

The impact of particle size of nanostructured lipid carriers on follicular drug delivery: a comprehensive analysis of mouse and human hair follicle penetration

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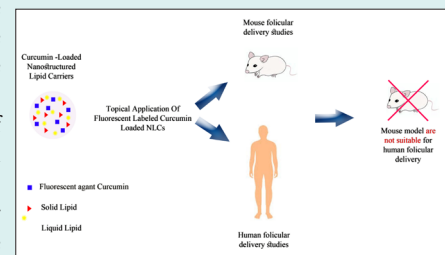
Abstract

Introduction: Follicular delivery is one of the targeted drug delivery methods aiming to target the hair follicles. The accumulation and retention time of targeted drugs is enhanced when nanoparticles are used as drug carriers. Particle size is one of the important factors affecting the penetration and accumulation of particles in the hair follicles, and there is a controversy in different studies for the best particle size for follicular delivery. Mouse models are mostly used in clinical trials for dermal, transdermal, and follicular delivery studies. Also, it is essential to investigate the reliability of the results between human studies and mouse models.

Methods: Curcumin-loaded nanostructured lipid carriers (NLCs), as a fluorescent agent, with three different particle size ranges were prepared using the hot homogenization method and applied topically on the mouse and human study groups. Biopsies were taken from applied areas on different days after using the formulation. The histopathology studies were done on the skin biopsies of both groups using confocal laser scanning microscopy (CLSM). We compared the confocal laser scanning microscope pictures of different groups, in terms of penetration and retention time of nanoparticles in human and mouse hair follicles.

Results: The best particle size in both models was the 400 nm group but the penetration and accumulation of particles in human and mouse hair follicles were totally different even for the 400 nm group. In human studies, 400 nm particles showed good accumulation after seven days; this result can help to increase the formulation using intervals.

Conclusion: The best particle size for human and mouse follicular drug delivery is around 400 nm and although mouse models are not completely suitable for follicular delivery studies, they can be used in some conditions as experimental models.



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Introduction

Follicular delivery is a drug delivery targeted to hair follicles for control and treatment of some conditions related to hair follicles like alopecia.¹⁻⁴ In this root of drug delivery, the drug substance bypasses the skin barrier via follicular units to reach the hair bulb *i.e.*, the targeted area. Follicular units are not some hollow shunts and are filled with a kind of lipid substance called sebum, which is made by sebaceous glands and is secreted to hair follicle units.^{3,5,6} Lipid-based nanoparticles can easily penetrate sebum and reach hair follicles.^{7,8} After deposition in hair follicles, the second important point of targeted delivery is to increase the residence time of drug substances in the targeted area, to release the targeted drug in a long period of time and avoiding of further penetration into skin layers and deeply to blood vessels. Lipid nanoparticles are shown to have good performance in penetration and retention in hair follicles.⁹ Solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) showed better results in delivery and accumulation in hair follicles.^{10,11} In addition to the hair follicle, the applied formulation to the scalp can be absorbed via other absorption roots (extra and intra-cellular roots)¹²⁻¹⁵ and reach the systemic circulation, which is a challenge in follicular drug delivery. The absorption of the drug to systemic circulation can be decreased by targeting the formulation to hair follicles. Particle size is one of the most studied parameters for optimizing the effectiveness of drug targeting hair follicles.^{13,16-18} It seems that particles smaller than 100 nm can easily penetrate skin layers and they are preferred for transdermal drug delivery.^{19,20} So if the particle size is selected larger than 100, the transdermal penetration will be decreased and other roots of drug transfer, *i.e.* follicular units can be used, but there is a controversy between the results of different studies on optimum particle size for follicular targeted delivery. Vogt A *et al* showed that particles with smaller sizes (20-40 nm) have a deeper penetration in hair follicles than larger particles (750 and 1500 nm).²¹ F. Knorr *et al* reported that larger particles (750-1500 nm) are preferred for follicular delivery than smaller particles (40 nm).²² Also, two other studies expressed that particles with smaller sizes (20 nm) are preferred to larger particles (200 nm),²³ and 40 nm particles are preferred to larger particles (130 nm).²⁴ All these studies have been done on laboratory animals, especially on mice. However, due to the hair type differences and density of hair between mice and humans, it seems that mouse models cannot be an appropriate model for hair loss and follicular delivery studies. Although some studies reported the penetration of nanoparticles into hair follicles,^{13,22} no research has focused on the retention and residence time of nanoparticles in the site of action *i.e.*, hair follicles, especially in humans which is one of the most important claimed advantages of nanoparticles for sustained drug release. This study aimed to compare the effect of particle size on penetration and

