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# Use of remote film loading methodology to entrap sirolimus into liposomes: Preparation, characterization and in vivo efficacy for treatment of restenosis

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ABSTRACT

The main objective of this study was to formulate an effective controlled-release liposomal drug delivery system for sirolimus (SIR), a potent antiproliferative and anti-inflammatory drug, to be used for the treatment of restenosis following local vascular delivery. Liposomes were prepared using remote film loading method and characterized with regard to entrapment efficiency (EE), size distribution and zeta potential. The effects of key formulation and proceeding variables on both EE and drug release were studied using a fractional factorial design. By means of this entrapment technique, 98% SIR incorporation was achieved. Nanoliposomes were found to have average size of 110 nm and zeta potential of −9 mV. Developed formulations were found to have prolonged drug release for up to 3 weeks in vitro; this was best fitted by the Higuchi model. Other scopes of this work were to determine the applicability of sirolimus-loaded nanoliposomes (SIR-L) as drug carriers for the treatment of restenosis and to evaluate the effect of the presence of rigid lipids on the in vivo efficacy of the liposomal carrier of SIR. In vivo studies in balloon injured rat carotid arteries revealed the potential of SIR-loaded liposomes as efficient local and controlled drug delivery systems to reduce restenosis.



## 1. Introduction

Coronary artery disease is the most common type of heart disease. Although surgical coronary interventions, such as percutaneous transluminal balloon angioplasty (with or without stent placement), effectively remove the atherosclerotic lesion and restore blood flow, a significant number of patients redevelop obstructions, a disease process known as restenosis (Burt and Hunter, 2006; Melikian and Wijns, 2008). Proliferation of smooth muscle cells from the arterial wall into the lumen of the artery is considered as a major cause of restenosis (Costa and Simon, 2005; Lee et al., 2004). Systemic administration of antiproliferative agents at acceptable doses suffer from subtherapeutic arterial drug levels and are not quite effective (Popma et al., 1991), whereas local drug delivery of these therapeutic agents via drug eluting stents (DESs) has significantly reduced restenosis because it provides higher localized arterial drug levels (Hunter, 2006; Salam et al., 2006).

However, despite excellent short-term and intermediate-term clinical outcomes of DESs, recent increasing concerns about late adverse events plague these devices. Stent thrombosis is a severe complication after stent implantation, owing to its high morbidity and mortality (Genereux and Mehran, 2009; Luscher et al., 2007). The cause of late stent thrombosis is postulated to be multifactorial. The questionable biocompatibility of the materials used for stent preparation seems to be one of the principal causal factors (Luscher et al., 2007; Steffel and Tanner, 2007). Both Cypher and Taxus stents use non-erodible polymers as platforms for retardation of drug delivery (Salam et al., 2006). Many of the synthetic polymers induce an exaggerated inflammatory response in vivo (Azarbal and Currier, 2006; van der Giessen et al., 1996). Furthermore, local infiltration of eosinophils suggests hypersensitivity reactions to the polymer carriers (Luscher et al., 2007; Nebeker et al., 2006). These complications can consequently result in late adverse cardiac events in humans, such as late stent thrombosis (Luscher et al., 2007; Steffel

*Abbreviations:* %DR, percent drug released; $\%DR_{10h}$, percent drug released after 10 h; $\%DR_{72h}$, percent drug released after 72 h; $\%DR_{10h\text{-}p}$, percent drug released after 10 h in the presence of plasma; $\%DR_{72h\text{-}p}$, percent drug released after 72 h in the presence of plasma; Chol, cholesterol; DES, drug-eluting stent; DSPC, distearoylphosphatidylcholine; DSPG, distearoyl-sn-glycerophosphoglycerol; EE, entrapment efficiency; EEL, external elastic lamina; EL, empty liposome; EPC, egg phosphatidylcholine; IEL, internal elastic lamina; PC, phosphatidylcholine; SIR, sirolimus; SIR-L, sirolimus entrapped liposome; $T_m$, transition temperature.

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and Tanner, 2007) or late in-stent restenosis (Wessely et al., 2005). The presence and type of polymeric coating are believed to contribute to the rate of in-stent restenosis and the thrombogenic potential of DES because polymers can lead to acute and chronic vascular inflammation, adverse tissue reactions, delayed vascular healing and a prothrombotic environment (Finn et al., 2005; Luscher et al., 2007; Steffel and Tanner, 2007). Other drawbacks have also been reported for DESs. Some recent reports indicate poor attachment between the DES and the arterial wall and, even worse, aneurysms (Ong et al., 2005).

On the other hand, 30–40% of critical lesions cannot be stented, mainly because they occur at branch sites or in small and tortuous vessels (Scheller et al., 2004); they also reocclude more easily following stent placement. Moreover, coronary bifurcations, which account for up to 15% of all current percutaneous coronary interventions, still represent a challenging lesions subset due to a high restenosis rate, especially at side branches (Melikian et al., 2004; Sukhija et al., 2008).

To eliminate the previously mentioned drawbacks and limitations of permanent implants, other methods for prevention of restenosis beyond the drug-eluting-stents strategy are in great demand, particularly in vessels less amenable to stent therapy. Non-stent-based local delivery of antiproliferative drugs by means of colloidal carrier systems may offer additional flexibility and efficacy in the entire range of applications. Among these systems, biodegradable polymeric nanoparticles have shown a certain degree of success (Banai et al., 2005; Reddy et al., 2008). Despite the progress of the knowledge in this field, present limitations of polymer-based nanoparticles include the following: (a) their acidic degradation products and low surface to content ratio, which may lead to toxic local acid concentrations (Hunter, 2006), and (b) during polymer biodegradation, by-products including initiators, catalysts and solvents that are crucial to polymer processing are released, which often reduces biocompatibility (Commandeur et al., 2006).

Offering the advantage of higher biocompatibility, nanoliposomes have been proposed as a promising alternative to polymeric nanoparticles for local vascular drug delivery allowing sustained drug release at the injured site over a prolonged period of time.

Sirolimus (also known as Rapamycin) is a macrolide lactone antibiotic with profound antiproliferative and anti-inflammatory effects (Gallo et al., 1999; Marx et al., 1995). Additionally, in vitro studies show that SIR inhibits platelet-derived growth factor-induced migration of human vascular smooth muscle cells from the media to the intimal region, without affecting their cytoskeletal components or their ability to bind collagen (Poon et al., 1996).

In light of these considerations, the aims of the present study were to develop SIR liposomal formulation, identify the predominant formulation parameters and explore the formula's application for the treatment of restenosis following local vascular delivery. To overcome the technical challenge of producing SIR-Ls by the conventional method, which involves the passage of lipid and drug mixture through the extruder apparatus at temperatures above $T_m$ that could lead to decrease in EE and drug degradation, a novel method of drug entrapment, termed "remote film loading," was employed for the loading of SIR into liposomes. This method includes preparing empty liposomes of desired lipid composition and size, and then the trapping of drug occurs following a few minutes of sonication. This entrapment technique was reported by Sadzuka et al. (2005) for the first time and was used for the effective entrapment of SN-38, a lipophilic drug, into liposomes. To our knowledge, however, the applicability and usefulness of this method for other insoluble drugs has not been reported yet.

A $2^{4-1}$ fractional factorial design was applied to assess the effects of three formulation variables (lipid to drug molar ratio, mol% cholesterol content and bilayer lipid composition) and one technological factor (sonication time) on the EE and release profile as the key parameters that may affect the performance of nanoliposome formulation in this application. This design considerably reduces the number of preparations in such a way that the information required is obtained in the most effective and precise ways possible, carrying out the necessary experiments and identifying the key variables for a better understanding of the process (Braun et al., 2006; Hamoudeh et al., 2007; Loukas, 1998). Eight different liposomal formulations with duplicates of the center point were prepared and characterized in terms of EE, release profile, size distribution and zeta potential. We also present the antirestenotic efficacy of the SIR-loaded nanoliposomes in the rat carotid artery balloon injury model.

## 2. Materials and methods

### 2.1. Materials

Sirolimus was kindly provided by Wyeth Pharmaceuticals (New York, USA). Purified egg phosphatidylcholine (EPC), distearoylphosphatidylcholine (DSPC) and distearoyl-sn-glycerophosphoglycerol (DSPG) were obtained from Lipoid GmbH (Switzerland). Cholesterol (Chol, purity >99%) was purchased from Sigma Chemical Company (St. Louis, MO, USA). Chloroform, methanol, HPLC-grade acetonitrile, Tween 80 and sodium chloride were supplied by Merck (Darmstadt, Germany). Cellulose dialysis tubing (molecular weight cutoff 12,000 Da) was from BioGene (USA).

### 2.2. Liposome preparation

SIR-Ls were prepared by the remote film loading method developed by Sadzuka et al. (2005) as a novel method for liposomal entrapment of SN-38, a lipophilic drug. This method involves the production of drug film and the subsequent addition to empty liposomes (ELs).

#### 2.2.1. Preparation of empty liposomes (ELs)

ELs were prepared by the lipid film hydration method (Hope et al., 1985). Briefly, the lipid mixture of the desired molar composition was dissolved in chloroform/methanol (4:1) and dried under reduced pressure in a rotary evaporator (90 rpm) at 65 °C to form a thin lipid film. Evaporation was continued for 2 h after the dry residue appeared, to completely remove all traces of the solvent. The film was then hydrated with 0.9% NaCl at 65 °C for 1 h. The obtained multivesicular suspensions were extruded (Northern Lipids, Vancouver, BC, Canada) five times through each of 200 and 100 nm pore size Nucleopore polycarbonate membranes (Whatman, UK) to produce samples with a narrow size distribution. The extrusion was carried out at 65 °C to maintain vesicles above phase transition temperature.

