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## Histological evaluation of follicular delivery of arginine via nanostructured lipid carriers: a novel potential approach for the treatment of alopecia

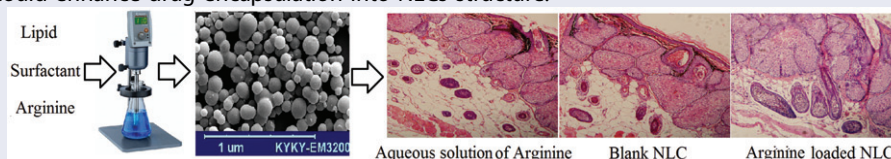
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### ABSTRACT

The use of lipidic nanoparticles can overcome the limitations of skin penetration of hydrophilic charged materials. Oleic acid as an ion pairing agent was used to enhance the encapsulation of positively charged arginine into lipophilic nanostructured lipid carrier (NLC). Histological study on the male hamsters was performed to assess the efficiency of drug delivery on hair growth. The accumulation of NLCs in the hair follicles was verified and *in vivo* studies showed more new hair follicle growth by utilizing arginine-loaded NLCs than arginine aqueous solution. It was concluded that employing ion pairing agent could enhance drug encapsulation into NLCs structure.



### ARTICLE HISTORY

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### KEYWORDS

Arginine; alopecia; nanostructured lipid carriers; dermal drug delivery; NLC

### Introduction

Hair loss, or alopecia, affects millions of men and women of all ages and frequently has significant social and psychological consequences. Alopecia decreases self-confidence and limits one's ability to enjoy life, which leads to some degree of depression and even suicide (Aggarwal et al. 2010; Kaufman 2002; Pérez-Ornelas et al. 2005; Sintov et al. 2000). Recently, a newly-proposed formulation was patented based on arginine for topical application to promote hair growth (Cifra et al. 2004; Fossel 2009, 2013; Mészáros and Fuentes 2006; Taylor et al. 2011). Arginine is an amino acid which acts as the physiological precursor for nitric oxide (NO) synthesis in animal cells. In blood vessels, NO released by endothelial cells activates guanylyl cyclase in smooth muscle cells, thereby elevating cellular cGMP concentrations and causing smooth muscle relaxation. NO is of interest where hair loss and growth are concerned because of its role as vasodilator in the stimulation of hair growth. NO is also involved in opening potassium channels, the activity mechanism of minoxidil, the well-known FDA approved medicine administered in alopecia. Topical drug delivery is an essential objective for drugs used in alopecia to maximize treatment efficiency and minimize the

associated side effects. However, penetration of the skin by charged and/or hydrophilic compounds is restricted. Obviously, the success of this strategy would depend on a suitable carrier, which could improve skin accumulation (Raj et al. 2015; Sintov et al. 2000; Tabbakhian et al. 2006). Small particles are increasingly being employed in dermatological and cosmetic products that are applied to the skin. Since epidermal lipids are found in high amounts within the penetration barrier, lipid carriers such as liposomes, solid lipid nanoparticles (SLNs), and nanostructured lipid carriers (NLCs) appear to be promising carriers by attaching themselves to the skin surface and allowing lipid exchange between the outermost layers of the stratum corneum and the carrier (Agrawal et al. 2015; Alnasif et al. 2014; Lauterbach et al. 2014). NLCs have recently attracted serious focus as a novel colloidal drug vehicle, because it has several advantages over the other mentioned carriers, including satisfactory stability, drug loading, and skin permeation and accumulation (Cheng-Ying et al. 2015; Gupta and Trivedi 2015; Weber et al. 2014). However, due to its high hydrophilicity, encapsulating arginine into NLC formulations can be challenging. To overcome this issue, the pairing of ions with a lipophilic material (oleic acid) was designed. This novel concept enhanced the encapsulation

of arginine into NLCs and was used for the follicular delivery of arginine for the treatment of alopecia.

## Materials and methods

### Materials

Arginine, o-phthaldialdehyde (OPA) and Poloxamer<sup>®</sup> 407 were supplied from Sigma-Aldrich Company (St. Louis, MO). Glyceryl palmitostearate (Precirol ATO-5<sup>®</sup>) was prepared from Gattefossé Company (Saint Priest, France). Oleic acid (Merck Chemicals, Darmstadt, Germany), and diethyl ether (KianKaveh Pharmaceutical and Chemical Company, Tehran, Iran) were used as received. Rhodamine B and 3-Mercaptopropionic acid were obtained from Merck Chemicals Company (Darmstadt, Germany).

### Preparation of arginine-loaded NLC

Nanostructured lipid carriers formulations were prepared by the hot melt homogenization method as described previously (Hamishehkar et al. 2014). Briefly, Precirol<sup>®</sup> (800 mg) and oleic acid (200 mg) were melted at about 75 °C. Subsequently, arginine (100 mg) was dissolved in water (0.7 mL) and added dropwise into the oil phase under stirring at 20,000 rpm by homogenizer [DIAX 900, Heidolph, Germany) for 15 min]. Subsequently, aqueous phase (containing Poloxamer<sup>®</sup> 1.5% w/v, 30 mL) was dropped into the oil phase, keeping the temperature at 75 °C and the same stirring rate. Finally, after 15 min, the hot formulations were allowed to cool down at room temperature. Each formulation was prepared and characterized in triplicate (Pezeshki et al. 2014).

