

## Fabrication and Evaluation of Spironolactone-Loaded Nanostructured Lipid Carries for Cardiac Tissue Regeneration

### Abstract

**Background:** Spironolactone (SP) is a lipophilic aldosterone receptor antagonist that few studies have reported its effect on cardiac remodeling. In addition, fewer researches have considered its influence on cardiomyocyte viability and potential benefits for myocardial tissue remodeling. **Method:** In this study, stearic acid (SA) (solid lipid) and oleic acid (OA) (liquid lipid) were utilized to produce nanostructured lipid carries (NLCs) (various ratios of SA to OA and water amount, F1: 80:20 [30 ml water], F2: 80:20 [60 ml water], F3: 70:30 [30 ml water], and F4: 70:30 [60 ml water]) containing SP and their particle size, polydispersity index, zeta potential, entrapment efficiency, and release profile were measured. The purpose of encapsulating SP in NLCs was to provide a sustain release system. Meanwhile, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay with different concentrations of SP-loaded NLCs (SP-NLCs) was conducted to evaluate the cytotoxicity of the NLCs on rat myocardium cells (H9C2). **Results:** Increase of oil content to 10 wt% reduced the particle size from 486 nm (F1) to 205 nm (F2). Zeta potential of the samples at around -10 mV indicated their agglomeration tendency. After 48 h, SP-NLCs with the concentrations of 5 and 25  $\mu$ M showed significant improvement in cell viability while the same amount of free SP-induced cytotoxic effect on the cells. SP-NLCs with higher concentration (50  $\mu$ M) depicted cytotoxic effect on H9C2 cells. **Conclusion:** It can be concluded that 25  $\mu$ M SP-NLCs with sustain release profile had a beneficial effect on cardiomyocytes and can be used as a mean to improve cardiac tissue regeneration.

**Keywords:** Cardiac tissue regeneration, nanostructures lipid carriers, spironolactone, sustain release

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### Introduction

Cardiac failure (CF) is one of the main reasons of death worldwide and it causes more than 50% of deaths.<sup>[1]</sup> Heart transplantation is the best therapy for the patients with CF, but there are not sufficient organ donors for those in need of transplantation.<sup>[2]</sup> Therefore, tissue engineering and drug delivery have been attracted great attention to reduce the risk of CF. Spironolactone (SP) has been orally administered to decrease cardiac remodeling by hampering aldosterone secretion in kidneys.<sup>[3,4]</sup> Apart from aldosterone blocking effect, SP has proliferative influence on cardiac cells.<sup>[3]</sup>

SP-loaded solid lipid nanoparticles (SLNs) and SP-NLCs were previously fabricated and they illustrated promising results in

drug loading efficiency (LE), particle size, and zeta potential but these formulations rarely investigated for cardiac tissue engineering. Shamma and Aburahma<sup>[5]</sup> studied SP-NLCs for follicular delivery of SP to treat Alopecia. The SP release of NLCs depicted a primary burst release followed by a sustained release. Confocal laser scanning microscopy affirmed the capability of delivering the fluorolabeled NLCs within the follicles.

Kelidari *et al.*<sup>[6,7]</sup> also examined the SP-NLCs and SP-SLNs to treat skin-related diseases such as acne vulgaris. They exhibited enhanced skin penetration and favorable outcomes in acne vulgaris therapy. Meanwhile, they illustrated that SLNs have lower loading efficiency.<sup>[8]</sup> There were several researches on SP benefit for skin diseases and follicular disorders

Mehrzad Falak<sup>1</sup>,  
 Mehdi Mehdikhani<sup>1</sup>,  
 Jaleh Varshosaz<sup>2</sup>,  
 Batool  
 Hashemibeni<sup>3</sup>,  
 Mehdi Ebrahimian-  
 Hosseinabadi<sup>1</sup>

<sup>1</sup>Department of Biomedical Engineering, Faculty of Engineering, University of Isfahan, <sup>2</sup>Department of Pharmaceutics, Novel Drug Delivery Systems Research Centre, School of Pharmacy, Isfahan University of Medical Sciences, <sup>3</sup>Department of Anatomical Sciences, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

