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Original Article

Preparation and Characterization of Thermoresponsive In-situ Forming Poloxamer Hydrogel for Controlled Release of Nile red-loaded Solid Lipid Nanoparticles

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Abstract

Preparation and characterization of thermoresponsive in-situ forming poloxamer hydrogel for controlled release of Nile red-loaded solid lipid nanoparticles. Nanoparticles (NPs) are cleared rapidly from systemic circulation and do not provide sustained action in most cases. To solve this problem, this investigation introduces an erodible in-situ forming gel system as potential vehicles for prolonged release of NPs. In this study, Nile red-containing SLNs were prepared by solidification of an oil-in-water microemulsion using stearic acid, surfactants and co-surfactants. SLN particles were then loaded in a Poloxamerthermoresponsive sol-gel matrix. Dialysis membrane and membrane-less diffusion method were used to study release of the fluorescent probe. Erosion test were carried out by gravimetric method and the medium was checked for zeta potential to investigate existence of intact SLNs. Sol-gel transition temperature was determined by stirring method. Release results showed high entrapment of Nile red in lipid matrix of SLN. Therefore, Nile red content in erosion medium was attributed to SLN particles. Zeta potential of SLNs remained unchanged after sol-gel loading (P>0.05). The correlated released amount of Nile red to dissolved gel weight implied erosion could be major mechanism of SLN release. Results also showed that SLN increase erosion rate of Poloxamer gel and its sol-gel transition temperature. The present study show that thermoresponsivePoloxamer gel can be used to control the release of NPs and those intact NPs are released from this system. The prepared formulation can be used for further investigations in vivo.

Key words: Controlled release, Erosion, in-situ forming sol-gel, solid lipid nanoparticles,Thermoresponsive.

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1. Introduction

Nanoparticles (NPs) are eliminated rapidly from the body, mainlyvia reticuloendothelial system[1]. As a result, sustained nanoparticle concentration and, therefore, prolonged drug delivery to the target organs cannot be

achieved easily. In order to solve this problem, this study investigates the possibility of application of erodible sol-gel systems as NP controlled release
system, using solid lipid NP (SLN). The growing interest in fabrication of SLN has been attributed to their biocompatibility, biodegradability, possibility of incorporation of hydrophilic and lipophilic compounds such as proteins and finally their enhanced drug stability during preparation and storage[2-4].

In-situ thermosensitive sol gel system have been gaining popularity and receiving considerable attention in recent years especially in area of controlled release of drug molecules[5]. Such thermoresponsive systems are applied as as a liquid (sol) that changes to a gel at body temperature. Over longer period of time, their cargo will release from gelled polymeric matrixes upon erosion. Poloxamer 407, also known as Pluronic or Lutrol® F127, which shows a sol-gel transition around body temperature [6]was used as sol-gel system here. It consists of poly(oxyethylene) (OE) and poly(oxypropylene) (OP)units and shows a reversible semi-solid gel-like structure at 37°C above a critical concentration of 20%[7, 8].Poloxamer 407 has been used in several studies for controlled delivery of various drugs such as peptides[9-11], genes[12], antibiotics like ceftiofur[13] and vancomycin[14] and anticancer like paclitaxel[15].

Loading of NP in Poloxamer 407 gels have been reported for controlled release of drug molecules in local [16, 17]and systematic applications[18, 19]. Even controlling the release of drug molecules by comprising lipidicNPs such as liposomes[20] and cubosomes[21] into Poloxamer 407 have been reported. Our group has recently introduced application of injectable thermoresponsive sol-gel systems for controlled release liposomeNPs [22]and has shown that liposomal hydrogels control the release of their cargo and provide prolonged tissue concentrations in-vivo, in comparison to plain liposomes.

The present investigation introduces SLN-containingPoloxamer 407 systems with different volumes of SLN dispersions for prolonged NP delivery.To trace the SLN NPs, a hydrophobicfluorescence dye,Nile red (NR), 9-diethylamino-5H-benzo [alpha]phenoxazine-5-one, was loaded into SLNs here. It is a solvatochromic dye and its spectral properties such as positon, shape and intensity varies with the nature of its surrounding microenvironment and solvents[23]. Therefore, it is mostly used in structural and localization study of multiphasic systems such as microemulsions[24]. Besides that, this vital stain is popular for intracellular lipid droplets study by fluorescence microscopy and flow cytofluorometry[25].It is highly fluorescent in organic medium and almost non-fluorescent in aqueous mediums because of its negligible water solubility [23].

