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Research Article

Nanostructured Lipid Carrier for Topical Application of N-Acetyl Glucosamine

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Abstract

Purpose: Hyperpigmentation occurs when melanin is overproduced in certain spots on the skin and is one of the most challenging skin conditions to treat. Although it is usually harmless, for cosmetic reasons, it is dreadfully bothersome to those who undergo it. It was reported that N-acetyl-glucosamine (NAGA) prevents melanin synthesis and alters the expression of numerous genes related to pigmentation. In spite of these advantages, NAGA cannot be employed in topical formulations due to its extremely polar characteristics. Nanoparticles, especially lipid-based ones, have been introduced as an efficient carrier for dermal drug delivery.

Methods: The aim of the present study was to load adequate hydrophilic NAGA to the lipophilic nanostructured lipid carriers (NLCs) for potential dermal application. Methods: NAGA-loaded NLCs were formulated, using hot homogenization technique, and the characteristics of the optimized formulation were analyzed by laser light scattering, X-ray diffraction, and scanning electron microscopy methods. Loading capacity percentage and in vitro release study were carried out by applying a validated HPLC method. The optimum formulation was utilized for the in vivo skin lightening evaluations in healthy volunteers.

Results: NAGA-loaded NLCs demonstrated promising results (the size of 190 nm, narrow size distribution, loading capacity of 9%, and appropriate NAGA release profile) suitable for dermal delivery. XRD results exhibited a dramatic reduction in the crystalline structure of encapsulated NAGA. Dermoscopy images indicated a considerable decline in melanin distribution pattern in the majority of the cases treated with NAGA-loaded NLCs.

Conclusion: Thus, this study has opened new horizons for the potential use of lipid based nanoparticles in the managing of hyperpigmentation.

Introduction

Glucosamine is used to decrease melanogenesis in melanocyte and, thus, has a potential to decrease hyperpigmentation by topical use. However, owing to the stability restrictions of glucosamine in skincare products, the stable derivative N-acetyl glucosamine (NAGA) is utilized which is a monosaccharide derived from glucose chemically produced by linking glucosamine and acetic acid. It inhibits the tyrosinase glycosylation, a step necessary in the production of melanin. Hyperpigmentation is a common skin disorder and has a considerable impact on the psychosocial quality of life, since skin color is well thought as a universal sign of youth and beauty.[1] Skin lightening creams available on the market mainly contain hydroquinone which causes skin irritation, localized contact dermatitis, and, in some cases, temporary or permanent discoloration of the skin.[2] Furthermore, studies on animals showed that prolonged use and high concentration of hydroquinone were carcinogenic, and the use of hydroquinone as a cosmetic ingredient was banned in the European Union since 2001.[3] Therefore, NAGA can be introduced as a good candidate to be used instead of hydroquinone. Generally, the molecules with the log p of 1-4 are suitable candidates for dermal drug delivery. It was reported that the molecules with the log p lower and higher than 1 and 4 will not properly penetrate into the stratum corneum of skin.[4] NAGA is a very hydrophilic characteristic with the log p of -3.3.[5] Solid lipid nanoparticles (SLNs) and nanostructure lipid carriers (NLCs) have attracted growing attention in pharmaceutical research for dermal and transdermal delivery enhancement.[6] Unlike liposomes and emulsions, lipid carriers possess a number of advantages, including protection of the

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incorporated active compound against chemical degradation, controlled release profile of the incorporated drug, and physical stability of colloidal system.[4,7,8] These particles possess additional valuable benefits such as biocompatibility and ease of industrial scalability. The formulations of both SLNs and NLCs are extensively employed for producing dermal and cosmetic products. These formulations indicate various favorable advantages such as skin hydration, smoothness, and occlusion as well as improved skin penetration and formulation appearance.[9-11] These advantages of lipid nanoparticles resulted in the creation of a number of cosmetic products, including sun blockers, herbal extracts, as well as many poly peptides and fatty acids.[12,13] However, one of the most serious disadvantages of these types of lipid-based carriers is insufficient drug loading, particularly hydrophilic drugs such as NAGA. Therefore, the objective of this study was to introduce a new method to load adequate amounts of very hydrophilic NAGA molecule into lipid-based nanoparticles for clinical application on healthy volunteers.

