Original Article

Optimization of LDL targeted nanostructured lipid carriers of 5-FU by a full factorial design

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Abstract

Background: Nanostructured lipid carriers (NLC) are a mixture of solid and liquid lipids or oils as colloidal carrier systems that lead to an imperfect matrix structure with high ability for loading water soluble drugs. The aim of this study was to find the best proportion of liquid and solid lipids of different types for optimization of the production of LDL targeted NLCs used in carrying 5-Fu by the emulsification-solvent evaporation method.

Materials and Methods: The influence of the lipid type, cholesterol or cholesteryl stearate for targeting LDL receptors, oil type (oleic acid or octanol), lipid and oil% on particle size, surface charge, drug loading efficiency, and drug released percent from the NLCs were studied by a full factorial design.

Results: The NLCs prepared by 54.5% cholesterol and 25% of oleic acid, showed optimum results with particle size of 105.8 nm, relatively high zeta potential of −25 mV, drug loading efficiency of 38% and release efficiency of about 40%. Scanning electron microscopy of nanoparticles confirmed the results of dynamic light scattering method used in measuring the particle size of NLCs.

Conclusions: The optimization method by a full factorial statistical design is a useful optimization method for production of nanostructured lipid carriers.

Key Words: 5-FU, LDL targeted nanostructured lipid carriers, optimization, structural parameters

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INTRODUCTION

5-FU a pyrimidine analogue inhibits the activity of thymidylate synthetase and is a wide spectrum anti-cancer that is used in different solid tumors of colon, liver, neck, head and breast cancers. In spite of its activity in breast cancer, it is inactivated by dihydropyrimidine dehydrogenase resulting in inadequate and incomplete absorption by gastrointestinal tract, very short half life due to its rapid metabolism and the toxic effects on bone marrow, un-selectivity on normal cells and the inherent or acquired resistance. For these reasons localized drug delivery by targeted therapy is suggested for this drug.[1-3] 5-FU is the drug of choice of colorectal cancer which is a very common cancer with incidence of about 13 5000 new cases in each year in the United States.[4]

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The adenomatus polyps and malignant cells replicate so fast and produce tumors that can spread to other sites.[2] Systemic chemotherapy of 5-FU causes many serious side effects and rapid multi drug resistance limits effective treatment of the disease. Targeted therapy for colorectal cancer allows for the local high concentration of chemotherapeutic drugs and reduces their side effects.[2]

Colloidal drug carriers have potential for targeting chemotherapeutic agents. Nanostructured lipid carriers (NLCs) are a new generation of lipid nanoparticles that improves drug loading and firmly incorporate the drug during storage. NLCs accommodate the drug because of their highly unordered lipid structures[5,6] and imperfect lipid matrix which provides space for drug molecules.

Expression of LDL receptor is higher in some cancer cells like leukemia, breast cancer and human lung cancer tissues.[7] Also in human colon carcinoma LDL receptor mRNA expression is significantly increased. Therefore, using lipid carriers containing cholesterol seems logic for targeted delivery of 5-FU in the treatment of this disease.[8]

The aim of the present study was designing LDL targeted NLCs of 5-FU and optimization their structural parameters.

MATERIALS AND METHODS

Materials

5-FU was provided from Sigma (USA), cholesterol, oleic acid, octanol, Tween 20, Tween 80, ethanol, acetone were from Merck Chemical Company (Germany). Soy lecithin S100 was from Lipoid (Germany) and cholesteryl stearate (CS) from Aldrich (USA).

Preparation of NLCs

5-FU had the highest solubility in octanol and oleic acid than other solvent lipids tested for solubility screening. Considering Table 1, the studied variables were: lipid type and concentration, oil type and content. NLCs were prepared from 59.5% ± 5% of lipid (cholesteryl stearate or cholesterol), 0.5% lecithin, 10% of PEG 40 stearate (as the pegylated lipid to help escaping NLCs from reticuloendothelial system), 20% ± 5% of oil (oleic acid or octanol) and 20 mg of 5-FU which were all dissolved in 5 ml of a mixture of acetone/ethanol by the ratio of 3:1. The mixture was then transferred to bath sonicator and warmed up to 50 ºC until a transparent phase was achieved. This organic phase was then slowly added to 25 ml of distilled water containing 0.5% Tween 80 while stirred on a magnetic stirrer during 15 minutes. The organic solvent was allowed to evaporate for one hour using a magnetic stirrer at a low speed rotation. After preparation of NLCs, the following parameters were studied as output responses to formulation variables: drug loading efficiency, particle size, zeta potential and release efficiency till 20 h.

