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Development and *In vitro*/*in vivo* evaluation of HPMC/chitosan gel containing simvastatin loaded self-assembled nanomicelles as a potent wound healing agent

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Development and *In vitro*/*in vivo* evaluation of HPMC/chitosan gel containing simvastatin loaded self-assembled nanomicelles as a potent wound healing agent

Abstract

The aim of this study was to develop hydroxypropyl methyl cellulose (HPMC) /chitosan gel containing polymeric micelles loaded with simvastatin (Sim) and evaluates its wound healing properties in rats. An irregular full factorial design was employed to evaluate the effects of various formulation variables including polymer/drug ratio, hydration temperature, hydration time and organic solvent type on the physicochemical characteristics of pluronic F127-cholesterol nanomicelles prepared using the film hydration method. Among single studied factors, solvent type had the most impact on the amount of drug loading and zeta potential. Particle size and release efficiency was more affected by hydration temperature. The optimized formulation suggested by desirability of 93.5% was prepared using 1 mg of Sim, 10 mg of copolymer, dichloromethane as the organic solvent, hydration time of 45 min and hydration temperature of 25^{o} C. The release of the drug from nanomicelles was found to be biphasic and showed a rapid release in the first stage followed by a sustained release for 96 h. The gel-contained nanomicelles exhibited pseudo-plastic flow and more sustained drug release profile compared to nanomicelles. In excision wound model on normal rats, the wound closure of the group treated by Sim loaded micelles-gel was superior to other groups. Taken together, Sim loaded micelles -gel may represent a novel topical formulation for wound healing

Key words: Wound, Simvastatin, Pluronic, Nanomicelles, Chitosan, *in vitro* release

Introduction:

Body skin has a protective function against foreign agents. Maintenance of integrity of the skin is crucial to avoid further complications such as infection, disability or even death [1]. Wound is the result of disruption of continuity of cells in normal anatomical structure of skin due to mechanical damages (eg; surgery), thermal condition (eg; burn) or physiological defect (eg; diabetes and malignancies) [2]. Wound healing process has different stages: hemostasis, inflammation, migration, proliferation and maturation phases [3].

Delay in the wound healing process converts acute wound to chronic one. In severe pathological conditions, the cascade of healing process lost and it is often associated with oxidative stress and subsequent chronic inflammation, result in increasing tissue destruction and necrosis rather than healing [4]. Simvastatin (Sim) is a HMG-COA reductase inhibitor, and is used conventionally for cholesterol reduction but recent studies demonstrated the potential of this agent for diverse pathologic conditions such as wound healing due to its antioxidant, anti-inflammatory and antibacterial properties. Previous studies showed that Sim play a key role in epithelialization by decreasing epidermal farnesyl pyrophosphate (FPP) levels and rising keratinocyte migration. It also has an angiogenesis effect by augmenting secretion of vascular endothelial growth factor (VEGF) in different cells; increase nitric oxide product and blood vessels regeneration [5-8]. However, the systemic bioavailability of Sim is very low (approximately 5%) after oral administration because of both low aqueous solubility and extensive first pass hepatic metabolism [9]. Moreover, the systemic administration of Sim can cause several adverse effects such as myopathy and liver problems [10]. Therefore, topical application of Sim can increase the accessibility of drug in wound area at lower systemic level and decrease the possible incidence of side effects. So far, many drugs have been incorporated in the hydrophilic gel matrices for topical delivery. Despite favorable advantages of these systems, the amount and homogeneity of hydrophobic drugs in the hydrophilic gel matrix is low and hydrophobic drugs tend to precipitate due to the incompatibility with the hydrophilic polymer network [11-12]. Encapsulation of medication into nanoparticles (NPs) is a feasible and advantageous means to dissolve difficulties such as insolubility of the drug and fast deterioration. These systems which act as a reservoir for the extended release of effective compound have been attracted increasing interest to decrease dosing frequency and drug toxicity. In addition, small size and high surface to volume ratio of nanoparticles (NPs) enable them to cross through skin barrier [13-15]. Among different nano delivery systems, core –shell fundamental structure of nanomicelles is so favored for carrying insoluble drug such as Sim because of the hydrophobic core characteristic. The nano encapsulation of Sim can increase its solubility and permit its controlled release. Pluronic is a nonionic amphiphilic copolymer composed of a triblock structure of poly (ethylene oxide)x-poly (propylene oxide)y-poly (ethylene oxide)x. Pluronic is extensively used in pharmaceutical formulations due to its unique properties such as biocompatibility, low toxicity and weak immunogenicity. The ability of pluronic micelles to increase drug solubility and bioavailability

of low soluble drug is already demonstrated [16-17]. It has been shown that pluronic F127 (PF127) and pluronic F68 (PF68) also accelerate wound treatment by inhibiting inflammation and stimulating growth factor expression [18]. But because of high critical micelle concentration (CMC) value, they are susceptible to dissociation in water upon dilution and they also suffer from low capacity to encapsulate large amounts of hydrophobic drugs [19-20]. In order to overcome this shortcoming, several strategies, including preparation of mixed micelles or conjugation of the hydrophobic moiety to pluronic was suggested [21-22]. In our previous study, a micellar system composed of cholesterol (Chol) bearing PF127 was prepared [19]. The CMC value of this copolymer was about 41 μg/mL [19], which was significantly lower than the CMC value reported for pluronic micelles (1–25 mg/mL) [23-24] and low molecular weight surfactants such as sodium dodecyl sulfate (2.3 mg/mL) [25]. This result indicated more stability of PF127-Chol micelles against dilution in aqueous solution. The biocompatibility of PF127-Chol micelles was also demonstrated in our previous study [26]. In this study, the lipophilic drug (Sim) is solubilized using PF127-Chol micelles and then the solubilized drug was incorporated in to chitosan/HPMC gel in order to use as a wound dressing. Chitosan is a natural polysaccharide composed of the D- glucoseamine and N-acetyl-D-glucoseamine. Chitosan is widely used in the biomedical fields because of the unique combination of attractive properties such as biodegradability, biocompatibility, non-antigenicity, non-toxicity [27]. Chitosan has pronounced wound healing properties due to its antimicrobial effect, haemostasis stimulation and acceleration of tissue regeneration. In recent times, several studies are presented on chitosan and modified chitosan (blends/composites/derivatives) for wound healing [27-29]. The limitation of chitosan gel is the relatively low mechanical strength, which could be overcome through combination with other excipients or polymers [28-29]. In study by Archana et al [30], chitosan–PVP–titanium dioxide nanocomposite was prepared and evaluated as a wound dressing material. The wounds treated with the prepared nano dressing showed higher healing rate than those treated with conventional gauze, soframycin skin ointment and chitosan treated groups. Another benefit of this system was reduction of product cost by using chitosan in combination with PVP due to lower price of synthetic polymer than biopolymer chitosan. In another report, Poonguzhali et al [31] prepared chitosan-PVP nanocellulose composites and evaluated its wound dressing application invitro. This material showed suitable water uptake, mechanical strength and antibacterial activity. You et al also [32] showed silver nanoparticle loaded collagen/chitosan

scaffolds as an excellent wound dressing material. Hydroxypropyl methycellulose (HPMC) is a semisynthetic nonionic water soluble polymer with distinct properties such as high swelling ability and surface activity. Increase in physicochemical feature of chitosan hydrogel (such as hardness, viscosity and bioadhesion) was demonstrated with the incorporation of HPMC [28].

Sim loaded PF127-Chol micelles were prepared by film hydration method and optimized. The optimized Sim loaded PF127-Chol micelles were then embedded in gels and finally the effectiveness of this system for wound healing was investigated.

Materials and Methods

Materials:

Sim was provided by Arya pharmaceutical company (Iran). Dichloromethane (DCM) and chloroform (CLF) with analytical grade were obtained from Merck Chemical Company (Germany). PF127, Chol, chitosan (Medium molecular weight) and dialysis bag (molecular cut off 12000 Da) were purchased from Sigma (US). HPMC was obtained from the DOW Chemical Company (US).

Methods

Preparation of Sim loaded polymeric micelle

PF127- Chol copolymer was prepared in our laboratory as described previously [19]. Polymeric micelles containing Sim were prepared by the thin film hydration method. In the production of nanomicelles, four different variables, including polymer/drug (P/D) ratio, hydration temperature, hydration time and organic solvent type each at 2 levels were studied by an irregular full factorial design. The variables, levels and the formulations resulted using irregular full factorial design are shown in Table 1-2. To determine the contribution effect of each factor as well as statistical analysis of irregular full factorial design, Design Expert Software (version 10, US) was used. The particle size, zeta potential, release efficiency and encapsulation efficiency of polymeric micelles were considered as responses. The optimum conditions were determined by irregular full factorial design to yield a heightened performance with the lowest possible effect of the noise factor.