#### 2.2.2. Remote film loading of liposomes

Standard solutions of SIR were prepared in methanol and then evaporated to form a thin layer film. Following the addition of ELs, the mixtures were sonicated for desired time at 60 °C using a high-energy bath-type sonicator (Starsonic, Liarre, Italy). The liposomal suspensions were allowed to stand at room temperature for 1 h. Unentrapped drug was separated by centrifugation at 14,000 rpm for 15 min (Immordino et al., 2003). For in vivo experiments, nanoliposomes were filtered through a 0.22 μm syringe filter to maintain their sterility.

### 2.3. Effect of variables

Influences of the different process parameters on both %EE and %DR were investigated by an experimental design methodology

**Table 1**
Investigated independent variables (factors) and their levels used in the experimental design.

| Variables | Unit | Range | Design levels | |
|---|---|---|---|---|
| | | | −1 | +1 |
| Chol proportion (*A*) | % | 10–25 | 10 | 25 |
| Sonication time (*B*) | min | 3–10 | 3 | 10 |
| DSPC to EPC molar ratio (*C*) | | 0–0.6 | 0 | 0.6 |
| Lipid to drug molar ratio (*D*) | | 30–60 | 30 | 60 |

using Design-Expert® software (Version 7, Stat-Ease Inc., Minneapolis, MN, USA). Based on the results obtained in preliminary experiments, Chol proportion (A), sonication time (B), DSPC to EPC molar ratio (C) and lipid to drug molar ratio (D) were selected as independent variables affecting %EE and %DR. Each factor was set at a high level and low level represented by a 1 or a −1 sign, respectively (Table 1). The EE, percent drug released after 10 h ($\%DR_{10h}$), percent drug released after 72 h ($\%DR_{72h}$), percent drug released after 10 h in the presence of plasma ($\%DR_{10h\text{-}p}$) and percent drug released after 72 h in the presence of plasma ($\%DR_{72h\text{-}p}$) were considered to be dependent variables. The effects of the four independent variables were studied through $2^{4-1}$ fractional factorial designs comprising 8 runs. To estimate the experimental error and possible response curvature, two center points were added, giving a total of 10 runs. All other production variables were kept at the same level. Each batch was prepared in duplicate, the analysis of EE and %DR was performed three times, and mean responses values were determined (Table 2). The liposome batches were produced in a random order to nullify the effect of extraneous or nuisance variables. Statistical comparisons were made with ANOVA. A difference with $P < 0.05$ was considered to be significant. Pareto charts, normal probability of effects and perturbation plots were used to analyze each response coefficient and compare the effect of all the factors.

### 2.4. Liposome characterization

The prepared liposomes were characterized in terms of EE, vesicle size, zeta potential and in vitro release in normal saline and in the presence of human plasma.

#### 2.4.1. Entrapment efficiency (EE)

Prepared lipid vesicles were separated from the free (untrapped) drug by centrifugation at 14,000 rpm for 15 min; in these conditions the liposomes remained suspended and the free drug precipitated. Small aliquots of liposomes (50 μl) were diluted in 950 μl methanol, were subjected to sonication until liposomes disruption and analyzed for SIR content by HPLC. The %EE was calculated from the amount of incorporated SIR divided by the total amount of drug used at the beginning of preparation multiplied by 100.

#### 2.4.2. Drug analysis

The amount of SIR in liposome formulations and in release medium (NaCl 0.9% containing 0.05% Tween 80) was determined using a validated HPLC method. The HPLC system consisted of a model K-1001 solvent delivery pump, a Wellchrom online degasser, a Rheodyne autoinjector equipped with a 100 μl loop, a model K-2600 UV detector (all from Knauer, Germany). Chromgate software (Version 317) was used to acquire and process all chromatographic data. The separation was achieved on a PerfectSil Target $C_8$ column, 4.6 mm × 250 mm, 5 μm particle size (MZ-Analysentechnik, Germany), using an isocratic mobile phase consisting of water and acetonitrile (25:75, v/v) as eluent at a flow rate of 1.5 ml/min with UV detection at 277 nm. The column temperature was kept at 35 °C. A linear response was observed over a concentration range of 0.020–2.000 μg/ml. The coefficients of variation for inter-day and intra-day assay were found to be less than 5.0%.

#### 2.4.3. Size distribution and zeta potential

Size and population distribution of EL and SIR-L were determined by dynamic light scattering measurements using a Malvern Zetasizer Nano ZS (Malvern Instruments, London, England). The analysis was performed at 25 °C after the dispersion had been diluted to an appropriate volume with NaCl (0.9%). The measurements were conducted in triplicate; results are presented as an average diameter of the liposome suspension (*z*-average mean).

Zeta potentials of the liposomes were measured in NaCl (0.9%) using 90 PLUS particle size analyzer with ZETA PALS system (Brookhaven Corp., Hostville, NY) at 25 °C.

#### 2.4.4. In vitro drug release

The in vitro release of SIR from the liposomal formulations was determined using cellulose dialysis tubing (molecular weight cut-off of 12 kDa). Liposomal solutions (0.5 ml) were placed in donor chamber and dialyzed against releasing medium (normal saline containing 0.05% Tween 80 (w/v) for achieving sink condition) at 37 °C, under 100 rpm magnetic stirring. Samples were removed at 1, 2, 4, 7, 10, 24, 34, 48 and 72 h. In the case of formulations 6 and 7, the samples were taken up to 21 days. To evaluate the effect of plasma proteins on drug release from liposomes, 0.5 ml of liposome suspension combined with 0.5 ml of human plasma was placed in the dialysis bag. Aliquots of release medium were withdrawn at predetermined time intervals up to 72 h. Each experiment was repeated in triplicate. In all cases, the incubated medium was replaced with a fresh one daily, and the samples were analyzed by HPLC. For the evaluation of release kinetics, the obtained release data were fitted into the first order, zero order and Higuchi equations. Selection of the best model was based on the comparisons of the relevant correlation coefficients. The data obtained were also fitted to the Ritger–Peppas model (Ritger and Peppas, 1987) for further mechanistic evaluation.

**Table 2**
The plan of fractional factorial design layout for SIR-loaded nanoliposomes with corresponding experimental results (mean ± SEM, $n = 3$).

| Formulation | *A* (%) | *B* (min) | *C* (ratio) | *D* (ratio) | %EE | $\%DR_{10h}$ | $\%DR_{72h}$ | $\%DR_{10h\text{-}p}$ | $\%DR_{72h\text{-}p}$ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 10.0 | 3.0 | 0.0 | 30 | 87.0 ± 1.7 | 13.2 ± 0.2 | 50.5 ± 0.2 | 16.1 ± 1.0 | 59.2 ± 0.1 |
| 2 | 25.0 | 3.0 | 0.6 | 30 | 73.4 ± 1.2 | 42.7 ± 1.1 | 63.2 ± 1.5 | 36.3 ± 2.6 | 70.6 ± 0.4 |
| 3 | 25.0 | 10.0 | 0.0 | 30 | 86.2 ± 0.5 | 26.4 ± 0.1 | 63.1 ± 1.2 | 24.4 ± 1.8 | 68.6 ± 1.8 |
| 4 | 17.5 | 6.5 | 0.3 | 45 | 89.3 ± 1.2 | 10.2 ± 0.3 | 45.7 ± 0.4 | 17.4 ± 1.1 | 61.0 ± 1.2 |
| 5 | 25.0 | 3.0 | 0.0 | 60 | 91.2 ± 0.9 | 20.7 ± 0.1 | 60.4 ± 3.7 | 20.6 ± 1.5 | 65.4 ± 1.7 |
| 6 | 10.0 | 3.0 | 0.6 | 60 | 83.5 ± 0.7 | 11.2 ± 1.3 | 43.9 ± 3.2 | 13.4 ± 1.6 | 59.0 ± 2.9 |
| 7 | 10.0 | 10.0 | 0.0 | 60 | 98.2 ± 1.4 | 9.0 ± 0.2 | 37.1 ± 0.4 | 10.6 ± 0.3 | 49.7 ± 0.4 |
| 8 | 10.0 | 10.0 | 0.6 | 30 | 85.7 ± 1.4 | 15.3 ± 0.1 | 57.7 ± 2.0 | 17.3 ± 1.6 | 63.6 ± 4.4 |
| 9 | 17.5 | 6.5 | 0.3 | 45 | 83.4 ± 1.2 | 12.9 ± 1.3 | 48.6 ± 0.5 | 19.4 ± 1.2 | 63.8 ± 0.6 |
| 10 | 25.0 | 10.0 | 0.6 | 60 | 87.8 ± 1.3 | 22.3 ± 1.2 | 39.7 ± 1.6 | 24.1 ± 0.5 | 67.8 ± 1.0 |

#### 2.4.5. Stability of formulations

The stability of liposomal formulations was evaluated at room temperature for a period of 30 days. At intervals, aliquots of samples were withdrawn, and the EE%, particle size and zeta potential of the liposomes were determined.

### 2.5. Animal experiments

#### 2.5.1. Animals

Male Sprague-Dawley rats weighing 350–400 g obtained from Razi Institute (Tehran, Iran) were used for animal studies. Animals were maintained in a controlled environment of 25 °C, 60% relative humidity and light cycle (7 AM to 7 PM), with free access to water and normal rat chow. Animals were allowed to acclimatize to animal facility condition for at least 1 week before starting the experiment. All protocols for this study were approved by the local ethics committee for animal experiment of Shaheed Beheshti University of Medical Sciences (Tehran, Iran).