### Address for correspondence:

Dr. Mehdi Mehdikhani,  
 Department of Biomedical Engineering, Faculty of Engineering, University of Isfahan, Isfahan, Iran.  
 E-mail: [me.mehdikhani@gmail.com](mailto:me.mehdikhani@gmail.com)

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therapy, but its effect on cardiomyocytes viability and proliferation has not been evaluated yet. In contrast, SP induced hyperkalemia and renal failure in patients receiving systemic SP.<sup>[9,10]</sup> Therefore, in this study, an attempt was made to design a SP containing formulation and its effect on cardiomyocytes was investigated *in vitro*. The American College of Cardiology, American Heart Association, and Heart failure society of America guidelines 2017 recommended that aldosterone antagonists (SP and Eplerenone) can reduce the risk of morbidity in those with HF and preserved ejection fraction.<sup>[11,12]</sup> Furthermore, SP can decrease collagen synthesis biomarkers in patients with resistant hypertension.<sup>[13,14]</sup>

NLCs are nanoparticles which the drugs are distributed in a binary blend of solid lipid and liquid lipid. The nanostructures of NLC can escalate the drug loading capacity. NLCs include a blend of solid lipid and liquid lipid that can adapt the space required for the entrapping drugs. The potency of this carrier system is strongly determined by drug EE, which is influenced by the ratio of the solid lipid and liquid lipid as well as particle size.<sup>[15]</sup> In a study, the effect of lipid ratio of SA and OA on the physical properties as well as the EE of diethylammonium diclofenac (DETA) with NLC system. DETA belongs to nonsteroid anti-inflammatory drugs. It has been vastly utilized in the treatment of osteoarthritis and rheumatoid arthritis. In the formulation of NLC-DETA, three different lipid ratios were used. The SA to OA ratios were determined at 60:40, 70:30, and 80:20. In this NLC system, DETA functioned as the drug, SA as solid lipid, OA as liquid lipid, and Tween 80 as surfactant constituents. The results depicted that the different ratios of OA and SA had no considerable influences on the viscosity and EE of NLC-DETA.<sup>[15]</sup>

In this research, an attempt was made to synthesize SP-NLCs by different SA to OA ratios and then the particle size, zeta potential, drug release profile, and cytotoxicity using cardiomyocytes were investigated.

## Methods

### Materials

SP was purchased from Mino Pharmaceutical Co (Tehran, Iran). Stearic acid (SA) (solid lipid), oleic acid (OA) (liquid lipid), and chloroform (solvent) were purchased from Merck (Germany). Pluronic F127 (surfactant), dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (USA).

### Nanostructured lipid carries fabrication

NLCs were synthesized in accordance with other similar studies with some modifications.<sup>[6,7,16,17]</sup> Briefly, SA (80% and 70%) and OA (20% and 30%) were melted at 70°C. SP (30 mg) was dissolved in chloroform (1 ml) and added

slowly to the mixture at 55°C before SA solidification and remained at 55°C for several minutes to form the organic phase. The aqueous phase was prepared by dissolving Pluronic F127 (1%) in deionized water and heating to 55°C on a stirrer. The organic phase was added drop wise to the aqueous phase and the solution was stirred for 5 min before going for sonication (Bandelin Sonopuls HD3200, Germany) in 2 min with TT13/FZ probe with 2 s pulse on and 1 s pulse off in an ice bath. The mixture was stirred for further 30 min to insure excessive chloroform evaporation. The solution was then frozen in – 20°C refrigerator before lyophilizing. The obtained samples were stored in 4°C refrigerator for further use.

### Nanostructured lipid carries characterization

#### Particle size and zeta potential

SP-NLCs particle size and zeta potential were determined after dilution to 1/10<sup>th</sup> of their initial volume and using a Zeta Sizer instrument (Malvern Nano Series Zen 3600, UK) with He-Ne lamp and 90° radiation angle. The prepared samples were evaluated in triplicates.

