 100 μl of MTT reagent (0.5 mg/ml per well) in DMEM solution was added to each well and after 4 h incubation, MTT solution was replaced with DMSO to dissolve formazan and was shaken for 20 min at room temperature. The absorbance was measured in a microtiter plate reader (MIOS Junior, Merck) at 570 nm. All tests were performed in three replicates and the percentage of viability was calculated as  $(Y/X) \times 100$ , where Y represents mean absorbance of sample and X is mean absorbance of control cells.

#### 3.3.2. LDH Leakage Assay

HeLa and HUVEC cells ( $4 \times 10^3$  cells/well) were seeded in 96-well and incubated for 24 h. Cells were treated with different concentration of RGD-MNL encapsulated eptifibatide, drug-free RGD-MNL, UNL encapsulated eptifibatide and drug-free UNL (50, 100 and 200 μg/ml) and incubated at 37 °C for 24 h. The cells were also incubated with free eptifibatide at the concentrations equivalent to the eptifibatide content of the nanoliposomes. For the LDH leakage assay, 125 μl of the cell free culture supernatant was added to 1 ml of working reagent of LDH commercial kit (Pars Azmoon Lot no, 94001) (containing NADH and pyruvate). LDH activity was assayed spectrophotometrically following the decrease in absorbance of NADH at 340 nm by LDH assay kit (Pars azmoon, Iran).

#### 3.3.3. Measurement of ROS

Intracellular generation of ROS was measured using 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) with a minor modified method previously reported (22). The DCFH-DA is non-fluorescent and very sensitive to several ROS that can be oxidized to a highly fluorescent 2, 7-dichlorodihydrofluorescein (DCF). HUVEC and HeLa cells were seeded in 96-well flat-bottomed microplate ( $6 \times 10^4$  cell/ml) and incubated for 24 h. RGD-MNL encapsulated eptifibatide, drug-free RGD-MNL, UNL encapsulated eptifibatide and drug-free UNL with various concentration (50, 100 and

200 µg/ml) were added into the wells and incubated for 24 h. The cells were also incubated in the presence of free eptifibatide at the concentrations equivalent to the eptifibatide content of the nanoliposomes. After that, the cells were washed with PBS and probed with 200 µL of DCFH-DA (15 µM) and incubated for 45 min at 37 °C, 5% CO<sub>2</sub> in the dark. Then, cells washed with PBS again and DCF fluorescence was measured using Varian Cary Eclipse fluorescence spectrophotometer (Mulgrave, Australia). Fluorescent intensities normalized with incubation of 50 mM H<sub>2</sub>O<sub>2</sub> with DCFH-DA as positive control (23).

### 3.4. Hemocompatibility analysis

#### 3.4.1. Hemolysis

Hemolysis induced by liposomal samples was assessed photometrically. Whole blood was collected from healthy volunteers in citrated tubes (3.8 % sodium citrate, 1 part sodium citrate, 9 part blood). Red blood cells (RBCs) were harvested by centrifugation at 1500 × g for 10 min. The plasma supernatant was removed and RBC washed using phosphate buffer saline (PBS) thrice. The RBC re-suspended in 1 ml PBS to a concentration of 20 % (v/v) and incubated with 0.5 ml liposomal formulations (200 µg/ml) at 37 °C for 2 h, followed by centrifugation at 1500 × g for 5 min. The hemoglobin released into the supernatant was spectrophotometrically detected at 540 nm wavelength. The observed hemolysis of RBC in PBS and in 1 % triton X-100 was used as spontaneous hemolysis and 100 % hemolysis control, respectively. The percentage of hemolysis was calculated as  $(Y-X)/Z \times 100$ , where Y represents mean absorbance of the supernatant of RBC incubated with liposomal samples and X and Z are mean absorbance of the supernatant of RBC incubated with PBS and 1 % triton X-100 respectively.

#### 3.4.2. Evaluation of the RBC Membrane Integrity

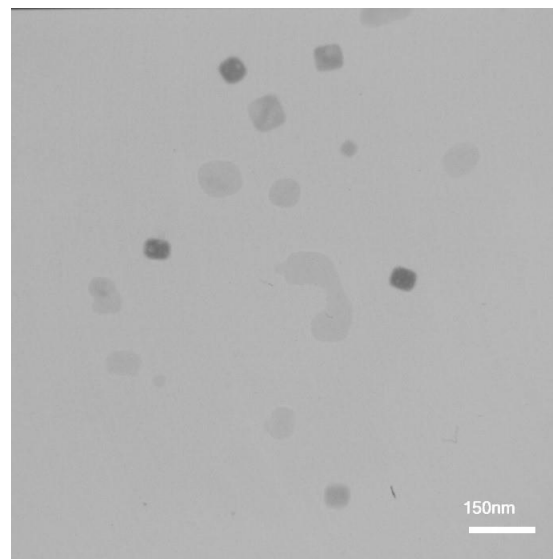
The activity of lactate dehydrogenase (LDH) released from RBC was induced by liposome treatment and assessed photometrically using LDH commercial kit (Pars Azmoon Lot no, 94001). The whole blood was centrifuged at 1500 × g for 10 min and leukocytes and plasma supernatant was discarded. RBCs were washed using phosphate buffer saline (PBS) three times. 0.5 ml of liposomal formulations (200 µg/ml) were incubated with 1 ml of RBC solution in PBS 20 % (v/v) at 37°C for 2 h, followed by centrifugation at 1500 × g for 5 min. The LDH released in the supernatant was photometrically detected at 340 nm. The spontaneous LDH release and 100 % LDH release controls prepared by incubation of RBC in PBS and triton X-100 (1 %), respectively.