#### 2.5.2. Balloon injury technique and local liposome delivery

All procedures were performed under sterile conditions. Rats were anesthetized with an i.p. injection of 50 mg/kg ketamine hydrochloride and 10 mg/kg xylazine, and then the ventral neck region was shaved and disinfected. Through a midline incision, the left external carotid artery, region of the bifurcation and the left common carotid artery were exposed. A 2F Fogarty balloon catheter (Edwards Lifesciences Corp., Irvine, CA, USA) was introduced into the external carotid artery through an arteriotomy and advanced proximally through the external carotid into the common carotid artery. The balloon was inflated with distilled water to an atmospheric pressure of 2 and was then gently withdrawn in a stepwise fashion to the entry point using a slight twisting motion. After three repetitions of this procedure, the endothelium was denuded (Tulis, 2007). Following removal of the catheter, two microvascular clips were used to clamp the proximal segment of common carotid and internal carotid arteries. A cannula was then inserted, and the segment of artery was irrigated with 0.15 ml of saline (control group, $n = 4$) or 0.15 ml of ELs (control group, $n = 4$) or with 0.15 ml of SIR-Ls (treated group, $n = 5$) at a mean pressure of 1 bar for 10 min. The external carotid artery was then ligated, and the wound closed. The clips were released, and blood flow was restored. Blood flow through the common and internal carotid arteries was confirmed by the presence of a strong pulse in both arteries.

#### 2.5.3. Tissue harvest and histological processing

Two weeks after balloon catheterization, animals were euthanized and the carotid arteries were flushed with saline and subsequently perfusion-fixed with 10% buffered formalin at mean arterial pressure via the left ventricle. Left common carotid arteries were harvested and subsequently processed for paraffin embedding. The segments were microtome sectioned. Each segment was stained with hematoxylin and eosin or Orcein-Giemsa stain. After staining the neointimal, medial and lumen areas, the neointima:media area ratio and the percent of luminal occlusion (% stenosis; evaluated as follows: neointimal area × 100/area bounded by IEL) for each arterial segment were calculated using a microscopical image analyzing system, Nikon Eclipse (Tokyo, Japan) and customized software. Histological differences were analyzed by two sample Student's *t*-test for differences in means (two-tailed) and the ANOVA test. Differences achieving $P < 0.05$ were interpreted as being statistically significant.

## 3. Results and discussion

Liposomes have represented a milestone in the field of innovative drug delivery systems for the encapsulation, prolonged and controlled delivery of active molecules to the site of action. Their attraction lies in their composition, which makes them biocompatible and biodegradable. Also, their structure and colloidal size along with a lack of immune system activation or suppression may be useful in various applications (Immordino et al., 2006; Metselaar et al., 2002). Considering all these desirable properties as well as the necessity for improving hemocompatibility, we aimed to develop and optimize the SIR loaded nanoliposomes to prevent restenosis by local arterial delivery.

### 3.1. Nanoliposome preparation

There are many methods of liposome preparation, and a suitable method is selected according to the physical properties of the entrapped drugs. For SIR liposomes, the preparation was first attempted using the thin-film hydration technique, which is the conventional and most common technique for liposome preparation, followed by extrusion. Surprisingly, we observed a strong decrease in EE during the extrusion process, and therefore we could not achieve a high ratio of SIR entrapped in the liposome. Additionally, stability studies showed that SIR was stable at high temperature (65 °C) for about 30–40 min (data not shown). Overall, the thin-film hydration method did not appear suitable for SIR liposome preparation, especially because we intended to evaluate the effect of the presence of rigid lipids (DSPC) on in vitro properties and in vivo efficacy of liposomal SIR. Next, the ethanol injection method (Meure et al., 2008) was tried, but it was not effective due to the relatively large particle size of the liposomes (around 240 nm) and the production of an inhomogeneous population of particles; thus, further extrusion was essential to achieve a mean particle size of 100 nm. The remote loading method is another approach for liposome preparation, and it is successfully used for drugs that are weak bases by exerting a transmembrane pH gradient (Webb et al., 2007). However, there are important drugs like SIR that do not have the weakly basic amino groups required for drug loading; therefore, they cannot be stably encapsulated and retained in liposomal carriers by this method. To overcome these problems and the challenge of producing liposomal SIR by the conventional method, which involves the passage of a mixture of the drug and lipids through the extruder apparatus at a temperature above the phase transition of the phospholipid, we investigate the remote film loading method. This method has been reported by Sadzuka et al. (2005) as a novel method for effective entrapment of SN-38, a lipophilic drug. However, to our knowledge, the applicability and usefulness of this method for other insoluble drugs has not been reported yet. This method involved the production of SIR film and the subsequent addition of preformed liposomes containing different lipid compositions. The drug was entrapped by sonication after a few minutes at temperatures above $T_m$, which resulted in a significantly higher EE compared to sonication at lower temperatures. The membrane permeability of PC-based vesicles is increased as temperature increases due to expansion in the vesicle area (Xiang and Anderson, 1998). The EE of SIR in liposomes by the remote film loading method was about 5–10 times that of the previously mentioned methods. This novel method of liposomalization extends the benefits of liposomal carriers to hydrophobic drugs and particularly could be advantageous for drugs with limited thermal stability, like SIR. As mentioned before, SN-38 was successfully entrapped by this method (Sadzuka et al., 2005), but the different parameters of the method were not elaborated.

### 3.2. Experimental design

Liposomal preparation as well as performing release studies require costly material and are time-consuming. Therefore, it is useful to employ an experimental design for screening the fac-

tors affecting the entrapment efficiency and release rate. The more efficient approach to study these factors and how they affect the examined responses, would be to vary the factors of interest in a $2^k$ full factorial design ($k$ factors at two level), that is, to try all possible combinations. This would work fine, except that the number of necessary liposomal preparations will increase geometrically. The use of a $2^{(k-n)}$ fractional factorial design can reduce considerably the number of preparations, give useful conclusions for the main effects and interactions between the examined factors, and shorten the time necessary for the development of effective formulations (González-Rodríguez et al., 2007; Loukas, 1998).

A fractional factorial design was therefore used to formulate and screen process parameters for the incorporation of SIR into a nanoliposome by the remote film loading method, which ultimately yields a formulation with maximum EE and minimum release rate. Such an experimental design enabled the evaluation of the effect of variables at two different levels on selected responses. First-order models were employed, and their adequacy was investigated by adding center points to the design data set to evaluate a possible response curvature.

### 3.3. Statistical analysis of experimental data

Because the lipid composition and concentration could have a significant impact on developing a therapeutically efficient liposomal carrier system, we examined different properties of the liposomes prepared at varying Chol proportions, DSPC to EPC molar ratios and lipid to drug molar ratios. We also considered sonication time as another process variable. The range of each variable was chosen so as to not only measure its effects on the studied responses but to also encompass all of the preparation conditions that were likely to be encountered during preparation process (Table 1). The ranges of Chol, DSPC, and EPC concentrations and lipid to drug molar ratios were initially determined to obtain stable liposomes devoid of aggregation, fusion and sedimentation (visual observation, data not shown). Increasing the Chol proportion and DSPC/EPC ratio to more than 25% and 0.6, respectively, and a further decreasing the lipid to drug molar ratio (lower than 30) caused instability of the formulations. Because the removal of Chol in the absence of pegylated lipids has introduced problems with regard to in vivo stability and the disposition of liposomes (Drummond et al., 2008), a further decrease in Chol content (lower than 10%) was not investigated. The extreme values assigned to the independent variables were checked in order to establish that nanoliposomes could be prepared under these conditions. DSPG at 10% was incorporated into all formulations because the inclusion of a small quantity of a negatively charged phospholipid was shown to have a positive influence on the EE. This observation was in accordance with the observations of Dai et al. (2005).

Following preliminary studies, a $2^{4-1}$ factorial design was used, and ten batches of SIR-Ls were prepared in duplicate and in a random order, varying of four independent variables, as shown in Table 2. After performing calculations of the responses (%EE, $\%DR_{10h}$, $\%DR_{72h}$, $\%DR_{10h\text{-}p}$, $\%DR_{72h\text{-}p}$), analysis of data was carried out using ANOVA, and the individual parameters were evaluated with the $F$-test.

Each response coefficient was studied for its statistical significance by Pareto charts as shown in Fig. 1. These graphs show the ANOVA effect estimates. The effects that pass through the $t$ limit line are designated as the significant coefficient, while a $t$ value of effect below the $t$ limit line is considered a statistically insignificant coefficient and should be removed from the analysis. Another useful, albeit more technical summary graph, is the normal probability plot of effects. From this kind of plot, the factors that contributed significantly to the selected responses would appear as outliners. Another equally advantageous tool used to compare the effect of all the factors is the perturbation plot (Fig. 2). The selected responses are plotted by changing only one factor over its range while holding all of the other factors constant. The slope or curvature in a factor shows the sensitivity of the response to that factor.

### 3.4. Drug entrapment efficiency

In the field of nanotechnology, EE is an important index to characterize drug delivery systems. A high EE would be beneficial in incorporating the required dose in the minimum volume, facilitating local administration. Here, the EE ranged between 73% and 98% (Table 2). The EE achieved was higher than that reported by the only published study on SIR liposomal formulation (maximum EE was 93% for the liposomal carrier with a mean particle size of 181 nm) (Rouf et al., 2009). Moreover, in the mentioned study, the utilized method for the removal of unencapsulated drug (dialysis against pure water) seems to not be efficient due to the limitation of pure water in the maintenance of sink condition in the case of the practically insoluble drugs like SIR.