For preparation of Sim loaded PF127-Chol micelles, 1-2 mg of Sim and 10 mg of PF127-Chol copolymer were dissolved in DCM or CLF as organic solvents. Afterward, organic solvent was

removed in the rotary evaporator under vacuum. The residual organic solvent remaining in the film was removed under vacuum overnight at room temperature. Finally, the resultant film was hydrated with 10 ml of deionized water through stirring at 280 rpm while the hydration temperature and hydration time was changed according to the experimental design. Then the dispersions were sonicated in ice bath by probe sonicator for 1min at 40W (2 s pulses of on and off).

Encapsulation efficiency

Nanomicelles were centrifuged (Microcentrifuge Sigma 30 k, UK) in Amicon microcentrifugation tubes (cutoff 10 000 Da, Ireland) for 10 min with 14000 rpm. The amount of drug in aqueous medium was measured by ultraviolet spectrophotometer (UV1200, Shimadzu, Tokyo, Japan) at 238 nm. Blank nanomicelles (without drug) were used as the control. The entrapment efficiency (EE) of Sim in nanomicelles was calculated using the following equation:

$$EE = \left(\frac{total\ amount\ of\ drug\ added - free\ drug}{total\ amount\ of\ drug\ added}\right) \times 100 \qquad \text{eq. 1}$$

Determination of particle size, poly dispersity index (PDI), zeta potential of micelles

The mean diameter and PDI and zeta potential of the prepared drug loaded micelles were determined by dynamic laser scattering method using Malvern nanosizer (ZEN3600, Malvern instrument Ltd, UK).

Morphology of nanomicelles

The morphological examination of selected micelles was done by field emission scanning electron microscopy (FE-SEM, Mira3 TESCAN, Czech Republic). Samples were mounted on a carbon-film-coated 200 mesh copper grid and were sputter-coated with gold prior to imaging.

***In vitro* release study of Sim from PF127-Chol nanomicelles**

The release of Sim from nano micelles was determined using the dialysis method (molecular cut off 12000 Da, Sigma, US). An appropriate amount of Sim loaded nanomicelles were poured into dialysis bag and then the membrane was immersed in phosphate buffer saline (PBS, pH 7.4) containing 10% ethanol to provide sink condition and was stirred at 300 rpm at 32^0C [33]. At different time intervals, an aliquot of release medium was removed and the same amount of fresh

medium was replaced. The amount of Sim released in each time was determined using ultraviolet spectrophotometer (UV1200, Shimadzu, Tokyo, Japan) at 238 nm. The parameter of release efficiency during 96 h (RE_{96}%) was used to compare release profile and calculated by equation 2:

$$RE_{96}\% = \frac{\int_0^t y.dt}{y100.t} \times 100 \qquad \text{eq. 2}$$

Where *y* is the released percent at time *t*.

Study the kinetic of drug release

Sim release data obtained from optimized formulation was studied by best curve fitting with different kinetic models such as Higuchi ($Qt/Q\infty = kt^{1/2}$), Hixson Crowell ($Q0^{1/3}-Q^{1/3} = kt$), first order ($Ln(1- Qt/Q\infty)=-kt$), zero order ($Qt/Q\infty = kt$) and Korsmeyer-Peppas model ($Q_t/Q\infty =kt^n$). In these equations, Q0 is the initial amount of drug in nanoparticles, Qt is the amount of drug released at time t, Q is remaining amount of drug in system, k is release constant and $Q\infty$ is total amount of drug loaded in nanomicelles that can be released after infinite time and n is release exponent describing the drug release mechanism. The best model was selected based on the highest correlation coefficient (R^2). The drug release mechanism was also studied using Korsmeier-Peppas model. For spherical samples, n= 0.43 indicates Fickian diffusion. When $0.43 < n < 0.85$, anomalous transport is dominant mechanism of drug release. If n value is close to 0.85, the drug release mechanism is case II transport [34-35].

Preparation of gel incorporated optimized nanomicelles loaded with Sim

The gels were prepared using chitosan or/and HPMC at 3% (W/W). In brief, a chitosan gel was prepared by dissolving appropriate amount of medium molecular weight of chitosan in 1% aqueous acetic acid. The pH was finally adjusted with NaOH until desired pH was reached. To prepare HPMC gel, the same amount of HPMC (15kMS) was weighed and dispersed in hot water (50° -90°C) on the stirrer. After 15 minutes, stirring was continued at room temperature. The composite gel was prepared from 3% of chitosan and HPMC solution (W/W) at 1:1 ratio. Then, the prepared gels were kept for overnight to swell at room temperature and remove air bubble before the experiment. To achieve gel formulations containing drug (0.5% W/W), an

appropriate amount of freeze dried Sim encapsulated PF127-Chol micelles was dispersed into the gels by magnetic stirring to make gels homogenous.

Bioadhesion test:

Bioadehesion was determined using a tensile strength machine (SANTAM STM-1, Iran). 0.5 g of each gel was spread on one of the disks (2.5×2.5cm) and a portion of hairless fresh skin of rat was attached to the other disk. The upper disk was brought into contact with the skin over the bottom disk under applying little pressure for one minute to form bioadhesive bond. Then the force of detachment required to break the adhesive bond between two disks was determined at the speed of detachment of 20 mm/min [36].

Rheology studies:

The flow curves of prepared gels were determined using computer controlled rotational concentric cylinder viscometer (Haake Rotovisco RV12 SVI coaxial viscometer, Germany) equipped with MV sensor system. To obtain plots of shear stress versus shear rate, the formulations were subjected to different shear rates (0.3–250 rpm).

***In vitro* release study:**

The *in vitro* release of Sim from different gel formulations was studied using dialysis method (molecular cut off 12000 Da, Sigma, US) as described above under *in vitro* release test of nanomicelles. The drug release mechanism of optimized gel containing nanomicelles was also studied using Korsmeyer-Peppas model. According to this equation, n value < 0.45 (0.5) represent the Fickian diffusion, 0.5<n<0.8 is Non-Fickian diffusion and if 0.8<n<1, a zero-order mechanism is governing the drug release mechanism from the gels [37].

***In vivo* experiments**

Animals

All procedures conducted in the present study were approved by the ethical committee of Isfahan University of Medical Sciences for the Care and Use of Laboratory Animals. 18 male Wistar rats of 8–10 weeks with a mean weight of 200 ± 20 g were employed. Dorsal hair of animals was shaved using hair depilatory cream one day before wound creation. Rats were anesthetized through intraperitoneal administration of a mixture of 70 mg/kg ketamine hydrochloride and 10

mg/kg xylazine hydrochloride before wound creation. An (1 × 1 cm^2) excision wound was surgically created on the back of animals using a round template. Animals were divided into four groups: Groups I, II, III and IV assigned as (I) animals were served as a negative wound control (II) animals were dressed up with blank gel with the same size as the skin wound area, (III) animals were treated with the gel containing Sim and (IV) animals were wrapped with a gel containing Sim loaded micelles (Sim-micelle-gel). After each dressing, the wound was covered by non-woven adhesive bandage to prevent rats from removing the dressings. Each animal was kept in a separate cage under controlled conditions of temperature 22±1^{0}C and a 12 h light/dark cycle. Dressings were changed by a new one every 3 days until 15 days. The wound closure, the percentage of initial wound area closed, was determined using following formula

$$Wound\ closure\ \% = \left(\frac{wound\ area\ on\ day\ [0] - wound\ area\ on\ day\ [n]}{wound\ area\ on\ day\ [0]}\right) \times 100 \qquad \text{eq.3}$$

where n is the number of days. Wound area was estimated by FIJI image analysis software. 3 animals from each group were sacrificed by formaldehyde on day 12 and 17. The entire wound and a 5 mm margin of the surrounded wounded area were excised for histopathological examination. Paraffin-embedded formalin fixed blocks were obtained from all samples and sections of all specimens were stained by haematoxylin–eosin (H&E) and Masson's trichrome methods. All prepared slides were inspected by a surgical pathologist who was unaware of the groups using an optical microscope (Olympus CX 21, Japan)

Results

Polymeric micelles containing Sim were prepared by film hydration method. Irregular full factorial design was employed to evaluate the effects of different variables and their interactions on the properties of nanomicelles. Table 3 shows the results of EE, particle size, zeta potential, PDI and RE of the studied formulations. Contribution of different studied parameters on Sim EE, particle size, zeta potential and RE in micelles is shown in Figure 1. The Design Expert® software suggested a mathematical equation to make predictions about the response for given levels of each factor. In this equation, the positive and negative signs signify the enhancing or lowering effect

Encapsulation efficiency of Sim in the nanomicelles

EE of Sim in PF127-Chol micelles was high and in all cases above 97%. Statistical analysis by Design Expert Software revealed that the most effective factor on EE was the interaction of P/D ratio and hydration time. However, among single studied factors, solvent type had the most impact on the amount of drug loading (Figure 1). Based on the information gained from the ANOVA analysis, hydration temperature and solvent type as well as the interaction of each two pairs of factors (interaction of P/D ratio and hydration temperature or solvent type, the interaction of solvent type and hydration temperature or hydration time) had a significant effect on obtained EE. Equation (4) is used to predict EE by changes independent parameter.

