Original Article

Streptokinase Loaded Solid Lipid Nanoparticles: Preparation and Charactrization

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Abstract

Thrombolytic therapy for acute myocardial infarction consists of recombinant streptokinase (rSK) or tissue plasminogen activator (tPA) and other similar enzymes. In this study, the potential of solid lipid nanoparticles (SLN) as nano-protein carriers of thrombolytic agents was investigated. SLN formulation for utilization in targeted enzymatic delivery of rSK was prepared using a combined technique. Response surface methodology (RSM) was employed to develop the best formulation and analyze the effect of different parameters on the efficient of the SLN-rSK formulation. The optimum condition of the formulation for SLN-rSK preparation was found to be at lipid concentration 0.5 mg/ml, surfactants ratio of 0.5, rSK concentration 4 mg/ml, hgomogenization time 20 min and speed 20000 rpm. The formulation characterization analyzed by SEM method showed that SLN-rSK formulation has a spiral appearance. The size of formulation was determined with DLS (<60 nm) as well as the entrapment efficiency percentage (EE%) calculated as 67.30%. The integrity assessment approved via SDS-PAGE, no significant change was observed between streptokinase and SLN-rSK formulation in terms of protein structure. Moreover, potency of SLN-rSK conjugates was statistically greater than streptokinase. It seems that high enzymatic activity of SLN-rSK conjugates can be a favorable way for delivering bioactive macromolecules with SLN. Therefore, the SLN-rSK showed to be non-cytotoxic, effective and stable formulation intended to be used as a non thrombolytic dosage form.

Keywords: Recombinant streptokinase (rSK); Acute myocardial infarction; Pulmonary embolism; Solid lipid nanoparticle; Drug delivery system; Cytotoxicity

Introduction

Recombinant streptokinase is an extracellular bacterial enzyme with a molecular weight of 47 kDa secreted by various strains of β-hemolytic *streptococci*. This single-chain polypeptide is a thrombolytic agent and one of the plasminogen activators. It interacts with human plasminogen and forms an active complex with a protease activity that transforms plasminogen to plasmin. Thus, it is used in the treatment of acute myocardial infarction (AMI) and pulmonary embolism (PE). Among different types of plasminogen activators, recombinant streptokinase (rSK) is the first as well as one of the best drugs introduced to the market for more than 55 years. It has high molecular weight and short life-time about 20-30 min due to immune response and enzymatic degradation in the blood stream. Recently, researchers showed that targeted delivery system is more efficient than systemic approaches. As appeared in past ponders, in such cases, medicate conveyance frameworks can be utilized to improve the medicate properties by expanding dissolvability, dynamic half-life, balance of pharmacokinetics and moving forward the security of medicate treatment. Subsequently, planning rSK details with a more noteworthy degree of target specificity may move forward its adequacy. As of late, SLN is considered as a novel pharmaceutical conveyance framework and a substitute carrier framework for other colloidal carriers. These nanostructures were presented in 1990 [1]. They are composed of a strong lipid center and emulsifiers with an estimate less than 1000 nm. The lipid center framework has an imperative part in controlling sedate discharge [2, 3]. In SLN, the utilization of physiological lipids improves the biocompatibility and security of their definitions [4]. Both hydrophilic and lipophilic pharmaceutical active ingredients

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can be incorporated into SLN [5]. Therefore, this study was conducted to develop a safe, stable rSK loaded SLN formulation with an acceptable EE% and suitable particle sizes to control the enzyme delivery based on some excellent characteristics of nanotechnology which contribute to overcome the aforementioned problems especially lipophilic excipients in SLN-loaded particles.

Materials and Methods

Materials

Cetyl palmitate (CP), Tween 80, Tween 20, sodium azide were purchased from Merck (Darmstadt, Germany). Anti-streptokinase antibodies (Bio Rad Life Sciences), Roswell Park Memorial Institute (RPMI) 1640, Fetal Bovin Serum (FBS), EDTA and trypsin were purchased from GIBCO (Gibco, Life Technologies GmbH, Karlsruhe, Germany). rSK was obtained from Pasteur Institute of Iran, and 3-4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich (Sigma, Deisenhofer, Germany). All other chemicals and reagents used in this study were of analytical grade.