## 4. Result

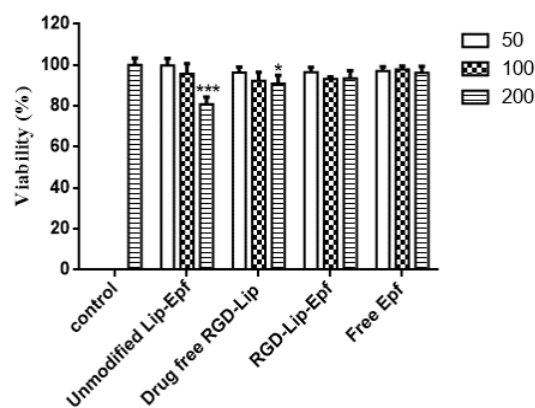
### 4.1. In vitro Cytotoxicity Assay

#### 4.1.1. MTT Assay

The cytotoxicity of prepared nanoliposomes (drug-free UNL, drug-free RGD-MNL, RGD-MNL encapsulated eptifibatide, UNL encapsulated eptifibatide and free eptifibatide) with the size of about 90 nm (Fig. 1) were examined using MTT assay on HeLa cell line. The results show that only UNL encapsulated eptifibatide at concentration 200 µg/ml has significant cytotoxicity on HeLa cells after 24 h ( $P < 0.001$ ). However, all other liposomal samples at various concentrations (50, 100 and 200 µg/ml) have not significant cytotoxicity on HeLa cells after 24 h ( $P < 0.001$ ) (Fig. 2).



**Figure 1.** TEM images of phospholipid-based bilayer vesicles containing eptifibatide prepared by freeze/thawing method.

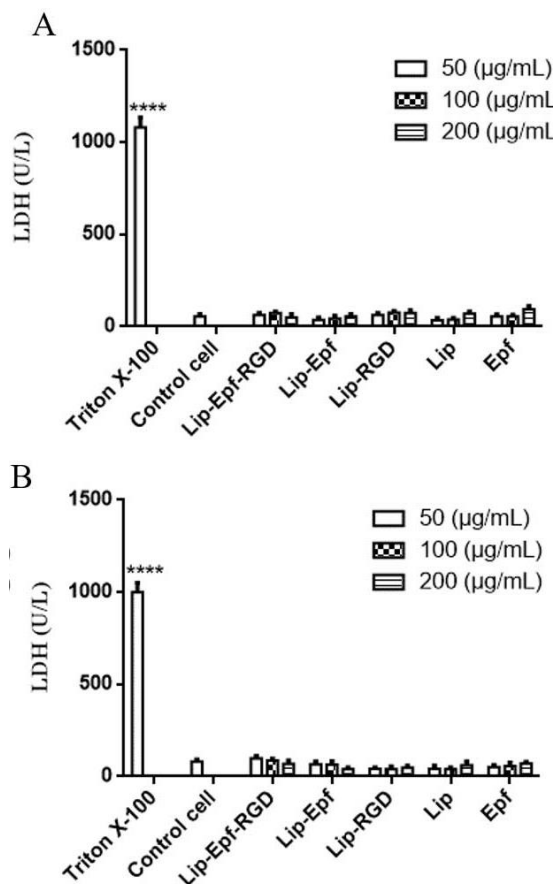


**Figure 2.** Cytotoxicity of nano-liposomes determined by MTT assay on HeLa cells after 24 h exposures. Data are expressed as percent of control mean ± SD of three independent experiments. \*, \*\*\* denotes a statistically significant ( $P < 0.05$ ,  $P < 0.001$ ) difference from the untreated control.

#### 4.1.2. LDH Release Assay

The adverse effect of drug-free RGD-MNL, RGD-MNL encapsulated eptifibatide, drug-free UNL, UNL encapsulated eptifibatide and free eptifibatide on membrane integrity of HUVEC and HeLa cells were assessed using the activity of released LDH enzyme into the culture supernatant (Fig. 3). The results revealed

that liposomal samples had no significant effect on LDH release from HUVEC and HeLa cells when compared with the control cells after 24 h incubation.



