Original Article

Novel Formulation for Recombinant *Streptokinase* by Solid Lipid Nanoparticle: A Light at the End of the Tunnel

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Abstract

Anticoagulants are used to prevent or treat thrombotic diseases. Some antithrombotic drugs like streptokinase (SK) are ones that cause destruction of the clot or thrombosis through fibrinolytic system. These drugs imitate the action of the plasminogen activator enzyme in the body. In this research, we have investigated the potential of solid lipid nanoparticles (SLNs) as the carrier of proteins of thrombolytic factors. The SLNs formula has been developed for use in targeted enzymatic delivery of recombinant streptokinase (rSK) using a combined technique. Response surface method (RSM) has been used to provide the best formula and to analyze the effect of different parameters on the efficiency of the SLN-rSK formula. According to the results of optimal formulation conditions for SLN-rSK preparation, lipid concentration is 0.5 mg/ml, surfactant ratio 0.5, rSK concentration 4 mg/ml, homogenization time 20 minutes and speed set at 20,000 rpm. The formulation properties analyzed by SEM showed that the SLN-rSK formula had a spiral appearance (<60 nm) and entrapment efficiency percentage (EE%) 67.3%. Structural integrity was approved by SDS-PAGE. In terms of protein structure, no significant change was observed between the formulation of streptokinase and SLN-rSK. In addition, the ability of SLN-rSK was statistically greater than that of streptokinase. It seems that the high enzymatic activity of the SLN-rSK could be a desirable way to deliver bioactive macromolecules with the SLN. Therefore, rSK-SLN was shown to have an effective, stable and non-cytotoxic formula and can be used for non-thrombolytic drug administration.

Keywords: Recombinant streptokinase, Acute myocardial infarction, Solid lipid nanoparticles, Drug delivery system, Cytotoxicity

INTRODUCTION

Thrombolytic diseases such as myocardial infarction are one of the leading causes of mortality in the societies, especially in developing countries. As diseases increase, the use of drug in the prevention and treatment of these diseases is expected to increase. Anticoagulants or fibrinolytic drugs are among the major drugs for the prevention and treatment of these diseases. Apart from blockage removal through surgery, the only treatment available is the administration of thrombolytic agents to eliminate clots. Fibrinolytic drugs including Plasminogen Activator may be seen in arterial or venous vessels. Streptokinase is the most current and cost-effective fibrinolytic drug for this purpose [1].

Plasminogen activators are a group of protolysis chemicals that are used to treat cardiovascular and cerebrovascular obstruction through the placement of plasminogen protein in plasmin [2]. There are three plasminogen activators, including rSK, urokinase, and tissue plasminogen activator (tPA). Among them, rSK is relatively cheaper than other thrombolytic agents and is most commonly used to open arterial blood clots in the heart blood vessels [3].

Due to the difficulties in developing and designing new drugs, more and more attention is being paid to the development of new drug systems for existing drugs. Reducing the size of drug carriers to nanomaterials has many benefits such as improved pharmacokinetic and biodistribution of drug agents due to increased ratio of surface area to volume, reduced toxicity by preferential aggregation at the target site, facilitating intracellular conductance and enhancing their life in cells. Nanomaterials are made of small

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units using the self-assembly method. The drug delivery system plays an important role in the development and development of drugs. Nanoparticles used in drug delivery are colloidal particles of 10-100 nm containing a therapeutic compound [4].

This therapeutic compound enters in the nanoparticle construction by dispersing it into a polymeric matrix, encapsulating it within the polymer membrane, covalently bonding it to the polymer, adsorbing at the particle surface, or encapsulating it into a structure such as liposomes.

Due to their lipid nature, solid lipid nanoparticles are used for the formulation and delivery of drugs that are less or less soluble in water. The drug can be placed in the solid lipid matrix and between the lipid fatty acid chains [5].