### Characterization of drug-loaded NLC

#### Particle size and zeta potential determination

Particle size and zeta potential of prepared NLCs formulations were analyzed by laser diffraction technique using a particle size analyzer (Nano ZS, Malvern, UK). Prior to measurement, NLCs formulation samples were diluted with double-distilled water. Each sample was measured in triplicate.

#### Scanning electron microscopy

The scanning electron microscopy (SEM) photographs of prepared NLCs formulations were obtained using scanning electron microscope MIRA3 TESCAN instrument (Tescan, Brno, Czech Republic) operating at 15 kV. The specimens were mounted on a metal stub with double-sided adhesive tape and coated under vacuum with gold in an argon atmosphere prior to observation using a direct current sputter technique (DST1, Nanostructured Coating Co., Tehran, Iran).

#### Encapsulation efficiency and loading capacity percent

The encapsulation efficiency percent (EE%) and loading capacity percent (LC%) values were expressed as the percentage of encapsulated drug to the added drug or to the used lipid, respectively. EE% was determined by first separation of the

un-entrapped drug by centrifugation method using Amicon<sup>®</sup> Ultra-15 with the MWCO of 100 kDa (Millipore, Schwalbach, Germany) tubes. The formulation was added to the upper chamber of the Amicon<sup>®</sup> tube and then the tube was centrifuged (3K30, Sigma, Ostrode, Germany) at 5000 rpm for 5 min. The clear solution in the bottom of Amicon<sup>®</sup> tube was used for the determination of unloaded arginine (Mohammadi et al. 2014). The concentration of arginine was determined by measuring the concentration of unloaded drug, after conjugation with OPA, using UV/Visible spectrophotometer (1601, Shimadzu, Kyoto, Japan) ( $\lambda_{\text{max}} = 336 \text{ nm}$ , linear in the range of 2–30  $\mu\text{g/mL}$  with  $R^2 = 0.9995$ ) (Kutlán et al. 2002). The EE% and LC% values of arginine-loaded NLCs formulations were calculated mathematically according to the following equation:

$$EE (\%) = \frac{W_{(\text{Initial drug})} - W_{(\text{Unloaded drug})}}{W_{(\text{Initial drug})}} \times 100$$

$$LC (\%) = \frac{W_{(\text{Encapsulated drug})}}{W_{(\text{Total lipid})}} \times 100$$

where,  $W_{(\text{Initial drug})}$  is the amount of initial added drug and  $W_{(\text{Free drug})}$  is the amount of unloaded drug detected in the lower chamber of Amicon<sup>®</sup> tubes after centrifugation of the NLCs formulations. Accordingly,  $W_{(\text{Encapsulated drug})}$  is the amount of loaded drug and  $W_{(\text{Total lipid})}$  is the amount of used lipid in the preparation process (Ghaderi et al. 2015). The formulations in the upper chamber of Amicon<sup>®</sup> tubes were rinsed five times by water to eliminate unloaded arginine. These rinsed formulations were used for the rest experiments.

#### X-ray diffraction study

In order to evaluate the effect of NLCs preparation process on crystallographic patterns of arginine and lipids, X-ray diffraction (XRD) analysis was performed using an X-ray diffractometer (D-5000, Siemens, Karlsruhe, Germany, 2° to 40°) to assess the crystalline structures of Arginine, Precirol<sup>®</sup>, Poloxamer<sup>®</sup>, and vacuum oven-dried powder of optimized NLCs formulation. The diffraction pattern was measured using a Cu-K $\alpha$  radiation source (30 mA and 40 kV).

#### Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) spectra of pure drug, oleic acid and their mixture were obtained separately using FTIR spectrometer (8400S, Shimadzu, Kyoto, Japan). The potassium bromide discs were prepared by compressing the powders at pressure of 15 tons for 10 min in hydraulic press. Scans were obtained at a resolution of 2  $\text{cm}^{-1}$ , from 4000 to 400  $\text{cm}^{-1}$ . Arginine was dissolved in water and added to heated oleic acid under homogenizer (the same situation as preparation of NLCs excluding the solid lipid). The arginine-oleic acid mixture was used to investigate the possible ionic interaction by FTIR experiment.

#### In vitro drug release study

The drug release study was conducted immediately after separation of free arginine from NLCs formulations. Samples of optimized formulation (1.5 mL) were placed in the dialyzer

and 120 mL of phosphate buffered saline (PBS) was used as dissolution medium ( $37 \pm 2^\circ\text{C}$ ). Samples from dissolution medium (2 mL) were collected at predetermined time intervals and replaced with equal volume of fresh medium and analyzed spectrophotometrically after derivatization at  $\lambda = 336\text{ nm}$ . Drug concentration was calculated using a standard calibration curve and expressed as cumulative released percent. The release study was performed in triplicates.