Preparation of rSK loaded SLN

rSK loaded SLN was fabricated using a previously described high Shear homogenization (HSH) microemulsion technique with a slight modification [6]. This technique was optimized, in particular, for the preparation of SLN-rSK through selecting five independent variables including concentration of lipid, surfactants, rSK, homogenization time and rate. Then, a full factorial design was used, where each independent variable (X_1 to X_5) was evaluated at three levels resulting in 45 different SLN-rSK formulations (SLN-rSK1-45). To prepare the SK loaded SLN formulations, lipid phase- *i.e.* cetyl palmitate- was heated to 50 °C and, subsequently, surfactant containing the rSK was added to the melted lipid while stirring to obtain a clear dispersion. The resulting emulsion was then homogenized using a high shear homogenizer [6, 7] (IKAULtra-Turrax, T25, Germany) at a predetermined homogenization speed and time, as mentioned in Table 1.

The obtained microemulsion was promptly spread in 2-8 °C double distilled water under magnetic stirring to solidify SLN-rSK [7]. Subsequently, the physicochemical properties including particle size and entrapment EE% as dependent variables were measured for each formulation (Table 1). To determine the best SLN-rSK formulation, the response surface methodology (RSM) was used.

Formulation characterization

Z-average diameter and polydispersity index (PDI) were measured using photon correlation spectroscopy (Zetasizer Nano Series, Malvern, UK) method at 90^0 scattering angle and 25 °C. To this aim, SLN-rSK samples were diluted to 0.1 (v/v%) with ultra-pure water at pH=5.5 [8]. Other properties, such as loading capacity percentage (LC%) and EE% were also evaluated using the methods further described in this section. In addition, particles diameter was calculated in triplicate by dynamic light scattering technique as previously described.

Scanning electron microscopy (SEM)

The morphology of prepared SLN-rSK formulations was analysed by scanning electronmicroscopy (NOVA NANOSEM 450 FEI model at an accelerating voltage of 15 kV) in high vacuum mode. A certain amount of sample (diluted with an appropriate volume of DDW, 1:100), was placed on the FE-SEM holder and coated with a layer of gold of 100 Å for 3 minutes under argon at a pressure of 0.2 atm.

Determination of the entrapment efficiency and drug loading capacity

The percentage of rSK encapsulated in the SLN was determined using a centrifugation method as described earlier by Ghadiri et al [7]. Briefly, a suspension of the optimized SLN-rSK formulation in fresh PBS (1X) at pH 7.4 containing 1% (W/V) SDS was centrifuged at 20000 rpm for 20 min (4 °C) and the resulting supernatant was collected. The amount of free (un-entrapped) rSK in the supernatant was measured using a UV spectrophotometer (UV-1601 PC; Shimadzu, Kyoto, Japan) at 595 nm following Bradford assay. The EE% was calculated using the following equation [9]:

$$EE\% = \frac{W_1 - W_2}{W_1} \times 100$$

Where W_1 is the initial amount of drug incorporated in the formulation and W_2 corresponds to the dry weight of free drug in the supernatant.

Meanwhile, the loading capacity of rSK was measured using the following equation [10]:

$$LC\% = \frac{\text{Total quanitiy of drug in SLN}}{\text{Quantity of Drug+Quantity of Excipients}} \times 100$$

In vitro release study

The in vitro release of rSK from the optimized SLN-rSK formulation was evaluated using a dialysis membrane (cutoff 10000 Da) in a dissolution medium comprising PBS (phosphate buffered saline) and 0.02% sodium azide. To this end, a 5 ml solution of the optimized SLN-rSK formulation was inserted into the dialysis tubes, previously soaked in double distilled water, and placed in 50 ml dissolution medium. The medium was placed in a thermostatic shaker bath at 37 °C at a rate of 60 times per min. After 0.5, 1, 2, 3, 4, 5, 6, 7, 8 and 12 hours of incubation, 0.5 ml of the dissolution medium was withdrawn and replaced with fresh dissolution medium [11]. The concentration of rSK in samples

was determined using Bradford assay followed by spectroscopic analysis at λ_{max} of 595 nm.

Evaluation of the structural integrity of the encapsulated rSK

To evaluate the effects of SLN-rSK fabrication conditions on the structural integrity of the encapsulated rSK, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. For electrophoresis, samples collected from the released rSK into PBS (1X) then, after 10-14 h were first mixed with 2X Laemmli sample buffer (BioRad, Hercules, CA, USA) thereafter, boiled for 4 min in the presence of SDS and β-mercaptoethanol. Subsequently, an aliquot of the denatured samples was loaded onto a 12.5% SDS polyacrylamide gel. Protein standards of determined molecular weight were also loaded to compare the electrophoretic mobility of encapsulated rSK with unformulated rSK. Finally, protein bands were visualized using silver nitrate staining method [12].