SLNs are produced in many ways such as high-pressure homogenization [6]. These nanoparticles were introduced as substitutes for colloidal carrier systems such as emulsions, liposomes and polymers because of their higher stability and cost of production [7]. Solid lipid nanoparticles not only show the benefits of other nano-carriers in drug delivery systems, but also lack some of their major disadvantages [8].

Optimization of formulations containing recombinant protein and the use of stabilizers is another approach that is of particular interest in the pharmaceutical industry. Hence, albumin (Alb) is used in the formulation of recombinant streptokinase [9]. The role of albumin in this medicinal product is to maintain the stability of rSK against the mechanisms of chemical degradation, proteolysis, deamination, oxidation, or physical alterations like aggregation [10].

To achieve long-lasting nanoparticle delivery systems in circulation, new technologies have been developed that improve the physicochemical properties of nanoparticles by modifying the surface area of nanoparticles.

Coating the surface of nanoparticles with hydrophilic polymers prevents the opsonization and prolongs the permeability of nanoparticles from a few seconds to several hours. Polyethylene glycol (PEG) is one of the polymers selected for the production of stealth nano-carriers. Evidence has shown that the shelf life of nano-carrier in the blood can be prolonged by coating their surface with neutral or zeutronic polymers and providing high hydrophilicity and flexibility [11, 12]. Therefore, surface pegylation of nanoparticles used as drug carrier reflects special importance to increase the half-life of a protein-based drug or medication such as streptokinase.

Streptokinase is the well-known fibrinolytic drug in the treatment of myocardial infarction. Despite the benefits and advantages of streptokinase over other thrombolytic drugs, its administration can be associated with problems such as stimulating the immune system and potential risk of viral contamination [13].

Different methods have been used to increase the half-life of thrombolytic agents. We can refer to complex acylation of plasminogen, streptokinase (known as the maltose binding protein connecting (MBP) to the N-terminal streptokinase), human serum albumin chemical integration to urokinase, mutation overexpression of glycosylation regions in the tPA plasminogen activator and the binding of polyethylene glycol to staphylokinase [14].

Lopez *et al* investigated the stability of recombinant dry ice formulation without albumin serum for streptokinase [13].

Ojalvo *et al* conducted a research to evaluate the prevalence of antibodies against C-terminal streptokinase in healthy blood donors. The results showed that in 306 out of 1008 individuals, the antibody against this peptide was detectable. The results of this research indicate the high prevalence of these antibodies in the population under study. The researchers claim that deletion of 42 amino acids from the C-terminal region of the *streptokinase* molecule reduces the problems of use of streptokinase by reducing the antigenic propertie [15].

In 2012 Tatyana *et al* investigated the application of pegylated liposome containing streptokinase in the diagnosis and treatment of cardiovascular failure; they identified the role of liposome pegylation in the stability of these nanoparticles [16].

In 2013, an artificial neural network model was investigated to identify and study the main factors affecting the loading efficiency of *streptokinase* complex with chitosan nanoparticles. For this purpose, the effect of three variables, namely chitosan concentration, pH buffer and enzyme concentration was tested as input and loading efficiency as output [17].

With a review of past researches, we can conclude that the use of solid nanoparticles due to its benefits in the design of a streptokinase drug is one of the new approaches that have not been employed so far.

Nowadays, the design of nanoparticles is a strategy to combat cardiovascular disease [18]. Due to successful experiments in the delivery of recombinant proteins with the help of solid lipid nanoparticles, we decided to design and evaluate a system of novel drug delivery attitude for the treatment of cardiovascular diseases that are an important factor in death.

In this research, we hope that, by preparation of SLNs containing streptokinase with the surface arrangement of PEG polymer, we can overcome the problems and disadvantages of the formulation and design of this drug through the nanotechnology. By increasing the systemic toxicity of the drug, and increasing its stability, we can design

a pegylated SLNs containing streptokinase drug in the drug delivery system.