Particle size and zeta potential measurements

The mean particle size and zeta potential of NLCs were measured by photon correlation spectroscopy (PCS) at a fixed angle of 90º (Zetasizer 3000 HS, Malvern Instrument, UK). Nanodispersion was suitably diluted to measure mean particle size and polydispersity index.

Entrapment efficiency and drug loading

To separate nanoparticles, a 600 µl of sample emulsion was centrifuged (Microcentrifuge Sigma 30k, UK) at 10,000 rpm for 5 min. The supernatant containing the free drug was diluted 40 times and analyzed spectrophotometerically (RF-5301 PC, Shimadzu, Kyoto, Japan) at λ_{max} 267.2 nm. The difference between the total and free drug shows the amount of the

Table 1: Different formulations of prepared NLCs (containing 20 mg 5-FU in 25 ml ofNLCs dispersion)

Formulation code	Octanol (OC) (%)	Oleic acid (OA) (%)	Cholesterol (C) (%)	Cholesteryl stearate (CS)(%)
$CS_{64.5}OA_{15}$	-	15	-	64.5
$CS_{59.5}OA_{20}$	-	20	-	59.5
$CS_{54.5}OA_{25}$	-	25	-	54.5
$C_{64.5}OA_{15}$	-	15	64.5	-
$C_{59.5}OA_{20}$	-	20	59.5	-
$C_{54.5}OA_{25}$	-	25	54.5	-
$CS_{64.5}OC_{15}$	15	-	-	64.5
$CS_{59.5}OC_{20}$	20	-	-	59.5
$CS_{54.5}OC_{25}$	25	-	-	54.5
$C_{64.5}OC_{15}$	15	-	64.5	-
$C_{59.5}OC_{20}$	20	-	59.5	-
$C_{54.5}OC_{25}$	25	-	54.5	-

encapsulated drug. The encapsulation efficiency (EE) of 5-FU in NLCs was determined as the ratio of the actual and theoretical loading by using the following equation:

$$EE(\%) = \frac{\text{Entrapped drug in NLSs}}{\text{Total aimount of drug added}} \times 100$$

Drug loading capacity (DL) was calculated according to the equation (2) as drug analyzed in NLCs versus the total amount of the drug, lipid, and oil excipients added for preparation:

$$DL\ (\%) = \frac{\text{Entrapped drug in NLCs}}{\text{Weight of NLCs (amount of drug + amount of lipid)}} \times 100$$

Drug release studies

One milliliter of dispersion was transferred to a dialysis bag (molecular weight cutoff 12000, Membra-Cel®, Viskase, USA). The sealed bag was put into a beaker of 70 ml of phosphate buffer solution (pH 7.4) containing 2% Tween 20. Samples were shaken horizontally in a shaker (Lab tech, Korea) at 37 ± 1ºC with 40 strokes per minute. Six hundred microlitres of the medium was taken at predetermined time intervals and the absorbance of free 5-FU was measured at λ_{max} 268.4 nm. The samples were returned to the test medium again. The parameter of release efficiency within 20 h (RE_{20}%) was used to compare the release profiles [9]:

$$RE\% = \frac{\int_0^t y.dt}{y_{100}.t} \times 100$$

Optimization of the formulation of NLCs

Computer optimization process by Design expert software (version 7.2, US) and a desirability function determined the effect of the levels of independent variables on the responses. All responses were fitted to the linear model. The constraints of particle size was $80.6 \leq Y_1 \leq 284.1$ nm with targeting the particle size on minimum, for zeta potential was $-29 \leq Y_2 \leq -7.7$ mV while the target was maximum absolute value of zeta potential, for loading efficiency the constraint was $22 \leq Y_3 \leq 51\%$ with the goal set at the maximum and the RE_{20}% constraint was $10.2 \leq Y_4 \leq 51.5\%$ with the target set at the maximum.