#### Entrapment efficiency

Samples were centrifuged (Eppendorf Centrifuge 5430, Germany) for 10 min at 7000 rpm and then filtered (Millipore 0.2 μ, Germany). The UV absorption of the resulting solution which contained the free unloaded drug was measured with spectrophotometer (Shimadzu, Japan) with the maximum wave length ( $\lambda_{max}$ ) of 241 nm ( $n = 3$ ) and compared to the previously obtained standard calibration curve of SP to determine the concentration of the free drug. To calculate the entrapment efficiency (EE) and loading efficiency (LE) in the NLCs, the following equations were used:<sup>[18]</sup>

$$EE\% = \frac{\text{Total drug} - \text{Free drug in centrifuged solution}}{\text{Total drug}} \times 100 \quad (1)$$

$$LE\% = \frac{\text{Wtrapped drug}}{\text{Wnanocarrier}} \times 100 \quad (2)$$

### *In vitro* release of spironolactone from nanostructured lipid carries

Drug release from NLCs was studied *in vitro* using dialysis bag method in phosphate buffer saline solution (PBS) (pH 7.4). For this purpose, 2 ml of NLCs dispersion was poured in a dialysis bags (cut off of 12000 Da). The bags were placed in 40 ml of PBS and stirred at 500 rpm. The temperature was adjusted at 37°C during the drug release test. At different intervals between 30 min and 144 h, the absorptions of the solutions were measured at  $\lambda_{max}$ . The drug concentration of each sample was determined using the concentration-absorption equation obtained from the standard curve in the PBS medium.<sup>[19]</sup>

The test was repeated three times for each formulation. The percentage of the release efficiency (RE%) was determined by calculating the percentage of drug released from the nanoparticles. The RE was calculated by eq. 3, which showed the ratio of area under the drug release versus time diagram in a definite time to the time which the drug released completely (100%).<sup>[20]</sup>

$$RE\% = \frac{\int_0^t y dt}{y_{100} \times t} \times 100 \quad (3)$$

### 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

H9C2 cells (rat myocardium) were purchased from Pasteur institute (Tehran, Iran) and cultured in DMEM/F-12/10% FBS media. The cells were then seeded in 24 well plates with 20000 cells per well.<sup>[19]</sup> Pure different concentrations of SP were added (5, 50 and 500  $\mu$ M) to the wells containing complete culture medium and incubated for 24 and 48 h. At each time interval, medium was removed and MTT solution in serum-free media (5 mg/ml) was added to each well. The plates were placed in an incubator for another 4 h. Then, the medium was removed and DMSO was added to dissolve the formazan crystals. Optical density (OD) was measured in a microplate reader (Hyperion, Florida, USA). Based on the previous studies<sup>[21]</sup> and the half maximal inhibitory concentration (IC<sub>50</sub>) test, SP-NLCs concentrations were determined (5, 25, 50  $\mu$ M) and the same procedure was conducted to evaluate their cytotoxicity. All of the samples were tested in triplicates. Control groups were prepared with cells being cultured in media containing no SP-NLCs. Cell viability was measured with the following equation, where OD<sub>s</sub> and OD<sub>c</sub> are sample and control group average optical densities, respectively:

$$\text{Cell visibility} = \frac{OD_s}{OD_c} \times 100 \quad (4)$$

## Results and Discussion

### Nanostructured lipid carries characterization

#### Particle size and zeta potential

Table 1 shows that a 10% increase in OA content caused the reduction of particle size to almost half the size. This decrease in particle size was in accordance with

some previous studies.<sup>[6,22]</sup> Wang *et al.*<sup>[23]</sup> synthesized buprenorphine-loaded NLCs, SLNs, and lipid emulsions (LE) with different oil/fatty ester ratios as injectable formulations of this drug and its prodrugs. The physicochemical characteristics illustrated that each formulation system (SLNs, NLCs, and LE) had detectable properties. Drug/prodrug release could be controlled by modifying the lipid carrier. SLNs demonstrated the prominent delivery system followed by NLCs and LE.