retention of encapsulated substance in the hair follicles by using a fluorescent substance encapsulated in NLCs in human and mouse study groups.

Materials

Curcumin, glycerol, Poloxamer188, and Miglyol were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA), and Precirol was supplied from Gattefossé (Saint Periest Cedex, France). Chloroform was purchased from Dr.Mojallali Iran and isopropyl myristate and isopropyl alcohol was purchased from (Merck KGaA, Germany).

Methods

Nanoparticle preparation

NLCs were prepared using the hot homogenization method; 400 mg of liquid oil (Miglyol), 550 mg of solid lipid (Precirol), and 2 mg of a lipophilic fluorescent agent, Curcumin, melted in a hot water bath and were homogenized (21000 rpm) via a high-speed homogenizer (Silent Crusher M, Heidolph, Germany) while an aqueous solution of surfactants (glycerol 500 μ L and poloxamer 188-250 mg) was added dropwise to the lipid phase. The prepared nano-emulsion was transferred to the refrigerator to form NLC (F3). The two other formulations after homogenization were probe sonicated (Sonics, Vibracell, Newtown CT, USA) for 5 and 12 cycles of 1 min sonication with 1 min rest intervals to form F2 and F1 formulations, respectively. The ultrasonic probe sonicator was set at 80% of amplitude with 0.5 cycles/sec (200 W, 24 kHz). The formulations were kept in the refrigerator until cooling and forming the final NLCs. The whole process was done in the absence of light to protect the fluorescent substance (Curcumin) from quenching. After the preparation of formulations, they were packaged in plastic containers avoiding light exposure, and freshly used in mouse and human study groups.

NLC characterization

Size distribution and morphology

Particle size distribution of prepared NLCs was assessed using a laser diffraction particle size analyzer (Wing SALD 2101, Shimadzu, Japan). The average particle size was measured as mean volume diameter, and the samples were measured in triplicate. The morphology and particle size of prepared NLCs were determined using a scanning electron microscope (SEM) (MIRA3, TESCAN, Czech Republic).

Entrapment efficiency

Entrapment efficiency (EE%) is a term for explaining the yield of the encapsulation process. It is defined as the percentage of added drug encapsulated in the carrier. EE% of loaded Curcumin in three different prepared formulations was calculated using ultraviolet (UV) spectroscopy (UV-2000, Shimadzu, Japan). To this end, freshly prepared formulations were centrifuged at

12000 rpm for 20 minutes and the unloaded Curcumin precipitated at the bottom of the falcon. The supernatant dispersion (Curcumin-loaded NLCs) was separated, then mixed with chloroform and kept in a shaker incubator at 37 °C, 200 rpm, and the loaded Curcumin was extracted and the EE% of three different formulations of Curcumin-loaded NLCs were calculated using UV absorptions in $\lambda_{max} = 259$ nm.

$$\%EE = \frac{\text{Weight of drug entrapped within nanoparticles}}{\text{Total drug added}} \times 100$$

All the calculations were done in triplicate and the results were reported as mean \pm standard deviation (SD). The EE% of these three formulations were statistically analyzed by one-way ANOVA test (IBM SPSS statistics 26 software).