From the ANOVA test, the model was found to be significant ($P < 0.05$), and the DSPC to EPC molar ratio ($C$), lipid to drug molar ratio ($D$) and sonication time ($B$) have a direct relationship with EE. The lack of fit and curvature were statistically insignificant; therefore, the first-order model chosen was considered to be a suitable model. The results of ANOVA for full and reduced models of responses confirmed the omission of non-significant terms. After removing the insignificant variables (Fig. 1A), the equation of %EE in terms of coded factors was simplified to the following equation:

$$Y1\,(\%EE) = 86.63 + 2.85B - 4.03C + 3.55D \quad (1)$$

The positive signs for the coefficients of terms $D$ and $B$ in Eq. (1) show that EE can be increased by an increase in $D$ and $B$. In contrast, the negative sign for the coefficient of term $C$ shows that EE increases by a decrease in the value of the $C$ variable, which is also evident from the perturbation plot (Fig. 2A). The highest value for the coefficient of the $C$ variable in Eq. (1) indicates that the DSPC to EPC molar ratio (i.e. variable $C$) is the most dominant factor concerning the EE. Indeed, a higher extent of incorporation was observed when using EPC-membranes as compared to DSPC-membranes. Because the entrapment of lipophilic drugs occurs mainly through partitioning into the membrane (Nallamothu et al., 2006), this higher incorporation might be explained by the lower rigidity of the EPC membrames; this could improve the interaction between the lipophilic and bulky molecules of SIR and the liposome membrane. Additionally, when using EPC, a high degree of miscibility between the lipid components and the drug may be provided (Desai et al., 2002).

Lipids are the major structural components of liposomes and therefore have great influence on fluidity characteristics of liposomal membranes. Depending on the chain length and the degree of saturation, lipids show different $T_m$ values. DSPC contains a saturated C18 fatty acid and forms rigid membranes. Liposomes composed of this lipid are in the gel state, whereas EPC liposomes have a mixture of phospholipids of different chain lengths and varying degrees of saturation and are in the liquid crystalline state; hence, regions of high bilayer disorder exist (Drummond et al., 1999). Because the characteristics of these lipid compositions differ widely; thus, there was significant influence of the lipid on the amount of drug incorporated.

As shown in Fig. 2A, contrary to DSPC proportion, the lipid to drug molar ratio (variable $D$) had a positive influence on the EE, which is in accordance with other published data (Nallamothu et al., 2006; Xiong et al., 2009). This result can be explained by the fact that increasing the lipid concentration level leads to an increase in the number of liposomes present per milliliter and, therefore, to an

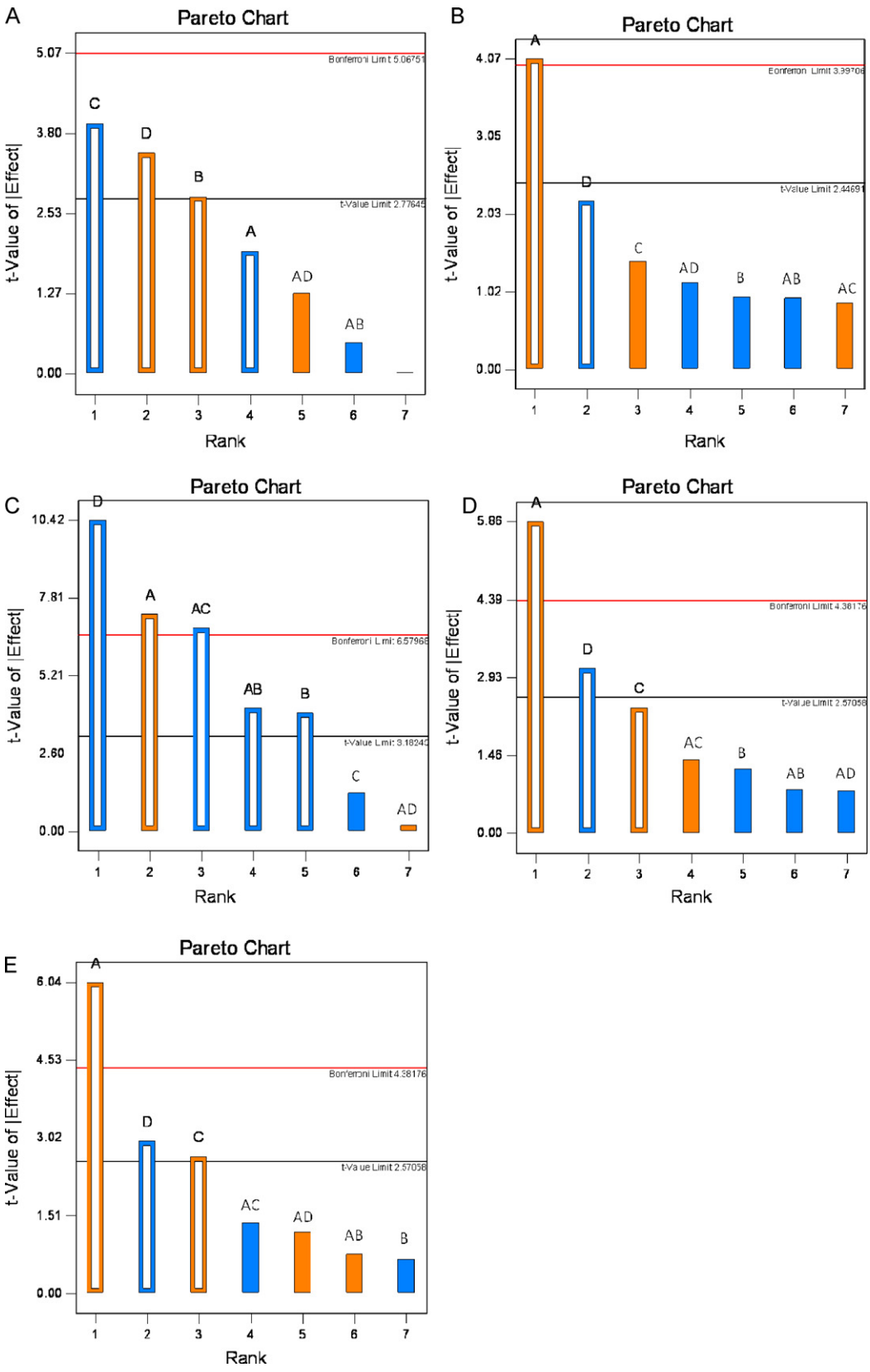



**Fig. 1.** Response coefficient significance study on (A) EE, (B) $\%DR_{10h}$, (C) $\%DR_{72h}$, (D) $\%DR_{10h\text{-}p}$ and (E) $\%DR_{72h\text{-}p}$ of SIR-entrapped liposomes by Pareto charts.

increase in the total volume for drug incorporation per milliliter of the formulation (Zuidam et al., 2003). Nevertheless, due to handling difficulties of suspensions at very high lipid concentrations, a lipid to drug molar ratio of 60 was chosen as the upper limit. As shown in Table 2, a fairly high EE (about 85%) was achieved by a lipid to drug ratio as low as 30; this ratio is much lower than that used in the previous report for the preparation of SIR-entrapped liposomes (Rouf et al., 2009).