Statistical analysis

SPSS software version 11.5 was used for all statistical analysis. Univariate analysis of data by a full factorial design was used for comparison between particle size, zeta potential, loading and release efficiency percent of 5-FU in different NLC formulations. A significant level of $P < 0.05$ was denoted significant in all cases.

Atomic force microscopy

Atomic Force Microscope (AFM) (Bruker, Nanos 1.1, Germany) was used to observe the morphology and also the particle size of NLCs. AFM images were obtained by measurement of the interaction forces between the tip and the sample surface. The experiments were done in air at room temperature (25 ºC) operating in contact mode on dried 20 µl droplet sample of the final suspension. The measurements were performed in different sample locations. The mean size of NLCs was obtained by processing the topographical AFM images with the AFM Nanos 1.1 software.

RESULTS AND DISCUSSION

Physical properties of NLCs

Twelve different formulations of NLCs were prepared [Table 2] from 5-FU by two types of lipids and two types of oils each in three different levels by emulsification solvent evaporation method.

Table 2 shows that the mixture of cholesterol/octanol used in NLCs caused smaller particle size than other lipids ($P < 0.05$). The results of particle size analysis

Table 2: Physical properties of prepared NLCs

Formulation code	Particle size (nm)	PdI	Zeta potential (mv)	Drug loading efficiency (%)	RE_{20}(%)
$CS_{64.5}OA_{15}$	226.5 ± 2.1	0.28	-14.2 ± 2.3	48 ± 2.3	51.53 ± 3.20
$CS_{59.5}OA_{20}$	130.9 ± 2.4	0.42	-26.0 ± 1.9	30 ± 1.7	48.37 ± 1.03
$CS_{54.5}OA_{25}$	135.3 ± 3.9	0.30	-9.1 ± 0.6	34 ± 1.0	48.35 ± 0.78
$C_{64.5}OA_{15}$	119.0 ± 0.9	0.12	-20.8 ± 1.1	30 ± 1.3	35.33 ± 0.89
$C_{59.5}OA_{20}$	102.0 ± 1.3	0.18	-29.0 ± 3.0	30 ± 1.7	32.69 ± 2.00
$C_{54.5}OA_{25}$	105.8 ± 1.9	0.23	-25.1 ± 0.7	38 ± 3.1	37.98 ± 1.98
$CS_{64.5}OC_{15}$	257.4 ± 27.9	0.39	-19.3 ± 1.4	38 ± 1.3	40.59 ± 1.77
$CS_{59.5}OC_{20}$	150.6 ± 8.8	0.44	-18.3 ± 1.4	51 ± 2.9	44.46 ± 2.11
$CS_{54.5}OC_{25}$	284.1 ± 16.8	0.48	-7.7 ± 1.6	29 ± 1.9	51.53 ± 2.82
$C_{64.5}OC_{15}$	105.5 ± 1.0	0.24	-20.1 ± 0.7	38 ± 2.3	32.94 ± 2.26
$C_{59.5}OC_{20}$	80.6 ± 0.5	0.21	-8.6 ± 1.0	22 ± 1.8	10.21 ± 1.31
$C_{54.5}OC_{25}$	110.1 ± 1.5	0.40	-14.5 ± 1.0	36 ± 2.6	48.37 ± 0.87

indicated that the change of the lipid type, from cholesterol to cholestryl stearate significantly increased the particle size [Figure 1a]. This effect may be attributed to the higher molecular weight of cholestryl stearate (653.12 g/mol) compared to cholesterol (386.7 g/mol)[10] or to surface active properties of cholesterol compared to cholestryl stearate[11] which in turn reduces the surface tension and consequently decreases the particle size.[12] Change of the oil type from oleic acid to octanol also caused growth in particle size [Figure 1c] Considering the structure of these two oils, octanol is a linear tough molecule that can not bend easily, but oleic acid has unsaturated structure that can be compacted more easily. When lipid content of NLCs was increased to 59.5% the particle size enlarged [Figure 1b] probably because of the increase in solid phase content and

