Design and Characterization of New Nano System for Delivery of Erythropoietin

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ABSTRACT

Erythropoietin (EPO) can increase cell maintenance during different damages of central nervous system as a strong cell protector. However, studies displayed that brain-blood barrier prevents the entrance of large proteins similar to EPO (30.4 kD) into the brain. The goal of this study was to find an alternative method for delivering of EPO to the brain to skip the blood brain barrier using design of new nanodrug delivery system. Then, an new cationic Gemini surfactant has been used in this study for preparation of EPO-loaded Gemini micelles. The physic chemical properties of nano micelles was investigated using Dynamic light scattering (DLS) and ζ-potential measurement, Morphology studies and \textit{in vitro} drug release. Preparation of EPO loaded Gemini micelles results indicate that there are appropriate interactions between EPO drug and Gemini micelles and that of the EPO loaded in to the Gemini nanoparticles. DLS results display that size of the Gemini-EPO nanoparticle is smaller than 150 nm. Morphology studies results have further shown that, micelles (EPO-loaded) having smooth regular surface and the sizes of TEM micrograph were reported between 100-150 nm. Also, \textit{in vitro} drug release results indicate that nanomicells (EPO-loaded) has been degraded in simulated early-time periods and released the drug into a simulated solution results also indicate decreasing stability structure of EPO-Gemini against temperature. In conclusion, the obtained results proposed that Gemini as a Nano carrier can bind to EPO with smooth surface micelles and size of 114 nm and can be considered as a candidate for drug delivery (EPO).

Keyword: Erythropoietin, Blood-brain barrier, Gemini, DLS, TEM, Drug release

INTRODUCTION

Erythropoietin (EPO) is a 30.4 kDa glycoprotein hormone containing 165 amino acids long after post-translational modification. The molecule of EPO has four glycosylation sites contain three N-linked and one O-linked acidic oligosaccharide side chains and sialic acid residues. EPO is manufacturing by peritubular capillary endothelial cells in the adult kidney and fetal liver [1]. The principal function of EPO is hematopoietic growth factor that regulation of erythropoiesis which increase of the O2-carrying capacity by boosting the number of RBCs and thus the hemoglobin concentration. EPO widely used as a standard therapy to treat renal or chemotherapy-related anemia [2-4].

In addition to the kidney and liver, EPO and the EPO receptor (EPOR) are also expressed in the central nervous system (CNS) (hippocampus, cortex, internal capsule, and midbrain). Also EPO and EPOR expressed in glial cells of brain, neurons, and endothelial cells that actively participate in neurogenesis and angiogenesis during brain injury including stroke [5]. In the recent time, the no hematopoietic functions of EPO in the CNS have been elucidated. EPO displays neuroprotective effects and it is considered as a promising candidate for the treatment of brain injury [6]. EPO via anti-apoptosis [7], promotion of neural regeneration [8], anti-inflammation [9], anti-neurotoxicity [10] and protecting the brain from edema [11] can improvement the harmful effects after brain injury.

In the last few decades, researchers have been focused on the affectivity of recombinant erythropoietin (r-Hu EPO) to protect neurons of CNS against injury [12,13]. However,
studies displayed that the effect of EPO are related to its direct effect within the CNS and this requires penetration of the blood-brain barrier (BBB). Therefore for reached this aim only should be upon administration of EPO in large doses, because EPO crosses BBB at a very slow speed [14]. The natural function of BBB, prevent enter of toxic substances into the CNS. So, small compounds (less than 400 Da) with appreciable lipid solubility can across BBB and enter to CNS. However, although lipophilic molecules may passively diffuse through the BBB, they can be quickly expelled back into the blood stream by P-glycoprotein (P-gp) and multidrug resistance protein family (MDR) locating on BBB endothelial cell membranes have also formed a molecular barrier to extrude various drugs from the brain [15-17]. However, the improvement of drug delivery in brain is still a formidable challenge.