### In vivo studies

The hamster flank organ has served as a model to study the therapeutic strategies against alopecia (Adachi and Kano 1972; Lucky et al. 1986). Rhodamine B-loaded NLCs formulation was contacted with hamster skin for 3 h and then hamster was scarified and skin was cut by a freeze-microtome (Frigocut® 2800 N, Leica, Bensheim, Germany) vertically in  $10\text{ }\mu\text{m}$  slices. The dye distribution in the skin was investigated by fluorescence microscopic (BX50, Olympus Corp., Tokyo, Japan) (Babaie et al. 2015). The Rhodamine B solution in the same concentration with NLC formulation was applied on the other side of hamster skin as a control group. In the next step, 20 young male hamsters were used for *in vivo* experiment (weighing 70–120 g). The hamsters' skin was shaved ( $1\text{ cm}^2$ ) on the dorsal side with the electrical razors. The hamsters were divided randomly into three groups ( $n = 6$ ). In the first group,  $200\text{ }\mu\text{L}$  of the arginine solution (the same arginine concentration in NLCs formulation) was applied on the left side flank organ of hamster twice a day for 8 weeks. In the second group,  $200\text{ }\mu\text{L}$  of blank NLCs formulations, as a control, were applied twice a day on the left side of the treated skin and in the third group, arginine-loaded NLCs formulation was administered. After 50 days, the animals were killed by over inhalation of anesthetic gases and the skin ( $1\text{ cm}^2$ ) was cut by a surgical blade. Samples were fixed in 10% phosphate buffer formalin for 24 h and then dehydrated in a descending series of ethyl alcohol, cleared in xylene and embedded in paraffin. Paraffin sections were cut vertically into slices ( $5\text{ }\mu\text{m}$  thickness) by a rotary microtome (Leitz, 1512, Wetzlar, Germany), mounted on slides, stained with hematoxylin-eosin (H&E staining) and examined under a light microscope (BX50, Olympus Corp., Tokyo, Japan) (Hamishehkar et al. 2014). The area of the projected image of hair follicles was determined by an expert pathologist with an image analyzer software (Scion Image, version Alpha 4.0.3.2, Scion Corporation, Frederick, MD). Evaluations were performed by two independent observations, with an agreement level of 92%. Data analysis was performed using Statistical Package for Social Sciences (SPSS) 20.0 (SPSS, Chicago, IL). Results were stated as the mean  $\pm$  standard deviations. The independent-samples *t*-test was used to compare follicle areas between untreated and treated skins on both sides of hamsters in three studied groups.

## Results and discussion

### Characterization of arginine-loaded NLCs formulations

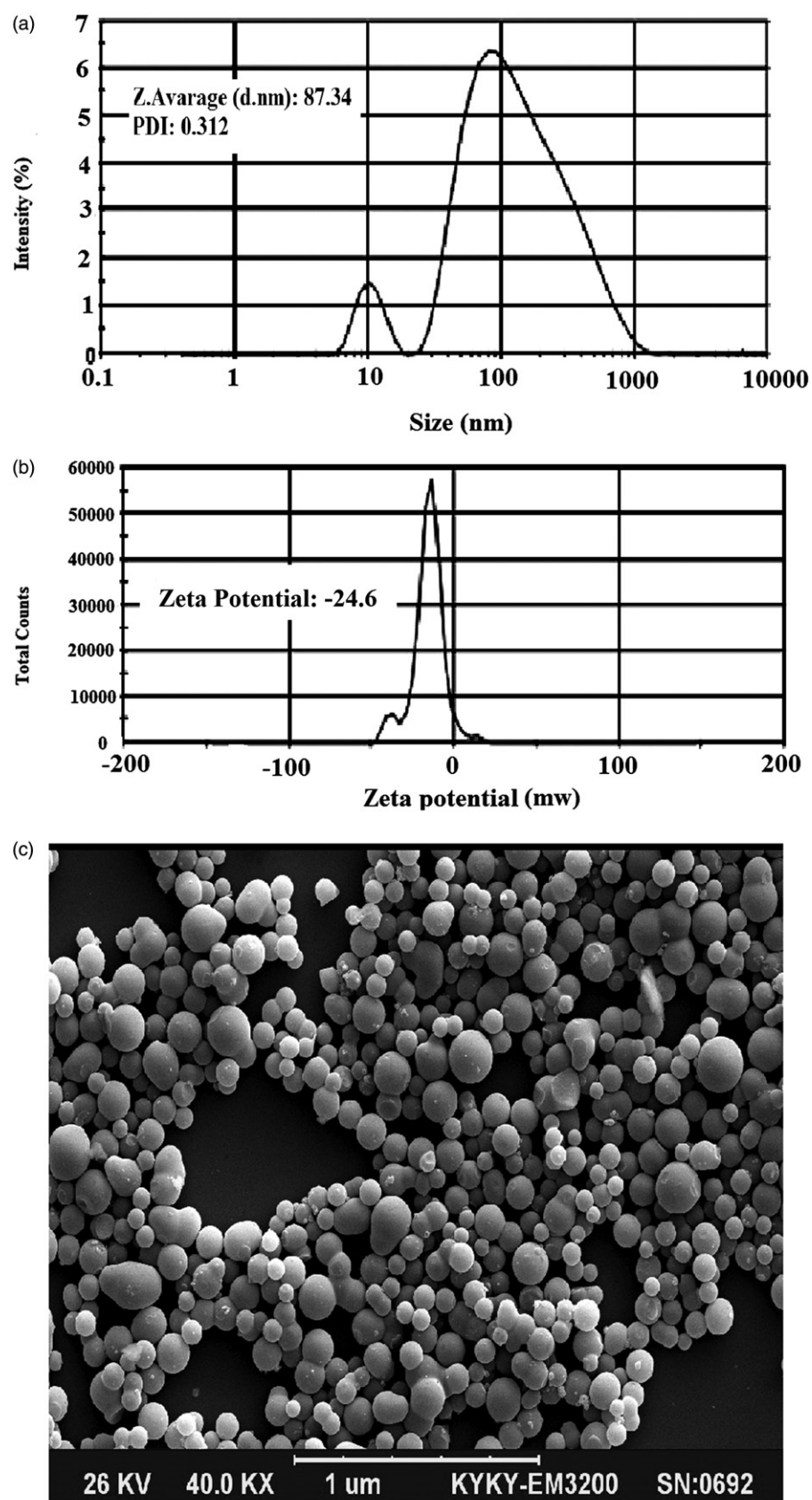
Arginine is not soluble in molten lipid, and its dispersion in molten lipid phase results in a low drug-loading capacity.