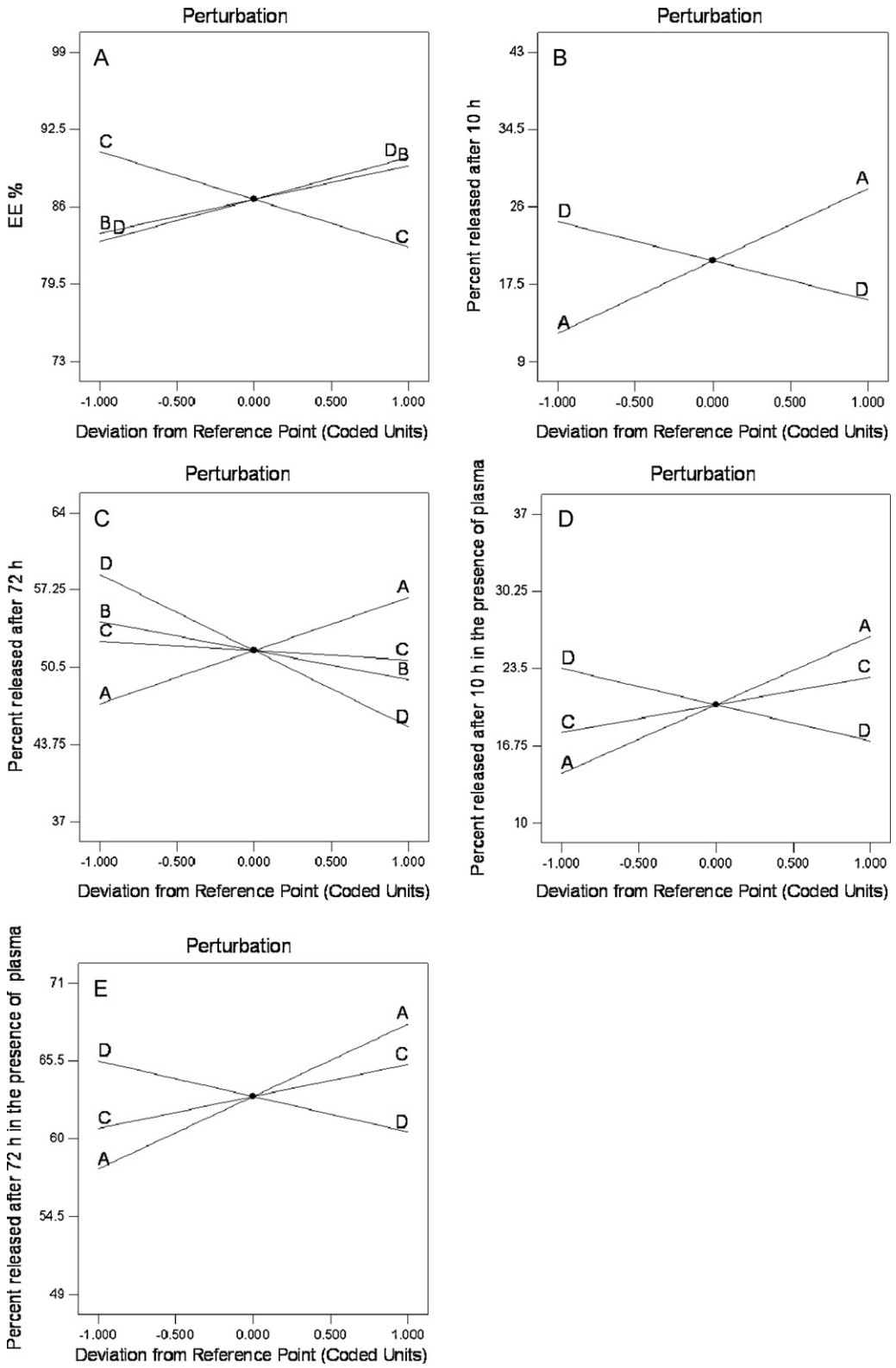
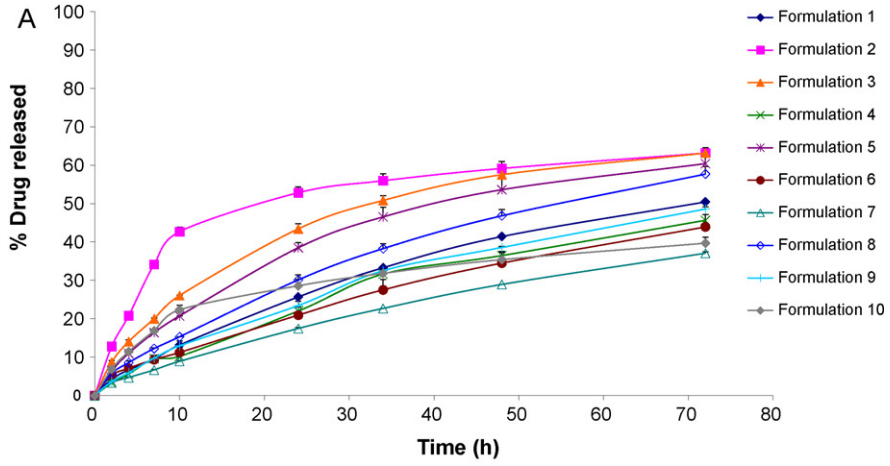
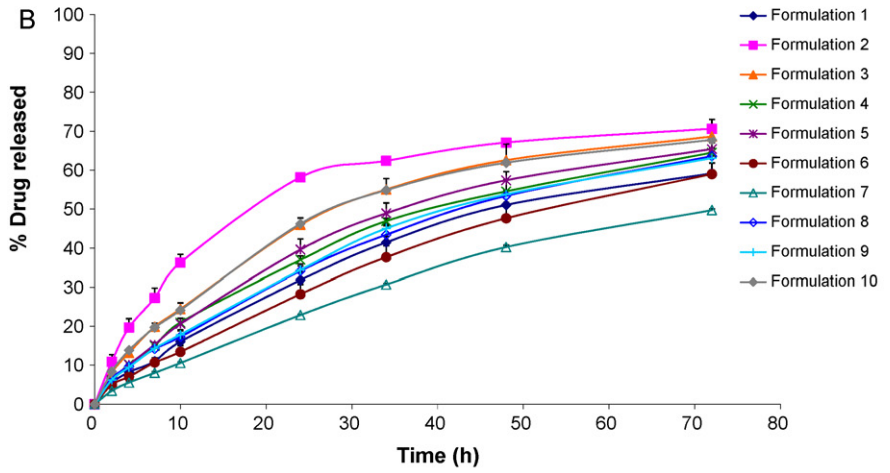



**Fig. 2.** Perturbation plots of different responses: (A) Chol proportion, (B) sonication time, (C) DSPC to EPC molar ratio, and (D) lipid to drug molar ratio.

The effect of the sonication time on the EE% was also studied, and it was found to be negligible. A longer sonication time showed only a minor positive effect on the EE (Fig. 2A).

Increasing the content of Chol (variable *A*) from 10% to 25% did not significantly affect the EE. The upper level for the Chol proportion was considered to be 25% because the higher Chol levels markedly affected the stability of SIR liposomes and resulted in rapid aggregation of vesicles in the trial runs in our preliminary studies. High levels of Chol have been reported to interfere with the close packing of lipids in the vesicles (Coderch et al., 2000), thereby reducing the encapsulation of the hydrophobic SIR. More-



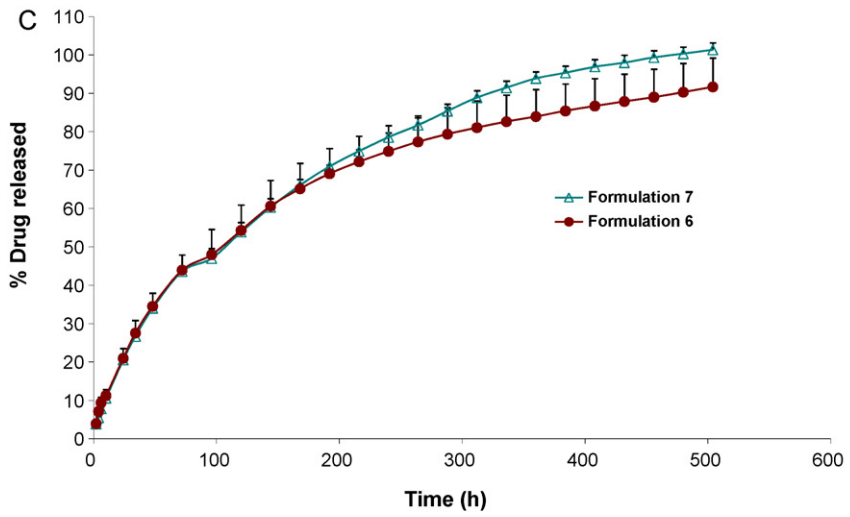




**Fig. 3.** In vitro release profile of SIR from different liposomal formulations in: (A) normal saline containing Tween 80 and (B) in the presence of plasma. (C) 3 weeks in vitro release profile of formulas 6 and 7 in normal saline containing Tween 80. Data represent means ± SEMs ($n = 3$).

over, Chol is known to increase membrane rigidity and packing density by accumulating in the molecular cavities formed by the phospholipid molecules assembled into bilayer vesicles (Semple et al., 1996), which may result in decreased bilayer partitioning and hydrophobic space available for the incorporation of hydrophobic drugs like paclitaxel (Zhang et al., 2005) and nystatin (Moribe et al., 1999).

### 3.5. Particle size and zeta potential

The mean particle sizes of EL and SIR-L, as measured with the particle size analyzer, were in the range of 94–126 nm, with a polydispersity index always lower than 0.2, which is proof that the samples were relatively monodispersed. Changes in particle size with changes in the operating variables were not significant.

**Table 3**
Kinetic fitting results of sirolimus released from drug entrapped nanoliposomes.

| Formulation | Correlation coefficient | | | $K^a$ ($h^{-0.5}$) | Peppas model | |
|---|---|---|---|---|---|---|
| | Zero order | First order | Higuchi | | $n^b$ | Correlation coefficient |
| 1 | 0.983 | 0.901 | 0.998 | 0.069 | 0.72 | 0.999 |
| 2 | 0.879 | 0.792 | 0.945 | 0.082 | 0.47 | 0.956 |
| 3 | 0.972 | 0.901 | 0.997 | 0.092 | 0.60 | 0.997 |
| 4 | 0.985 | 0.884 | 0.996 | 0.062 | 0.76 | 0.998 |
| 5 | 0.980 | 0.908 | 0.998 | 0.089 | 0.68 | 0.998 |
| 6 | 0.992 | 0.934 | 0.995 | 0.056 | 0.62 | 0.996 |
| 7 | 0.991 | 0.920 | 0.996 | 0.049 | 0.70 | 0.998 |
| 8 | 0.983 | 0.908 | 0.998 | 0.076 | 0.67 | 0.998 |
| 9 | 0.986 | 0.880 | 0.998 | 0.064 | 0.75 | 0.999 |
| 10 | 0.918 | 0.815 | 0.975 | 0.045 | 0.47 | 0.976 |

[a] Release rate constant calculated based on the Higuchi model.
[b] Diffusion exponent calculated based on the Peppas model.

Zeta potential is the electric charge on the surface of a particle, which creates an electrical barrier and acts as a 'repulsive factor' and prevent the aggregation of the spheres. The zeta potential of empty vesicles and drug loaded liposomes were found to be in the range of (−10 to −8) and (−8 to −5), respectively, due to the presence of the negatively charged DSPG. The aggregation of neutral liposomes is brought about by Van der Waals interactions. Small concentrations of charged lipids can provide sufficient electrostatic repulsion to prevent the aggregation of the particles upon the addition of hydrophobic drugs to the membrane (Drummond et al., 2008). Small quantities (e.g., 10%) of negatively charged cardiolipin or phosphatidylglycerol have been used to stabilize hydrophobic drugs, such as paclitaxel or the highly active camptothecin, SN-38 (Sharma and Straubinger, 1994; Zhang et al., 2004).