EE = +98.48-0.18 P+0.24 T - 0.075 H +0.29 S +0.36 PT + 0.62 PH + 0.60 PS - 0.41 TH
eq.4

Where P ,T ,H ,S are P/D ratio, hydration temperature, hydration time and solvent type respectively.

The effects of different studied parameters on EE of nanomicelles are shown in Figure 2.

Particle size

The particle size and size distribution of Sim loaded micelles were measured using dynamic light scattering method. The average particle size of the prepared micelles was between 145 and 283.33 nm. The PDI varied between 0.328 and 0.455. As shown in Figure 1, the most effective factor on the particle size of Sim loaded polymeric micelles was hydration temperature. P/D ratio, temperature and solvent type along with interaction of P/D ratio and hydration time also had a significant effect on the particle size of nanomicelles ($p < 0.05$). The best fitted equations to data for estimation of particle size by changes of the independent factor is introduced as below:

Particle Size=+228.29-14.90 P - 31.18 T + 4.28 H + 27.79 S -5.09 PT -20.22 PH - 11.12 PS - 10.03 TH -13.62 HS eq.5

The effects of different studied parameters on particle size of nanomicelles are shown in Figure 3.

In vitro Release study

The *in vitro* release behaviors of Sim loaded micelles in phosphate buffer solution were investigated and exhibited in Figure 4.

The RE_{96}% is directly related to the release rate of the drug from NPs. The bigger the value of RE_{96}% indicates the faster release rate of the drug. The calculated RE_{96}% values for each formulation are shown in Table 3. The effect of each factor on the obtained RE_{96}% is given below:

Release Efficiency = + 38.45 + 1.13 P - 6.11 T + 5.28 H - 3.13 S -2.38 PT - 3.42 PS -1.95 TS-5.93 HS eq.6

Based on the analysis using Design Expert Software, the most effective parameter on RE_{96}% was hydration temperature. Other effective factors on RE_{96}% were solvent type, hydration time, and interaction between each two pair factors shown in equation.6 ($P < 0.05$). Figure 5 depicts the effect of different levels of each studied parameter on RE_{96}% of Sim loaded micelles. To study the release kinetic and mechanism of Sim from optimized micelles, release data were fitted to different kinetic models and the results were shown in Table 3. Based on the highest R^2 values, Sim release data fitted better with Korsmeier-Peppas release model. The kinetic exponent was found to be between 0.43 and 0.85 representing that the release was mainly controlled by anomalous diffusion mechanism or diffusion coupled with erosion.

Zeta potential of micelles

Prepared micelles had negative surface charge within the ranges from -2.89 to -18.36 mV. Statistical analysis of zeta potential data with Design Expert Software demonstrated that all studied factors and interactions of P/D ratio and hydration time or solvent type, the interaction of hydration temperature and solvent type had a significant effect on zeta potential. As indicated in Figure 1, zeta potential was mostly influenced by the interaction of P/D ratio and hydration time. Among single studied factors, solvent type had the most impact on the zeta potential. Equation (7) is used to predict zeta potential by changing the independent parameters.

Zeta potential =-11.70 - 1.08 P -1.08 T + 1.09 H -1.09 S + 2.38 PH + 1.16 PS + 0.49 TH + 1.55 TS eq.7

Figure 6 depicts the effect of different variables on zeta potential of nanomicelles.

Optimization

The influence of the levels of independent variables on the responses was assessed using computer optimization process by Design Expert Software (ver. 10, US). All responses were fitted to the linear model. The constraint of particle size, zeta potential, EE, was between 145 and 283.33 nm, -2.89 and -18.36 mV and 97.18 and 99.88 respectively with targeting these variables in the range of the obtained results. The RE_{96}% constraint was between 22.92-63.55 % with the target set at the maximum of the obtained data of Table 3, for PDI, it was between 0.328 - 0.455 while the target was set on minimum. Based on the modeling generated by Design Expert software, the optimized micelle formulation suggested by desirability of 93.5% was P10T25H45D that was prepared using 1 mg of Sim, 10 mg of copolymer, DCM as the organic solvent when hydration time and hydration temperature were 45 min and of 25^0 C respectively. The optimized formulation exhibited a particle size of 248 nm, 98.06% EE, zeta potential of –6.37 mV, drug release efficiency of about 63.55% at 96 h and PDI of 0.34. As it can be seen in Figure 7, the Sim-loaded optimized PF127-Chol micelles were spherical with smooth surface.

Rheology and bioadhesion studies

The rheological behavior of all gel formulations was evaluated. It was shown in Figure 8, the gel containing Sim loaded nanomicelles exhibited pseudo-plastic behavior because viscosity value was not constant with increasing shear rate. In the case of chitosan/HPMC gel, the gel-embedded nanomicelles exhibited higher shear stress levels at each level of shear rate compared to the chitosan gel and HPMC gel. This finding indicates that incorporation of HPMC in chitosan based gelling system increases the viscosity. Bioadhesive strengths of chitosan, HPMC and chitosan/ HPMC mixture gel measured by tensile strength tester were about 30.94 ± 2.59 N, 37.75 ± 2.24 N and 44.55 ± 3.15 N respectively. Since HPMC/chitosan gels showed higher viscosity values and bioadhesive strength, this formulation was chosen for further animal studies.

Drug release from the gels containing the optimized nanomicelles

The release profiles of gels containing Sim loaded nanomicelles are shown in Figure 9. The drug release data from HPMC/chitosan gel containing nanomicelles were fitted to zero-order, first-order, Higuchi and Hixon-Crowell and Korsmeyer-Peppas release equations and the results are shown in Table 4. Release of Sim from chitosan/HPMC gel containing nanomicelles exhibited

high correlation with Higuchi diffusion model. In addition; the Korsmeyer-Peppas value was 0.4919 confirming that drug release was controlled with Fickian diffusion.

In vivo wound healing study

For histological examinations, wounded tissues from different treated groups were stained with H & E and Masson's Trichrome. The representative photographs of wounds obtained for each treatment group on 0, 3, 7, 10, 12, and 17 days post wounding are shown in Figure 10. The percentage of wound closure on 7, 10, 12, 17 days post wounding is illustrated in Figure 11. As shown in Figure 11, the difference in wound closure among groups received gel with and without drug did not reach the statistical significance as experiment went on. By day 7 and 10, a significant increase in wound closure was observed in rats treated with Sim –micelles -gel as compared with those treated with gel containing drug and blank gel ($p < 0.05$). On day 12, the percentage of wound closure of rats treated with Sim - micelle- gel was significantly greater than that of rats treated with blank gel ($p < 0.05$). In contrast, no significant difference was found in wound closure of rats treated with gel containing encapsulated Sim (96.25 ±1.39) and free Sim (86.38 ± 6.27) ($p = 0.06$). On day 17, all wounds were closed completely in groups treated with Sim -micelle -gel.

Representative histological images of wounded tissue of each treated group on the 12th and 17th days of post wounding are shown in Figure 12. Wound treated with normal saline showed marked necrotic tissue, hemorrhage, severe inflammatory cells infiltration on day of 12. However, tissue section from the wounds of rats treated with gel containing drug and without it, revealed less inflammation and necrotic areas compared with normal saline groups. On day 12, in the normal saline and blank gel treated groups, the epidermis wasn't clearly formed. Where as in rats treated with gel containing Sim, very thin epithelium was formed. Compared with all groups, a significant increase of reepithelization and considerable decrease of inflammation following administration of Sim -micelle- gel was revealed. As shown in Figure 12, Collagen content was highest in wounds treated with Sim -micelles- gel on day 12 of post wound healing. The wound tissue from Sim -micelle- gel treated group had compact and well- ordered collagen fiber, however loose and irregular reticular arrangement of collagen was seen in other groups. On day 17, less inflammatory cell infiltration in granulation tissues and more numerous collagen proliferations were seen in the blank gel, Sim in gel and normal saline treated wounds. In these groups, defective epithelialization of the wound is seen while in the Sim- micelle- gel treated

wounds, skin appendages such as hair follicles, sebaceous glands were regenerated and skin wound was covered with a well formed epidermis with normal epithelium. Inflammatory cells in Sim- micelle- gel treated wound were also absent. In the animal experiments, Sim- micelles- gel showed fastest healing rate among all of the groups and had better reconstruction of skin tissue than the other groups.