Therefore, to maximize drug encapsulation and loading capacity into NLCs, drug was dissolved in the aqueous phase and added into the molten lipid phase to form the initial W/O emulsion. Then, the external aqueous phase containing polymeric surfactant was added to form the W/O/W emulsion. After cooling down the formulation, emulsion droplets were converted to nanoparticles. The formed nanoparticles were stabilized by the presence of a polymeric surfactant (Poloxamer®). The particle size distribution, SEM image, and zeta potential of the optimized formulation are shown in Figure 1. The uniform distribution of NLC particles was clearly illustrated by SEM imaging (Figure 1(c)). The uniform size distribution enhances the predicted and reproducible skin penetration of drug following the administration of dosage form (Alomrani et al. 2015; Alvarez-Román et al. 2004; Dragicevic-Curic et al. 2004; Iannuccelli et al. 2014). EE% and LC% values of optimized NLC formulations were  $68 \pm 3.4\%$  and  $10 \pm 1.2\%$ , respectively. In the face of the high aqueous solubility of arginine ( $148.7\text{ mg/mL}$ ), these high EE% and LC% values are important and valuable achievements.

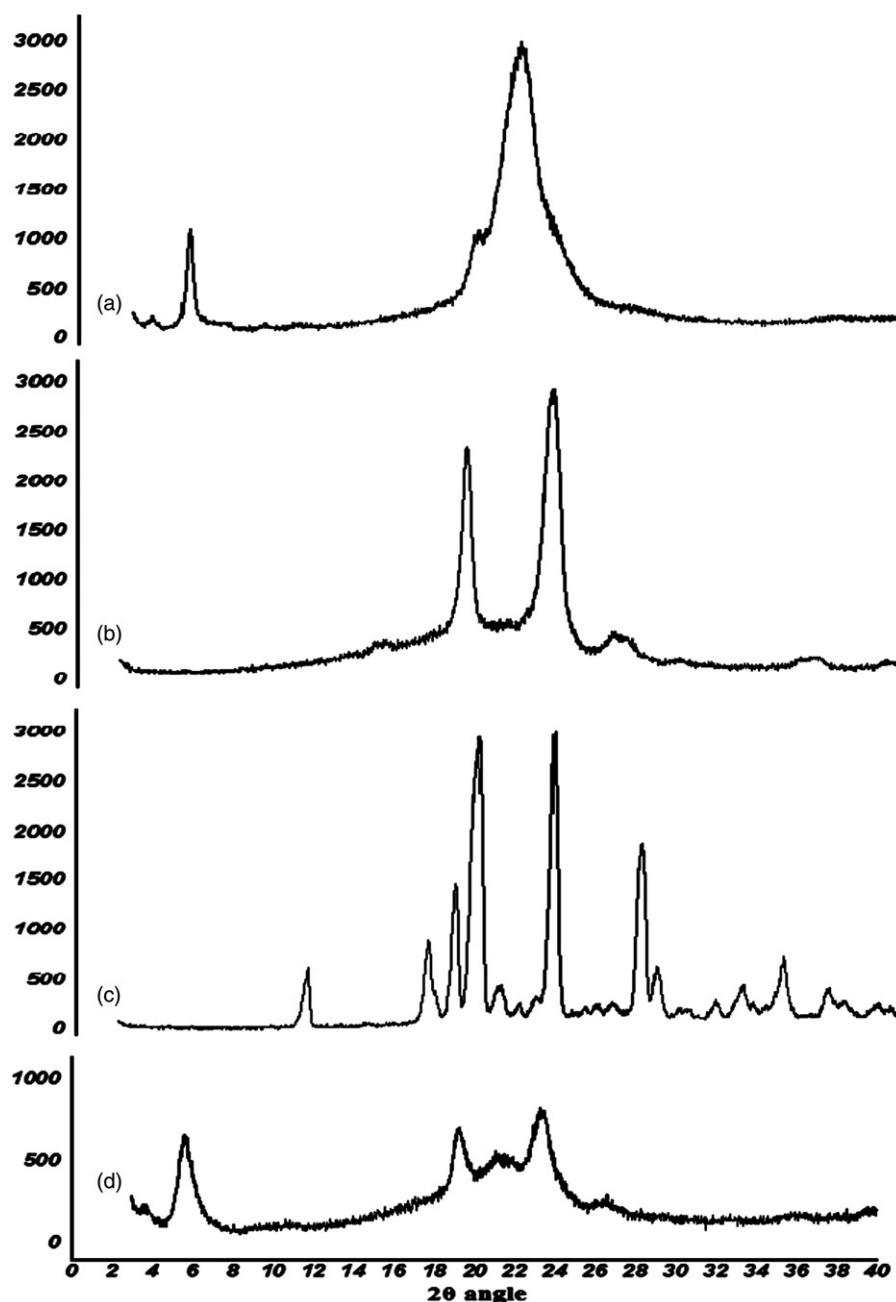
The crystalline state of the nanoparticles was analyzed using the X-ray diffraction method (Figure 2). The diffraction pattern of arginine exhibited several sharp peaks, indicating the crystalline nature of arginine. The characteristic main peaks for arginine ( $2\theta = 11.24, 17.15, 18.47, 19.52, 23.28, 27.53, 28.3, 32.47$ , and  $34$ ) were omitted from the XRD pattern of NLC formulations compared with pure arginine, suggesting that arginine was uniformly and molecularly distributed in the lipid matrix. The diffraction pattern of Precirol® in NLCs was much weaker than that of bulk lipid ( $2\theta = 5.3, 2\theta = 19.5$ , and  $2\theta = 21.5$ ), which indicates that Precirol® in the arginine-loaded NLCs was partially recrystallized and formed less-ordered crystals. These types of less-crystalline structures would contribute to a higher drug-loading capacity. Both pieces of evidence regarding the conversion of crystalline drug to a