Materials and Methods

Preparing the SLN containing rSK

The SLNs containing rSK were obtained by high shear homogenization (HSH) technique with slightly modifying [19, 20]. To prepare the SLN formula containing rSK, the lipid phase, namely cetyl palmitate, was heated to 50° C and then the surfactant (Tween 20/80) containing rSK was slowly added to the fused lipid. This step was performed on heater-stirrer. The obtained emulsion was then homogenized using a high shear homogenizer at a predetermined homogenization speed and time; the results section has summarized it.

The obtained microemulsion was immediately dispersed in double distilled water at 2-8 °C by magnetic stirrer to crystallize SLN-rSK. Then physicochemical properties including particle size was measured as dependent variables for each formula. Response surface methodology (RSM) was used to determine the best SLN-rSK formula.

Determination of physicochemical properties of synthesized nanoparticles

Zetasizer was used to measure the mean particle size. The device we used in this study was Malvern's; the basis of measuring particles is dynamic light scattering (DLS) and the basis of measuring surface potential is electrophoretic principles.

Avarage diameter and polydispersion index (PDI) were measured using zetasizer nano series, Malvern, UK at a dispersion angle of 90^0 and a temperature of 25°C. For this purpose, SLN-rSK samples were diluted to 0.1 (v/v %) with very pure water at pH = 5.5. In addition, they were calculated by the particle diameter dynamic light scattering technique in three versions.

The surface morphology of the SLN-rSK formulas prepared by scanning electron microscope was analyzed in high vacumm. A certain amount of sample (diluted with a suitable volume of double distilled water, 1:100) was placed on the FE-SEM receiver and covered with a layer of 100 A^0 gold for 3 min at 0.2 atm pressure under argon stream.

Studying Kinetics of Streptokinase Release from Nanoparticles

For this purpose, drug release from solid lipid nanoparticles for zero, first and Higuchi kinetics was investigated.
The following equation is used to calculate the zero degree kinetics:

Equation 1 $$C = C_0 - Kt$$

C_0 = amount of total antigen present in the system at time zero;
C = amount of antigen present in the system in time t;
t = time;
K = zero-order release speed constant.

The following equation is used to compute the first-degree kinetics:

Equation 2 $$LnC = \ln C_{.} \cdot Kt$$

C_0 = amount of total antigen present in the system at time zero;
C = amount of antigen present in the system in time t;
t = time
K = first-order release speed constant.

The following equation is used to compute the Higuchi kinetic model:

Equation 3 $$C = Kt\frac{١}{٢}$$

In this model, the diagram of the cumulative dissolved drug versus time, straight line is of a positive slope, the slope value was equal to the Higuchi speed constant (K).

Evaluation of the biological activity of recombinant streptokinase and solid lipid nanoparticles containing recombinant streptokinase

Biological activity is the plasminogenolytic activity (enzyme activity of streptokinase in converting plasminogen to plasmin) or its proteolytic activity (proteasome).

The biological activity of streptokinase was evaluated by chromogenic assay using S-2251 kit. This kit contained the chromogenic substrate S2251 (Val-leu-lys-p-nitroaniline.2HCl) that converts plasminogen to plasmin. In this method, streptokinase generates active plasmin and facilitates hydrolysis of S-2251. Therefore, the optical absorption of the solute is increased by the concentration of streptokinase. Finally, the light absorption of microplate wells was read immediately using an ELISA reader at 405 nm [21].

The results of microplate readings were analyzed by Parlin software using Parallel Line Assay and the biological activity of Streptokinase enzyme and its Nano-conjugate with Streptokinase were determined with Streptokinase compared to standard enzyme.

Cytotoxicity assay using MTT method

At this stage, fibroblast cells of mouse L-929 connective tissue with properties ATCC® TIB-202™ were used; they were prepared from the research and production complex of pasteur institute of Iran. For this purpose, L-929 cells were first transferred to a cell culture flask containing RPMI 1640 culture medium with 10% fetal bovine serum (FBS), penicillin (100 µg/ml) and streptomycin (100 µg/ml). They were incubated in incubator at 37° C and 5% CO_2.