Discussion

Core-shell type nanomicelles would form in aqueous solution at concentrations above the CMC. The micelle core serves as a cargo space for lipophilic drugs, thereby may increase the solubility and stability of lipophilic drugs into aqueous system. Loading of hydrophobic drugs into micelle core is driven by hydrophobic interactions and covalent/non-covalent bond formation. Micelle loading efficiency of block copolymer micelles depends on the (1) the affinity of the loaded drug with core-forming polymer, (2) the content of hydrophobic core (3) hydrophobicity of drug and (4) drug - drug interaction [38]. In present study, PF127-Chol micelles proved to be able to encapsulate Sim with a very high efficiency due to sufficient compatibility of the drug and block forming core of the micelles. To make nanomicelles using the film hydration method, volatile organic solvents that can dissolve copolymer should be considered. DCM and CLF are commonly used organic solvents in film hydration method. As shown in Figure 2.a, CLF produced micelles with higher EE compared with DCM. The selected solvent can influence the physicochemical properties of the PF127-Chol nanomicelles, due to its effect on the extent of mixing between the drug and copolymer and the extent of the chain relaxation of copolymer molecules [39]. By increasing hydration temperature, the encapsulation efficiency increased ($p < 0.05$) (Figure 2.b). Wei et al [40] showed that by increasing the temperature, drug loading of PTX in Pluronic P123/F127 mixed polymeric micelles increased. This could be attributed to the ability of solid pluronic to form a soft gel at high temperature, which may be more favorable for drug loading. In addition, increasing temperature could increase thermal agitation. As a result, amount of drug molecules loaded in core of micelles increased [41]. The increase of drug content led to greater EE (although not significant) when micelles were prepared by DCM (Figure 2.c). However, when CLF was used EE decreased following increasing the drug content ($p > 0.05$) (Figure 2.d). Particle size is an important parameter that should be controlled and evaluated because it can influence physical stability and release pattern of drug from NPs [42]. Based on the results of the design of experiment, by increasing temperature from 25^{o}C to 50^{o}C, the particle

size of PF127-Chol micelles decreased ($p < 0.05$) (Figure 3.a). This observation is well correlated to Kim, et al [43] study, who demonstrated that by increasing temperature, the particle size of Pluronic/PCL block copolymeric nanospheres decreased. Increasing temperature decreased micellar hydration and caused to form compact and shrinkage micelle structures due to increase in intra and intermolecular chain interaction. Another important parameter with significant effect on the particle size was drug content. As the amount of drug loading increased, the particle size of the micelles increased too ($p < 0.05$) (Figure 3.b). This finding was in accordance with the previous study of Saadat et al [41] and Hu et al [44] who showed an increase in particle size with increasing paclitaxel feeding ratio. This could be due to increase of core size of the micelles due to more drug accommodation. The organic solvent type used to form micelles, significantly affected the size of the micelles too. As described earlier, nanomicelles prepared by CLF had higher EE compared with those prepared by DCM which in turn can increase the particle size of micelles (Figure 3.c). Changing the hydration time from 15 to 45 min resulted in an increase, though not significant, in particle size of nanomicelles (Figure 3.d).

The release of a drug from nanomicelles was found to be biphasic and showed a rapid release in the first stage followed by sustained release for long time. The release of Sim located on the interface between micelle core and the corona or within corona caused the initial burst release and subsequent slow release was due to diffusion of Sim from inner core into outer aqueous environment. This release pattern has been reported for most polymeric micelles [45-46]. Normally, drug release can take place in several processes including diffusion through the polymer matrix, release by polymer degradation and solubilization, or diffusion through microchannels that exist in the polymer matrix [46]. The application of the correct mathematical model allows us to simplify the complex release process and to gain insight into the release mechanisms. Analysis of release data of optimized nanomicelles containing Sim showed both diffusion and erosion mechanisms play role in Sim release from nanomicelles (non-Fickian diffusion mechanism).

Effect of formulation variables on drug release from the prepared nanomicelles was also investigated. RE_{96}% decreased with changing organic solvent from DCM to CLF ($P < 0.05$) (Figure 5.a). This change was parallel with increase in particle size and EE. The smaller the particle size, the faster the release of drug. As particle size increased the surface area of nanomicelles decreased and the length of the diffusion path increased, thereby, Sim release rate

was decreased. This finding was confirmed by other studies including that of Taymouri and co-workers [47] who reported that decrease of particle size of nanomicelles increased release rate of docetaxel. By increasing the hydration temperature from 25°C to 50°C, the RE_{96}% value also decreased (Figure 5.b). This change, although reduced the particle size, was associated with increasing EE. Increasing loading amount of drug in micelles enhanced the interaction of Sim with hydrophobic moiety in polymeric micelles resulting in a reduction of the release rate. Kim et al [48] also reported similar results in incorporation of indomethacin in polymeric micelles of poly (epsilon-caprolactone)/methoxy poly (ethylene oxide). Zeta potential is a very good index that determines stability or aggregation of NPs in dispersion. Strong repulsive interactions between the NPs resulted from high surface charge guarantees their stability in colloidal dispersion. The prepared micelles had negative surface charge within the ranges from -2.89 to -18.36 mV (Table 3).

In Figure 6.a, b, c, we showed that increasing the temperature, P/D ratio and decreasing the hydration time from 45 min to 15 min in the production process of the micelles, increased the absolute value of zeta potential to more negative values. All these changes reduced the particle size of nanomicelles. As the particle size decreased, the surface charge density increased and consequently the absolute value of zeta potential increased. This finding was in agreement with previous report of Varshosaz, et al [25] who showed increasing the absolute value of zeta potential with decreasing the particle size of folate targeted dextran stearate polymeric micelles. Changing the solvent type from DCM to CLF significantly increased the absolute value of zeta potential too (Figure 6. d).

Incorporation of Sim loaded nanomicelles in gel resulted in a decreased release rate compared with Sim loaded nanomicelles because of additional barriers provided by gel matrix. In gel-containing nanomicelles, at first the drug molecules must be released out of micelles, then diffuse through and out of the gel matrix. This finding is, of course, in accordance with that reported by several other studies. For instance, Sohrabi et al [49], observed that addition of moxifloxacin niosomes in chitosan gel resulted in much slower drug release rate. Drug release from the HPMC/chitosan gel embedded nanomicelles followed the Higuchi square root law. Higuchi's model has been based on the Fick's Law and describes the release of drug within the delivery system based on diffusion. In this case, the cumulative amount of the released drug is proportional to square root of time. This mechanism indicates that the release rate is dependent

on drug diffusion rate from the polymeric matrix. Bioadhesion is an important parameter that should be considered for topical formulations because it can influence on retention at site of action and therapeutic outcome [49]. The bioadhesion of HPMC/chitosan gel was found to be significantly higher than that of both chitosan and HPMC gel alone, which makes it more applicable due to increasing drug retention at site of action. The obtained result from this study was in good agreement with the result of previous study that showed incorporation of HPMC in chitosan gel significantly increased its bioadhesion [28]. In continuous shearing, all prepared formulations showed shear-thinning behavior which is required to allow gentle gel application over the skin [49]. Psudoplastic behavior of semisolid containing nanocarriers was also confirmed by some other studies [49-50]. The presence of HPMC in chitosan gel enhanced viscosity of the formulation possibly due to formation of physical entanglements such as hydrogen bonds between two polymers that caused an increased resistance to polymer deformation [51]. In agreement with our study, Chelladurai and coworker [52] showed addition of HPMC in both chitosan and pectin based gelling systems increased the viscosity and gel strength.