As shown in Table 1, the reduction of total lipid concentration in the aqueous phase, contributed to the decrease in particle size and zeta potential (more negative amounts) as well. Few studies have been conducted to evaluate the effect of total lipid concentration on NLCs size and zeta potential. The reduction of particle size with increase of aqueous phase volume was probably due to the existence of larger space which could hinder agglomeration of lipid clusters and the particles with lower sizes can be formed.

Zeta potentials more than +30 mV and <30 mV are indicative of stable particles.<sup>[24,25]</sup> This suggests that particles with absolute values lower than 30 mV are susceptible to agglomeration which was the reason for freeze drying of the samples in our study. This low zeta potential might be related to the type of surfactant. Kelidari *et al.*<sup>[6]</sup> reported the raise in zeta potential absolute values of NLCs with the increase of OA. In fact, the negative nature of synthesized NLCs was attributed to the OA negative charge that attributed to the presence of carboxylic acid groups. Hendradi *et al.* showed that increasing the amount of OA in the NLCs formulations diminished the size of NLCs particles.<sup>[15]</sup> Furthermore, Khalil *et al.*<sup>[8]</sup> confirmed that adding liquid lipid to lipid matrix, reduce the particle size. This result was related to the greater molecular mobility of the matrix after liquid lipid addition and consequently enhanced the formation of the small particles.

Akhoond Zardini and *et al.* showed that liquid oil could be dispersed in a lipid matrix (Matrix type) and decreased particle size or made core-shell type nanocarriers that in this type, particle size raised with oil addition. Similar to our present research because the particle size of their nanocarriers declined with oil addition, therefor synthesized NLCs were likely matrix type.<sup>[26]</sup>

Particles with lower size have more surface area and higher amount of surface charge and zeta potential.<sup>[27]</sup> This can be

**Table 1: Formulation compositions, particle size, polydispersity index, and zeta potential of the prepared spironolactone - nanostructured lipid carries**

Formula	Stearic acid (mg)/ oleic acid (mg)	Water (ml)	Pluronic F127 (mg)	Particle size (nm)	PDI	Zeta potential (mV)
F1	80/20	30	300	486±101.59	0.631±0.06	-9.36±1.05
F2	80/20	60	600	414±64.02	0.507±0.03	-12.70±2.16
F3	70/30	30	300	285±14.57	0.425±0.03	-9.46±0.50
F4	70/30	60	600	205±22.86	0.515±0.04	-12.40±2.74

PDI: Polydispersity index

observed for the samples with the same lipid composition and different particle size in Table 1. It was showed that in the samples with the same lipid composition and different particle size (F1 and F2 as well as F3 and F4), with decreasing the particle size for each composition the absolute value of zeta potential was increased (zeta potential increased from 9.36 and 9.46–12.70 and 12.40 for F1/F2 and F3/F4, respectively).

Hu *et al.* prepared and characterized SA NLCs by solvent diffusion technique in an aqueous system. In this research, SA NLCs with various OA content were successfully synthesized. As OA amount rose up to 30 wt%, the prepared particles illustrated considerable smaller size. There was no trend for the zeta potential changes by increasing the OA content of the nanoparticles.

### Entrapment efficiency

EE and loading efficiency of the synthesized formulations are shown in Table 2. All the compounds had a high EE. For instance, EE% of F1 and F2 formulations was measured at 100%. This high EE could be related to the lipophilic nature of SP and the imperfections in NLCs due to the presence of oil.<sup>[5,28]</sup> Furthermore, F3 and F4 (i.e., formulations prepared with higher content of OA) had lower EE and drug loading than those of F1 and F2. Shalaby *et al.*<sup>[29]</sup> studies showed the most effective parameter on EE of PEG/PLA nanoparticles was polymer to drug ratio. In addition, the higher molecular weight could lead to higher EE. Likewise, using higher amount of solid lipid in NLCs formulation caused coarser particles synthesis with higher viscosity and drug LE.<sup>[30]</sup> Higher EE of F1 and F2 formulations could be attributed to their larger particle size and higher molecular weight.

### In vitro spironolactone release studies

Figure 1 illustrates the release profile of SP from NLCs during soaking in PBS for 72 h. Table 2 shows the percentage of SP released at 72 h ( $RE_{72}\%$ ), too.