### 3.6. In vitro drug release study

The release profile of SIR from liposomal formulations in normal saline (containing 0.05% (w/v) Tween 80) and in the presence of human plasma are shown in Fig. 3A and B, respectively. No significant burst effect was observed. All profiles showed biphasic behavior, and the release rate of the primary phase was faster than the steady release phase. Considering that SIR is mainly associated within the bilayer lipid structure of the liposomes, the initial release is primarily ascribed to drug detachment from the outer lamellae and drug adsorbed on or close to the surface of the particles. Furthermore, the smaller particles of nanoliposomes are associated with a smaller diffusion path, so drug that is accessible to dissolution medium interface can diffuse easily. The later, slow release results from sustained drug release from the inner lamellae and is assumed to be controlled by the diffusion rate of the drug across the liposomal bilayer. The drug release was faster when liposomes were incubated in human plasma (Fig. 2B), a result that could be attributed to the presence of several host-derived factors, including albumin protein; these proteins could displace the phospholipids in the liposomes, resulting in a faster rate of drug leakage (Ramana et al., 2010).

#### 3.6.1. Effect of variables on the drug release

A summary of the effects of the different parameters on the average release values after 10 and 72 h is presented in Table 2. These time intervals were chosen because the effects caused by the variations in the different parameters were clearer after longer time periods.

The values of percent drug released for the 10 batches showed a wide variation, indicating that the release profile is strongly dependent on the selected variables. The significance of the variables affecting the drug release rate can be verified from the Pareto chart shown in Fig. 1. In Fig. 2, an overview of the effects of the different variable parameters on the percent drug released values is given.

The insignificant terms were neglected from full models, and the equations for reduced models (Eqs. (2)–(5)) were obtained following multiple regressions of each dependent variable ($\%DR_{10h}$ and $\%DR_{72h}$ in buffer and $\%DR_{10h\text{-}p}$ and $\%DR_{72h\text{-}p}$ in plasma) and significant terms ($P < 0.05$).

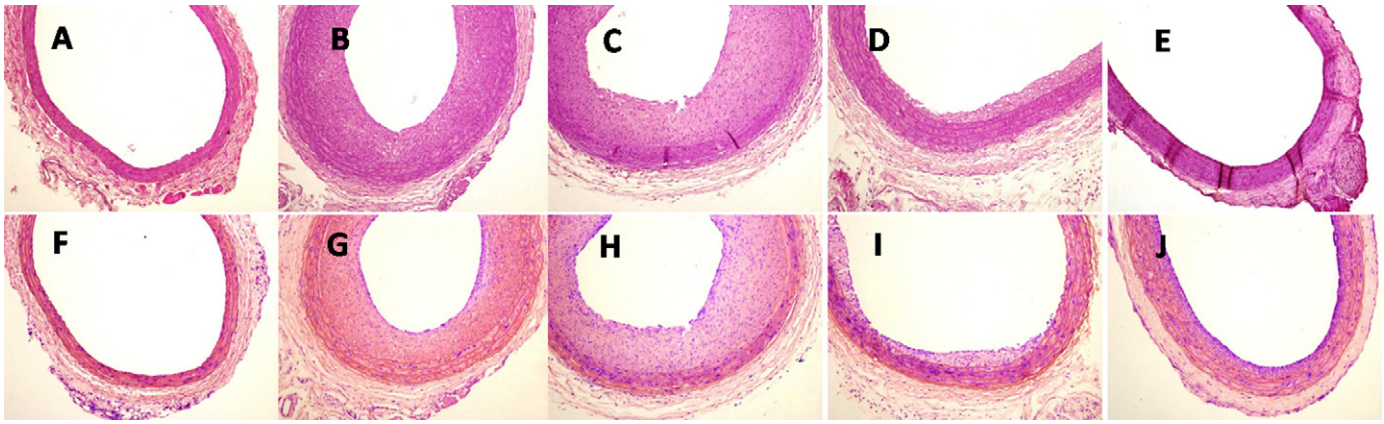



**Fig. 4.** Representative photomicrographs of rat common carotid arteries taken 14 days after balloon injury. The upper figures indicate the results of hematoxylin-eosin staining, and the lower ones are the results of elastic fibers staining (Orcein-Giemsa). (A) and (F): uninjured/untreated group, (B) and (G): saline group, (C) and (H): empty liposomes, (D) and (I): formula 7 group, and (E) and (J): formula 6 group. Neointimal area and % stenosis are markedly reduced in vessel segments treated with sirolimus-loaded nanoliposomes as compared to control segments treated with saline or empty liposomes under similar conditions.

**Table 4**
Stability of nanoliposomes at 25 °C.

| Formulation | Entrapment efficiency (%) Mean ± SEM | | Size (nm) Mean ± SEM | | Zeta potential (mV) Mean ± SEM | |
|---|---|---|---|---|---|---|
| | Day 0 | Day 30 | Day 0 | Day 30 | Day 0 | Day 30 |
| 6 | 83.5 ± 0.7 | 79.7 ± 2.4 | 111 ± 2.4 | 122 ± 2.0 | −8 ± 1.5 | −5 ± 1.7 |
| 7 | 98.2 ± 1.4 | 90.4 ± 0.9 | 105 ± 3.3 | 128 ± 3.8 | −7 ± 1.9 | −6 ± 2.1 |

$$Y2\,(\%DR_{10h}) = 20.10 + 7.93A - 4.03D \quad (2)$$

$$Y3\,(\%DR_{72h}) = 51.95 + 4.65A - 2.55B - 6.67D - 2.64AB - 4.35AC \quad (3)$$

$$Y4\,(\%DR_{10h\text{-}p}) = 20.35 + 6.00A + 2.42C - 3.17D \quad (4)$$

$$Y5\,(\%DR_{72h\text{-}p}) = 63.00 + 5.13A + 2.26C - 2.51D \quad (5)$$

From the equations and perturbation plots, it becomes noticeable that the Chol content (variable *A*) and the lipid to drug molar ratio (variable *D*) were the most influential parameters; they had a positive and a negative impact on the release rate, respectively. The main effect of sonication time was negligible.

It is well accepted that membrane composition governs drug release rate. It is clear from the previously mentioned equations that in the case of $\%DR_{10h}$, the Chol content was the most dominant factor. However, after 72 h, the lipid to drug molar ratio (variable *D*) was considered to be the major contributing factor to the drug release rate. Overall, as indicated by perturbation plots (Fig. 2), the higher lipid to drug molar ratio and the lower Chol proportion were necessary to minimize the drug release rate.

Incorporation of a higher Chol concentration in liposomal formulations increased the SIR release rate. The planar and rigid ring system of Chol is thought to reside in the outer layer of the fatty acyl chain region, where it tends to restrict the motion of chains in liquid crystalline bilayers (Lagerquist et al., 2001; Robinson et al., 1995), which may increase the tendency of SIR to partition into the release medium. Moreover, an increase in Chol content beyond a certain concentration can disrupt the regular structure of the liposomal membrane (El-Samaligy et al., 2006).

To simulate the highly complex composition of the physiological fluids, including the presence of proteins, on the release of SIR from the liposomes, SIR-L combined with human plasma was placed in the dialysis bag. After incubation in plasma, the situation was different (Figs. 1 and 2), and in addition to the content of Chol and the lipid to drug molar ratio, the release rate was dependent on the DSPC proportion. The effect of the Chol proportion was found to be the most prominent as indicated by the observed values for the respective coefficients (Eqs. (4) and (5)). The in vitro release of the current formulations in the absence and presence of physiologically relevant concentrations of protein together suggest that these formulations may not self-aggregate or adsorb significant amounts of protein following arterial administration. In this way, these studies provide a preliminary indication of the behavior of the formulation in vivo.

### 3.6.2. Release kinetics

To investigate the release kinetics, data obtained from the in vitro release tests were fitted into the zero-order and first-order models, also into the square-root of time model (Higuchi model, $M_t/M_\infty = kt^{0.5}$). The model with the largest correlation coefficient was judged to be the best apparent model for the release data. Based on the results (Table 3), the release of SIR from nanoliposomes followed Higuchi's controlled release model, as has been previously reported for drug-loaded liposomes (Vali et al., 2008). This result suggests that the drug transport out of the liposomes was driven mainly by a diffusion-controlled mechanism.

Data obtained from in vitro release studies were also fitted to the Ritger–Peppas model ($M_t/M_\infty = kt^n$) for further mechanistic evaluation. As shown in Table 3, the value for *n* (diffusional exponent characteristic of the release mechanism) was between 0.47 and 0.76, suggesting a non-fickian diffusion process and anomalous transport (Ritger and Peppas, 1987). The correlation coefficient values for this model indicate a good model fit. No changes were observed in the release kinetics of SIR from nanoliposomes by varying different factors.