Wound healing is an essential response to tissue injury. After injury, wound space is covered with granulation tissue which is manifested with numerous vessels and fibroblasts. In the healing process, granulation tissue is replaced by fibrous tissue, which is associated with reepithelialization and appearance of cutaneous adnexa. In present study, the gel without drug significantly improved wound healing compared with normal saline. This could be attributed to potential healing effect of chitosan [28, 53]. The incorporation of Sim in gel didn't give any significant improvement in healing process compared to gel alone. This finding might be due to failure of the drug to penetrate skin efficiently [54]. In agreement with us, the study conducted by El-Refaie et al, [54] also showed equal healing effect of curcumin and curcumin in hyaluronic acid gel after 7 and 10 days. Lin et al [55], also found that the wound closure efficiency of the chitosan dressing alone and chitosan dressing with curcumin was similar at different time intervals. The wounds treated with Sim- micelles -gel were healed faster than those treated with gel containing drug and without it. Sim- micelle- gel induced complete re-epithelialization and more organized collagen deposition in dermis than did gel with and without Sim. These observations showed that the present nanomicelles in HPMC/Chitosan gel promoted tissue reconstruction. This could be due to controlled release of drug from micelles which allows Sim

to continually contact with the wound surface and maintain an effective concentration to promote wound healing. In addition, small size and high surface-to-volume ratio of nanomicelles, facilitating increased passage through biological barriers [14, 56].

Conclusion

In the present study, we have successfully prepared a novel drug delivery system for Sim, a HPMC/chitosan gel containing PF127-Chol micelles. The nano formulations were prepared by film hydration method and optimized using irregular full factorial design. Wound healing experiment using rats showed faster rate of wound healing for gel loaded with PF127-Chol micelles. The results indicate the obtained composite gel has great potential for topical applications at the site of wounds

Acknowledgments

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Declaration of Interest

The authors report no conflicts of interest.

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Table1**.**Different factors and their levels investigated by irregular full factorial design in production of nanomicelles loaded with Sim.

	Levels	
Studied variables	I	II
Polymer/Drug ratio	5	10
Solvent type	C	D
Hydration temperature (^{o}C)	25	50
Hydration time (min)	15	45

D: Dichloromethane C: Chloroform

Table2. Composition of different studied Sim loaded nanomicelles by irregular full factorial design

Formulations	Polymer/Drug ratio	Solvent Type	Hydration temperature (^{o}C)	Hydration time (min)
P10 T50H45D	10	D	50	45
P10T25H45D	10	D	25	45
P5T50H45D	5	D	50	45
P5T25H45D	5	D	25	45
P10T50H15D	10	D	50	15
P5T25H15D	5	D	25	15
P10T25H45C	10	C	25	45
P5T50H45C	5	C	50	45
P10T50H15C	10	C	50	15
P10T25H15C	10	C	25	15
P5T50H15C	5	C	50	15
P5T25H15C	5	C	25	15

P: Polymer/Drug ratio T: Hydration temperature H: Hydration time D: Dichloromethane C: Chloroform

Table3. Physical properties of different prepared Sim loaded nanomicelles

Formulations	Drug loading efficiency%	Particle size (nm)	Zeta potential, (mV)	PDI	Release efficiency,RE_{96} %
P10 T50H45D	97.97±0.49	145.60±6.65	-11.03±0.61	0.41±0.07	52.01±2.99
P10T25H45D	98.06±0.45	248.67±46.06	-6.37±0.89	0.34±0.09	63.55±2.59
P5T50H45D	98.03±0.42	204.66±27.46	-10.93±0.25	0.45±0.04	46.14±1.90
P5T25H45D	98.65±0.40	276±12.34	-7.01±0.58	0.33±0.03	49.45±0.47
P10T50H15D	98.82±0.002	168.00±3.60	-18.36±0.93	0.45±0.06	27.51±0.53
P5T25H15D	99.45±0.26	187.01±12.28	-2.89±0.44	0.50±0.09	28.50±1.09
P10T25H45C	99.67±0.26	242.67±29.50	-10.74±0.99	0.41±0.08	42.92±2.02
P5T50H45C	97.18±0.45	261.66±12.5	-13.73±0.25	0.36±0.06	31.18±0.74
P10T50H15C	99.88±0.07	240.65±12.05	-17.70±1.13	0.42±0.01	22.92±1.56
P10T25H15C	97.46±0.37	283.33±16.25	-16.53±1.27	0.44±0.01	44.32±1.63
P5T50H15C	99.16±0.15	252.66±28.50	-11.96±0.20	0.61±0.08	33.01±0.66
P5T25H15C	99.04±0.59	285.50±14.84	-13.10±1.01	0.32±0.03	43.63±2.06

Table 4. Correlation coefficient (R^2) obtained from curve fitting of Sim release data from optimized nanomicelles and HPMC/chitosan gel containing optimized Sim loaded nanomicelles

Formulations	Kinetic models(r^2)				Korsmeier-Peppas parameters	
	Zero order	Higuchi	Hixson-Crowell	First order	n	r^2
Optimized Sim loaded nano micelles	**0.7266**	**0.8901**	**0.5761**	**0.8336**	**0.4666**	**0.9486**
Gel containing optimized Sim loaded nanomicelles	**0.8206**	**0.9492**	**0.9258**	**0.8865**	**0.4919**	**0.9377**

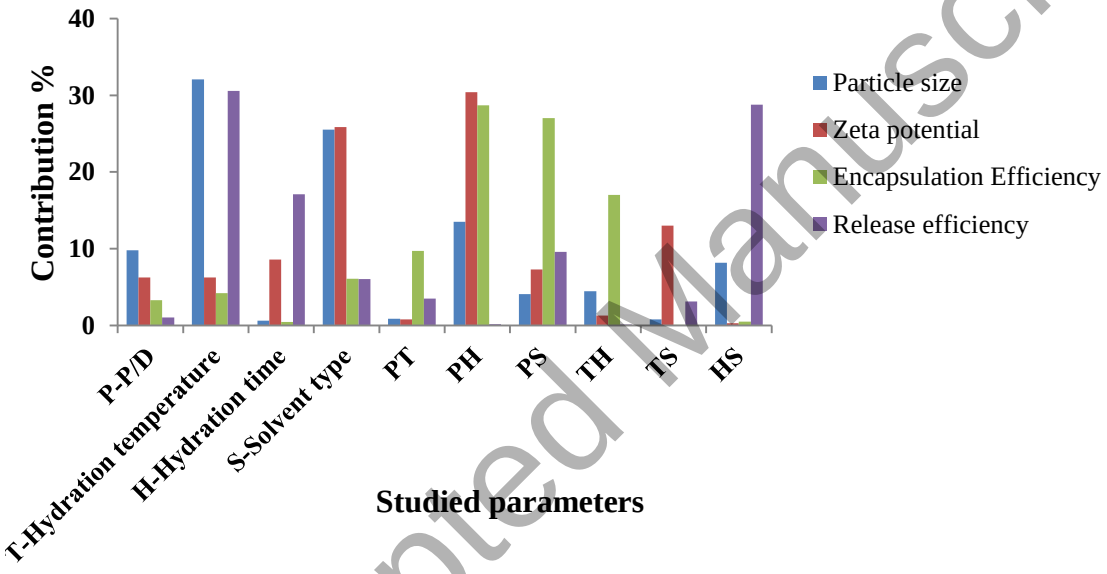

Figure1. Contribution of different studied parameters on Sim encapsulation efficiency, particle size, zeta potential and release efficiency in micelles