Biological activity determination

Biological activity of the encapsulated rSK was quantified by a colorimetric assay using human plasminogen and a synthetic chromogenic substrate, S2251™ (Chromogenix-Instrumentation Laboratory, Milan, Italy). The chromogenic method is an endpoint strategy employing a chromogenic substrate (S2251)(Val-leu-lys-p-nitroaniline.2HCl); hromogenix, Milan, Italy), whereas rSK changes over plasminogen to plasmin in solution, within the nonappearance of fibrin. In this strategy, a given concentration of rSK comes about in direct production of active plasmin leading to quick hydrolysis of S-2251, consequently coming about measurement within the optical density (OD) of the solution expanding exponentially. After forming the whole sum of product, its OD is corresponding to the rSK concentration. Substrate solution preparation was set by including 1 ml of 0.5 M Tris-HCl with pH of 7.4 at 37 °C with 1 ml of 3 mM S-2251 (reconstituted in water) and 5 µl of 10% Tween 20 (Usb®,USA). This course of action was kept up at 37 °C and, promptly before utilization, 45 µl of plasminogen solution (1 mg/ml) were included as well. Different dilutions of rSK for the dose-response curve preparation were arranged in 10 mM Tris-HCl with pH of 7.4 containing 0.1 mM NaCl and 1mg/ml albumin, over the run 4.0, 2.0, 1.0 and 0.5 IU/ml, then kept up at 37 °C in a microtiter plate. Plasminogen actuation was started by mixing 60 µl of rSK solution with 40 µl of substrate. Concentrations of rSK within the actuation curve were, hence, 2.4, 1.2, 0.6 and 0.3 IU/ml. The response recording was permitted to continue for 20 min and after that halted by expansion of 50 µl of 50% acetic acid and consequently the OD was measured at 405 nm [13].

In Vitro cytotoxicity assay

Mouse connective tissue fibroblast cell line (L-929) was obtained from Pasteur institute (Tehran, Iran). L-929 cells (ATCC® TIB-202™) were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin (100 µg/ml) in a 5% CO_2 humidified incubator at 37 °C [11].

To evaluate cell viability following exposure to unprocessed rSK, SLN and SLN-rSK, the MTT assay was performed. To this end, L-929 cells with a concentration of 2×10^4 cells/cm^2 were seeded in a 96-well plate and grown at 37 °C in an atmosphere of 5% CO_2 in RPMI medium. After reaching confluence of 70-80%, cells were incubated with different dilutions of SLN-rSK, SLN and rSK (corresponds to 1:10, 1:20, 1:40, 1:80 and 1:160) for 24 to 48 h. The SLN, SLN-rSK and rSK solutions were previously filtered through 0.2 µm filter units to minimize contamination. After 24 and 48 h incubation, 20 µl MTT (5 mg/ml) was added to each well followed by another 4 h incubation at 37 °C, with 5% CO_2. Subsequently, the medium was withdrawn and replaced with 150 µl dimethyl sulfoxide (DMSO) to dissolve MTT formation, then the absorbance was measured at 570 nm after passing 30 min incubation. Untreated cells were used as negative control. Using the following equation, the percentage of cell viability was calculated [14]:

$$\text{viability (\%)} = \frac{\text{Optical density of threated cells}}{\text{Optical density of unthreated cells}} \times 100$$

Stability studies

The optimized SLN-rSK formulation was subjected to stability studies according to International Conference on Harmonization [15] Q1A (R2) guidelines [16]. Three fresh samples of prepared SLN-rSK were maintained in sealed vials at 25±2 °C/60±5% RH placed in a humidity and temperature control stability chamber. The samples subjected to stability test were dispersed and analyzed for physical characteristics, enzyme biological activity, pH and particle size at intervals of 1, 3 and 6 months, respectively.

Statistical analysis

Comparisons between groups were analyzed by one way ANOVA using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Statistical significance was determined as $P \leq 0.05$. RSM analysis was carried out to evaluate the effect of the five previously mentioned formulation using RSM.

Results

Formulation characterization

Initially, the physicochemical characteristics (particle size and EE%) of all 45 formulations prepared by varying 5 independent variables including cetyl palmitate (CP) concentration (X_1), surfactants (X_2), rSK concentration (X_3), homogenization time (X_4) and homogenization speed (X_5) were determined (Table 1).