Materials and Methods

Materials

N-acetyl glucosamine and Poloxamer® were purchased from Sigma Aldrich Company (USA). Precirol® (glycerol palmito setarate) and Miglyol® were obtained from Gattefosse (France) and BASF (Germany) companies, respectively. Tween® 80 and acetonitrile were prepared from Merck Company (Germany).

Preparation of NAGA-loaded SLNs and NLCs

To prepare SLN, Precirol® (1 g) was melted at 80 °C and homogenized prior to addition of drug solution. NAGA was dissolved in one mL of aqueous solution containing Poloxamer® and Tween® 80, as surfactant, and added dropwise to the melted homogenized lipid mixture. Then the 50 mL of aqueous solution containing Poloxamer® (1%) and Tween® 80 (1%) was added under homogenization rate of 20000 rpm (Silent Crusher M, Heidolph, Germany). The procedure for NLC preparation is the same as SLN except that 300 mg of Miglyol® (an oil) was co-melted with 700 mg of Precirol® (solid lipid).

Characterization of NAGA-loaded lipid nanoparticles

Determination of entrapment efficiency (EE) and loading capacity (LC):The of EE and LC percent values were presented as the percentage of encapsulated drug to the used drug or lipid, respectively according to the following questions:

$$EE\ (\%) = \frac{W_{(loaded\ drug)}}{W_{(Initial\ drug)}} \times 100$$

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

Where, $W_{(Initial\ drug)}$ is the amount of initial NAGA used and $W_{(loaded\ drug)}$ is the amount of free NAGA determined after the NLC formulations centrifugation in the lower chamber of Amicon® Ultra-15 tube (Merck Millipore Ltd., Ireland). Accordingly, $W_{(Entrapped\ drug)}$ is the amount of NAGA which is loaded in nanoparticles and $W_{(Total\ lipid)}$ is the total amount of lipid used in the preparation of NLC.[14,15] EE was determined by separation of the un-loaded NAGA by centrifugation method using the Amicon tube with the molecular weight cutoff of 100 kDa[16] To separate unentrapped drug from nanoparticles, 5 mL of sample was added to the upper chamber of Amicon tube and centrifuged (Universal 320, Pole Ideal Tajhiz Co., Iran) at 4000 rpm for 20 min. The nanoparticles remained in the upper chamber and unentrapped NAGA passed through the filter to the lower chamber. The amount of entrapped NAGA in the lower compartment of Amicon® tube was determined using validated High performance liquid chromatography (HPLC) technique after sufficient dilution.[17] Then, for several times, 5 mL water was added to the remained formulation on the upper chamber of the Amicon® tube and centrifuged to remove unloaded NAGA. The rinsed optimized formulation was used for the rest *in vitro* and *in vivo* experiments.

NAGA analysis

NAGA was analyzed using a covalent bounded poly amine chromatographic HPLC column which is especially optimized for the separation of mono and oligosaccharides employing a reversed-phase HPLC technique by Knauer apparatus, utilizing a sensitive variable wavelength ultraviolet spectrophotometric detector (set at λ_{max}=205 nm). The samples were eluted using a mobile phase (acetonitrile: water, 70: 30 v/v) at a flow rate of 1 mL/min. In this condition the retention time was 5.8 min. Calibration curve was linear in the concentration range of 2.5–100 µg/mL (R^2=0.999). There was no interference with formulation components and the samples were stable throughout the study period.

Size distribution

Particle size of NLC formulation was analyzed using laser diffraction method by particle size analyzer (SALD 2101, Shimadzu, Japan). This method measured the mean diameter of the bulk population based on the volume mean diameter (VMD). Size distribution index (Span) was calculated according to the following equation:

$$Span = \frac{D_{90\%} - D_{10\%}}{D_{50\%}}$$

Where $D_{10\%}$, $D_{50\%}$ and $D_{90\%}$ specify the percentage of particles having 10, 50 and 90% of the diameter lower than or equal to the given value.[16] Each sample was measured in triplicate and formulation samples were diluted with double-distilled water.