Accelerated stability investigation

To evaluate the stability of optimized formulations of streptokinase antigen, we followed ICH Q1A (R2) as international guidelines [22]. To investigate the stability of solid lipid nanoparticles containing streptokinase antigen, we applied the accelerated stability assay. In this method for six months, the formulation prepared at 25 ± 2° C/60 ± 5% RH was placed under refrigeration at 25 ± 2° C/60±5% RH. Sodium bromide salt with a solubility of 1.17 g/ml was used to create the required relative humidity in the environment. The samples were periodically tested at 0, 1, 3, and 6 months to investigate the stability of the formulation and possible changes in the appearance, pH, biological activity and particle size.

Statistical analysis

Comparisons of groups were analyzed using GraphPad Prism 5. Statistical significance was set at $P \leq 0.05$. RSM analysis was performed to evaluate the effect of the five formulations mentioned above using RSM.

Results

Optimized formulation of SLN-rSK

First, the physicochemical properties (particle size and EE%) of all 45 formulas prepared by 5 different independent variables including cetyl palmitate (CP), concentration (X_1), surfactant (X_2), rSK (X_3) concentration, homogenization time (X_4) and homogenization rate (X_5) were determined.

The optimal formulation was obtained by achieving a particle size between 200-250 nm namely 210 nm, 0.218 PDI (Table 1) and EE% by 67.30%. Similarly, the optimal formulation of SLN-rSK was obtained by 0.5 mg/ml, CP: 0.5 mg/ml, ratio of surfactant compound at 1:2 and 4 mg/ml rSK, homogenized at 20,000 rpm for 20 minute (Table 1).

Table 1. 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

Morphological studies

Depending on particle size, PDI, and zeta potential, scanning electron microscopy can be performed to further evaluate the shape and surface of the nanoparticles. Clearly, SLNs were spherical in shape. It was also found that SLNs were almost scattered with the lowest density (Figure 1).

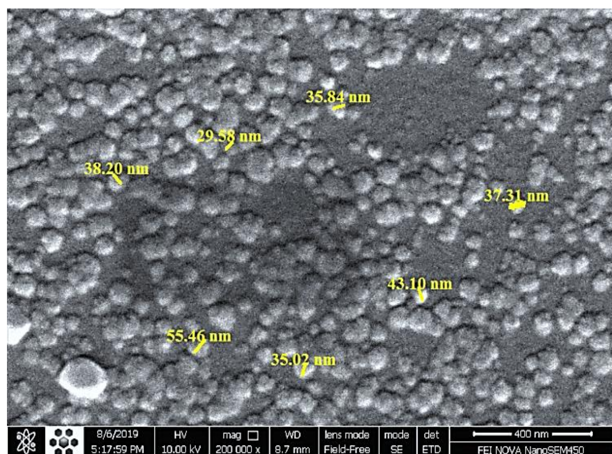

Figure 1. SEM micrograph of the SLN formulation prepared by high shear homogenization method.

Calculation of entrapment efficiency and loading capacity (LC)

The entrapment efficiency method was used to measure the percentage of absorbed drug in nanoparticles. In this method, the amount of drug released in buffer medium was calculated. The concentration of free rSK in supernatant was calculated by Bradford method. It should be noted that the initial concentration of streptokinase was 4 mg/ml and was also measured by the Bradford method (Figure 2).

EE% and LC% of SLNs were estimated to be 67.3% and 71.6%, respectively, indicating a successful entrapment of streptokinase into lipid solid nanoparticles.