**Figure 3.** Effects of liposomal samples on LDH release from (A) HUVEC and (B) HeLa cell lines during treatment for 24 h. Data are shown as mean  $\pm$  SD (n=3) significantly (\* $P < 0.05$ , \*\*\*\* $P < 0.0001$ ) different relative to untreated control cells. (Lip=Liposome, Epf = eptifibatide, RGD=GRGDSPA ligand).

#### 4.1.3. ROS Assay

The generation of ROS in HeLa and HUVEC cells in the presence of liposomal samples was measured using the fluorescent marker DCFH-DA. Exposure of HUVEC and HeLa cells to three different concentrations (50, 100 and 200  $\mu\text{g}/\text{mL}$ ) of all liposomal samples and free eptifibatide showed that the liposomal samples not only had significant effect on the formation of free radical in both cells, but the samples significantly decreased ROS formation in the cell line ( $P < 0.05$ ) (Fig. 4).

### 4.2. Hemocompatibility Analysis

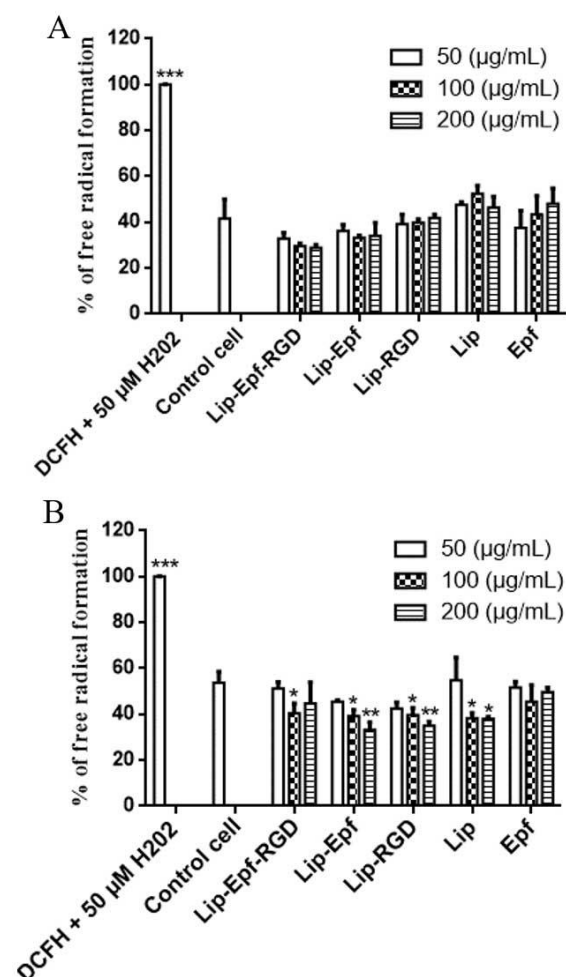
#### 4.2.1. Hemolysis

The potential of nanoliposomes to cause hemolysis is an important feature for their development and *in vivo* applications. Therefore, the ability of liposomal samples drug-free RGD-MNL, RGD-MNL encapsulated eptifibatide, drug-free UNL and UNL encapsulated eptifibatide to cause human RBC lysis was evaluated. The results of the hemolysis revealed that both UNL and RGD-MNL had no significant effect on the release

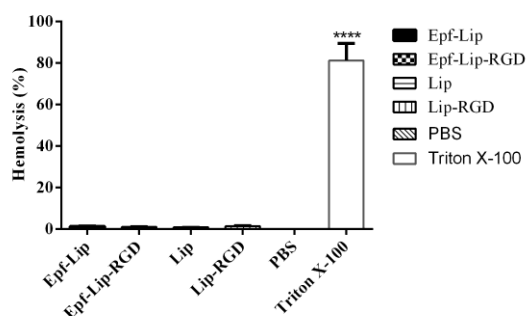
of hemoglobin from RBC into the supernatant compared to negative control (Fig. 5) ( $P < 0.05$ ). Therefore, not-hemolytic effect of RGD-MNL may be confirmed (24, 25).

#### 4.2.2. Evaluation of the RBC Membrane Integrity

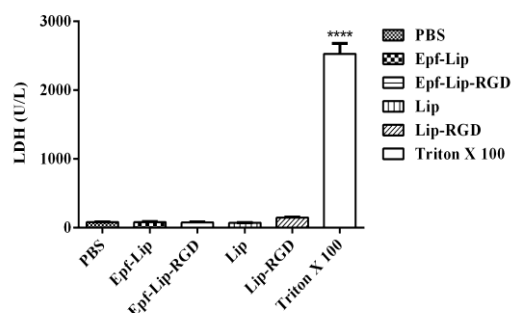
The membrane integrity of RBC in the presence of drug-free RGD-MNL, RGD-MNL encapsulated eptifibatide, drug-free UNL and UNL encapsulated eptifibatide was assessed by measuring the activity of LDH enzyme released into the supernatant from RBC and the results are presented in Figure 6. The presence of liposomal samples in blood have no significant effect on LDH released from RBC compared with LDH released from RBC incubated for 2 h in PBS at 37°C. Consequently, these results confirmed the results obtained by hemolysis assay.