Scanning Electron Microscopy (SEM)

The photographs of prepared NLCs were obtained by scanning electron microscope (MIRA3, TESCAN instrument, Czech Republic). Samples were mounted on a metal stub with double-sided adhesive tape. Subsequently, samples were coated under vacuum in an argon atmosphere with gold (DST1, Nanostructured coating co., Tehran, Iran).

X-ray diffraction (XRD) study

In order to assess the effect of preparation process on crystallographic patterns of NAGA and lipid, XRD analysis was performed using an X-ray diffractometer (D-5000, Siemen, Germany, 2° to 70°) to assess the crystalline structures of NAGA, Precirol®, Poloxamer®, physical mixture and optimized NLCs formulation. The diffraction pattern was measured using a Cu-Kα radiation source (30 mA and 40 kV) .

In vitro drug release study

Immediately after separation of free NAGA from optimized NLCs formulations, drug release study was performed using dialysis cellulose membrane (MWCO 12 KDa, Sigma, USA) which was mounted on the Franz-type diffusion cells (HDT6, Erweka, Germany). Available diffusion area and the receptor compartment volume were 3.14 cm^2 of 24 mL, respectively. Two milliliter of the formulation was located in the donor compartment and the receptor medium (phosphate buffer saline, PBS) was stirred with Teflon-coated magnetic stirring bars (700 rpm) and the temperature set on 32 ± 1 °C. To prevent evaporation during the study the sampling port and donor chamber were covered by parafilm. At predetermined time intervals (1, 2, 3, 4, 5 and 6 h) samples (0.5 mL) were obtained from the receptor compartment and the amounts of permeated drug into receptor compartment were determined by HPLC apparatus and cumulative released percent was expressed. To maintain a constant volume, the same volume of PBS was replaced. All experiments were repeated three times in different days in three diffusion cells and.

In vivo assessment of NAGA-loaded NLC formulation on human volunteers

Before the study, each volunteer filled in a consent form. It was an eight-week double blind, vehicle control, pilot study which was done on fifteen subjects. The NLCs formulation containing NAGA were applied every night, to the inferior forearm left hand of first five volunteer (group I, test group). In the second group (group II, negative control) blank NLCs were applied once every night to the left hand forearm. In the next five volunteer (group III, positive control) a NAGA solution was applied to estimate whether NLC shows superior lightening effect than NAGA or not. A sunscreen SPF15 was applied every next morning (Zinc oxide 15% in Vaseline). Pictures from chosen areas were captured at the beginning and the end of the study (8 weeks). For each volunteer pictures from chosen area were captured by dermatoscope device (TES-TB PORTABLE, brand KC TECHNOLOGY, South Korea) and compared and ranked by dermatologist from zero to 4 (zero= no lightening effect is seen, 1= less than 25% increase in skin lightening, 2= 25-50% increase and 4= 100% increase in skin lightening is seen). The NAGA concentration in NLC and solution formulations was 3.6 mg/mL.

Statistical analysis

Statistical analysis was done using a two related samples test Wilcoxon (SPSS, version 22.0, Chicago, IL, USA). P value of <0.05 was considered significant.

Results and Discussion

Preparation of NAGA-loaded NLCs

The size of formulated SLNs and NLCs accompanied by NAGA entrapment indexes are summarized in Table 1. Although NAGA-loaded SLN formulation (SLN1) showed a promising size, EE and LC percentage was very disappointing.

Table 1. Characteristics of the prepared formulations

-	NAGA[a] (mg)	Size (nm)					Span	EE[e] (%)	LC[f] (%)
		NMD[b]	VMD[c]	$D_{90\%}$[d]	$D_{50\%}$[d]	$D_{10\%}$[d]			
SLN[g]1	50	36 ± 5	128 ± 11	201 ± 12	134 ± 14	70 ± 5	1.1 ± 0.2	0.4 ± 0.05	0.02± 0.01
SLN2	200	19 ± 3	483 ± 51	1124 ± 45	491 ± 41	136 ± 7	2.9 ± 0.4	5.0 ± 0.9	1.00 ± 0.1
NLC[h]1	200	72 ± 9	191 ± 4.0	285 ± 21	195 ± 20	111 ± 21	0.9 ± 0.2	44 ± 3.6	8.80 ± 1.3
NLC2	400	765 ± 24	863 ± 37	1077 ± 57	873 ± 45	670 ± 58	2.5 ± 0.4	55 ± 2.6	22.0 ± 2.4