In-vitro release study

As the targeted area of these nanoparticles is hair follicles, we needed a media that simulates the physicochemical aspects of hair follicles. So, the oily media composed of isopropyl-myristate and isopropyl alcohol was chosen according to an article published by Rancan et al.²⁵ All calculations were done in triplicate and the results were reported as mean \pm SD.

In-vivo studies

Mouse follicular delivery studies

Twelve C57BL/6 mice were selected for *in-vivo* studies and randomly assigned into four groups (n = 3 in each). Three formulations of Curcumin-loaded NLCs with different size distributions (F1 = 50 nm, F2 = 400 nm, F3 = 1000 nm) were applied topically on the dorsal area of mice skins. These formulations were the same in oil/lipid ratio, lipid and fluorescent agent amount, and morphology. Then, 600 μ L of each formulation F1-3 and a control dispersion of Curcumin in distilled water was topically applied (200 μ L/mouse) and carefully rubbed in a circle with 1-cm diameter for about 2 minutes (group 1 = F1, group 2 = F2, group 3 = F3, group 4 = control solution). After 30 minutes, one mouse from each group was randomly selected and sacrificed. The formulation applied area was punched and transferred to normal saline 0.9% solution and the treated skin of the second and third mice of each group were punched one and three days after applying the formulations, respectively.

Human follicular delivery studies

Twelve volunteers participated in this study. All volunteers were selected from patients referred to the skin clinic of Sina Hospital in Tabriz, Iran with lipoma, and the healthy skin above the lipoma was supposed to be removed anyway. We used this healthy skin on the lipoma mass as a sample. We used Curcumin as a fluorescent agent because it is a natural and safe product extracted from turmeric; so, it is not hazardous for human study cases. Moreover, Curcumin is lipid soluble and easily encapsulated in

NLCs. Then, 3 ml of each formulation was topically applied in a group of three volunteers (1 mL/volunteer) and carefully rubbed in a circle with a 2-cm diameter for about 2 minutes (group1 = F1, group2 = F2, group3 = F3, group4 = control Curcumin solution). After 30 minutes, one of the volunteers in each group was randomly selected and the formulation applied area was punched and transferred to normal saline 0.9% solution, and the treated skin of the second and third volunteers of each group were punched one and seven days after applying the formulations, respectively.

Confocal laser scanning microscopy (CLSM)

After punching the skin, slides were made using the cryosectioning method. These slides were studied using a confocal laser scanning microscope to show the penetration and retention of Curcumin-loaded NLCs with different particle sizes. The intensity of fluorescent light shows the number of particles that reach the skin tissue, especially hair follicles. If the light intensity is high after three days in the mice and after seven days in human cases, it can be said that there is a high retention time of particles in the skin or hair follicles.

Results and Discussion

NLC characterization

Size distribution and morphology

Particle size distribution of prepared NLCs was characterized by DLS and SEM. The results of the particle size analyzer are shown in Fig. 1 and Table 1 (F1 = 52 nm, F2 = 349 nm, and F3 = 867 nm with narrow size distribution). The SEM images showed that the particles have the same shape and morphology, and the estimated size of each particle group was approved. We chose these particle size ranges according to previous studies. While in some studies larger sizes (more than 700 nm) were preferred,²² in some others smaller sizes (40 nm) were reported as better sizes.²¹ We prepared particles in three different size ranges to compare their accumulation and retention time.

Encapsulation efficiency

The results of EE % were 94.7 ± 0.9 %, 94.2 ± 0.4 % and 96.6 ± 0.7 % for F1, F2 and F3 respectively. The prepared formulations had an EE% of more than 94%, which is due to the high lipophilicity of Curcumin and the lipophilic structure of NLCs that can encapsulate the lipophilic compounds in a high percentage. In some published studies the ability of NLCs to encapsulate the lipophilic drugs has been confirmed²⁶⁻²⁸ and even in a study the EE% for Beclomethasone was more than 99%²⁸ and according to statistical analysis, one-way ANOVA test, which had done by IBM, SPSS statistics 26, there is no significant differences ($P > 0.05$) between the EE% of these formulations, then the results of light intensity in *in-vivo* studies will be comparable i.e. the more light intensity is the more penetration.