2. Materials and Methods

2.1. Materials

Stearic acid was purchased from Kirish Pharma GmbH (Salzgitter, Germany); Lipoid E80 (egg phosphatidyl choline 80%) was obtained from Lipoid (Ludwigshafen, Germany), taurocholate sodium salt and Tween® 80 were purchased from Fluka (Buchs, Switzerland). Poloxamer 407 (Lutrol® F127) was obtained from BASF (Ludwigshafen, Germany). Nile redwas purchased from Sigma Aldrich (St. Louis, USA). All other chemicals used were of analytical grade.

2.2. Preparation of SLNs

SLNs were prepared by amicroemulsion method employed by Gasco[26] and a formulation introduced by Bucca *et al.*[27]. The microemulsion composed of stearic acid as the lipidic(oily) phase, egg phosphatidyl choline as surfactant, taurocholate sodium as cosurfactant and distilled deionized water as the continuous phase[27]. Stearic acid (mp = 65°C) was melted and heated to 70°C to prepare the oily phase. NR was dissolved inthe melted oily phase (total lipid content of 1.54 g) Hot (70°C) aqueous surfactant solution was then added to the hot oily phasedropwise, while was being stirred in a water bath (Dorsa, Iran), until a clear homogenous oil-in-water (o/w)microemulsion was obtained. Microemulsion dropletswere then solidified into SLN particles by dispersing the warm microemulsion in cold distilled water containing Tween®80 (0.5% w/w) as the stabilizer, at a ratio of 1:10 (microemulsion: cold water, v/v) under mechanical stirring at 2500 rpm for 2h. (Heidolf, RZR1, Germany).

The SLNs were then purified by centrifugal filtration using 5000 Da cut-off Vivaspin 2 (Sartorius AG, Germany) filters. Particles were then dispersed in deionized water and stored in refrigerator until use. Possible un-trapped NR was removed by dialysis against phosphate buffer (pH 7.4) overnight at room temperature using12 kDa cut-off cellulose acetate membranes.

2.3. Characterization of SLNs

To evaluate the NR solvatochromism inside SLNs,NPs were dissolved in methanol and the spectrumofNR was recorded at excitation/emissionwavelengths of530/630nm by Hitachi F4500spectrofluorometer (Japan).Then the shape and emission position werecompared to NR methanol standard spectrum.

Before quantification of NR content of NPs, dye extraction from SLN was performed using a protocol adapted from literature[28].Briefly, 500µL of SLN dispersion was dissolved intetrahidrofuran (THF) (1:3 v/v). After complete evaporation of THF, selective lipid precipitation in methanol (1:2v/v) was performed at 4°C. After centrifuge, 250 µL of supernatant was analyzed by HPLC-fluorescent detector. The efficacy of this extraction protocol was more than 90%. Chromatographic separation was carried out with a NovapakC18

column (150×3.9 mm, 4µm) (Waters,USA) using a Waters separation module with a Waters dual fluorescent detector at excitation /emission wave length of 559/630 nm detection. The elution was performed with an isocratic mixture of methanol/water (90/10 v/v) with a flow rate of 2 mL/min. With this protocol, NR retention time was 3.25 min. Quantification is obtained by comparison between the NR observed area underpeak with a calibration curve obtained under the same condition for serial dilution of NR methanolic standard solutions.

Phospholipid content of SLN dispersions was also measured by Stewart method[29]. The encapsulation efficiency, defined as NR/phospholipid ratio of purified SLNs divided by initial NR/phospholipid ratio, was then calculated using NR and phospholipid content data.

Particle size and zeta potential of SLN dispersions were measured by Malvern Hydro 2000 SM particle size analyzer (Malvern, UK) and Malvern ZetasizerNano instrument (NanoZS, Malvern, UK) respectively.

2.4. Preparation of SLN-Loaded Sol-Gel System

Poloxamer407 solution (20%, w/w)was prepared according to the "cold method" described by Schmolka[30]. For preparation of SLN-loadedPoloxamer solution, 50% of water was replaced with SLN dispersion keeping the Poloxamer content at the constant value of 20% (w/v). Higher amounts of SLN dispersion did not provide proper gels, as discussed in our previous work[1].

2.5. Characterization of SLN-Loaded Sol-Gel System

Sol-gel transition temperature of the system was obtained by stirring magnet bar method[31] performed over the temperature range of 5 to 50°C. Briefly, in each experiment, 5 ml Poloxamer solution (with or without SLN) was first placed in a glass vial and the vial was placed in a hot/cool water bath (Dorsa, Iran). The water bath temperature was then increased step-by-step, while the contents were being stirredat 200 rpm using a 2 mm magnet bar. The temperature, at which the magnet bar stopped moving, was considered as the sol-gel transition temperature.