### 3.7. Selected formulations for further in vitro and in vivo studies

Based on the obtained results (Table 2), formulation 7, with the highest EE and slower drug release, was considered to be the most suitable formulation. It is worth mentioning that the in vitro release results were consistent with those of the EE; i.e., the liposomes with an EPC:Chol:DSPG (8:1:1) molar ratio and the highest loading efficiency (formulation 7) showed the lowest drug release percentage. It is believed that an ideal antirestenotic drug carrier should deliver drug for at least 3 weeks to prevent smooth muscle cell migration and proliferation (Acharya and Park, 2006). Therefore, the release profile of SIR from this formulation was investigated for up to 21 days, as shown in Fig. 3C. During the 21-day period, the entire SIR content was released from the system at a reasonable sustained rate. Because we wanted to study the effect of DSPC proportion

**Table 5**
Comparison of histomorphometric measurements of balloon-injured rat carotid arteries among treatment groups 14 days post-procedure (mean ± SEM).

| Parameters | Saline | Empty liposome | Formula 7[a] | Formula 6[b] |
|---|---|---|---|---|
| EEL area[c] ($mm^2$) | 0.140 ± 0.006 | 0.145 ± 0.001 | 0.161 ± 0.012 | 0.156 ± 0.010 |
| IEL area[d] ($mm^2$) | 0.104 ± 0.006 | 0.104 ± 0.003 | 0.119 ± 0.008 | 0.111 ± 0.010 |
| Neointima area ($mm^2$) | 0.052 ± 0.003 | 0.060 ± 0.005 | 0.017 ± 0.003 | 0.020 ± 0.004 |
| Media area ($mm^2$) | 0.036 ± 0.001 | 0.041 ± 0.002 | 0.042 ± 0.005 | 0.045 ± 0.002 |
| Lumen area ($mm^2$) | 0.052 ± 0.008 | 0.044 ± 0.007 | 0.102 ± 0.009 | 0.091 ± 0.014 |
| Neointima/media area ratio | 1.459 ± 0.148 | 1.483 ± 0.150 | 0.412 ± 0.091 | 0.432 ± 0.101 |
| % Stenosis | 50.52 ± 5.30 | 57.81 ± 5.52 | 14.41 ± 2.79 | 18.57 ± 5.70 |

[a] Lipid/drug ratio: 60, Chol:EPC:DSPG 1:8:1.
[b] Lipid/drug ratio: 60, Chol:EPC:DSPC:DSPG 1:5:3:1.
[c] Area bounded by external elastic lamina.
[d] Area bounded by internal elastic lamina.

on in vivo efficacy of formulation, we also studied the long-term release characteristics of formula 6 (Fig. 3C).

*3.7.1. Stability study of selected liposomes*

The physical and chemical stabilities of formulations 6 and 7 were evaluated at room temperature. The liposomes were stable for at least 1 month at this temperature, with negligible changes in EE, size and zeta potential. The results of the stability study are shown in Table 4.

*3.7.2. Effects of local delivery of sirolimus-loaded nanoliposomes on neointimal hyperplasia following vascular injury*

To avoid long-term complications after stent placement, a therapeutic strategy that is focused on preventing hyperplasia through intraluminal delivery of colloidal drug carrier systems at the target artery using a catheter might be more effective. Toward this goal, we investigated the effects of local application of SIR-L in balloon-injured rat carotid arteries.

As Fig. 4 shows, balloon injury induced a marked increase in the neointimal area and resulted in stenosis compared to uninjured/untreated vessels. The saline control and ELs demonstrated a comparable extent of hyperplasia, indicating that nanoliposomes themselves do not cause any change in the proliferative response. The effects of local intramural therapy with SIR-Ls on neointimal hyperplasia compared to vehicle treatment and EL administration in injured rat carotid arteries are shown in Fig. 4 and Table 5.

Local delivery of formulations 6 and 7 showed a significant reduction in the neointimal formation ($P < 0.005$), the neointima area to media area ratio ($P < 0.005$) and the percentage of stenosis ($P < 0.01$) as compared to the controls. The luminal area of the SIR-L treated group was also significantly enlarged ($P < 0.05$). However, the medial area was unchanged, suggesting that the medial wall can retain its integrity despite the administration of SIR (Fig. 4 and Table 5). SIR-Ls used in these experiments were designed with the following combination of properties, which are required for a safe and efficacious drug carrier: (1) small-sized particles and a flexible structure that could penetrate the arterial wall, (2) fully biocompatible components, and (3) sustained drug release properties.

Lipid composition and bilayer fluidity can have a considerable impact on the biodisposition of both the liposome and the associated drug. Although the effects of bilayer composition on the clearance and efficacy of drug-incorporated liposomal carriers following intravascular administration have been studied over the years, to our knowledge, these have not been studied for the local delivery of nanoliposomes at the injured arteries yet. The choice of lipids can affect the local fate of a liposomal drug, either directly by affecting the clearance of the liposomes from the target site or indirectly through modulation of the rate of drug release. To evaluate the role of the phospholipid component on the antirestenosis efficacy of SIR nanoliposomes, formulations 6 and 7 were selected for in vivo studies. Results revealed that there were no significant differences between histomorphometric measurements of the two groups.

## 4. Conclusion

The present study focused on the preparation and characterization of liposomes for sirolimus, a potent antiproliferative drug, by the remote film loading method. The efficacy of the prepared nanoparticles for the treatment of restenosis following local vascular delivery was also evaluated. The fractional factorial design employed in this study, successfully identified the predominant formulation parameters that influenced both EE and the release profile. The size of the liposomes ranged from 94 to 126 nm, and the maximum drug loading was 98%. In vitro release studies showed that nanoliposomes could successfully control the release of SIR for up to 3 weeks. Local administration of the particles was associated with a significant decrease in neointimal formation and the percentage of stenosis. The results show that the intramural delivery of SIR-entrapped nanoliposomes offers a promising approach for the treatment of restenosis after angioplasty. By combining an efficient drug carrier administered locally at the injured blood vessel and the sustained drug release properties of the biocompatible lipid-based nanoparticulates, this strategy could provide a safer and more effective alternative to current clinically used therapies for restenosis after angioplasty.

## Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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## References

Acharya, G., Park, K., 2006. Mechanisms of controlled drug release from drug-eluting stents. Adv. Drug Deliv. Rev. 58, 387–401.
Azarbal, B., Currier, J.W., 2006. Allergic reactions after the implantation of drug-eluting stents: is it the pill or the polymer? J. Am. Coll. Cardiol. 47, 182–183.
Banai, S., Chorny, M., Gertz, S.D., Fishbein, I., Gao, J., Perez, L., Lazarovichi, G., Gazit, A., Levitzki, A., Golomb, G., 2005. Locally delivered nanoencapsulated tyrphostin (AGL-2043) reduces neointima formation in balloon-injured rat carotid and stented porcine coronary arteries. Biomaterials 26, 451–461.
Braun, E., Wagner, A., Furnschlief, E., Katinger, H., Vorauer-Uhl, K., 2006. Experimental design for in vitro skin penetration study of liposomal superoxide dismutase. J. Pharm. Biomed. Anal. 40, 1187–1197.
Burt, H.M., Hunter, W.L., 2006. Drug-eluting stents: an innovative multidisciplinary drug delivery platform. Adv. Drug Deliv. Rev. 58, 345–346.
Coderch, L., Fonollosa, J., De Pera, M., Estelrich, J., De La Maza, A., Parra, J.L., 2000. Influence of cholesterol on liposome fluidity by EPR. Relationship with percutaneous absorption. J. Control. Release 68, 85–95.
Commandeur, S., van Beusekom, H.M., van der Giessen, W.J., 2006. Polymers, drug release, and drug-eluting stents. J. Interv. Cardiol. 19, 500–506.
Costa, M.A., Simon, D.I., 2005. Molecular basis of restenosis and drug-eluting stents. Circulation 111, 2257–2273.
Dai, C., Wang, B., Zhao, H., Li, B., 2005. Factors affecting protein release from microcapsule prepared by liposome in alginate. Colloids Surf. B Biointerfaces 42, 253–258.
Desai, T.R., Wong, J.P., Hancock, R.E., Finlay, W.H., 2002. A novel approach to the pulmonary delivery of liposomes in dry powder form to eliminate the deleterious effects of milling. J. Pharm. Sci. 91, 482–491.
Drummond, D.C., Meyer, O., Hong, K., Kirpotin, D.B., Papahadjopoulos, D., 1999. Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. Pharmacol. Rev. 51, 691–743.
Drummond, D.C., Noble, C.O., Hayes, M.E., Park, J.W., Kirpotin, D.B., 2008. Pharmacokinetics and in vivo drug release rates in liposomal nanocarrier development. J. Pharm. Sci. 97, 4696–4740.
El-Samaligy, M.S., Afifi, N.N., Mahmoud, E.A., 2006. Increasing bioavailability of silymarin using a buccal liposomal delivery system: preparation and experimental design investigation. Int. J. Pharm. 308, 140–148.
Finn, A.V., Kolodgie, F.D., Harnek, J., Guerrero, L.J., Acampado, E., Tefera, K., Skorija, K., Weber, D.K., Gold, H.K., Virmani, R., 2005. Differential response of delayed healing and persistent inflammation at sites of overlapping sirolimus- or paclitaxel-eluting stents. Circulation 112, 270–278.
Gallo, R., Padurean, A., Jayaraman, T., Marx, S., Roque, M., Adelman, S., Chesebro, J., Fallon, J., Fuster, V., Marks, A., Badimon, J.J., 1999. Inhibition of intimal thickening after balloon angioplasty in porcine coronary arteries by targeting regulators of the cell cycle. Circulation 99, 2164–2170.
Genereux, P., Mehran, R., 2009. Are drug-eluting stents safe in the long term? CMAJ 180, 154–155.
González-Rodríguez, M.L., Barros, L.B., Palma, J., González-Rodríguez, P.L., Rabasco, A.M., 2007. Application of statistical experimental design to study the formulation variables influencing the coating process of lidocaine liposomes. Int. J. Pharm. 337, 336–345.
Hamoudeh, M., Salim, H., Barbos, D., Paunoiu, C., Fessi, H., 2007. Preparation and characterization of radioactive dirhenium decacarbonyl-loaded PLLA nanoparticles for radionuclide intra-tumoral therapy. Eur. J. Pharm. Biopharm. 67, 597–611.