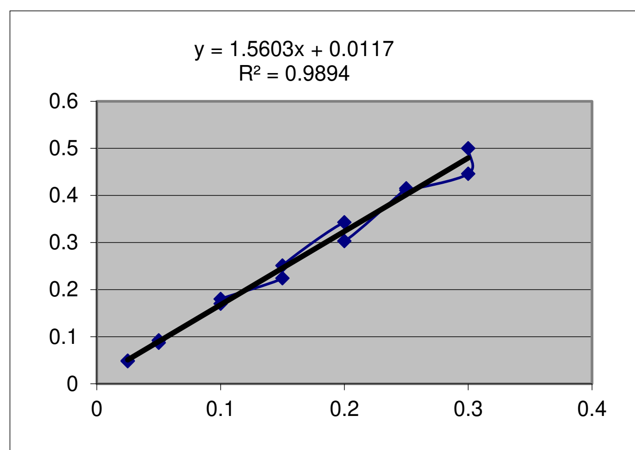

Figure 2. Standard curve of protein concentration by the Bradford method

Equation 4 $$Y = 1.56X + 0.0117$$

Using the slope formula of the curve and absorbance of the read samples, which Y is the number, and by its substitution in the formula, the X is the free rSK concentration in the supernatant was calculated.

Absorption of free rSK in supernatant after centrifugation was 1.54.

X = 0.98 mg/ml is rSK concentration in supernatant.

Determination of biological activity

The biological activity of rSK released from SLNs was evaluated by a colorimetric assay. It was found that the rSK loaded in the SLNs completely retained its biological activity after formulation and encapsulation in the SLNs. The biological activity of rSK and SLN-rSK was 205647.3 and 211396.44 IU/ml, respectively.

In vitro cytotoxicity Assessment

By evaluating the anticoagulant effect of rSK loaded on SLNs, the toxicity against L-929 results showed that the viability of mouse fibroblast L-929 cells in the presence of free SLN-rSK and rSK decreased significantly compared to cells exposed to SLNs ($P \leq 0.05$). However, blank SLNs showed no significant toxicity against L-929 compared to SLN-rSK. Macrophage cell viability of blank SLNs at 1:10 dilution (400 μg/ml), was significantly (80.6%) lower than that of SLN-rSK (Figure 3).

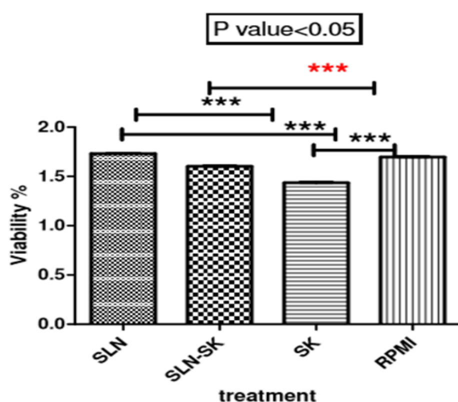

Figure 3. Cell viability test using mouse fibroblast L-929 prepared by high shear homogenization (n=3)

Kinetic study of streptokinase release from solid lipid nanoparticles

a. Zero-order kinetic model

In zero-order kinetics, a fixed amount of drug is released per unit time, and the percent diagram with an amount of drug released versus time is a straight line. In other words, in the zero-order model, the diagram of amount of drug dissolved against time is a straight line with a positive slope; the value of the slope is equal to the dissolution speed constant.

b. The first-order kinetic model

In first-order Kinetics, the speed of release is related to the amount of drug remaining in the system and the percentage of drug remaining in the system is released per time unit. If drug release is based on first-order kinetics, the diagram of the logarithm of the percentage of drug remaining against time is a straight line, from whose slope we can determine the first-order release speed constant.

In cases where the percentage of drug released against time is not a straight line, the first-order model is used; in this model, the logarithm of the drug remaining is drawn against time, and a straight line is obtained with a negative slope.

c. Higuchi model

In this model, the percent diagram of drug released against time square is a straight line with a positive slope whose slope value is equal to the dissolution constant of the obtained correlation coefficients shows that the percent release diagram follows the Higuchi model.

According to the different scientific models such as the Higuchi, the zero-order energy, the first-order active, 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. The results were summarized in table 2.