Table 1. Particle size analyzer results for F1, F2 and F3 formulations

	Modal diameter (nm)	Mean volume diameter (nm)	D10%* (nm)	D50%* (nm)	D90%* (nm)
F1	56	52	42	52	63
F2	355	349	266	42	484
F3	757	867	481	825	1691

*D10%, D50%, and D90% are the equivalent volume diameter at 10%, 50%, and 90% cumulative volume, respectively.

In-vitro release study

The release media in follicular drug delivery cannot be regarded as an aqueous medium as the hair follicles are lipophilic units. So, we selected an oily environment as release media. Also, the main challenge for in-vitro simulation of the release environment for topical drug delivery is that the skin and hair follicle units are in a solid state and the common release studies are done in liquid media. The release study in such oily media only shows whether the encapsulated fluorescent agent can be released easily from NLCs or not. The release profile of Curcumin in hair follicles is not the same in the in-vitro study; the slow-release profile of Curcumin is due to the accumulation of NLCs in hair follicles. The F1, F2, and F3 formulations had released the encapsulated fluorescent agent, curcumin, up to $92.3 \pm 0.8\%$, $94.5 \pm 1.3\%$, and $94.1 \pm 0.5\%$ respectively in the first 30 minutes, which means that, all the formulations had released more than 92% of the encapsulated compound in the first 30 minutes

of the release study to an oily environment composed of isopropyl alcohol – isopropyl-myristate (1:4) in sink condition. Rancan et al²⁵ used isopropyl-myristate as release media. As Curcumin is not soluble in this solvent, we added isopropyl alcohol to the release media so that Curcumin can be solved easily. According to the nearly complete release of Curcumin in 30 minutes, the loaded fluorescent agent can release freely from the carrier without degrading or complexing with the carrier.

In-vivo studies

Confocal laser scanning microscope

Curcumin is an antioxidant agent extracted from the rhizome of turmeric (*Curcuma longa*),²⁹⁻³² and because of its fluorescent activity, it has been used as a fluorescent agent in several studies.^{31,33-36} In our study, the excitation wavelength of curcumin was 543 nm and the emission wavelength was 576 nm, but it differs with pH and solvent.^{31, 37}