2.6. Evaluation of Nile RedLeakage from SLNs

NR-containing SLN dispersion was diluted with phosphate buffer (25 times), transferred into a dialysis tubing (12 KDa) and release of NR into phosphate buffer (receptor phase) wasstudiedat 25 and 37°C over 6h. Samples were withdrawnand their NR contentswere evaluated by HPLC analysis as described above.

[1]G. Dorraj and H. R. Moghimi,"*Preparation of SLN-containing thermoresponsive in-situ forming gel as a controlled nanoparticle delivery system and investigating its rheological, thermal and erosion behavior,"*I. J. P. R. (2014)in press.

2.7. Release of SLN andNile RedfromGel; Gel Erosion Test

The amount of dissolved gel was measured by a gravimetric method and the amount of NR in the eroded medium was evaluatedafter gel erosion. In this experiment, 1 mL of the SLN-containing Poloxamer sol was put into a pre-weighed empty glass tube in a water bath at 37°C until a clear gel was formed. 2 mL of erosion medium (phosphate buffer, pH 7.4) with the same temperature carefully was layered over the gel surface. At specific timepoints, the release medium was removed and replaced with a fresh medium and the amount of dissolved gel was calculated. The total amount of NR released into medium was monitored over 6h. Higher time points were not considered for this study as more than 80% of NPs are released from gel over 6 h erosion. Eroded samples were then analyzed for NRas described above. The same procedure was performed for SLN-free Poloxamer and SLN-free Poloxamer contain same amount of Nile red.

2.8. Statistical Analysis

Data were expressed as the means of three separate experiments ± SD. Statistical comparisons were made by ANOVA followed by Post Hoc Tukey test and T-test using IBM SPSS Statistics 20. P values of less than 0.05 were considered statistically significant.

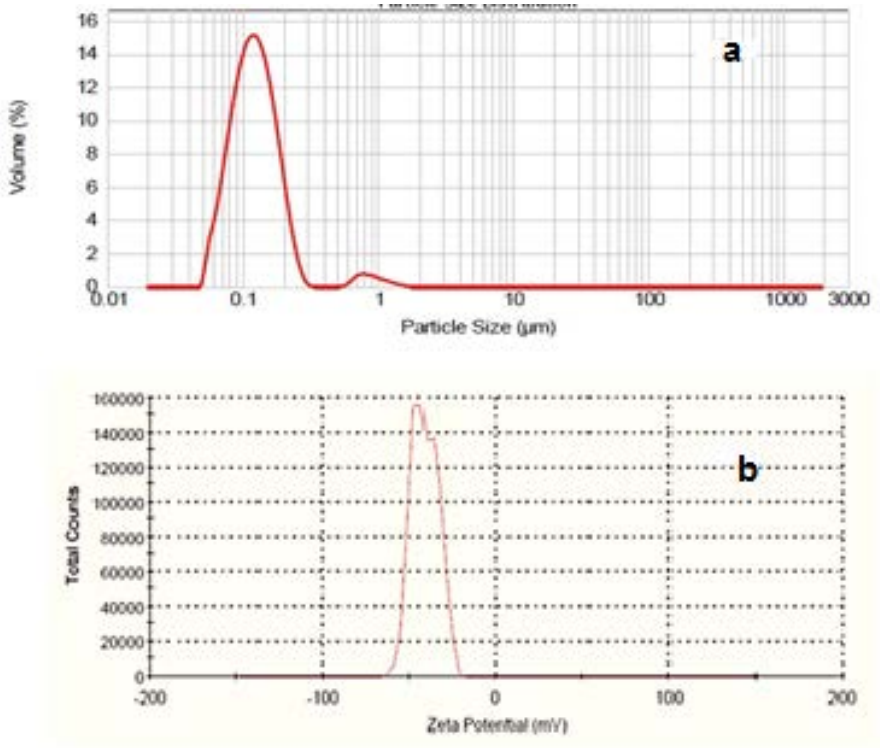

Figure 1. Hydrodynamic size measured by particle size analyzer (a) and zeta potential (b) of freshly prepared free SLN-NR at room temperature.