Table 1. Independent and dependent variables of experimental design matrix for SLN formulation

Run	Independent variables					Dependent variables	
	Lipid (x1): mg/ml	Surfactants (x2: Tween 20/80): ratio	rSK concentration (x3): mg/ml	Homogenization time (x4): min	Homogenization speed (x5): rpm	Size (nm)	EE (%)
1	0.5	1.5	4	20	20000	227.36	58.3
2	1	1	5	15	20000	243.14	56.74
3	0.5	1.5	6	20	18000	197.5	60.1
4	1.5	0.5	6	20	20000	272.67	39.74
5	1.5	1.5	4	20	18000	278.5	55.76
6	0.5	0.5	4	10	20000	258.66	50.25
7	1	1	5	15	19000	312.11	34.52
8	1	1.5	5	15	19000	267.7	65.81
9	1	1	5	10	19000	340.1	28.19
10	0.5	0.5	6	20	20000	287.63	34.6
11	1	0.5	5	15	19000	248.25	36.78
12	0.5	1.5	6	10	18000	250.5	6.11
13	1	1	5	15	19000	288	54.29
14	1.5	1.5	6	10	18000	304.69	53
15	1.5	1.5	4	10	18000	350.77	53
16	1.5	0.5	6	10	18000	264.3	47.75
17	0.5	0.5	4	20	20000	258.4	51.7
18	1	1	6	15	19000	214.66	37.22
19	0.5	0.5	4	10	18000	209.25	42
20	0.5	0.5	6	10	20000	224.81	49
21	1.5	0.5	4	10	18000	252	32
22	1	1	5	20	19000	223	55.34
23	1.5	1.5	6	20	20000	290.7	48.38
24	1.5	0.5	4	20	18000	206.64	54
25	1.5	0.5	6	20	18000	289.11	37.9
26	0.5	1	5	15	19000	192.67	59.8
27	1	1	5	15	18000	191.84	32.16
28	0.5	1.5	4	20	18000	297.36	45.19
29	1	1	4	15	19000	312.53	61.7
30	1.5	1.5	6	20	18000	261.44	32.84
31	1.5	0.5	4	10	20000	274.75	57.36
32	1.5	1.5	4	10	20000	316.32	50.5
33	0.5	1.5	6	10	20000	219	36.43
34	0.5	1.5	4	10	18000	299.7	49
35	1	1	5	15	19000	289	33.76
36	0.5	0.5	6	10	18000	211.37	60.12
37	0.5	1.5	4	10	20000	252	71
38	0.5	1.5	6	20	20000	217.5	46.55
39	0.5	0.5	4	20	18000	165.3	79
40	1.5	1.5	6	10	20000	237	52.96
41	0.5	0.5	6	20	18000	184.9	32.19
42	1.5	1.5	4	20	20000	260.1	61.48
43	1.5	0.5	4	20	20000	218	69.57
44	1.5	0.5	6	10	20000	194	53.43
45	1.5	1	5	15	19000	192	62.74

As a result represented in Table 2, the optimum formulation by obtaining a particle size between 200-250 nm, i.e. 210 nm (Figure 1) and a PDI of 0.218 (Figure 2) and a high EE% of 67.30% were obtained. Accordingly, the optimized SLN-rSK formulation was prepared using a ratio of "0.5 mg/ml CP: 0.5 mg/ml of the surfactants mixture in the ratio of 1:2 and the 4 mg/ml rSK, homogenized at 20000 rpm for 20 min.

Table 2. Optimum conditions for SLN-rSK formulation

Process condition					Result	
Lipid (x1): mg/ml	Surfactants (x2): ratio	rSK concentration (x3): mg/ml	Homogenization time (x4): min	Homogenization speed (x5): rpm	Size (nm)	EE(%)
0.5	0.5	4	20	20000	224.15±20.01	67.30±1.34