molecularly distributed one and the reduction in the crystalline status of lipids interpret the findings of the current study in achieving high drug loading (10%) for the hydrophilic and ionic drug arginine into NLC formulations. In addition to these advantages, a decrease in lipid crystallinity may show a drawback in the gradual instability of NLC structures becoming a crystalline one during the storage period of the formulation. However, this procedure depends on the crystal change kinetic rate. Probably, the rate would be low enough to guarantee formulation stability during the shelf life of the product.

The FTIR experiment was carried out to investigate the possible ion-pair interaction between arginine and oleic acid (Figure 3). The peak associated with the N-H stretch ( $3285\text{ cm}^{-1}$ ) in arginine was shifted to a higher value ( $3310\text{ cm}^{-1}$ ) in the mixture of arginine-oleic acid. Moreover, the peaks corresponding with the C=O O-H bends (carboxylic acid) at  $1709\text{ cm}^{-1}$  and  $949\text{ cm}^{-1}$  in oleic acid were shifted to  $1695\text{ cm}^{-1}$  and  $1012\text{ cm}^{-1}$ , respectively, in the mixture of arginine-oleic acid. These changes indicate the interaction of arginine and oleic acid in the formulation. This finding explains how a hydrophilic material (such as arginine) can be uniformly distributed in the lipophilic (Precirol®) matrix as concluded from the XRD experiment. The high LC%





**Figure 1.** Particle size distribution (a), zeta potential value (b), and scanning electron microscopy image of optimized formulation (c).



**Figure 2.** The X-ray diffractions pattern of precirol (a), Poloxamer<sup>®</sup> (b), arginine (c), and nanostructured lipid carrier formulation (d).

value reported during the encapsulation study was also elucidated by the result of the FTIR experiment. Oleic acid is an oil phase emulsifier, which existed in melted lipid phase (20% of oil phase). The water droplets containing arginine was surrounded by oleic acid to make a primary w/o emulsion. This could also increase the chance of arginine-oleic acid interaction for observed elevated EE and LC values for arginine.