The release test was conducted for 3 days and no significant differences could be observed in the release profile. F1 and F3 NLCs showed the lowest and highest release rate in 72 h.

All samples illustrated a starting burst release followed by a reduction of diagram slope. This behavior was observed in

**Table 2: Average loading efficiency, entrapment efficiency, and total amount of drug released from nanostructured lipid carries after released at 72 h of the prepared formulations**

Formula	EE (%)	LE (%)	$RE_{72}$ (%)
F1	99.08±0.48	29.94±0.11	71.00±2.95
F2	97.40±1.74	29.22±0.40	80.60±2.34
F3	89.04±0.20	26.71±0.05	86.00±4.23
F4	81.57±3.48	24.47±0.80	75.20±2.60

LE: Loading efficiency, EE: Entrapment efficiency,  $RE_{72}\%$ : Released at 72 h

similar studies and could be related to the starting release of un-entrapped drug from nanoparticles surface followed by a steady release from particles core.<sup>[6]</sup>

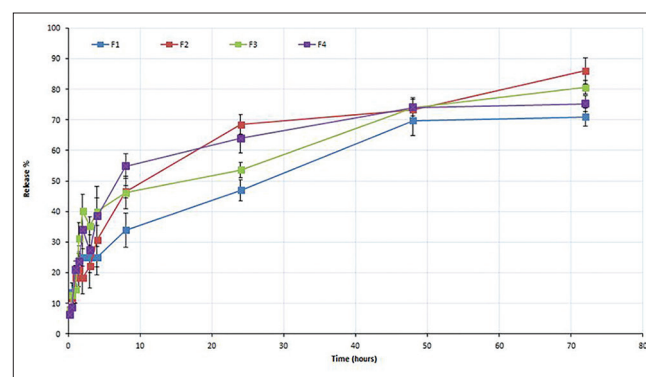
It is obvious that EE was not 100 percent and a portion of drug was free and not entrapped. However, the whole calculation related to the drug release was performed based on the encapsulating drug measurement and had no relation with the free drug. In fact, primarily, the samples were poured in the dialysis bags followed by soaking in distilled water to discharge the free drug. Then, the dialysis bags were put in PBS solution to evaluate the drug release of different samples in different time periods according to entrapped drug in the NLCs. Overly, all formulations indicated relatively similar release profiles and the amount of drug release was more than 70% of the entrapped drug after 72 h.

### Selection of the optimum formulation of nanostructured lipid carries

Optimum formulation can be selected based on the parameters such as release rate, loading efficiency, surface charge, and particle size of the NLCs. In this study, zeta potential did not have any role in the selection criteria since all formulations had zeta potentials with absolute values lower than 30 mV. EE was acceptable for all samples and did not play an important role in selection of the optimum formulation of NLCs. In addition, F4 had lower release rate than other formulations except F3 and this could result in more sustained release of the drug. Therefore, F4 was chosen as the optimum formulation.

### 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Figures 2 and 3 show the cell viability of H9C2 cells in the presence of free SP and SP-NLCs after 24 and 48 h of cell culture, respectively. Figure 2 shows that SP was cytotoxic in all concentrations except 5  $\mu$ M on the 1<sup>st</sup> day. After 48 h, all the concentrations of free SP reduced cell survival significantly ( $P < 0.05$ ). Furthermore, it was obvious that cytotoxicity directly related to SP concentration at each



**Figure 1: In vitro release of spironolactone from spironolactone nanostructured lipid carries in phosphate buffer saline containing 0.1% of SDS**