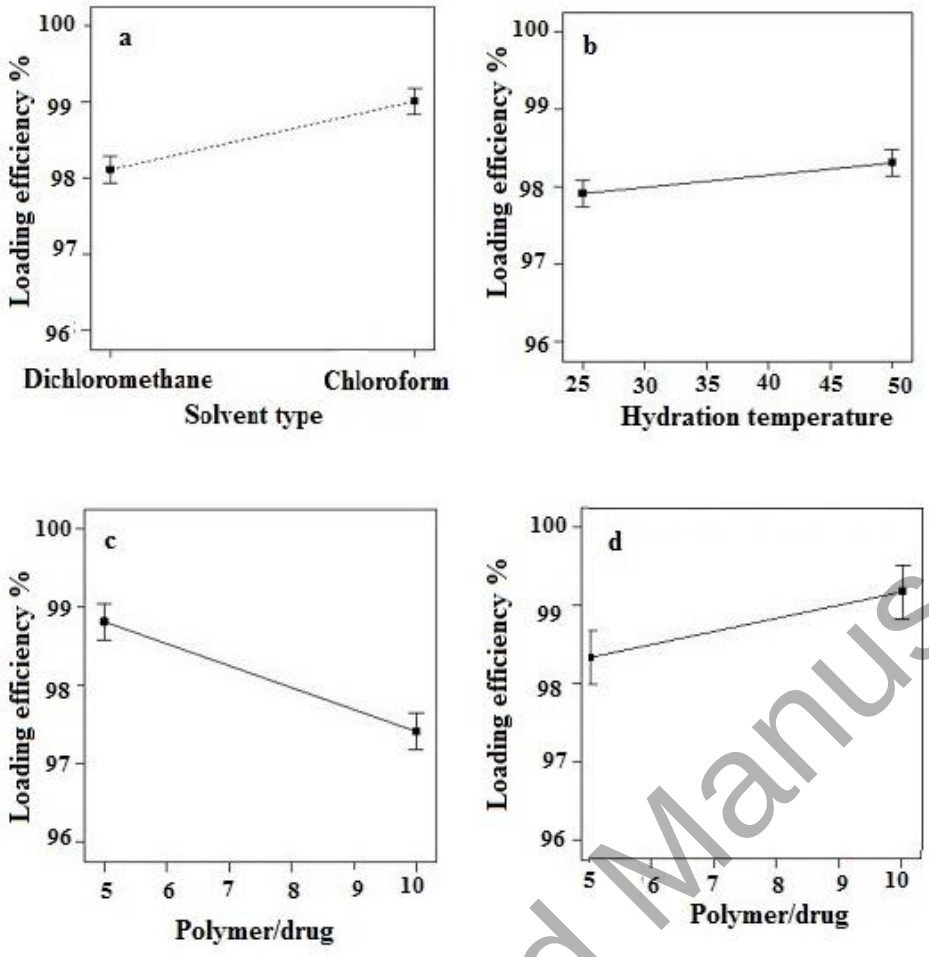

Figure2. The effects of different studied parameters on loading efficiency of nanomicelles

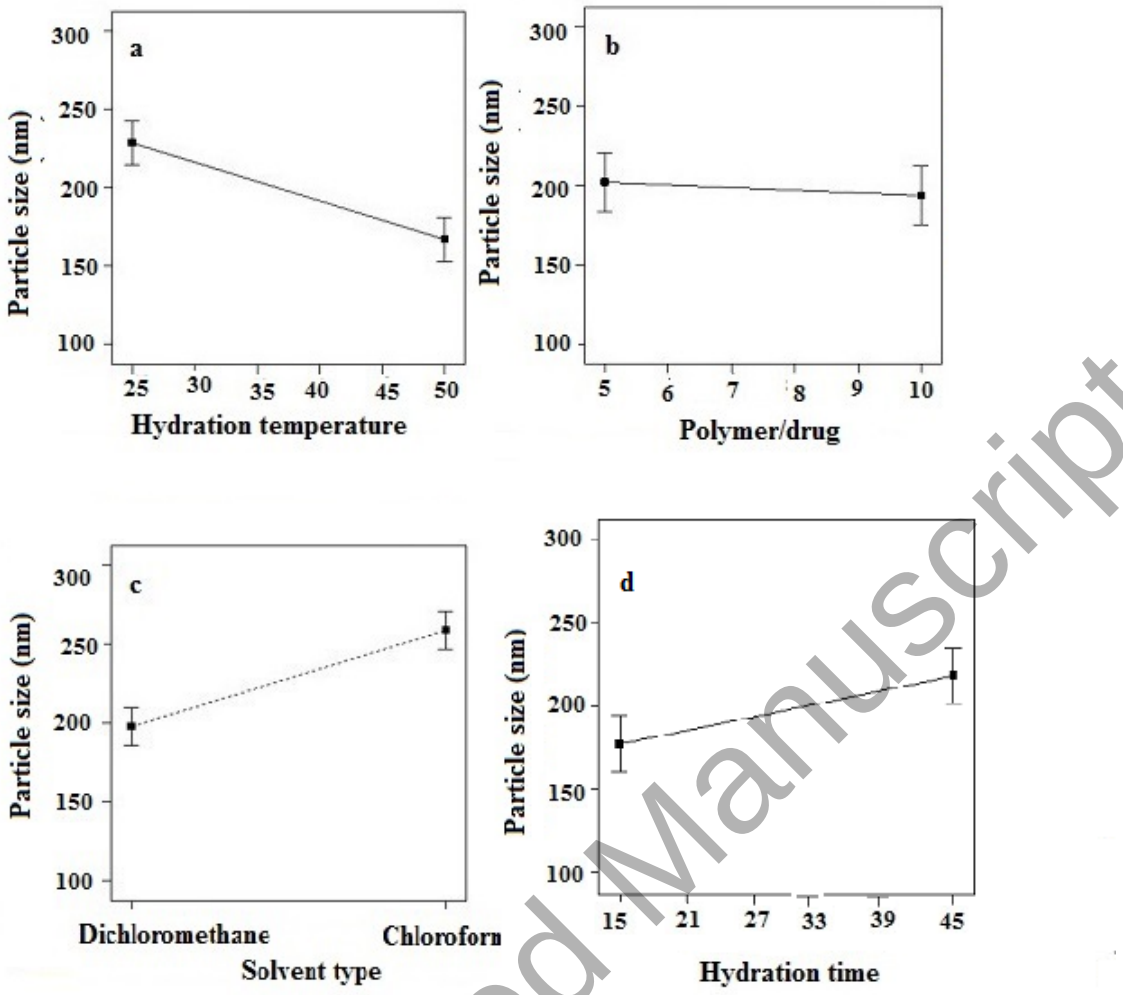

Figure 3.The effects of different studied parameters on particle size of nanomicelles

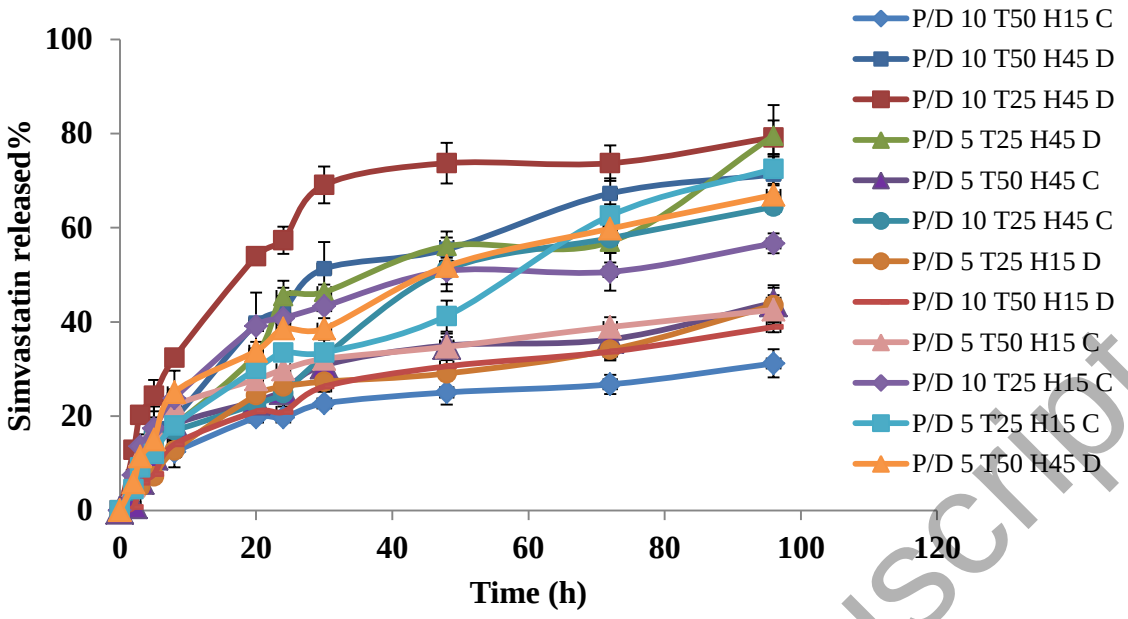

Figure 4. Release profiles of Sim from different studied formulations

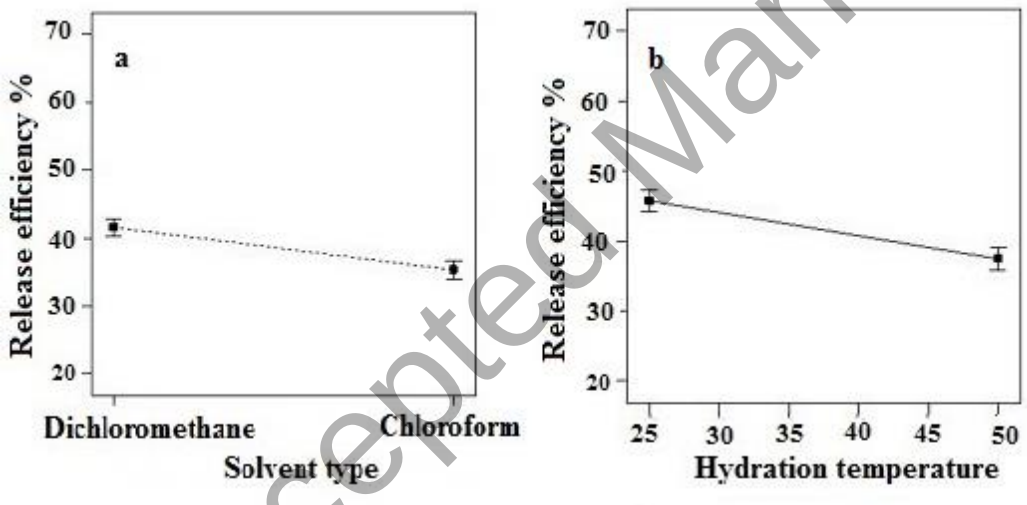

Figure 5. The effects of different studied parameters on release efficiency of Sim loaded micelles.