[a] N-acetyl- glucosamine; [b] Number mean diameter; [c] Volume mean diameter; [d] D10%, D50% and D90% indicate the percentage of particles having 10, 50 and 90% of the diameter lower than or equal to the given values based on volume diameter; [e] Encapsulation efficiency; [f] Loading capacity; [g] Solid lipid nanoparticles; [h] Nanostructured lipid carriers

To augment these values, we tried to add higher NAGA amounts in the first step of SLN production to improve drug entrapment. Therefore, with the aid of surfactant (Poloxamer® and Tween®), 200 mg of NAGA was dissolved in one milliliter of distilled water. Although EE and LC values increased, they still were not enough. Accordingly, we decided to replace NLC with SLN as NAGA carrier. It was claimed that NLCs show a more encouraging future in drug loading than do SLNs due to imperfect lipid

structure because of oil incorporation in solid lipid structure.[18,19] NLC1 was prepared in the identical conditions of SLN2 except that 300 mg Miglyol® (oil) was replaced with 300 mg of Precirol® (solid lipid). Accordingly, NAGA's EE and LC percentage values were improved dramatically. In addition to improved NAGA entrapment, size and size distribution values also decreased compared to SLN2 formulation. It was reported that NLC particles have a lower size than SLNs in the identical preparation conditions.[20-24] To achieve the most desired result, NAGA was dissolved in the highest possible amounts (400 mg) in one milliliter of water with the aid of surfactants (NLC2). Even though NAGA entrapment indexes (EE and LC) very hopefully increased, size and size distribution values rose (the same as what happened in the case of SLN formulations). Hence, NLC1 was selected as the optimized formulation for further in vitro characterization and in vivo study. Figure 1 depicts the size pattern of optimized formulation (NLC1) extracted from particle sizer device in both volume (VMD) and number (NMD) mean diameters.

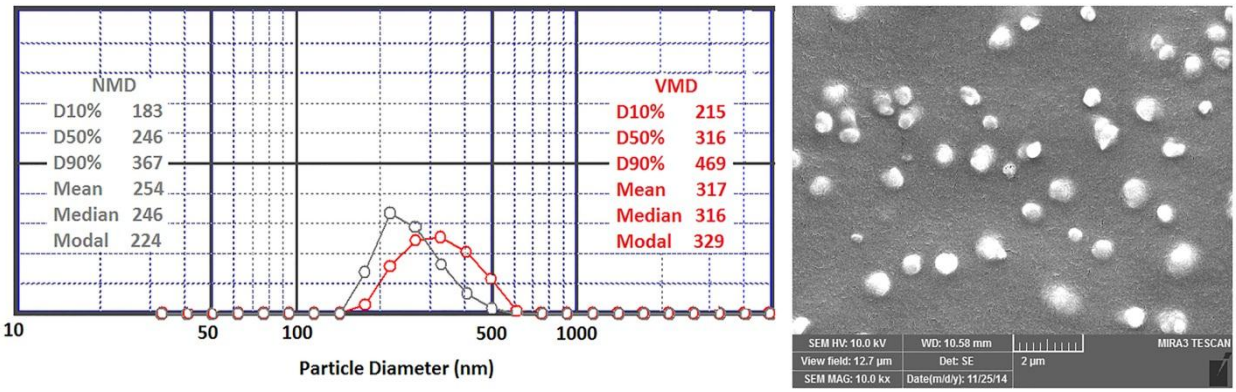

Figure 1. Left) Particle size distribution pattern based on volume mean diameter (VMD) (red line) and number mean diameter (NMD) (gray line) and right) scanning electron microscopy image of the optimized nanostructured lipid carrier formulation (NLC2).