### In vitro drug release

Figure 4 shows the release profile of arginine from the NLC formulation. The release profile indicates that arginine was released from the NLCs in an almost zero-order kinetic pattern after about 3 h of lag time. To analyze the release kinetics of arginine from NLCs, the data of release were subjected to the following equations: zero-order equation:  $Q_t = K_0 \cdot t$ , where  $Q_t$  is

the percentage of drug released at time  $t$  and  $k_0$  is the release rate constant; first-order equation:  $\ln(100 - Q_t) = \ln 100 - k_1 \cdot t$  where  $k_1$  is the release rate constant; Higuchi's equation:  $Q_t = K_H \cdot t^{1/2}$ , where  $K_H$  is the Higuchi release rate constant; Hixson-Crowell:  $(100 - Q_t)^{1/3} = 100^{1/3} - k_{HC} \cdot t$ , where  $k_{HC}$  is the Hixson-Crowell rate constant (Hamishehkar et al. 2015b). Mean percent error and  $R$ -squared values for zero-order, first-order, Higuchi, and Hixson-Crowell were 11.01 and 0.994, 212.46 and 0.932, 31.96 and 0.978, and 83.10 and 0.965 indicating the fit of zero-order kinetic to arginine release pattern. The release procedure was completed after 24 h (98.5% of loaded arginine).

### In vivo studies

Figure 5(a) and (b) shows the skin penetration capability of Rhodamine B solely in the solution after 3 h of administration

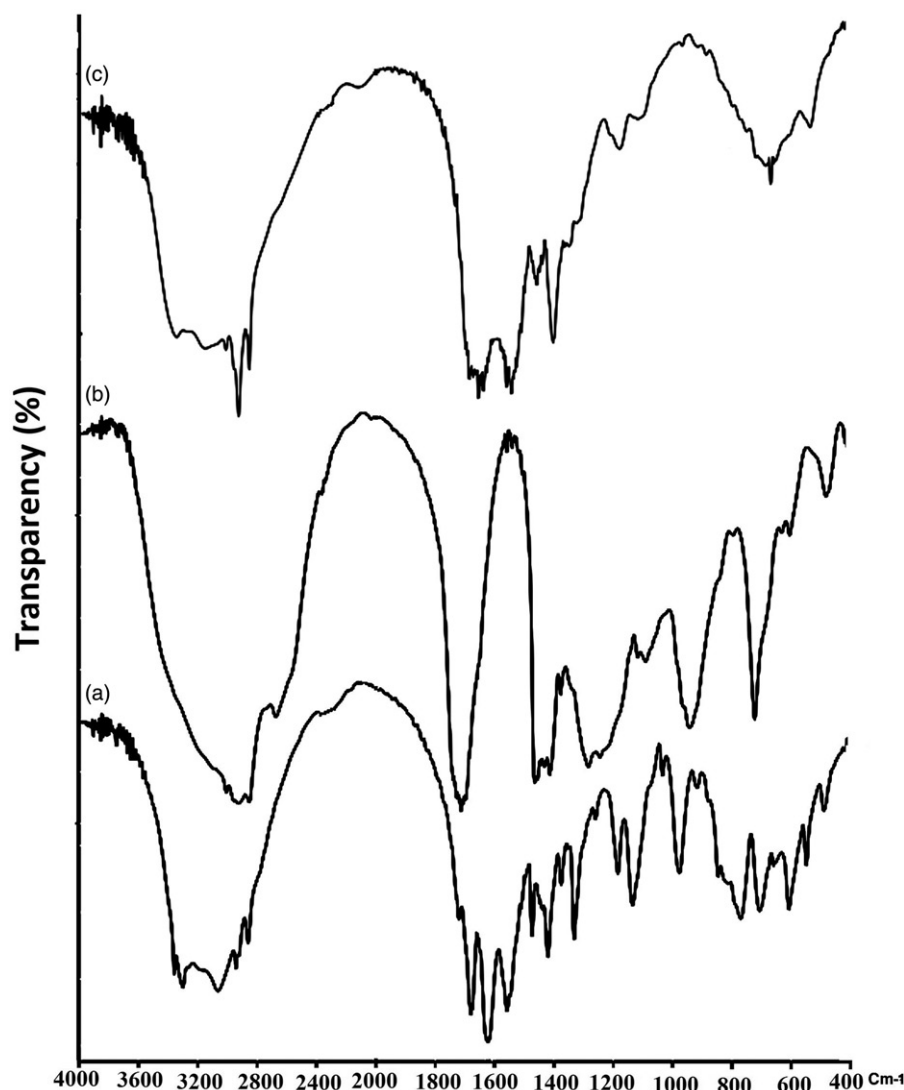


Figure 3. The Fourier transform infrared spectroscopy pattern of arginine (a), oleic acid (b), and the mixture of arginine-oleic acid (c).