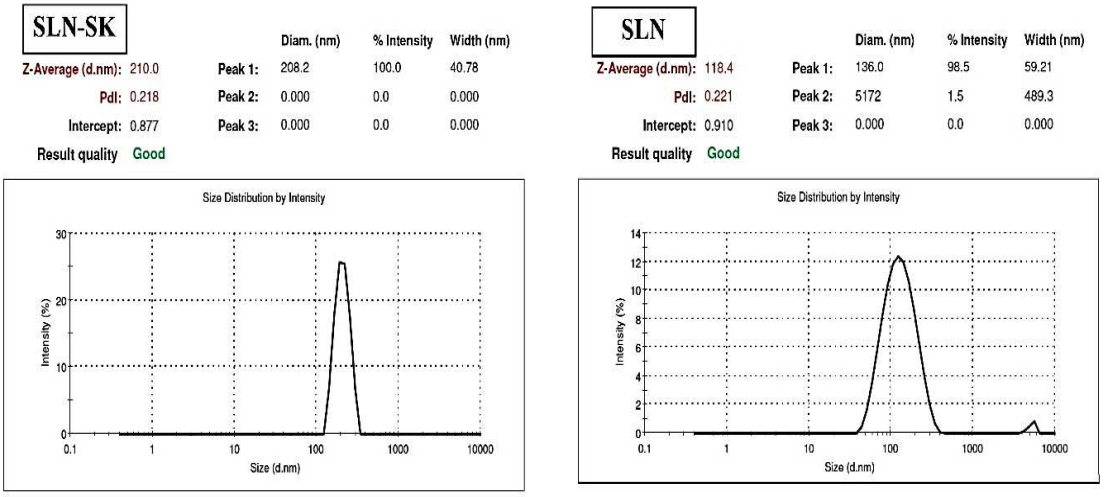

Figure 2. Particle size dispersion of SLN-rSK and SLN formulation

Morphological studies

In consistence with the results of particle size, PDI and zeta potential, scanning electron photomicrographs can be obtained to further evaluate the shape and surface morphological properties of the nanoparticles. The obtained SEM micrographs from freshly prepared SLN-rSK optimized formulation clearly demonstrated that SLN are of spherical shape. It was found that SLN was almost dispersed with minimum aggregation (Figure 3).

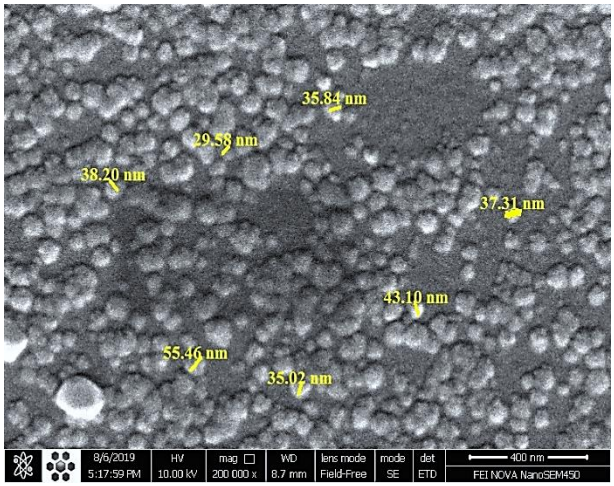

Figure 3. SEM micrograph of the SLN formulation prepared by high shear homogenization method.

Entrapment efficiency and drug loading capacity

The percentage of the drug adsorbed on the surface or entrapped within the nanoparticles is defined as entrapment efficiency [17]. This value is calculated by measuring the amount of free drug in the dispersion medium [18]. Using the centrifugation method in combination with Bradford assay,

the EE and LC% of SLN was estimated to be 0 and 71.6% respectively, demonstrating a successful encapsulation of rSK into SLN formulation.

In vitro drug release

The *in-vitro* evaluation of rSK release from SLN was carried out using the dialysis method. The *in-vitro* release study was accomplished in PBS (PH=7.4) at 37 °C and the obtained results were converted into a plot showing the relation between the cumulative drug release and time (Figure 4). According to this plot, the prepared SLN-rSK formulation released 96.9% of the drug in almost 12 h. Meanwhile, the resulting plot showed a biphasic drug pattern including a rapid release of the drug at the primary phase followed by a constant rate of release. The initial rapid drug release can be interpreted as due to the higher pH of the neutral medium compared to the rSK isoelectric point [15], generating a repulsion between rSK and cetyl palmitate which in turn leads to the breakdown of SLN [19].

Table 3. Different kinetic model for the *in vitro* release of different SLN-rSK formulations, prepared by high shear homogenization method (mean±SD, n=3) at room temperature (25 °C)

Mathematical release kinetics	Equation	r^2 value
Zero-order	$C_t=C_0+K_0t$	0.6041
First-order	$LogC=LogC_0+K_t/2.303$	0.7797
Higuchi	$Q=K_H\sqrt{t}$	0.8267

According to the different scientific models such as the Higuchi, the zero-order energy and the first-order active [18], the drug release data obtained from the dialysis method was best fitted into the Higuchi model (r^2=0.8267) meaning that the drug release follows the diffusion-controlled mechanism. According to the obtained formula from the Higuchi model (Y=9.88x+10.41), the prepared SLN-rSK was able to release 96.9% of its drug in 12 h.