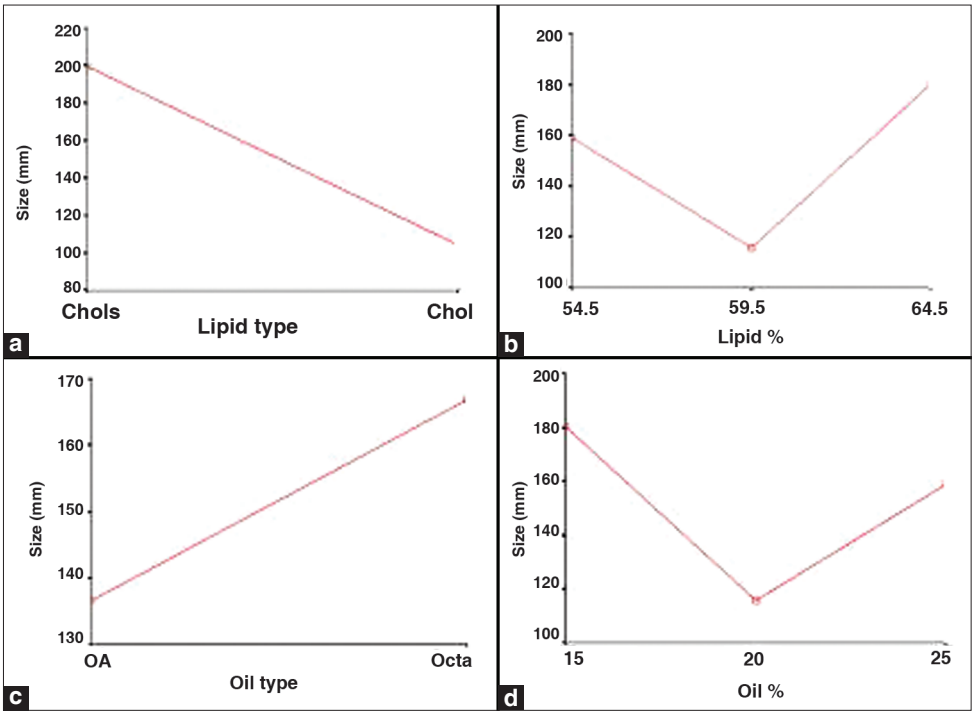

Figure 1: Effect of (a) lipid type, (b) lipid percent, (c) oil type, and (d) oil percent on particle size of NLCs

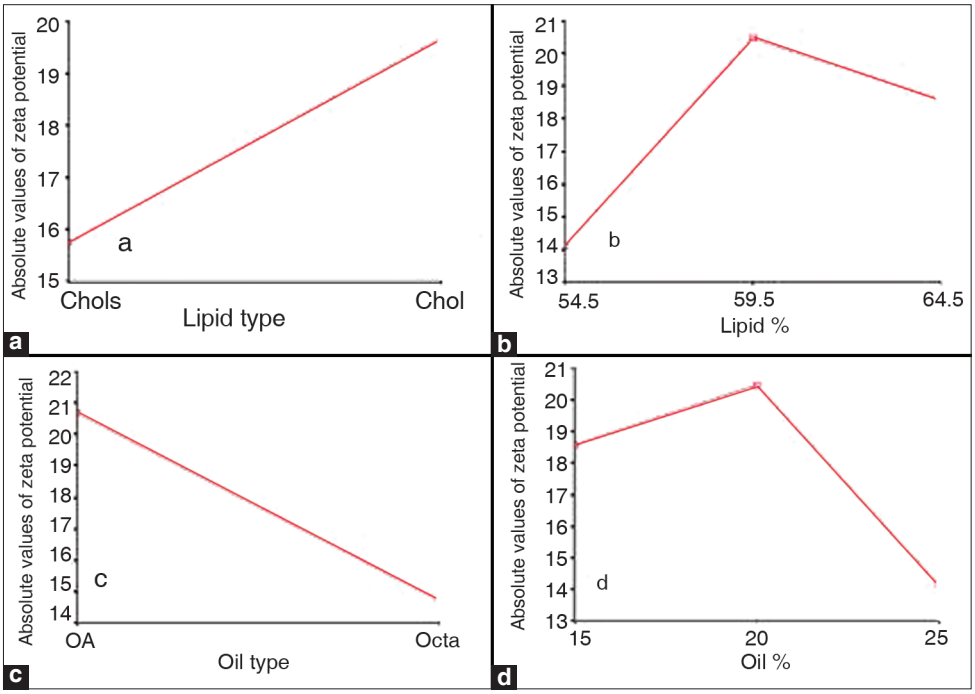

Figure 2: Effect of (a) lipid type, (b) lipid percent, (c) oil type, and (d) oil percent on absolute zeta potential values of NLCs