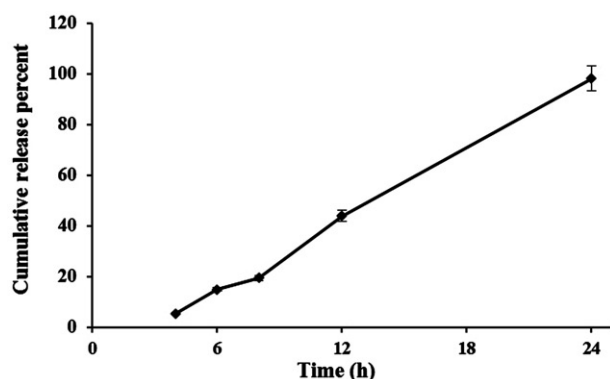


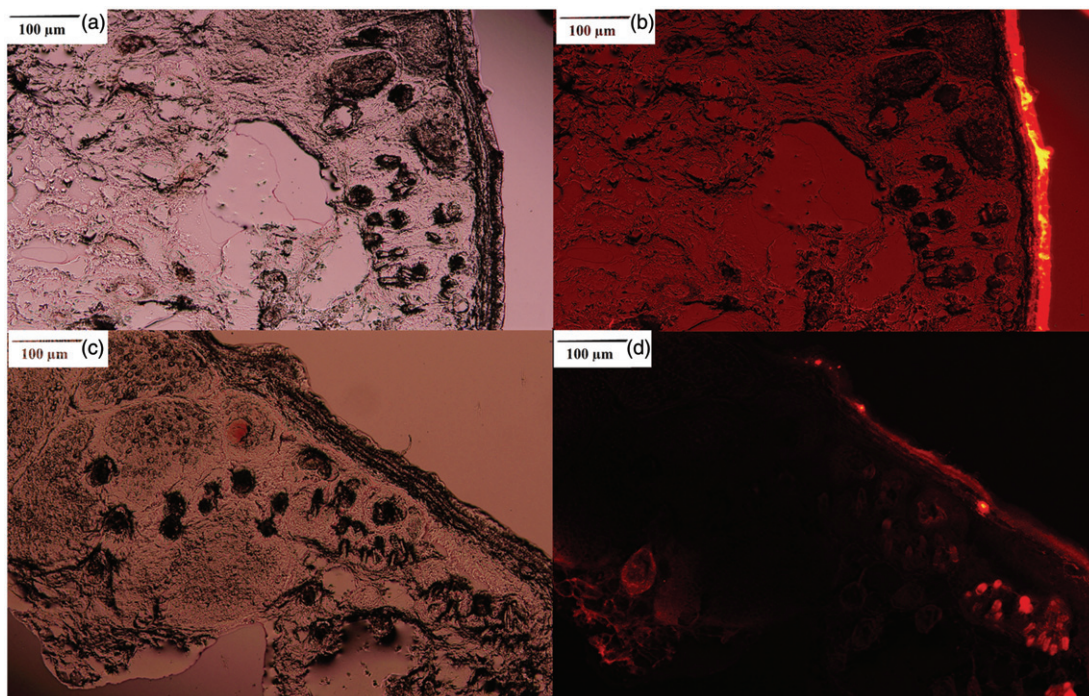
Figure 4. *In vitro* drug release profile of arginine-loaded nanostructured lipid carrier formulation (Data are presented as mean  $\pm$  SD).

on skin. The results illustrate that, although the physicochemical characteristics of Rhodamine B (MW of 422 and  $\log P$  of 1.95) are suitable for skin penetration (MW  $<500$  and  $\log P$  of 1–4), no evidence of skin penetration of Rhodamine B was observed, even after 3 h. A minor penetration of Rhodamine B solution might be due to its close contact with the

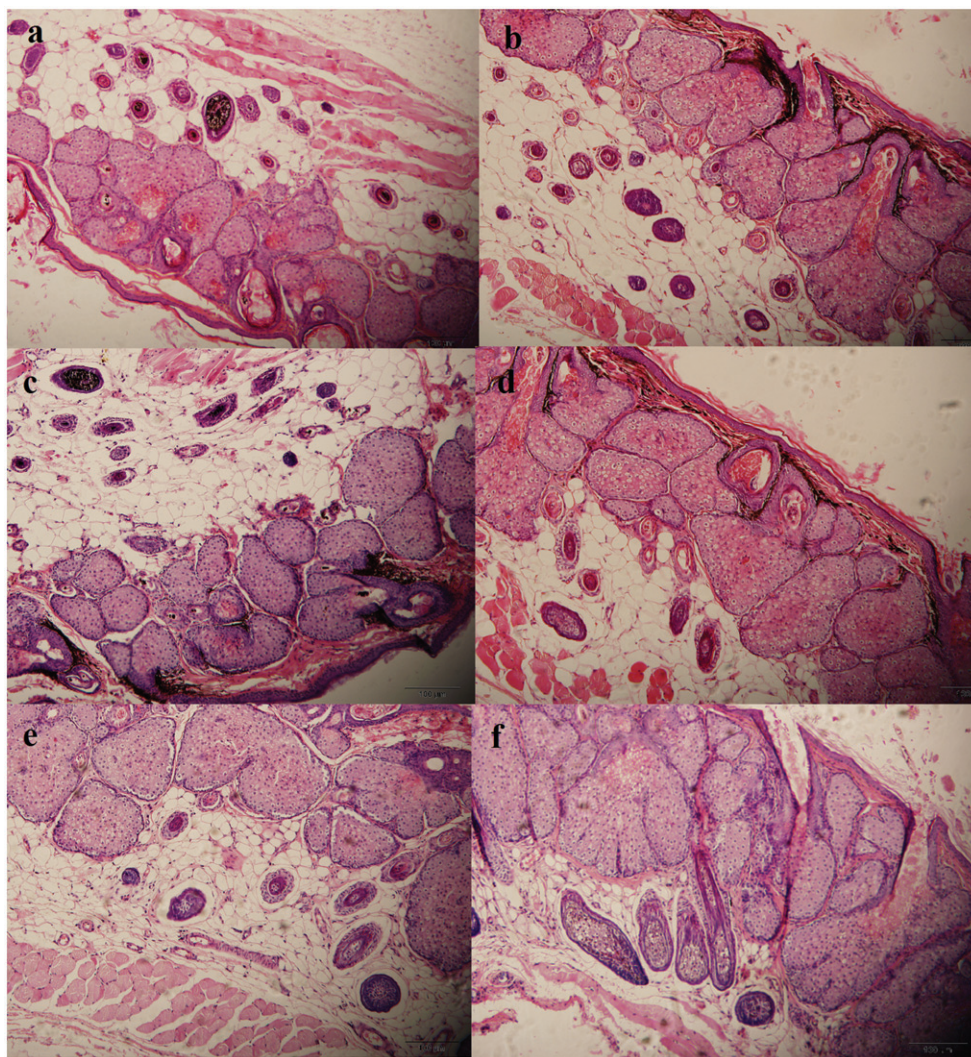
superficial junctions of the SC and the furrows between the corneocyte islands and hair follicles, allowing superficial spreading of the active agents (Ghanbarzadeh et al. 2015). However, the follicular accumulation of Rhodamine B-loaded NLCs (Figure 5(c) and (d)) compared to unpenetrated Rhodamine B in solution proves the key role of NLCs in efficient follicular drug delivery.