The small difference between VMD and NMD shows the size homogeneity of the nanoparticles. Lipid particles with a small size guarantee the near contact with the stratum corneum and, therefore, enhance drug penetration in the skin. The findings of the current study revealed that the optimized NLCs were in the appropriate size range for deep skin penetration. This result contradicts the report by Patzelt et al., who showed that reductions in particle size (643 nm, 470 nm, 300 nm, and 122 nm) led to a significant reduction in particle penetration depth.[25,26] Transportation through the skin was limited to molecules with certain properties (molecular weight < 500 Dalton, moderate lipophilicity with octanol-water partition coefficient between 10 and 1000, and melting point < 200 °C).[4,27] However, additional means is usually required to increase transportation through the skin. The molecular weight, log p, and melting point of NAGA are 221 Dalton, -3.2, and 211 °C, respectively.

XRD study

The X-ray patterns of NAGA, Poloxamer® and Precirol®, as well as prepared physical mixture and NAGA-loaded NLCs of the formulation are shown in Figure 2.

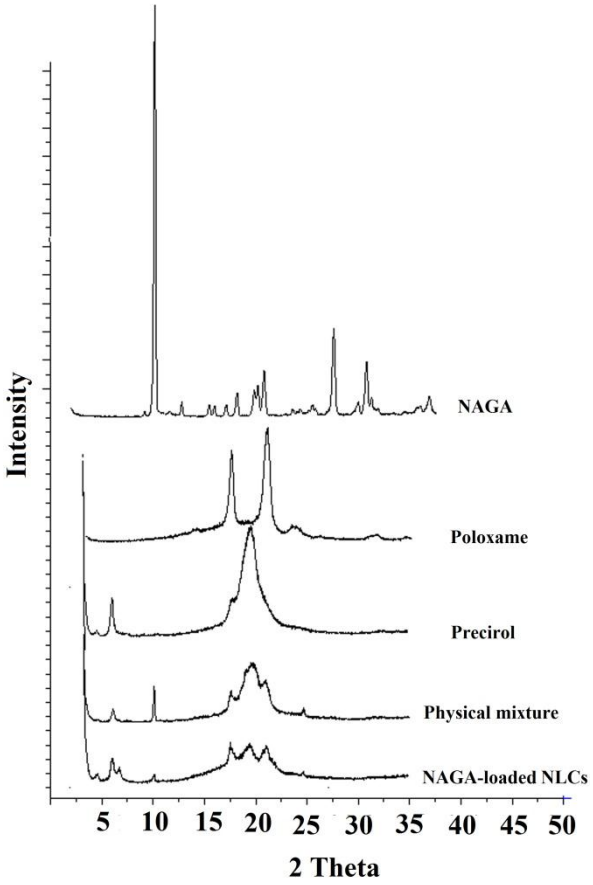

Figure 2. X-ray diffraction pattern of pure N-acetyl-glucosamine (NAGA), Poloxamer®, Precirol®, physical mixture and NAGA-loaded nanostructured lipid carriers (NLCs).

Precirol® showed distinct peaks at 2θ=19, 23, and 24, and Poloxamer® displayed two peaks at 2θ=19 and 23. Pure NAGA powder showed distinct peaks at 2θ=10 and 27.6, indicating the high crystalline nature of the NAGA. The same peaks were similarly shown in the case of physical mixture. However, in the NAGA-loaded NLCs, these peaks were observed with reduced intensity. These results indicated that NAGA was loaded in the nanoparticles in the reduced crystalline state. Decreasing the crystalline status of lipid and exchanging crystalline drug for the lower crystalline one verified our findings. This indicates the suitable drug integration into NLCs and the absence of possible upcoming leakage of NAGA from NLCs. Liquid lipids with different fatty acids form NLCs with low organized crystalline structure and, thus, guarantee a better loading capacity for drug lodging. [26] Therefore, liquid lipids are better solubilizers for drugs than solid lipids.

In vitro drug release study

Figure 3 illustrates that more than 90% of the loaded NAGA was released during 6 h in an almost rapid onset of release.

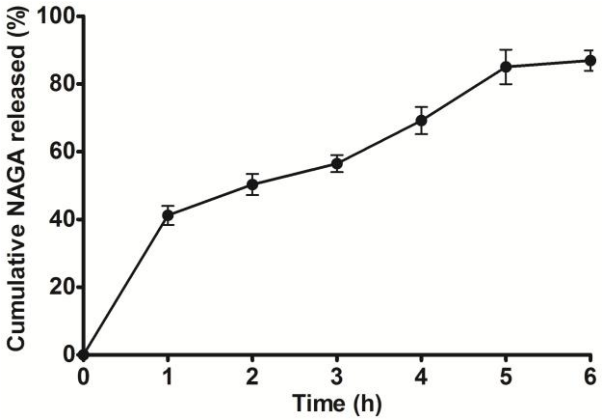

Figure 3. The release profile of NAGA from the optimized nanostructured lipid carrier formulation (NLC2).