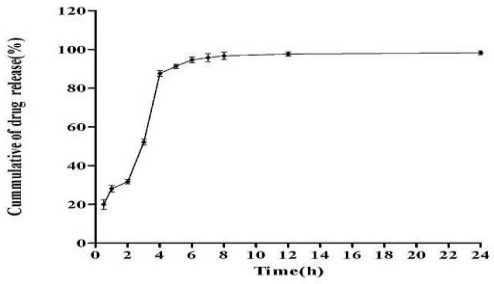

Figure 4. In vitro drug release profiles of rSK from SLN-rSK formulation

Integrity of the loaded rSK into SLN

The integrity of the released drug into PBS buffer medium after 10-14 h was evaluated using SDS-PAGE. According to previous studies, the molecular weight of native streptokinase has been determined to be 47 kDa [20, 21]. The SDS-PAGE analysis of the obtained rSK revealed an intact protein band with a molecular mass of approximately 47 kDa, as compared to the standard sample (Streptase®) obtained from ZLB Behring GmbH (Figure 5).

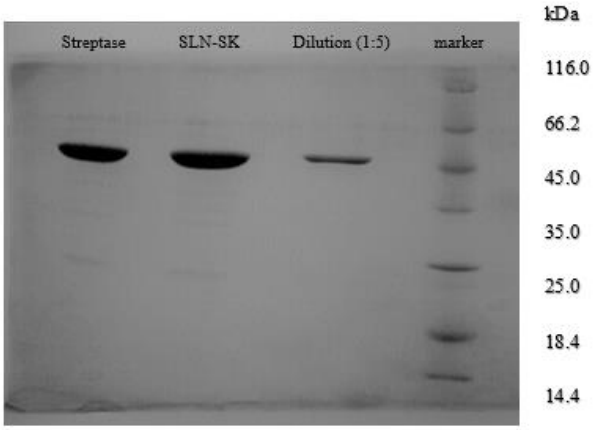

Figure 5. SDS-PAGE (12.5%) analysis of the loaded rSK into SLN

Conversely, the SDS-PAGE analysis of the released rSK showed a single band similar to the unformulated rSK, demonstrating that rSK preserved its integrity and showed no aggregate or disruption after encapsulation into SLN and its subsequent release (Figure 5).

Determination of the biological activity

In addition, the biological activity of both the unprocessed and the released rSK from SLN was assessed employing a chromogenic strategy as depicted in "Materials and Methods" section. It was found that the rSK loaded into the SLN completely retained its biological activity after formulation and encapsulation into the SLN. Biological activity of rSK and SLN-rSK were shown to be 211563.84 and 211396.44 IU/ml, respectively.

Evaluation of the formulation cytotoxicity

In order to assess the anti-thrombosis effect of rSK-loaded SLN, its cytotoxic activity was assessed against L-929 cell line using MTT assay. Our results indicated that the viability of L929 mouse fibroblast cell line in the presence of SLN-rSK and free-rSK was significantly reduced in a dose-dependent manner as compared to SLN-exposed cells (*P*-value≤ 0.05). However, the blank SLN did not show any significant cytotoxicity against L-929 as compared to SLN-rSK formulation and blank. The viability of macrophage cells in the ratio of 1:10 dilution, corresponding to 400 µg/ml, blank was significantly lower (80.6%) than that of SLN-rSK (89.9%, *P*-value≤ 0.05) with the same concentration (Figure 6).

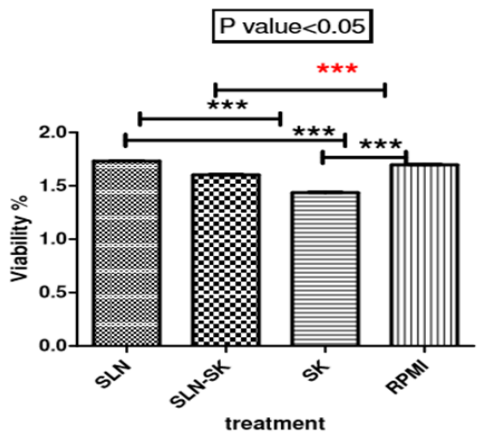

Figure 6. Cell viability test using mouse fibroblast L-929 prepared by high shear homogenization (n=3)

Stability studies

Accelerated stability studies were carried out on optimized SLN-rSK formulation with respect to specific activity, pH, size and transparency parameters. The biological activity of the loaded SLN-rSK varied from 211396.44 to 206648.50 IU/ml after 6 months, indicating that the drug can maintain its activity while being encapsulated in the nanoparticle for a considerably sufficient period. It is worthy to note, upon storage of SLN-rSK, no significant alterations in size, pH, purity, specific activity and transparency was observed. These parameters were found to be stable at 25±2 °C/60±5% RH within 6 months storage (Table 4) [16].