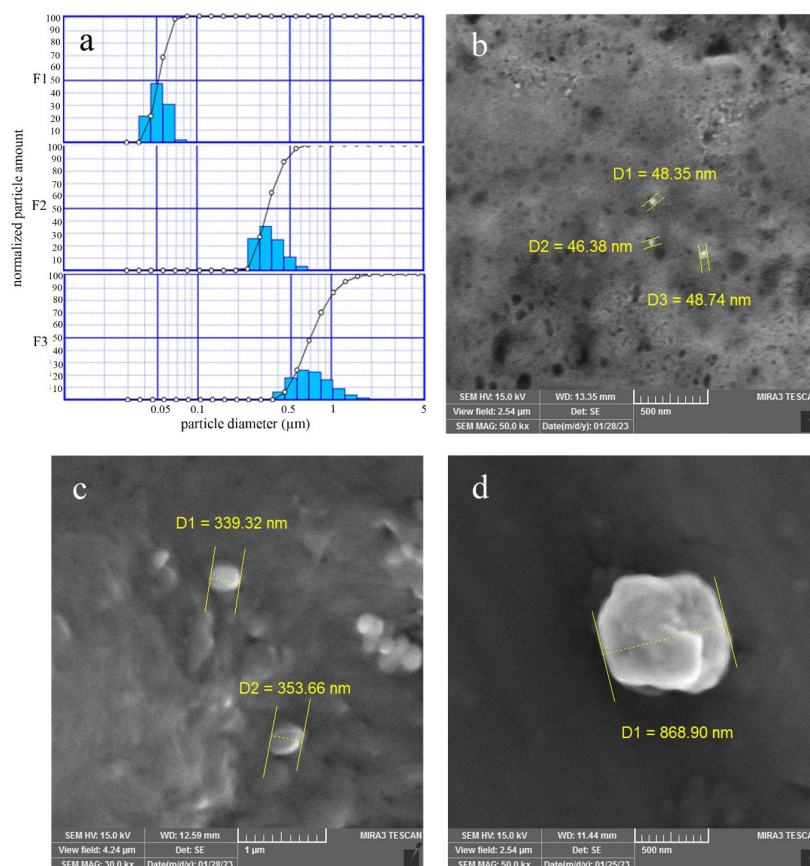


Fig. 1. Particle size distribution (a) and SEM results (b-d) of F1, F2, and F3 formulations

We selected curcumin as a fluorescent agent because it is safe for human studies³⁸⁻⁴⁰ and it has a lipophilic structure. So, it can be encapsulated in a high percentage in lipid carriers like NLCs.^{38,41,42}

CLSM images of each group are shown in Figs. 2 and 3. These images show the penetration of each group size of NLCs in human and mouse hair follicles. The intensity of light shows the number of penetrated particles compared to the other groups. In human study cases, the NLCs with particle sizes of 50 and 1000 nm (Fig. 2), the particles entered into the skin tissues after 30 minutes and a small ratio penetrated the hair follicle, but there was no selectivity in penetration. After seven days, the retention time of particles in follicles was lower than surrounding cells for 1000 nm particles and the 50 nm particles approximately disappeared. For 400 nm particles, at 30 minutes, and one day after applying the F2 formulation, the penetration of particles was very high, and follicles had high light intensity after seven days. So, the retention time of 400 nm particles in follicles was more than that of seven days. Among the three particle size ranges, 50 nm was not appropriate for follicular drug delivery because of the very low retention time of these particles

in hair follicles, and a size range larger than 400 nm was appropriate for follicular drug delivery. Also, the bigger the size the lower the penetration. Particles with 400 nm had more penetration to hair follicles and retention time (even after seven days); so, the formulation will have more effectiveness with minimum intervals of usage (every 7-10 days). However, the results were a little different in the mice. The group treated with 1000 nm and 50 nm particles (Fig. 3) had good penetration but after three days they disappeared. Of note, 30 minutes after applying the formulations, penetration of particles to the skin tissue and hair follicles was good, but the intensity of fluorescent light decreased after one day; this means that the particles had penetrated through the hair follicles cells to lower layers of skin tissue. Furthermore, there was no selectivity between hair follicles and other surrounding cells; it means that the same amount of drug reaching the hair follicles may reach other cells and even the systemic circulation. After days, the fluorescent color disappeared, which means that penetration is good, but retention time is low. Even for 50 nm particles, the hair follicles were colored, but the surrounding cells showed more fluorescent color, indicating that particles penetrated to other skin tissue