3. Result and Discussion

3.1. Physicochemical Characterization of SLN andSLN-Gel

Free SLN-NRshowed particle size of 121.3±2.1 (mean ± SD, n=3), as measured by particle size analyzer (Figure 1). These particles carried a negative charge of -41.6 ± 7.2 mV (mean ± SD, n=3) (Figure 1).

The NR fluorescence is very sensitive to its microenvironment[32]. The SLN formulation described here was based on an oil-in-water emulsion. After solidification of this microemulsion, SLNs were composed of solid lipid core (stearic acid) which is stabilized by phospholipid and taurocholate sodium. In such matrix, emission peak of SLN-NRand SLN-NR loaded gel showed sharper peak in comparison to methanolic standard peak (Figure 2).Results showed that a redshift of about 10 nm is observed after loading of NR into SLNwhich should be due to increased hydrophobicity of dye environment.This finding is in agreement with what has been reported in the literature[24, 25].

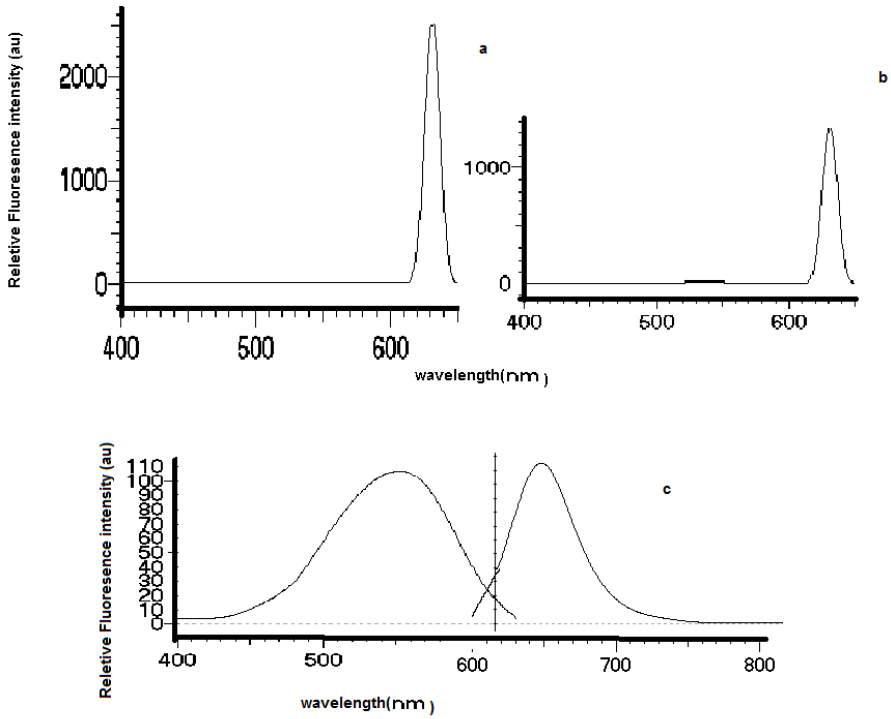

Figure 2. Emission spectra of SLN-Nile red before (a) and after (b) sol-gel incorporation compared to excitation/emission spectra of methanolic dye.

The encapsulation efficiency of NR is SLN was calculated to be of 75% ± 3.2 here. After sol-gel incorporation,encapsulation efficiency was decreased to 62% ± 3.4.

Sol-gel transition studies showed that Poloxamer solution (sol) turns into a gel at 28 ± 0.1°C. Incorporation of SLN-NR into this

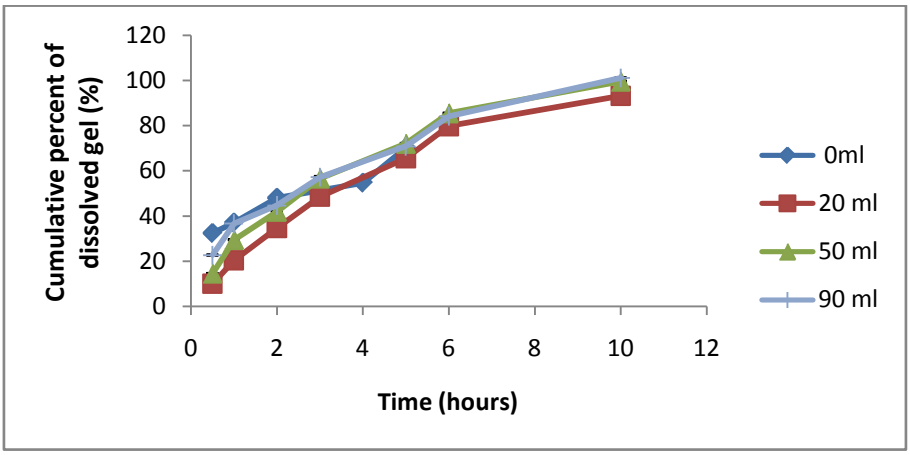

Figure 3. Erosion test results of 1 g of Poloxamer 407 20%w/v at 37°C after incorporation of 20-50-90 ml of SLN-NR dispersion. Data are mean±SD (N=3).

system increased transition temperature to 30 ± 0.2 °C ($P<0.05$). This shift is favorable in terms of handling at room temperature and clinical application of the system.