Table 4. Accelerated stability results in different time intervals (day)

Quality characteristics	Acceptance limit	Time (months)			
		0	1	3	6
Appearance	Colloidal Transparent yellow	Colloidal Transparent yellow	Colloidal Transparent yellow	Colloidal Transparent yellow	Colloidal Transparent yellow
Biological activity (IU/ml)	≥140000	211396.44	211375.4	211324.76	206648.50
pH	6.8-7.5	6.91	6.90	6.93	6.93
Size (nm)	210	210	211	216	221

Discussion

According to the World Health Organization (WHO) report, 17.9 million people die from cardiovascular diseases (CVDs) annually, an estimated 31% of all deaths worldwide [22, 23]. More than 75% of CVD deaths occur in low-income and middle-income countries [22]. Also, this organization has reported that the heart attacks and strokes are 85% reason of CVD deaths [22]. Thrombosis is the most common pathology agent of CVDs that it occurs due to the thrombus (blood clot) formation and subsequently blockage of blood vessels [23]. Plasminogen activators are a bunch of proteolytic chemicals that utilized for treating cardiovascular and cerebrovascular blockages by enacting the protein plasminogen into plasmin. There are three agent plasminogen activators counting rSK, urokinase and tissue plasminogen activator (tPA) [24]. Among them, rSK is relatively inexpensive than other thrombolytic agent and has the most use in dissolving arterial thrombi, particularly in cardiac blood vessels [24, 25].

rSK enzyme formulations are usually stabilized with human serum albumin (HSA) [26]. The HSA plays a critical role to secure the active ingredient of rSK chemical against known potential chemical and physical degradation mechanisms [27]. The use of this agent has been limited such as the formation of rSK/HSA complex with high molecular weight that can enhance the immune response against streptokinase, consequently impose the potential risk of viral contamination [28]. Therefore, the design and construction of the streptokinase enzyme formulation without HSA is highly desirable. In this research, HSA-free recombinant streptokinase was used for avoiding mentioned problems.

However, the short biological life-time of the recombinant rSK enzyme (about 20–30 min) and some side effects such as allergic reaction and fever, systemic fibrinogenlysis and bleeding are factors that have limited its clinical use [24, 25, 29]. Bilal *et al*. reported that allergic reactions (p= 0.044) occur during the infusion of rSK as a thrombolytic agent in patients with acute myocardial infarction [27]. In addition, in 2018, Aslanabadi et al. evaluated the complications of streptokinase therapy in patients with acute ST-elevation myocardial infarction (STEMI). Their results indicated the drug allergy is responsible for the main adverse drug reaction that creates by rSK in patients with STEMI [30]. To overcome these problems, there are a variety of strategies to increase circulation time or decrease immunogenicity features of this

plasminogen activator. Various studies have been conducted using liposomes incorporating enzymes as a delivery system to increase the enzyme half-life, preserve their catalytic activity, prolong their clearance and to protect them from degradation *in vivo* [29, 31, 32]. In recent years, a wide variety of liposomal formulations incorporating rSK have been studied by researchers in order to maximally promote the plasma stability, half-life and the thrombolytic activity of rSK in addition to its targeted delivery to thrombus [33]. Liposome-encapsulated rSK nanoparticles have demonstrated to increase the absorption rate of subconjunctival hemorrhage (SH) with no side effects in rabbits due to the minimal systemic absorption [29]. In 2011, Vaidya et al. provided rSK carrying liposomes containing surface anchored RGD peptide to selectively target the activated platelets entrapped in the clot. Their results demonstrated that peptide conjugation, due to generating some kinds of membrane destabilization, led to faster release of rSK *in vitro*. Meanwhile, a higher retention of rSK was observed within the carrier leading to the maximum release of the drug at the target site. According to these authors, the mechanism of drug release at the target site was based on the shear stress impacts generated by the blood vessels narrowing at the clot area [25]. As compared to liposomes, chitosan chains have shown to form a strong electrostatic interaction with thrombolytic agents including streptokinase. Recently, Shamsi et al. reported the preparation of chitosan-entrapped rSK nanoparticles (CSNPs) using a couple of techniques including microfluidics and bulk mixing. The obtained results indicated that the CSNPs prepared by microfluidic technique, in comparison to bulk mixing, achieved a more uniform morphology and resulted in the controlled release of rSK *in vitro* and higher rSK amidolytic activity *in vivo* [34]. According to the model provided by Baharifar et al., the cytotoxicity of CSNPs was a function of nanoparticle size, wherein the smaller particles led to more toxic effects [2]. Table 6 represents a comparison between the different types of particles incorporating rSK, prepared for the drug delivery purposes.