**Figure 4.** Quantification of oxidative stress in (A) HUVEC and (B) HeLa cells treated with different concentrations liposomal samples during a 24 h period.  $\text{H}_2\text{O}_2$  was used as a positive control of oxidative stress and was correlated to 100%. Data are shown as mean  $\pm$  SD (n = 4) significantly (\* $P < 0.05$ , \*\* $P < 0.01$ ) different relative to untreated control cells. (Lip = Liposome, Epf = Eptifibatide, RGD = GRGDSPA ligand).



**Figure 5.** Membrane integrity assessed by release of hemoglobin in whole blood samples treated with liposomal samples. Data are shown as mean  $\pm$  SD (n=3) significantly (\*\*\*\* P<0.0001) different relative to untreated control (Lip=Liposome, Epf = Eptifibatide, RGD=GRGDSPA ligand, PBS= phosphate-buffered saline).



**Figure 6.** LDH release from red blood cells after liposomal treatment. Triton X-100 (2 % v/v) was considered as 100 % of cell damage. Data are shown as mean  $\pm$  SD (n=3) significantly (\*P<0.05, \*\*\*\*P<0.0001) (Lip=Liposome, Epf=Eptifibatide, RGD=GRGDSPA ligand).

## 5. Discussion

Cytotoxicity of liposomal samples was investigated by MTT, ROS generation and LDH release assays. The MTT results showed that the samples have not significant cytotoxic effect on HeLa cell line. In our previous study, we evaluated the cytotoxicity of RGD-modified nanoliposomes on HUVEC cell lines using MTT assay and showed that liposomes do not have any cytotoxicity effect on HUVEC cells (20). Many factors such as surface charge of nanoliposomes can effect on the cytotoxicity. It was previously reported that cationic liposomes are more toxic compared with neutral and negative charge liposomes (26). The toxicity of cationic liposomes is due to induce the formation of reactive oxygen intermediate, destroy plasma membrane integrity and increase the number of autophagosomes (27). The effect of size and concentration of nanoliposomes are less compared to surface charge (28). In this study, RGD-MNL comprised a neutral phospholipid (DSPC) and had unilamellar vesicles (with size about 90 nm). Dokka *et al.* evaluated the cytotoxicity of differently charged liposomes and demonstrated that cationic liposomes were more toxic than neutral/negative liposomes and multivalent liposomes were more toxic compared with monovalent liposomes (27, 29). The results of ROS generation by the exposure of nanoliposomes to HeLa and HUVEC cell lines show no increase in ROS generation for both

normal and cancerous cells. In the case of cancerous cells, the exposure of nanoliposomes causes a significant decrease of ROS generation (P<0.5). Cancerous cells naturally cause more ROS generation compared with normal cells due to their growth and proliferation rate (30). ROS generation decrease in the presences of liposomes may be due to membrane regeneration effect of liposomes on cell compartments involved in ROS generation like mitochondria. The LDH release assay also demonstrated that liposomal samples had no significant effect on membrane integrity of HUVEC and HeLa cells.

Another important feature in the development of liposomal formulation for *in vivo* applications was hemocompatibility. Therefore, the effect of RGD-MNL on RBC lysis and LDH release was investigated. The results demonstrated that RGD-MNL had no adverse effect on hemolysis and membrane integrity of RBC. It was previously reported that cationic particles resulted in adverse effect on hemolysis and membrane integrity of RBC, while negative or neutral charged particles had a negligible effect on blood cells (25, 31). Kuznetsova NR. *et al.* also evaluated the effect of liposomes loaded with methotrexate and melphalan and decorated sialyl Lewis on hemolysis of RBC and showed that liposomal formulations are inert toward the blood major cellular components (24).

## 6. Conclusions

In conclusion, the hemocompatibility and cytotoxicity tests proved that RGD-MNL encapsulated eptifibatide has not significant adverse effect on the cells. All above mentioned factors such as surface charge and size of liposomes determine the cytotoxic effect of liposomes on cells. The slight effect of RGD-MNL encapsulated eptifibatide on cell can be due to the lowest liposome concentration (200  $\mu$ g/ml) used in this work. Our finding showed that, RGD-MNL encapsulated eptifibatide has an acceptable potential capability for delivery of antithrombolytic drugs.

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