3.2. Leakage Test of Nile Redfrom SLN

A negligible amount of dye (less than 2%)was detected in the receptor medium after leakage test from SLN particles dispersed in phosphate buffer. These results indicate that NR is entrapped in SLNs and does not release into the aqueous receptor phase significantly. NR is hydrophobic compound; its log P is reported to be around5 [25], and is expected to remain in lipophilic SLN particles. Besides this, SLN solid matrix provides a diffusional barrier against NR release. However, release values of as high as 20-30% has been reported for NR release from oily core in NLC[33].

3.3. Gel Erosion

Figure 3shows the erosion behavior of plain and SLN-loaded Poloxamer gel.Plain gel showed higher erosion at initial samples (higher

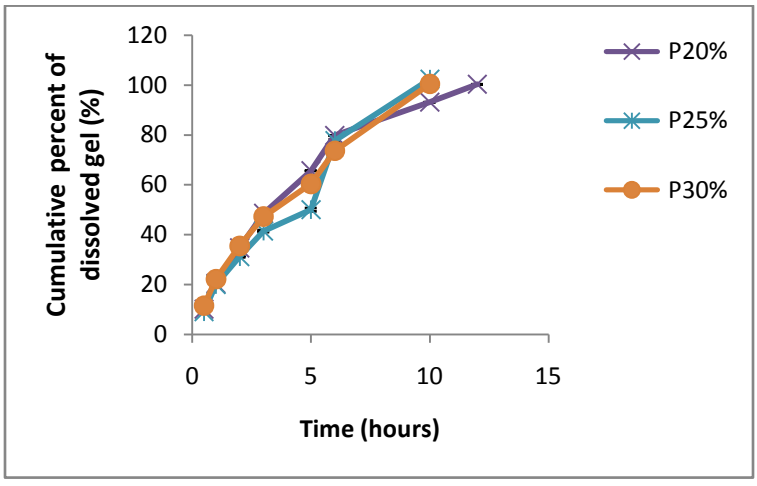

Figure 4. Effect of Poloxamer concentration on erosion of SLN-containig systems.

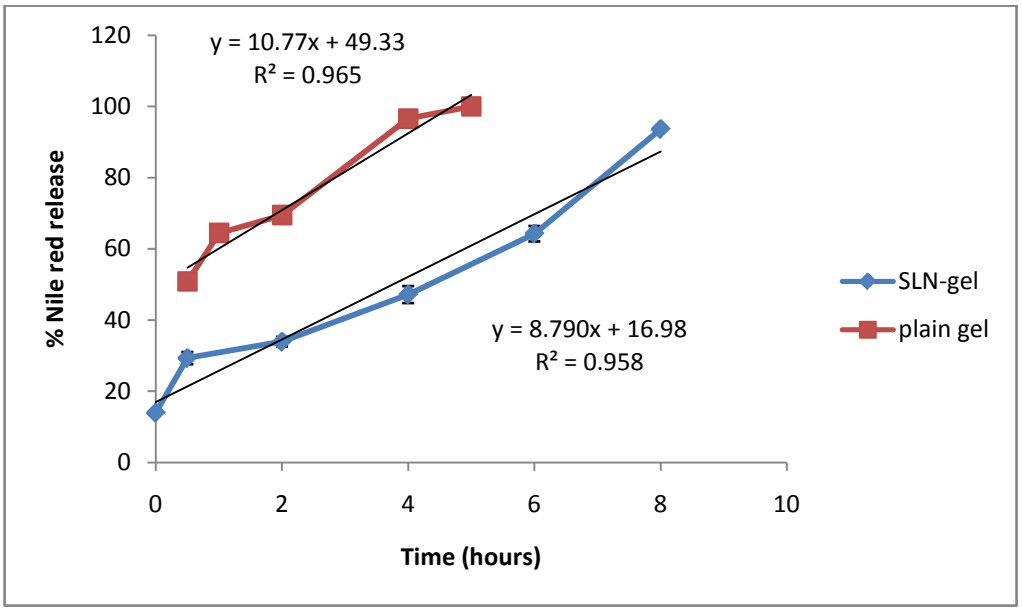

Figure 5. In-vitro release of NR from SLN-containing Poloxamer 407 (20%w/v) gel at 37°Cas a function of time (hour) compared to NR release from plain gel in the same concentration. Data are mean±SD (N=3).