Nanoparticles employ as a solid submicron-sized drug carriers that may or may not be biodegradable and defined as a vector for hold drugs from destruction throughout the circulation [18]. Also, nanoparticles by decrease systemic side results by free drugs (may exhibit different physical and chemical properties) and for their controllable self-assembly, high drug loading capacity, enhanced brain drug accumulation and increased circulation half-life can causes a dramatic increase in their ability to penetrate into tissues and their cellular uptake [19-20]. Most of the studies display that nanocarriers are nanosized systems and can carry multiple drugs [21].  Polymeric biodegradable nanoparticles by entrapped, encapsulated, adsorbed or dissolved drugs allow drugs release at the target site [22].

Notable point, the problem of instability of most protein drugs under conditions including: denaturation upon exposure to heat, aggregation, degradation, shearing, organic solvents, etc. These problems limited the drug delivery of EPO by nanoparticles [23].

Recently, surfactants have gained more attentions and many protein-surfactant interaction studies were conducted. Interaction between surfactants and proteins may results to protein stabilization or denaturation [24,25]. Amphiphilic cationic Gemini contains two quaternary ammonium positive head and two hydrophobic alkyl tails which have shown good surface activity, low critical micelle concentration (CMC) and high solubilizing ability [26,27].

Two hydrophobic chains of the surfactant cause protein unfolding by interacting with the nonpolar amino acid residues and eliminating unfavorable solvent contacts [28]. Cationic Gemini surfactant can interact with hemoglobin, Amyloid β-peptide, insulin and other proteins [29]. Another benefit of these Gemini surfactants is delivering of drugs and genes [30,31].

In the present study, EPO was encapsulated in Gemini as polymeric cationic amphiphilic conformations with two quaternary ammonium positive head and two hydrophobic alkyl tails. Then, the synthesized nanomicelles including drug of EPO were characterized for their physico-chemical properties such as particle size, zeta potential, particle shape and in vitro release profile.

MATERIALS AND METHODS

Erythropoietin was obtained from Pharmaceutical Research Center, Sinagen (Tehran, Iran). EPO was purchased in injection vials 4000 IU/0.5 ml (IU, international units). The new cationic Gemini surfactant (S6, MW of 4867 Da) was synthesized according previous reports [32]. Sodium phosphate buffer, pH 7.40 (PBS) was applied as a solvent for this study. All the solutions were prepared using de-ionized water [33].

Preparation of EPO-loaded Gemini Micelles

EPO was loaded into Gemini micelles via an incubated method. In order to synthesize the EPO-Gemini nanomicelles, 2 test tubes were prepared each containing 1 mg ml\(^{-1}\) of Gemini-bufffer solutions whit pH 7.2. The test tubes containing the Gemini solutions were then placed on a shaker for 10 min at a speed of 70 rpm, at two different temperatures of 25 and 37 °C. At intervals of 10 min, for a total duration of 100 min, 50 µl of EPO was added to the Gemini solutions. The final molar ratio of EPO that was mixed with the Gemini solution was 1/2 and 1/4. Then, the final concentration solutions were stirred on a shaker for 4 h with a speed of 70 rpm, at temperature of 37 °C.

Dynamic Light Scattering and ζ-potential Measurements

Size and zeta potential measurements of EPO-micelles
were carried out by Zeta-plus Instrument (90Plus/BIMAS, Zetaplus, USA) at wavelength of 660.0 nm, 25.0 ± 0.1 °C and angle of 90. The length of cell path was 1.0-cm for each measurement. In order to ensure the homogeneity of the sample solutions, the test tubes were initially sonicated for 30 min, prior to taking any measurements. Then, each sample was measured by 5 individual runs. The outlier data were taken and the mean was recorded. The corresponding diameters (Dₐ) and the zeta potentials were calculated carried out based on Stoke-Einstein relationship and the Smoluchowski model, respectively, by the instrument [34,35].