Hope, M.J., Bally, M.B., Webb, G., Cullis, P.R., 1985. Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. Biochim. Biophys. Acta (BBA): Biomembr. 812, 55–65.
Hunter, W.L., 2006. Drug-eluting stents: beyond the hyperbole. Adv. Drug Deliv. Rev. 58, 347–349.
Immordino, M.L., Brusa, P., Arpicco, S., Stella, B., Dosio, F., Cattel, L., 2003. Preparation, characterization, cytotoxicity and pharmacokinetics of liposomes containing docetaxel. J. Control. Release 91, 417–429.
Immordino, M.L., Dosio, F., Cattel, L., 2006. Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. Int. J. Nanomed. 1, 297–315.
Lagerquist, C., Beigi, F., Karlen, A., Lennernas, H., Lundahl, P., 2001. Effects of cholesterol and model transmembrane proteins on drug partitioning into lipid bilayers as analysed by immobilized-liposome chromatography. J. Pharm. Pharmacol. 53, 1477–1487.
Lee, M.S., David, E.M., Makkar, R.R., Wilentz, J.R., 2004. Molecular and cellular basis of restenosis after percutaneous coronary intervention: the intertwining roles of platelets, leukocytes, and the coagulation-fibrinolysis system. J. Pathol. 203, 861–870.
Loukas, Y.L., 1998. A computer-based expert system designs and analyzes a $2(k-p)$ fractional factorial design for the formulation optimization of novel multicomponent liposomes. J. Pharm. Biomed. Anal. 17, 133–140.
Luscher, T.F., Steffel, J., Eberli, F.R., Joner, M., Nakazawa, G., Tanner, F.C., Virmani, R., 2007. Drug-eluting stent and coronary thrombosis: biological mechanisms and clinical implications. Circulation 115, 1051–1058.
Marx, S.O., Jayaraman, T., Go, L.O., Marks, A.R., 1995. Rapamycin-FKBP inhibits cell cycle regulators of proliferation in vascular smooth muscle cells. Circ. Res. 76, 412–417.
Melikian, N., Airoldi, F., Di Mario, C., 2004. Coronary bifurcation stenting. Current techniques, outcome and possible future developments. Minerva Cardioangiol. 52, 365–378.
Melikian, N., Wijns, W., 2008. Drug-eluting stents: a critique. Heart 94, 145–152.
Metselaar, J.M., Mastrobattista, E., Storm, G., 2002. Liposomes for intravenous drug targeting: design and applications. Mini Rev. Med. Chem. 2, 319–329.
Meure, L.A., Foster, N.R., Dehghani, F., 2008. Conventional and dense gas techniques for the production of liposomes: a review. AAPS PharmSciTech 9, 798–809.
Moribe, K., Maruyama, K., Iwatsuru, M., 1999. Encapsulation characteristics of nystatin in liposomes: effects of cholesterol and polyethylene glycol derivatives. Int. J. Pharm. 188, 193–202.
Nallamothu, R., Wood, G.C., Kiani, M.F., Moore, B.M., Horton, F.P., Thoma, L.A., 2006. A targeted liposome delivery system for combretastatin A4: formulation optimization through drug loading and in vitro release studies. PDA J. Pharm. Sci. Technol. 60, 144–155.
Nebeker, J.R., Virmani, R., Bennett, C.L., Hoffman, J.M., Samore, M.H., Alvarez, J., Davidson, C.J., McKoy, J.M., Raisch, D.W., Whisenant, B.K., Yarnold, P.R., Belknap, S.M., West, D.P., Gage, J.E., Morse, R.E., Gligoric, G., Davidson, L., Feldman, M.D., 2006. Hypersensitivity cases associated with drug-eluting coronary stents: a review of available cases from the Research on Adverse Drug Events and Reports (RADAR) project. J. Am. Coll. Cardiol. 47, 175–181.
Ong, A.T., McFadden, E.P., Regar, E., de Jaegere, P.P., van Domburg, R.T., Serruys, P.W., 2005. Late angiographic stent thrombosis (LAST) events with drug-eluting stents. J. Am. Coll. Cardiol. 45, 2088–2092.
Poon, M., Marx, S.O., Gallo, R., Badimon, J.J., Taubman, M.B., Marks, A.R., 1996. Rapamycin inhibits vascular smooth muscle cell migration. J. Clin. Invest. 98, 2277–2283.
Popma, J.J., Califf, R.M., Topol, E.J., 1991. Clinical trials of restenosis after coronary angioplasty. Circulation 84, 1426–1436.
Ramana, L.N., Sethuraman, S., Ranga, U., Krishnan, U.M., 2010. Development of a liposomal nanodelivery system for nevirapine. J. Biomed. Sci. 17, 57.
Reddy, M.K., Vasir, J.K., Sahoo, S.K., Jain, T.K., Yallapu, M.M., Labhasetwar, V., 2008. Inhibition of apoptosis through localized delivery of rapamycin-loaded nanoparticles prevented neointimal hyperplasia and reendothelialized injured artery. Circ. Cardiovasc. Interv. 1, 209–216.
Ritger, P.L., Peppas, N.A., 1987. A simple equation for description of solute release. I. Fickian and non-fickian release from non-swellable devices in the form of slabs, spheres, cylinders or discs. J. Control. Release 5, 23–36.
Robinson, A.J., Richards, W.G., Thomas, P.J., Hann, M.M., 1995. Behavior of cholesterol and its effect on head group and chain conformations in lipid bilayers: a molecular dynamics study. Biophys. J. 68, 164–170.
Rouf, M.A., Vural, I., Renoir, J.M., Hincal, A.A., 2009. Development and characterization of liposomal formulations for rapamycin delivery and investigation of their antiproliferative effect on MCF7 cells. J. Liposome Res. 19, 322–331.
Sadzuka, Y., Takabe, H., Sonobe, T., 2005. Liposomalization of SN-38 as active metabolite of CPT-11. J. Control. Release 108, 453–459.
Salam, A.M., Al Suwaidi, J., Holmes Jr., D.R., 2006. Drug-eluting coronary stents. Curr. Probl. Cardiol. 31, 8–119.
Scheller, B., Speck, U., Abramjuk, C., Bernhardt, U., Bohm, M., Nickenig, G., 2004. Paclitaxel balloon coating, a novel method for prevention and therapy of restenosis. Circulation 110, 810–814.
Semple, S.C., Chonn, A., Cullis, P.R., 1996. Influence of cholesterol on the association of plasma proteins with liposomes. Biochemistry 35, 2521–2525.
Sharma, A., Straubinger, R.M., 1994. Novel taxol formulations: preparation and characterization of taxol-containing liposomes. Pharm. Res. 11, 889–896.
Steffel, J., Tanner, F.C., 2007. Biological effects of drug-eluting stents in the coronary circulation. Herz 32, 268–273.
Sukhija, R., Mehta, J.L., Sachdeva, R., 2008. Present status of coronary bifurcation stenting. Clin. Cardiol. 31, 63–66.
Tulis, D.A., 2007. Rat carotid artery balloon injury model. Methods Mol. Med. 139, 1–30.
Vali, A.M., Toliyat, T., Shafaghi, B., Dadashzadeh, S., 2008. Preparation, optimization, and characterization of topotecan loaded PEGylated liposomes using factorial design. Drug Dev. Ind. Pharm. 34, 10–23.
van der Giessen, W.J., Lincoff, A.M., Schwartz, R.S., van Beusekom, H.M., Serruys, P.W., Holmes Jr., D.R., Ellis, S.G., Topol, E.J., 1996. Marked inflammatory sequelae to implantation of biodegradable and nonbiodegradable polymers in porcine coronary arteries. Circulation 94, 1690–1697.
Webb, M.S., Johnstone, S., Morris, T.J., Kennedy, A., Gallagher, R., Harasym, N., Harasym, T., Shew, C.R., Tardi, P., Dragowska, W.H., Mayer, L.D., Bally, M.B., 2007. In vitro and in vivo characterization of a combination chemotherapy formulation consisting of vinorelbine and phosphatidylserine. Eur. J. Pharm. Biopharm. 65, 289–299.
Wessely, R., Kastrati, A., Schomig, A., 2005. Late restenosis in patients receiving a polymer-coated sirolimus-eluting stent. Ann. Intern. Med. 143, 392–394.
Xiang, T.X., Anderson, B.D., 1998. Influence of chain ordering on the selectivity of dipalmitoylphosphatidylcholine bilayer membranes for permeant size and shape. Biophys. J. 75, 2658–2671.
Xiong, Y., Guo, D., Wang, L., Zheng, X., Zhang, Y., Chen, J., 2009. Development of nobiliside A loaded liposomal formulation using response surface methodology. Int. J. Pharm. 371, 197–203.
Zhang, J.A., Anyarambhatla, G., Ma, L., Ugwu, S., Xuan, T., Sardone, T., Ahmad, I., 2005. Development and characterization of a novel Cremophor EL free liposome-based paclitaxel (LEP-ETU) formulation. Eur. J. Pharm. Biopharm. 59, 177–187.
Zhang, J.A., Xuan, T., Parmar, M., Ma, L., Ugwu, S., Ali, S., Ahmad, I., 2004. Development and characterization of a novel liposome-based formulation of SN-38. Int. J. Pharm. 270, 93–107.
Zuidam, J.Z., de Vrueh, R., Crommelin, D.J.A., 2003. Characterization of liposomes. In: Torchilin, V.P., Weissig, V. (Eds.), Liposomes, a practical approach. Oxford University Press, New York, pp. 31–78.