The results of the histological studies for the aqueous solution of arginine, blank NLCs, and arginine-loaded NLCs, with respect to their ability to influence hair growth, are shown in Figure 6. The histological qualitative (Figure 6) and quantitative (Table 1) assessment demonstrated the enlargement of hair follicles area (Figure 6(f)) than untreated skin (Figure 6(e)) after 8 weeks. No significant change between untreated (Figure 6(a) and (c)) and treated (Figure 6(b) and (d)) flanks in the skin histology of control groups (aqueous solution of arginine and blank NLCs) were observed (Table 1). The comparative results of Figure 6(c) and 6(d) also verify the safety of the used NLC formulations for topical administration, at least for 8 weeks. The lipidic structure of the newly-developed nanocarriers might be responsible for their capability for safe action in topical administration.





**Figure 5.** Rhodamine B penetration into hamster skin: staining of hamster skin following the application of 0.001% Rhodamine B solution (a: light microscopic image, b: fluorescent microscopic image) and 0.001% Rhodamine B-loaded nanostructured lipid carrier (c: light microscopic image, d: fluorescent microscopic image).



**Figure 6.** Effect of aqueous solution of arginine (b), blank nanostructured lipid carrier (NLC) (d) and arginine-loaded NLC (f) on the hair follicles compared with untreated skin (a, c, and e, respectively).



**Table 1.** The histological effect of arginine-loaded nanostructured lipid carrier (NLC) on hair follicles.

| Studied groups                 | Mean $\pm$ SD <sup>a</sup> | Independent t-test results |                 |          |
|--------------------------------|----------------------------|----------------------------|-----------------|----------|
|                                |                            | t                          | Df <sup>b</sup> | P values |
| 1 Untreated                    | 1046 $\pm$ 315             | 1.34                       | 14              | 0.200    |
| Treated with arginine solution | 1260 $\pm$ 292             |                            |                 |          |
| 2 Untreated                    | 1180 $\pm$ 570             | 1.29                       | 15              | 0.216    |
| Treated with blank NLC         | 1456 $\pm$ 425             |                            |                 |          |
| 3 Untreated                    | 1738 $\pm$ 819             | −3.11                      | 9.7             | 0.011    |
| Treated with arginine-NLC      | 4413 $\pm$ 668             |                            |                 |          |

<sup>a</sup>Standard deviation.<sup>b</sup>Degree of freedom.

The merit of using lipid nanoparticles in follicular drug delivery has been previously reported (Puglia et al. 2008). The authors of the current report also had a successful experience on the delivery of flutamide to hair follicles by means of SLNs (Hamishehkar et al. 2015a).

## Conclusions

Improving the skin penetration of hydrophilic and/or charged drugs by nanoparticulate carrier systems has attracted increasing interest. The Precirol®-oleic acid based arginine-loaded NLC formulations showed appropriate drug EE% and LC% values. The importance of the ion-pair interaction between carrier composition and drug was verified by XRD and FTIR experiments. *In vivo* studies indicated the efficient performance of the developed NLC particles. The presented oleic acid-based NLC formulations may be introduced as a successful template carrier for dermal delivery of positively charged hydrophilic drug, especially amino acids and peptides which have recently become popular in cosmeceutical sciences.

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## Disclosure statement

The authors report no conflicts of interest.

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