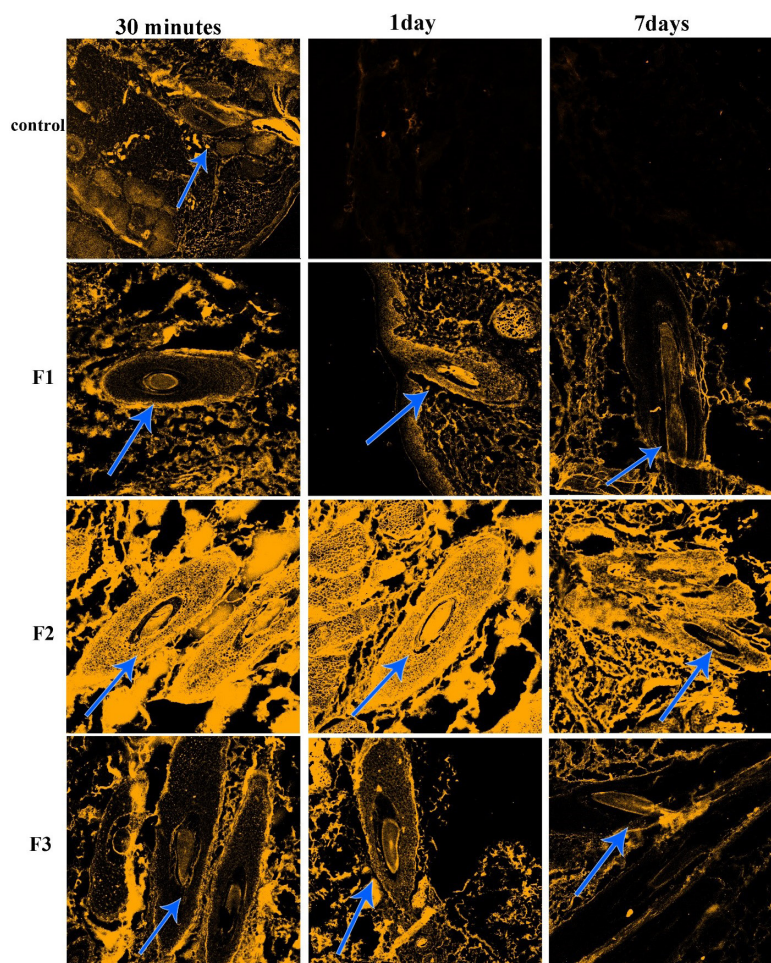


Fig. 2. CLSM images of human scalp skin punched from skin areas treated with control Curcumin solution and F1, F2, and F3 30 minutes, 1 day, and 7 days after treatment. The hair follicles are shown with arrows.

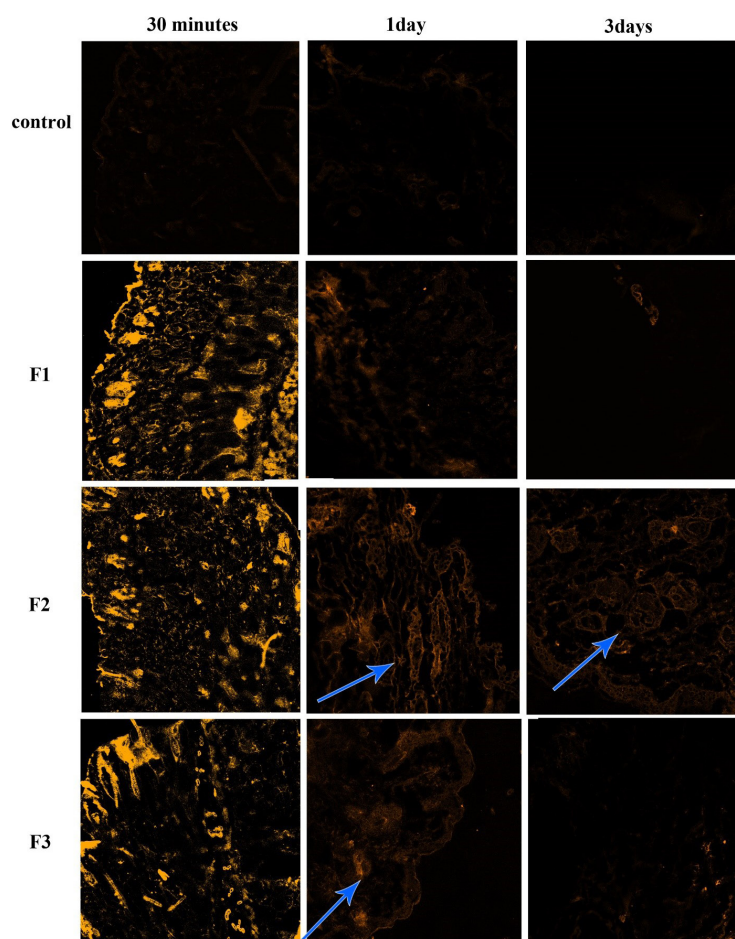


Fig. 3. CLSM images of mice dorsal skin punched from skin areas treated with control Curcumin solution and F1, F2, and F3 30 minutes, 1 day, and 3 days after treatment. The hair follicles are shown with arrows.