The extremely sustained release might prevent the adequate accumulation of NAGA for the therapeutic efficacy, while too rapid drug release might cause the chance of NAGA systemic absorption.[28,29] This might interpret why we could not detect NAGA in the receiver phase of Franz diffusion cell in ex vivo experiments by excised rat skin. Furthermore, the very low UV molar absorptivity of NAGA did not provide enough sensitivity for determination of small amounts of passed NAGA through rat skin even with HPLC technique. Due to the presence of endogenic NAGA in skin, the determination of skin- deposited NAGA was not possible.[30] Therefore, after *in vitro* release study, we directly fulfilled the *in vivo* study on human volunteers.

In vivo study on human volunteers

Blank NLCs and NAGA solution did not cause any significant difference in skin melanin density after 8 weeks of treatment (Figure 4II and III). Although dermoscopy pictures demonstrated a considerable reduction in melanin distribution pattern in most cases treated with NAGA-loaded NLCs (Figure 4I), the statistical analysis did not show a significant difference (p=0.09). This result might be due to the small population of the subjects, narrow scoring grade, or short period of treatment. To decrease melanogenesis and inhibit melanin production in melanocyte, NAGA should penetrate into the bottom layer (the stratum basale) of the skin's epidermis (the location site of melanocytes in skin).[31,32] This means that NAGA should pass the most resistant skin barrier for penetration of materials, i.e. stratum corneum, which is more difficult for very polar substances such as NAGA. It was shown that NLC particles in the small sizes can penetrate into stratum corneum.[33]

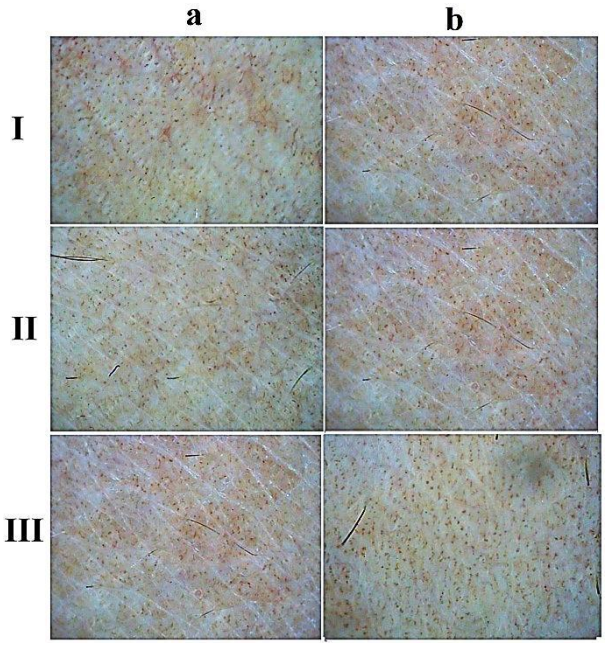

Figure 4. Polarized dermoscopy images before treatment (a), after 8 weeks of treatment (b), treated with N-acetyl-glucosamine (NAGA)-loaded nanostructured lipid carriers (NLCs) (I), blank NLC (II), and NAGA solution (III).

Conclusion

NAGA-loaded NLCs were prepared in the appropriate characteristics (high NAGA loading indexes, small size, and suitable NAGA release profile) for dermal delivery. Dermoscopy image demonstrated a considerable reduction in melanin distribution pattern; however, visual examination by dermatologists indicates an insignificant difference in the test group compared with the control group. We are very intended to the developed formulation to show promising results by increasing the investigated population, optimizing scoring grades, and increasing the treatment period of time. Our proposed formulation will pave the way for introducing new horizons in the management of hyperpigmentation.

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Ethical Issues
Not applicable.

Conflict of Interest
The authors declare no conflict of interests.

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