burst) in comparison to SLN-loaded systems. During gelation in Poloxamer 407, interaction of hydrophilic section of polymer poly(oxyethylene), (OE)with hydrophobic section poly(oxypropylene), (OP) formsmicellararrangement[34, 35]. The present data show that SLN particles affect micellar arrangement and therefore release at initial; stages. Erosion is linear with time in all systems (zero-order) and the slope is slightly higher in SLN-containig systems.

It has been reported that Poloxamer concentration affects erosion rate over the range of 20-30% [32]. Our studies, however, showed that same Poloxamer concentration does not affect erosion behavior of the system in SLN-containig systems (Figure 4).

3.4. Stability of SLN after Sol-Gel Incorporation

Stability of SLN in sol-gel system was also investigated by zeta potential measurement after erosion of gel in phosphate buffer at 37°C. The observed zeta potential values for eroded samples in SLN-containing systems showed slightly increase (-36.2±10.1 and -31.8± 3.75 for 3 and 8 hours eroded samples respectively) compare to corresponding initial value of free SLNs (-41±7.2) ($P>0.05$). It could be due to remained polymeric shell around SLNs after sol-gel.

3.5. Release of SLN and NR from gel

The cumulative amount of NR detected in the release medium after gel erosion (Figure 5) showed a linear relationship with time (r2> 0.95) (Figure 5), indicating that release of SLNs from gel follows a zero-order kinetic at 37°C.

The whole detected NR in erosion medium after dissolution is considered to be due to SLN release as NR leakage from NPs was seen to be negligible, as described above.

Release of NR from plain gel was alsolinear behavior with time.However, release from plain gel showed decreased release time and higher burst release compare to SLN-loaded system (Figure 5). Decrease in release rate at the presence of SLN was significant ($P<0.05$).

3.6. Correlation betweenGel Dissolution and Probe Release

Figure 6 illustrates relationship between dissolved gels (measured by gravimetric method) and released Nile red(measured by HPLC) and there is a good correlation between these two parameters. These data indicate that the main mechanism behind probe release is gel erosion and influence of other mechanisms such as diffusion is low.

4. Conclusion

Present results show that it is possible to control the release of NPs by erodible sol-gel systems. SLN particles are stable in such a system. As SLN release is controlled by gel erosion, the release duration can be tailored by changing gel properties. Nile red also proved to be a good probe for intact SLN release studies. Increased sol-gel transition temperature in the presence of SLN also makes the system favorable for handling and clinical applications. Further studies are in progress in our laboratories to investigate application of these systems in-vivo.

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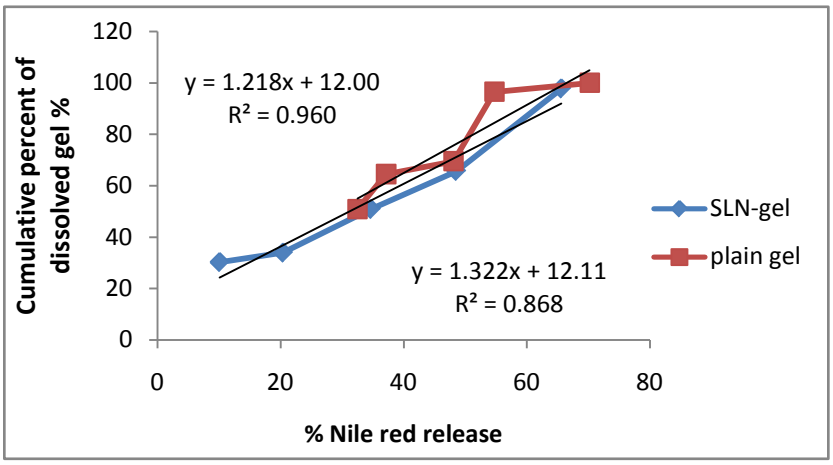

Figure 6. Relationship between gel erosion (measured by gravimetric method) and NR release (HPLC method) for SLN-containmig Poloxamer gel at 37°C.

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