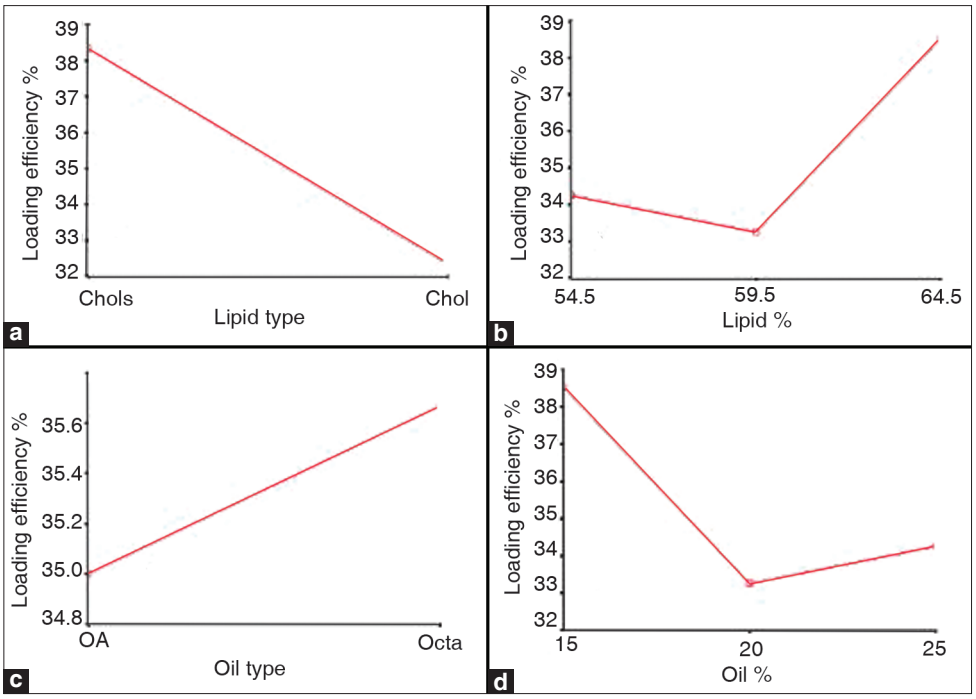

Figure 3: Effect of (a) lipid type, (b) lipid percent, (c) oil type, and (d) oil percent on loading efficiency of NLCs

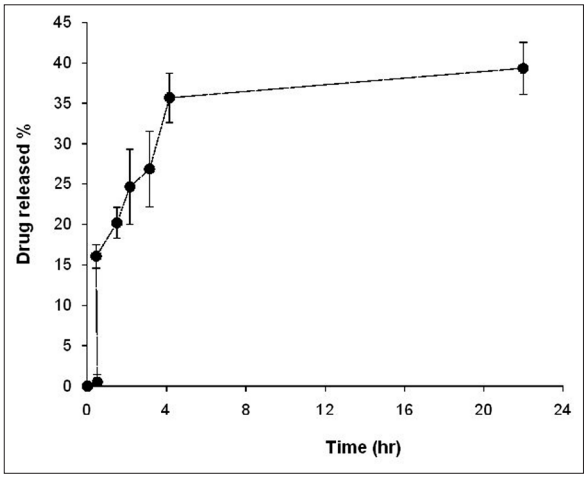

Figure 4: 5-Fu release profile from NLCs of $C_{54.5}OA_{25}$ in phosphate buffer solution (pH 7.4) ($n = 3$)

viscosity of their core.[13] Increasing the oil content of NLCs from 15% to 20% decreased the particle size [Figure 1d] possibly due to the reduction of core viscosity of NLCs and enhancing their fluidity. This caused continuous reduction of the surface tension and production of particles with smooth surface and small sizes.[14] However, further increasing the oil content disrupted the wall of NLCs and caused their aggregation and size growth [Figure 1d] Hu *et al*.[15] showed that as oleic acid content increased up to 30 wt%, the obtained particles showed pronounced smaller size and more regular morphology in spherical shape with smooth surface.