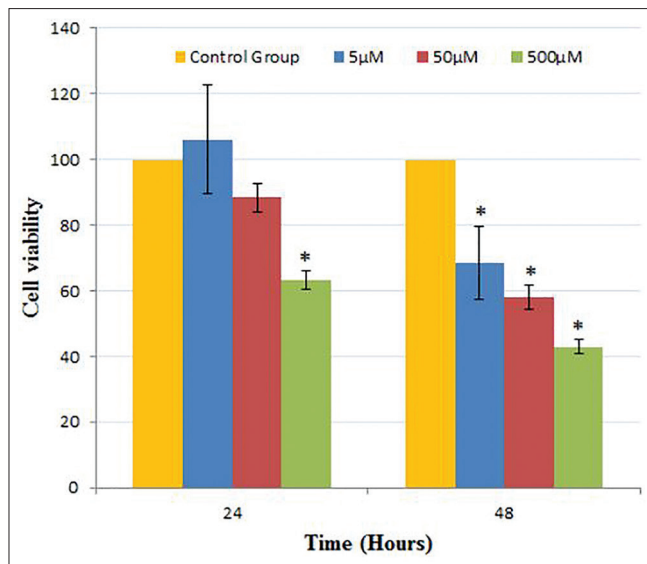


Figure 2: H9C2 cells viability after 24 and 48 h of contact with pure spironolactone (\* $P < 0.05$ )

time interval since the increase in SP concentration from 5 to 500 µM followed by less cell survival. Based on these results, the proposed suitable concentration of NLCs concentration in cytotoxicity test was <50 µM.

Figure 3 represents cell survival in media containing loaded and blank NLCs. It was observed that cytotoxic effect of the blank NLCs with different concentrations was relatively similar to the control group and nontoxicity was confirmed. It may be concluded that the designed nano-lipid carriers were being tolerated by living systems because of the fact that their compounds were fabricated by biocompatible substances.<sup>[31]</sup> The unloaded 5 µM and 25 µM samples (red and yellow columns) showed some more degrees of cell viability compared to control group after 24 h but overall the difference was not significant ( $P > 0.05$ ).

The SP-NLCs showed no significant difference with control group on the 1<sup>st</sup> day (except 5 µM) but on the 2<sup>nd</sup> day the cell viability changed dramatically for all of the samples. The 5 and 25 µM samples increased cell viability to almost twice the amount in the 1<sup>st</sup> day. Sustained drug release from the NLCs did not allow the sudden increase in the concentration of SP in the culture medium and kept it below the toxic level. On the other hand, while 50 µM concentration of SP-NLCs had better cell survival than control group on the 1<sup>st</sup> day, they reduced the cell viability to the levels comparable with free SP samples [Figure 2]. This suggests that SP-NLC with 25 µM concentration had the optimum effect on H9C2 cells.

## Conclusions

After 48 h of cell culture, SP-NLCs with the concentrations of 5 and 25 µM illustrated significant improvement in cell viability while the same amount of free SP was cytotoxic. SP-NLCs with higher concentration (50 µM) depicted

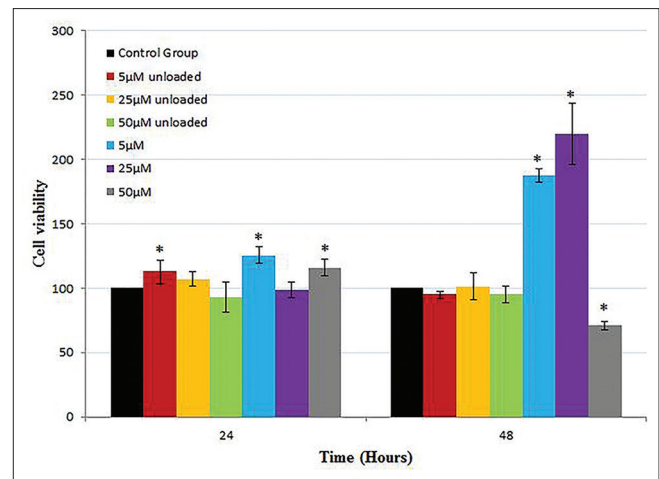


Figure 3: H9C2 cells viability after 24 and 48 h of contact with spironolactone nanostructured lipid carries in cell culture medium (\* $P < 0.05$ )

cytotoxic effect on H9C2 cells. As a consequence, 25 µM SP-NLCs showed a beneficial effect on cardiomyocytes and could be used as a mean to improve cardiac tissue regeneration and it deserved further *in vitro* and *in vivo* investigations.

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## Conflicts of interest

There are no conflicts of interest.

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