Table 5: Comparison between the different types of particles incorporating rSK as a drug delivery system

Nano/micro particles	Size	*In vitro* drug release	Entrapment efficiency (EE%)	Details	Reference
Liposome	100-120 nm	40% in 12 h	7–18%	Surface conjugation with RGD peptide	[35]
Chitosan	67 ± 13 nm	> 60% in <50 h	> 90%	prepared by microfluidic technique	[34]
PEG microspheres	0.802 ± 0.023µm	≈100% in >1 h	84.4 ± 3.1%		[36]
Chitosan	526 ±20 nm		43 ± 3		[37]
EPC/PEG-liposomes	<200 nm		32.0±10.2		[38]
Liposome	≈ 115 nm	13.5 ± 0.96% in 36 h	18 ± 1.3%.	Surface conjugation with RGD peptide	[25]
SLN	200-250 nm	96.9 % in 12 h	67.30%		present study

SLN as a thrombolytic drug carrier have been explored by various research groups due to their unique properties such as the ability of incorporating hydrophilic and lipophilic drugs, enhanced physical stability, inexpensive compared to liposomes and ease of synthesis [7]. Also, SLN can be produced without using the toxic solvents in large scale [39]. There are different methods for producing of SLN such as high shear homogenization and ultrasound, cold homogenization, hot homogenization, high-pressure homogenization, solvent emulsification/evaporation, and microemulsion [40]. Among the mentioned methods, the microemulsion is an easy technique that does not request very sophisticated apparatuses or high-energy input and avoids the employment of organic solvents [40]. Therefore, the SLN was prepared using a modified high shear homogenization microemulsion method in this research [41]. Photon correlation spectroscopy (PCS) and Laser diffraction are the most techniques that are applied for SLN characterization such as particle size and zeta potential [42]. The PCS method is based on the dynamic scattering of laser light due to the brownian motion of particles in solution/suspension. This technique is appropriate for characterization of particles in the range of 3 nm to 3 mm [42]. The properties of SLN-rSK including the intensity weighted mean hydrodynamic size and the PDI were measured by PCS technique.

Qi et al. prepared catalase-loaded SLN by the double emulsion method and solvent evaporation technique for achieving a narrow size distribution and a high loading of the biologically active enzyme against proteolysis. The activity of free catalase lost at 30% of H_2O_2-degrading activity after being incubated with Proteinase K within 1 h, while Catalase-loaded SLN remained active for 24 h [43]. In 2018 triclosan-SLN hybrid structure has been used by Pratibha et al for targeted triclosan delivery into the rSK in order to prevent infection. Their results revealed that the optimized

formulation was stable over 90 days. Moreover, *in vitro* permeation studies on porcine ear rSK indicated that SLN improved the triclosan delivery into the rSK and can direct the agent towards hair follicles. These properties display SLN suitable potential as a delivery system for the antiseptic dermal carrier [44]. In 2019, Ghaderkhani et al. improved antibacterial function of Rifampicin on *Brucella abortus* using SLN as a drug delivery system. In comparison with free Rifampicin, the antibacterial activity of Rifampicin-loaded SLN was statistically significant ($p<0.05$) in bacterial and cell culture media. Also, the antibiotic release of SLN continued for 5 days [45]. In the same year, Hosseini and his coworkers represented a Doxycycline-SLN formulation as promising tool against *Brucella melitensis* inside macrophages [46]. The survival of the microscopic organisms interior the macrophages is the foremost vital reason for the backslide of brucellosis, subsequently the microbes are ensured against the safe framework and the drug delivery mechanism is disrupted [46]. The time required for the antibiotic complete release and its antibacterial effectiveness was reported 72 h. Also, the effects of Doxycycline-SLN formulation on the bacteria enclosed in macrophages was remarkably higher than the free Doxycycline (3.5 fold in Log unit) [46].

In the present study, taking advantage of the features of SLN, the release of rSK enzyme has been strongly continued over 12 h, during which the release of enzyme arrived at 98%. This result indicated that SLN as a delivery system can significantly improve the biological half-life of the enzyme. Besides, the cetyle palmitate nanoparticles produced by high shear homogenization microemulsion technique exhibited remarkable entrapment efficiency 67.30%, leading to release enzyme in higher doses due to its new properties in nanometer scale. Hence, it may be concluded that streptokinase loaded SLN can serve as an appropriate candidate for improving the delivery of thrombolytic agents.

Conclusion

Here, a novel drug delivery system attitude is reported based on rSK loaded-SLN intended to be effective for myocardial infarction and pulmonary embolism. Our results indicated that the SLN-rSK as a cost-effective formulation could be considered in proper dosage with an acceptable biocompatibility and stability features. Nevertheless, further investigation must be performed to evaluate the efficiency of formulation *in vivo* as well as the scale up probability and probable large scale manufacture condition in the future.

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