**Morphology Studies**

The morphology characteristics of the prepared nanomicelles were examined using transmission electron microscope (TEM) (EM10C, Zeiss, Germany) with the accelerating voltage of 80 kV. A drop of the Gemini-EPO solution was placed on a Formvar-coated copper grid (75 × 300-mesh) and stained with 2% (w/v) uranyl acetate [36].

**In Vitro Drug Release**

The release assay of EPO from Gemini nanomicelles was conducted using certain weights of nanoparticles were dispersed in PBS, pH 7.2 and incubated in a shaker at 37 °C at 70 rpm speed. Each sample taken at 0.25, 0.50, 1, 2, 3, 4, 6, 8, 24, 48 and 72 h and replaced by 1 ml of BPS preheated at 37 °C. Then, EPO concentration was determined by centrifuged of samples for 30 min at 10000 rpm at 4 °C and finally, the supernatant of each sample assayed by Bradford method. The dilution was taken into account while calculating the cumulative amount released at each time point.

**RESULTS AND DISCUSSION**

The clinical applications of EPO are limited due to its poor water solubility, low stability, large size and severe side effects. Also, one of the major limitations of this drug for the neurodegenerative is that EPO cannot cross the blood-brain barrier, because of its large size. However, in the presence of Gemini as a nano carrier of EPO drug, the solubility and stability of the complex are significantly increased. Also, our previous results indicate that there are appropriate interactions between EPO drug and Gemini [37]. Then, EPO can load into the Gemini nanomicelles.

**Particle Size Distribution and Zeta Potential Analysis of the EPO-Gemini Nanomicells**

The main aim of this study was to production of new stable nanomicell in order to drug delivery of EPO for the treatment of neurodegenerative diseases and investigate its physicochemical properties such as the size and ζ-potential of the newly synthesized nanomicells.

At first, the influences of the molar ratio of EPO to Gemini on the properties and stability of Gemini nanomicells were studied. Results of the effects of two various molar ratios of Gemini/EPO represented in Table 1 and Fig. 1. At high concentration of EPO, the bigger particle size has formed and it can be seen a narrower size distribution. The results emphasize that the alteration in concentration of Gemini and EPO can play an important role in the formation and size of nanomicells. Also, results obtained in Table 1, display that the size of the Gemini-EPO nanoparticle is smaller than 150 nm, whereas, the synthesized nanomicells using Gemini in previous research, had a larger diameter (approximately 200 nm) [38,39]. As a result, our synthesized nanomicells can be a good option for participating in the delivery of the drug through the blood-brain barrier to treat of brain damage. Therefore, our results are suggested that Gemini-EPO nanomicells that produced at molar ratio of 2, ([Gemini]/[EPO] = 2) and temperature 37 °C are suitable candidates for the usage in drug delivery to brain. Zeta potential measurements are the other important factor should be considered for the optimizing of nanomicells characterization. This method is very important for understanding and controlling the properties of colloidal suspensions. [40]. Data obtained from the Zeta potential study display that Gemini-EPO nanomicells at the molar ratio 2, ([Gemini]/[EPO] = 2) and temperature 37 °C have a positively surface charge (+12.81 mV) (Fig. 2) [32,41]. As a result, in the presence of Gemini, because of the cationic nature of Gemini, the value of the ζ-potential of the nanomicells is determined by the electrostatic interaction that occurs between Gemini and EPO. Furthermore, Gemini surfactant has a critical role in regulating the charge distribution on the Gemini-EPO nanomicells owing to its
electrical properties. Also, this result might be suggested increasing in the stability and solubility of the Gemini-EPO nanomicells. Because the positive charge of the particles creates an electrostatic repulsion force between the two adjacent particles, it prevents the particles from coming close together and doing coagulation.