Zeta potential is a key factor in stability of nanoparticles. All NLCs had negative charge with the greatest zeta potential seen in $C_{59.5}OA_{20}$ (NLCs with cholesterol 59.5% and oleic acid 20%) [Table 2]. Changing the lipid type from cholestryl stearate to cholesterol increased in the absolute value of zeta potential [Figure 2a] possibly due to increase in the negative charge density which happens as a result of reduction in particle size.[16] Changing the oil type from oleic acid to octanol had a reverse effect on zeta potential.[Figure 2c] This may be interpreted to the presence of a carboxylic acid group in oleic acid which gives more negative zeta potential compared to octanol.[17] Both lipid and oil content of NLCs (up to 20%) caused increase in zeta potential but further increase of lipid phase decreased the zeta potential significantly [Figures 2b,d] which may be attributed to the size changes of the NLCs. As mentioned before disrupting the wall of NLCs happened with size growth when the content of oils increased to 20% [Figure 1d]. The size reduction causes higher charge density on the surface of particles and consequently the enhancing the zeta potential while growth in particle size reduces the zeta potential of the NLCs.

The greatest loading efficiency of 5-FU was observed in $CS_{59.5}Oc_{20}$ (NLCs with cholestryl stearate 59.5% and octanol 20%) [Table 2] which may be due to higher partitioning of drug to the lipid/oil mixture. Changing cholestryl stearate to cholesterol and octanol to oleic acid led to reduction of the loading efficiency [Figures 3a,c] due to the reduction of the particle size of NLCs. One may conclude that many imperfections are produced in

the structure of NLCs by increasing the oil content from 20 to 25% which offers greater space to accommodate the drug [Figure 3d]. Previous studies[15] showed that, NLCs exhibited improved drug loading capacity compared to SLNs and the drug loading capacity increased with increasing oleic acid content without any comparison between oleic acid and other liquid lipids.

In vitro drug release

The release efficiency of 5-FU from different NLCs after 20 hours (RE_{20}%) was calculated from release profiles as depicted in Table 2. A sustained release behavior was seen in release tests and after 20 h about 35%--45% of the drug was released [Figure 4]. The diffusion of drug out of the NLCs was affected by the amount of 5-FU loading capacity.

Figure 5a shows that changing the lipid type from cholestryl stearate to cholesterol decreased the RE_{20}% which may be due to the formation of hydrogen bond between the fluorine atom of 5-FU and OH groups of cholesterol. Formation of this bond is more probable compared to formation of hydrogen bond between the fluorine of 5-FU and oxygen atom of ketone group of cholestryl stearate. Octanol reduces the RE_{20}% comparing to oleic acid [Figure 5c] probably because of the enlargement of particle size of NLCs and consequently reduction of their surface area. As Figure 5d shows increasing the oil content from 20% to 25% increased the RE_{20}% due to reduction of the