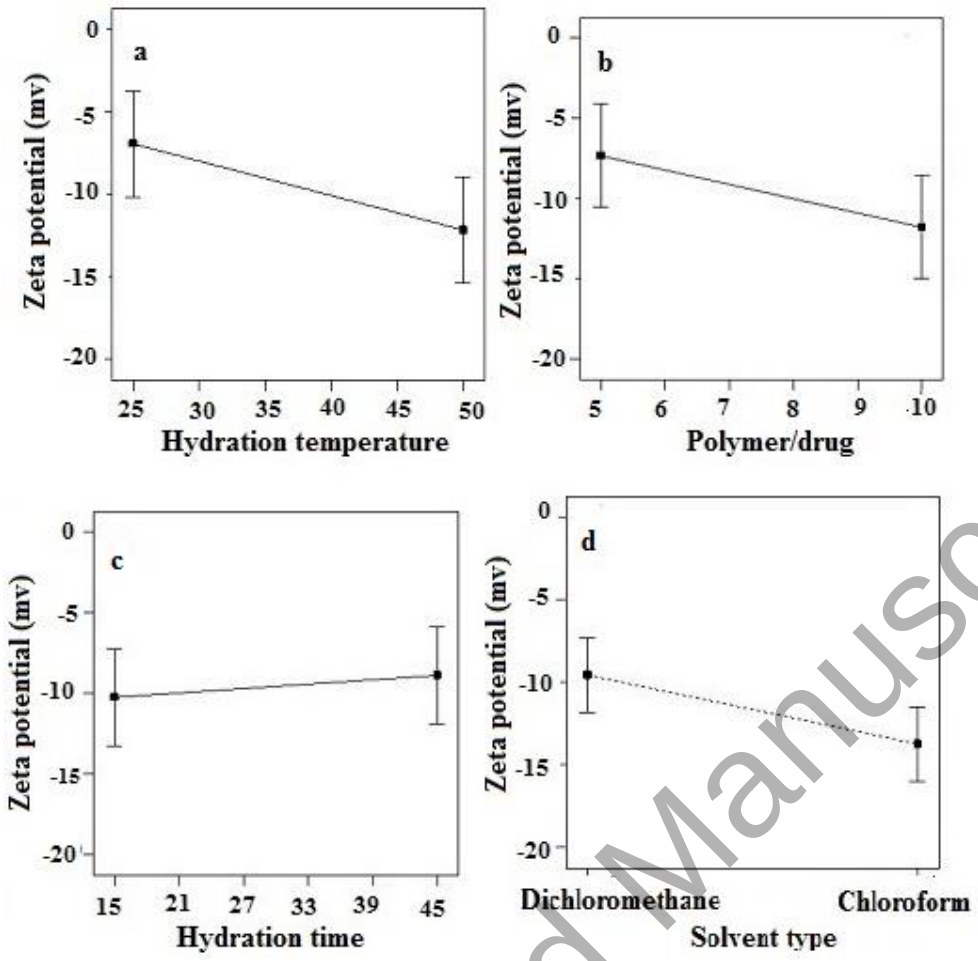

Figure 6 .The effects of different studied variables on zeta potential of nanomicelles

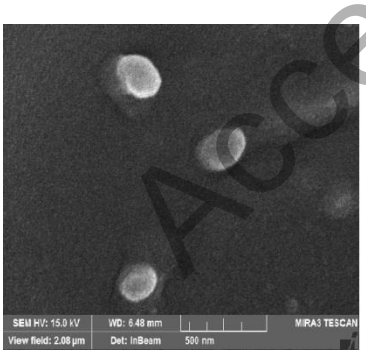

Figure 7. Morphology of Sim-loaded optimized PF127-Chol micelles

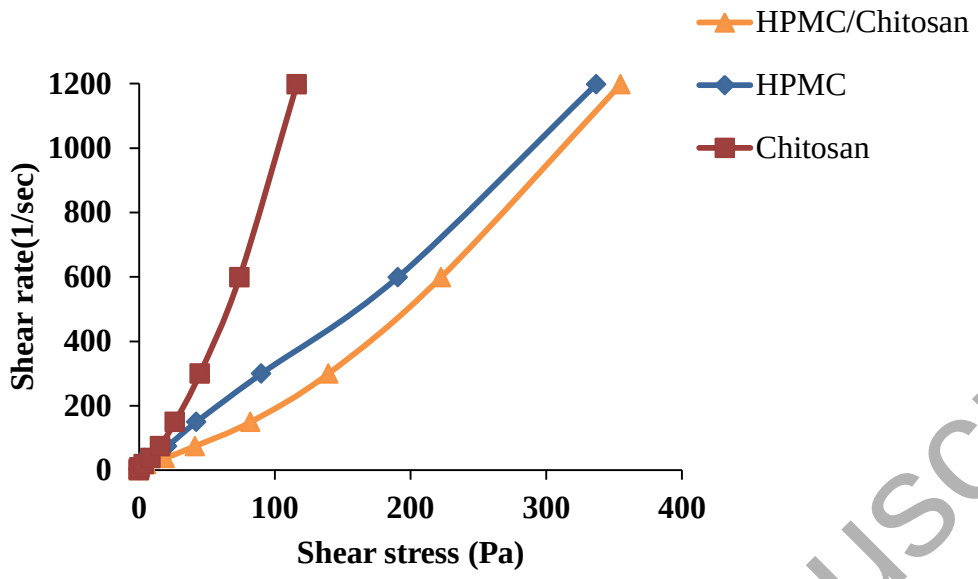

Figure 8.Reological behavior of different gels containing Sim loaded nanomicelles