**Morphology Results**

The shape and size of the Gemini-EPO nanomicells have great important in drug delivery. The morphological characteristics of the Gemini nanomicells including EPO were investigated using TEM. TEM negatively stained image of Gemini-EPO nanomicells represented in Fig. 3. It was clear from the image that nanomicelles (EPO-loaded) are homogeneously distributed, well separated, spherical in shape and having smooth regular surface. The sizes of TEM micrograph were between 100-150 nm. Also Gemini-EPO nanomicells are distributed separately and completely homogeneously. While in research by Fayed et al., the mean particle size diameter for the polymeric nanoparticles (EPO-loaded) produced was 225.9 ± 3.8 nm [42] and in research by Pirhaghghi et al., the micelles (insulin-Gemini) are spherical with the size distribution between 101-140 nm [33]. The TEM results have a good agreement with the DLS

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean diameter (nm)</th>
<th>Mean zeta potential (mv)</th>
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<tbody>
<tr>
<td>[Gemini]/[EPO] = 2</td>
<td>133.5</td>
<td>+12.81</td>
</tr>
<tr>
<td>[Gemini]/[EPO] = 4</td>
<td>114.1</td>
<td>+19.02</td>
</tr>
</tbody>
</table>
Fig. 2. The average surface charge of Gemini nanoparticles containing EPO.

Fig. 3. TEM image of Gemini-EPO nanomicells in PBS (pH, 7.2) (negative-staining, magnification: 20000x).
In order to determine and measure the capability of Gemini-EPO nanomicells for transfusion and delivering drug to the target's position and estimating the maximum benefit of drug release at the target's position, the in vitro release studies was performed [42]. In vitro studies were performed rather than in vivo studies of oral drug delivery due to the fact that it is a much more direct estimate of the

**In Vitro Release Results**

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product efficiency and also reduces the cost of the study [43]. In the present study, thermodynamic factors were studied for affecting the mechanism release of erythropoietin from Gemini surfactant nanomicelles. Gibbs free energy is one of the thermodynamic parameters that are effective in the delivery of the drug to the target. Gibbs free energy determines the amount of EPO release from Gemini nanomicelles to simulated conditions of brain were calculated according to the following equation (Eq. (1)) [44].

$$\Delta G^0 = 2.303RT \log \frac{C_T}{C_I}$$

Where $C_T$ and $C_I$ indicate the concentration of total EPO and the concentration of EPO, respectively in each of the simulated time intervals. $R$ and $T$ show the global constant of gases and temperature in Kelvin [44]. Gibbs free energy changes for release of erythropoietin from the Gemini surfactant nanomicelles is shown in Fig. 4. After the simulated time intervals, the amount of drug release from the Gemini surfactant nanomicelles decreases. Therefore, the results indicate that Gemini nanomicelles containing EPO has been degraded in simulated early-time periods and released the drug into a simulated solution.

Also, the percent of EPO release from Gemini nanomicelles at different times (up to 72 h) are shown in Fig. 5. As it is shown in Fig. 5, this result confirms the Gibbs free energy changes and shows that by increasing the time, the amount of erythropoietin release from the Gemini nanomicelles increases. But the highest amount of drug (about 80% of the encapsulated drug) is removed in the first 10 h from the nanomicelles. As a result, the release of erythropoietin in agreement with the previous research, starts initial with an delay and an explosion occurred [41].

**CONCLUSIONS**

In this work, a novel biodegradable Gemini nanomicells containing EPO was designed and characterized in order to the simulation of drug delivery for brain. The results obtained from the DLS and Zeta potential measurements have shown that at the molar ratio 2 from [Gemini]/[EPO] the synthesized nanomicells are soluble and have the smallest size, colloidal stability and solution homogeneity. The results obtained from the Zeta potential study have confirmed that the results obtained from the TEM have shown the spherical shape of Gemini nanomicells containing EPO and also confirmed the result of DLS studies. Also, in vitro release studies indicated that the maximum release occurred in the 10-hours interval at pH 7.2. Finally, according above results it can be concluded that the Gemini-EPO nanomicells can be a very promising candidate for the usage in drug delivery for brain damages treatment.

**REFERENCES**