cells more than hair follicles. In other words, the targeting and accumulation of particles failed for 50 and 1000 nm. For 400 nm particles, the penetration of particles to the skin tissue was as good as the other formulations after 30 minutes. After one day, although the light intensity decreased, the follicles had more fluorescent color than surrounding cells, and accumulation of particles was evident even after three days. After three days, the fluorescent color decreased in all formulations but F2 was better than the others, and the targeting and accumulation of particles were seen in F2. In the mouse model, the best result was for 400 nm, even though it was not a great result for follicular delivery; it was relatively preferable to the other two particle sizes.

Some studies reported that nanoparticles have the potential to accumulate in the hair follicles of mice,⁴³⁻⁴⁵ and human hair follicles,⁴⁶⁻⁵⁴ but no study has shown the duration of nanoparticle accumulation in hair follicles. Almost all these studies have simply studied the accumulation of nanoparticles in hair follicles a few hours after applying the nanoparticles to skin samples. In addition, most of the human studies have been done *ex-vivo* on punched and isolated skin.⁵⁵ In some other studies, the mouse models were not appropriate for human follicular delivery studies due to differences between mice

and human hair follicles; for example, the follicle size in humans is larger than in mice and the hair types are different. Also, the follicle size in humans changes from vellus to terminal hair (and vice versa), that is not true about mice; the anagen cycle of hair in mice is only 2-3 weeks whereas in humans it lasts 3-5 years.⁵⁶ However, there are several studies using mice as hair follicular drug delivery model⁵⁷⁻⁶¹ instead of direct study on a human due to the ease of working on mice and the financial and ethical aspects of the study on humans. In our study, despite the differences in accumulation and selectivity of nanoparticles with different sizes, in the mouse model and human hair follicles, the 400 nm particles had better results in both mice and humans. However, the accumulation of nanoparticles in mice was lower than in humans; human hair follicle samples showed an accumulation of 400 nm particles more than seven days with high light intensity.

Conclusion

400 nm NLCs are the best particle size for human hair follicular drug delivery and they can be used as carriers for targeted drug delivery of anti-hair loss drugs and other conditions that need delivery of drugs to hair follicles. In mouse models 400 nm particles were able to penetrate hair follicles better than smaller and larger

Research Highlights

What is the current knowledge?

√ The lipid nanoparticles can accumulate in hair follicles and release the encapsulated drug in a long period of time.

What is new here?

√ The best particle size for accumulation of nanostructured lipid carriers is around 400 nm both in mouse and human.

particles too; but the accumulation of NLCs was not as good as the accumulation of them in human hair follicles, the penetration and accumulation of NLCs of each size in human hair follicles was much better than mouse study models. As shown in Figs. 2 and 3, for 400 nm particles, a little correlation is seen between human and mouse study groups. Accordingly, mouse models for this particle size range can be used as animal models for follicular drug delivery studies. However, regarding the other two sizes, there are no correlations. So, mouse models for these two particle sizes are inappropriate for follicular drug delivery studies.

The limitation of this study was the lack of *in vivo* analysis of particle penetration to the systemic circulation for different size ranges to better compare the NLCs with different sizes in mouse and human study groups. This can help in better decision-making to choose the best particle size of lipid nano-carriers for hair follicle-targeted drug delivery.

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Competing Interests

Authors have no conflict of interest to declare.

Ethical Statement

All the *in-vivo* study protocols were approved by the Ethics Committee of Tabriz University of Medical Sciences, Tabriz, Iran (code: IR.TBZMED.REC.1399.406).

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