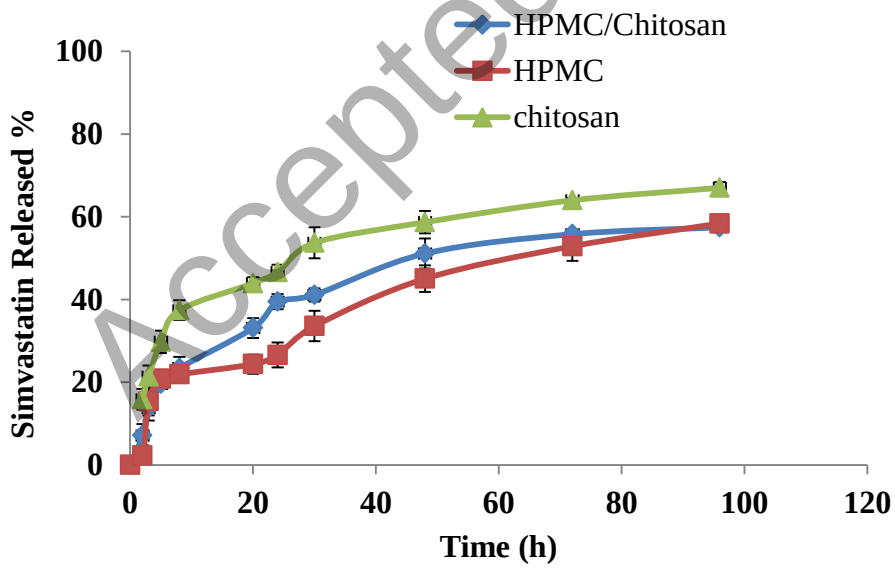

Figure 9. Release profiles of Sim from different gels containing nanomicelles

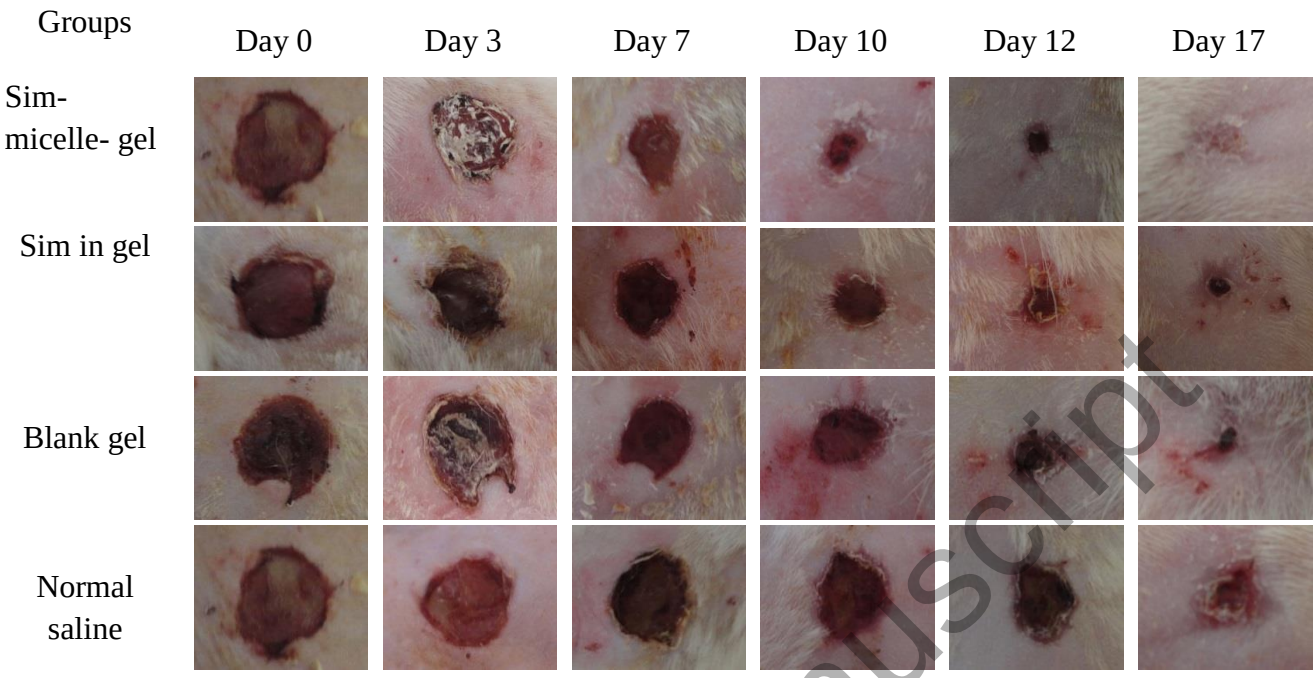

Figure 10. Images of wounds treated with normal saline, blank gel, Sim in gel and Sim - micelle - gel on days of 0, 3, 7, 10, 12, 17 post wounding

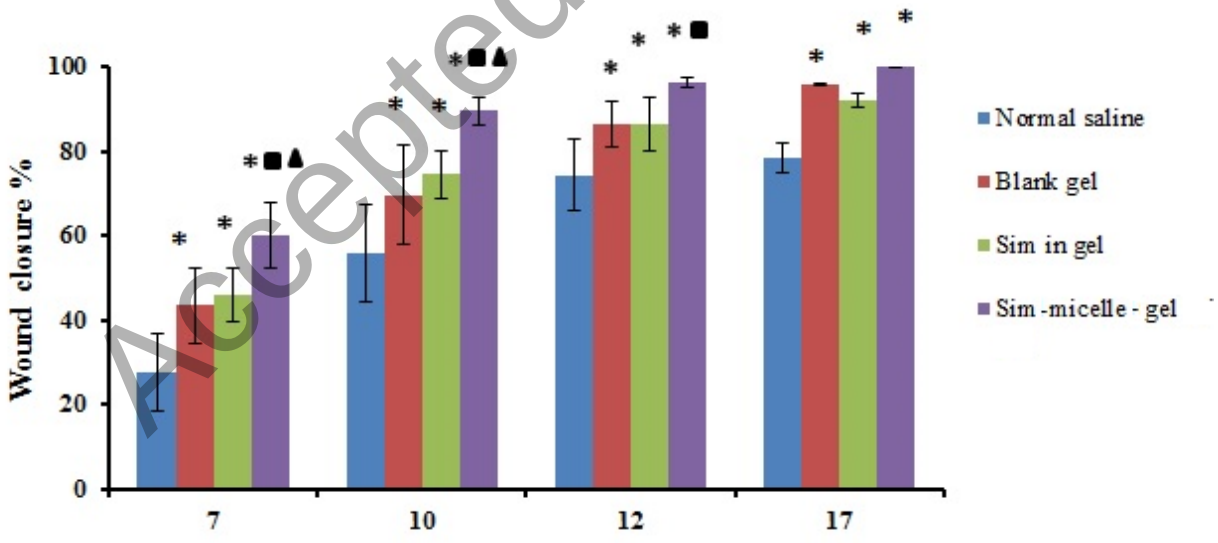

Figure 11. The influence of Sim –micelle - gel, blank gel, Sim in gel and normal saline on wound closure. * $p < 0.05$ vs . normal saline control group, ■ $p < 0.05$ vs . group treated with blank gel, ▲ $p < 0.05$ vs . group treated with Sim in gel

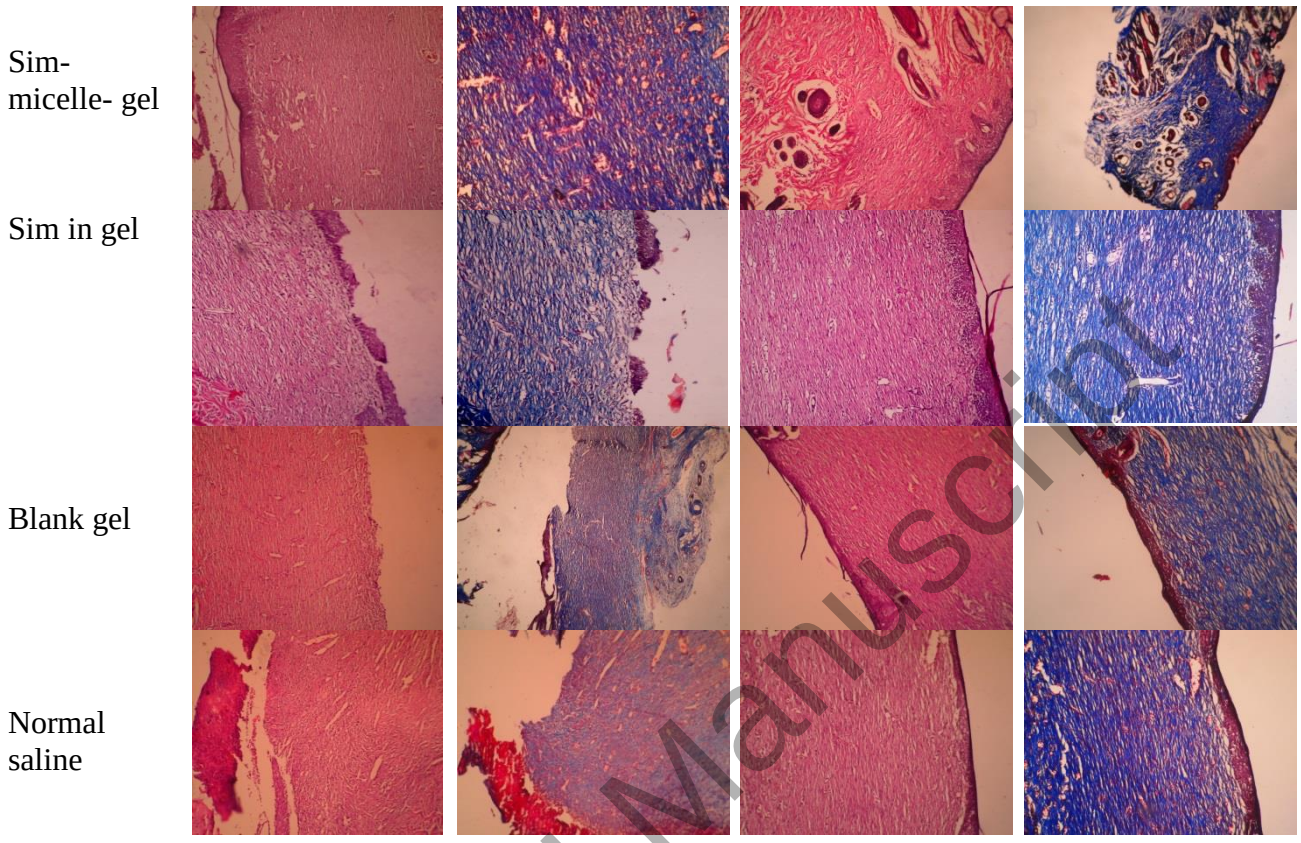

Figure 12. Representative histological images of wound in four treatment groups on day 12 and 17