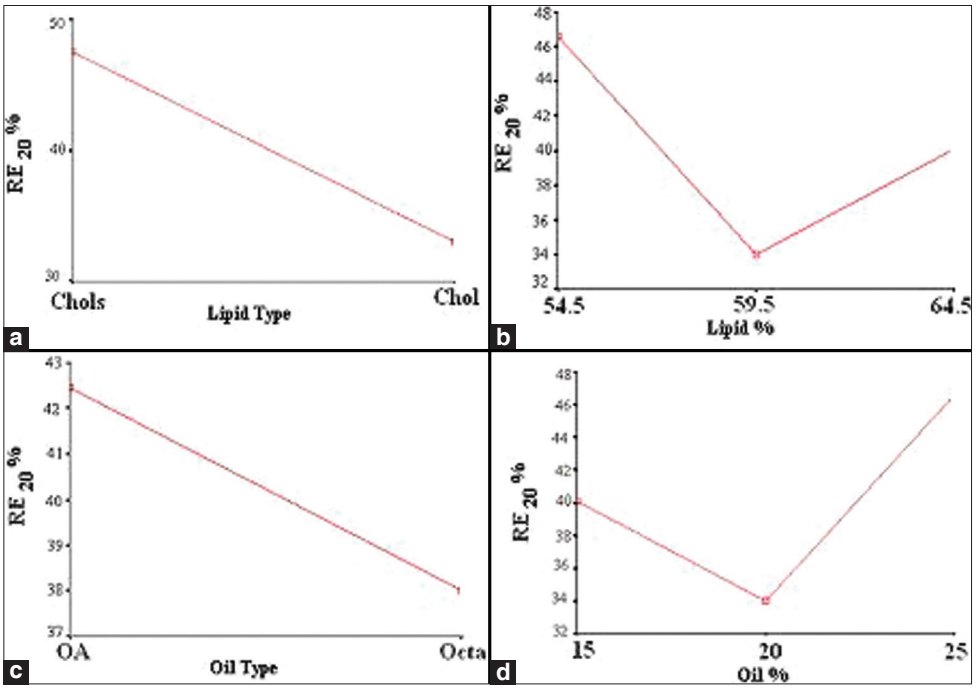

Figure 5: Effect of (a) lipid type, (b) lipid percent, (c) oil type, and (d) oil percent on release efficiency of NLCs over 20 hours

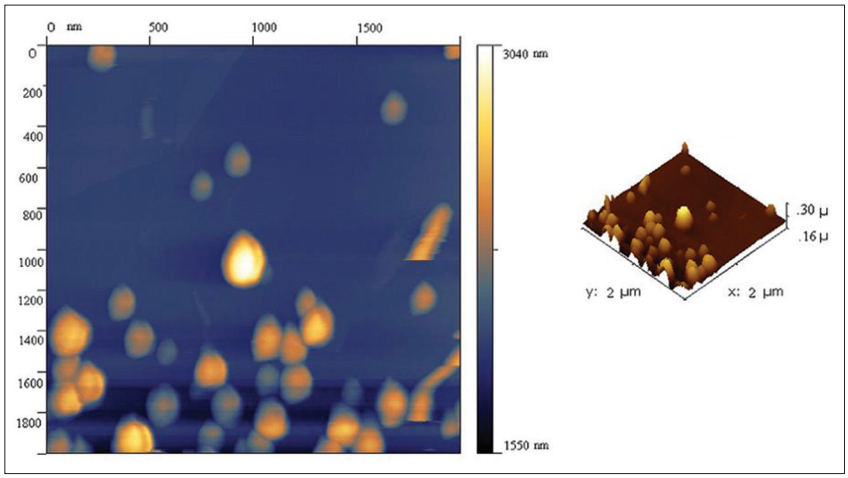

Figure 6: AFM micrographs of optimized NLCs of $C_{54.5}OA_{25}$

viscosity of the NLCs and so making them leaky. Not only the type of emulsifier can affect the drug release from NLCs but also the type of oil and its concentration are important. Reports show that incorporation of lipids in lipid nanoparticles has caused to the higher drug loading capacity and improved *in vitro* sustained drug release behavior.[18]

Optimization of NLC formulation

Considering the data of Table 2 optimization was carried out by Design Expert software and NLC formulation of $C_{54.5}OA_{25}$ (NLCs with cholesterol 54.5% and oleic acid 25%) was suggested as the optimum NLC. This formulation showed the particle size of 105.8 nm, a good stability due to a relative high zeta potential of –25 mV, an acceptable drug loading and release efficiency of 38%. These actual results were in close accordance with the predicted values by the software.

Atomic force microscopy

Figure 6 shows discrete particles of NLCs dispersed as spherical and round shape particles with about 100 nm diameter and show little or no aggregation. Photon correlation spectroscopy similarly demonstrates the particle size of NLCs.