Table 2. 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 model	$Q=K_H\sqrt{t}$	**0.8267**

Stability assessment

The biological activity, pH, size and appearance characteristics of the SLN-rSK formulation were studied and measured according to ICH Q1A (R2) international guidelines at specified times of 0, 1, 3 and 6 months. The biological activity of rSK loaded on solid lipid nanoparticles changed after 6 months from 211375.4 IU/ml to 211324.76 IU/ml. It indicates that the drug can maintain its biological activity while remaining encapsulated in the nanoparticles. It should be noted that there was no significant change in size, pH, biological activity and transparency at the mentioned times of SLN-rSK. Evaluation of these parameters was carried out according to ICH guidelines at 25±2°C/60±5% RH and the following results were obtained (Table 3).

Table 3. 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

The free concentrations were studied to determine the stability of the optimal SLN-rSK formulation in terms of specific activities, pH, size and transparency parameters. The biological activity of rSK loaded after 6 months changed from 211396.44 IU/ml to 206648.50 IU/ml. It indicates that the drug could maintain its activity while remaining encapsulated in the nanoparticles for a sufficient period. We should note that the storage of SLN-rSK showed no significant change in size, pH, purity of activity, and transparency. These parameters were almost constant for 6 months of storage at 25±2° C/60±5% RH (Table 3).

Discussion

Thrombolytic drugs are currently used as a standard treatment for patients with acute myocardial infarction.The rSK formulation is usually prepared with human serum albumin (HSA) as an Important excipiant [23]. HSA plays an important role in protecting rSK from chemicals and physical degradation [24]. The use of this preservative increases the molecular weight of rSK/HSA [13]. This can rase the immune response to streptokinase as well as the potential risk of viral infection. Therefore, the formulation of streptokinase drug without HSA is under survey. In this research, rSK without HSA was used to prevent these problems.

However, the short biological life of the rSK enzyme (approximately 20-30 minutes) and some of its side effects such as sensitivity, fever, and bleeding are factors that limit its use in medicine [25]. Bilal reported that an allergic response (p = 0.044) occurs during injection of rSK as a thrombotic agent in patients with acute myocardial infarction [24]. In recent years, researchers have been investigating the various types of liposome formulations with rSK to maximize plasma stability, half-life, and thrombolytic activity of rSK, as well as its targeted delivery to the clot [14]. Liposome-encapsulated rSK nanoparticles have been shown to increase the speed of absorption of subconjunctival hemorrhage (SH) in rabbits, without any side effects due to minimal systemic uptake [25]. According to the researchers, the mechanism of drug release at the target site is based on the effects of shear stress created by blood vessels at the clot location [26]. Compared to liposomes, chitosan chains have strong electrostatic interactions with thrombolytic agents including streptokinase. According to the model proposed by Baharifar et al, the cytotoxicity of CSNPs was a function of the size of the nanoparticles in which smaller particles had further toxic effects [27].

SLNs as thrombolytic drug carriers have been studied by numerous research groups due to their unique properties such as their ability to combine hydrophilic and lipophilic drugs, increased physical stability, lower cost than liposomes, and ease of combination [20].

There are various methods for producing SLNs. Microemulsion method is an easy method that does not require very complicated devices or the inputs of high energy; it avoids the use of organic solvents [28]. Therefore, SLNs were prepared using high shear homogenization (HSH) in this research [29].

In this research, the release of rSK enzyme continued for 12 hours using the properties of SLNs, during which the enzyme release was 96.9%. This result showed that SLNs as a delivery system can significantly improve the biological half-life of the enzyme. In addition, cetyl palmitate nanoparticles produced by high shear homogenization microemulsion showed a significant entrapment efficiency (67.3%), which resulted in the release of the enzyme at higher doses due to its novel nanoscale properties. Therefore, we can conclude that SLNs loaded with rSK can be considered as a potential suitable candidate to improve delivery of thrombolytic agents.

Conclusion

In this article, a new drug delivery system based on SLNs containing rSK is reported to be effective for heart attack and embolism. Our results showed that SLN-rSK as a cost-effective formula can be considered with the appropriate dosage, stability and biocompatibility characteristics. However, further studies need to be carried out to evaluate the *in vivo* effect of this formulation, and its potential for scale-up in the future.

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