CONCLUSIONS

In the present study, NLC formulations of 5-FU were prepared and optimized for particle size, zeta potential, drug loading efficiency and release efficiency by studying the effect of lipid type and concentration, oil type and percent. The selected formulation consisting of 54.5% cholesterol and 25% of oleic acid, 20 mg 5-FU, 0.5% of lecithin, 10% of PEG-40-stearate was the optimum NLC formulation due to its suitable particle size (105.8 nm), low polydispersity index (PdI of 0.23), the good stability due to relatively high zeta potential (–25 mV), an acceptable drug loading efficiency and release efficiency of 38%.

REFERENCES

1. Yassin AE, Anwer MK, Mowafy HA, El-Bagory IM, Bayomi MA, Alsarra IA. Optimization of 5-flurouracil solid-lipid nanoparticles: A preliminary study to treat colon cancer. Int J Med Sci 2010;7:398-408.
2. Li S, Wang A, Jiang W, Guan Z. Pharmacokinetic characteristics and anticancer effects of 5-Fluorouracil loaded nanoparticles. BMC Cancer 2008;8:103-21.
3. Boyer J, Maxwell PJ, Longley DB, Johnston PG. 5-Fluorouracil: Identification of Novel Downstream Mediators of Tumour Response. Anticancer Res 2004;24:417-43.
4. Cappell MS. The pathophysiology, clinical presentation, and diagnosis of colon cancer and adenomatous polyps. Med Clin North Am 2005;89:1-42.
5. Shidhaye SS, Vaidya R, Sutar S, Patwardhan A, Kadam VJ. Solid lipid nanoparticles and nanostructured lipid carriers—innovative generations of solid lipid carriers. Curr Drug Deliv 2008;5:324-31.
6. How CW, Abdullah R, Abbasalipourkabir R. Physicochemical properties of nanostructured lipid carriers as colloidal carrier system stabilized with polysorbate 20 and polysorbate 80. Afr J Biotechnol 2011;10:1684-9.
7. Vitols S, Gahrton G, Bjorkholm M, Peterson C. Hypocholesterolemia in malignancy due to elevated LDL receptor activity in tumor cells: Evidence from studies in patients with leukaemia. Lancet 1985;2:1150-4.
8. Niendorf A, Nägele H, Gerding D, Meyer-Pannwitt U, Gebhardt A. Increased LDL receptor mRNA expression in colon cancer is correlated with a rise in plasma cholesterol levels after curative surgery. Int J Cancer 1995;61:461-4.
9. Costa P, Sousa Lobo JM. Modeling and comparison of dissolution profiles. Eur J Pharm Sci 2001;13:123-33.
10. Benson HA, Sarveiya V, Risk S, Roberts MS. Influence of anatomical site and topical formulation on skin penetration of sunscreens. Ther Clin Risk Manag 2005;1:209-18.
11. Rai R, Srinivas CR. Photoprotection. Indian J Dermatol Venereol Leprol 2007;73:73-9.
12. Patel M, Jain SK, Yadav AK, Gogna D, Agrawal GP. Preparation and characterization of oxybenzone-loaded gelatin microspheres for enhancement of sun screening efficacy. Drug Deliv 2006;13:323-30.
13. Wissing S, Muller R. The development of an improved carrier system for sunscreen formulations based on crystalline lipid nanoparticles. Int J Pharm 2002;242:373-5.
14. Pardeike J, Hommoss A, Muller RH. Lipid nanoparticles (SLN, NLC) in cosmetic and pharmaceutical dermal products. Int J Pharm 2009;366:170-84.
15. Hu FQ, Jiang SP, Du YZ, Yuan H, Ye YQ, Zeng S. Preparation and characterization of stearic acid nanostructured lipid carriers by solvent diffusion method in an aqueous system. Colloids Surf B:Biointerfaces 2005;45:167-73.
16. Jenning V, Thunemann AF, Gohla SH. Characterisation of a novel solid lipid nanoparticle carrier system based on binary mixtures of liquid and solid lipids. Int J Pharm 2000;199:167-77.
17. Muller RH, Radtke M, Wissing SA. Nanostructured lipid matrices for improved microencapsulation of drugs. Int J Pharm 2002;242:121-8.
18. Shen J, Sun M, Ping Q, Ying Z, Liu W. Incorporation of liquid lipid in lipid nanoparticles for ocular drug delivery enhancement. Nanotechnology 2010;21:025101.

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