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# Preparation and Characterization of Nanostructured Lipid Carrier (NLC) and Nanoemulsion Containing Vitamin D for Enrichment of Dairy Products

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Research Article

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## ABSTRACT

Nanostructured Lipid Carriers (NLC) and nanoemulsions are carriers to improve bioavailability, stability and solubility of bioactive compounds. The main objective of this paper was to develop NLC and nanoemulsion for the enrichment of dairy products of vitamin D.

NLC containing Precirol and nanoemulsion containing vegetables oil was prepared and characterized for polydispersity index, particle size, zeta potential, particle shape, crystal properties, stability, encapsulation efficiency and releasing.

NLC showed sizes in the range of 123.4 to 210.6 nm and nanoemulsion 137.6 to 171.6 nm. Optimum NLC and nanoemulsion formulation was selected for other analysis (such as morphological study, encapsulation efficiency, thermal analysis and release study. Scanning and transmission electron microscopy revealed that particles had approximately spherical shape. In gastric simulated solution (pH=1.2) 9.3% of vitamin D from NLC and 29.6% from nanoemulsion released which indicated that NLC and nanoemulsion can protect vitamin D against acidic conditions.

The results suggest that NLC and nanoemulsion could be a promising carrier for the fortification of food to improve bioavailability of vitamin D.

## INTRODUCTION

In recent years, technologies have emerged in the food industry which leads to high quality products and high shelf life. Including this technologies are nanoencapsulation or microencapsulation [1]. In this technique, a variety of vitamins, antioxidants, oils, enzymes, microorganisms can be coated by biopolymer compounds such as carbohydrates, proteins, and fats.

Lipid based nanocarriers are useful for delivering water insoluble compounds. Examples for lipid nanocarriers are nanoemulsions, solid lipid nanoparticles (SLN), nanostructure lipid carriers (NLC) and nanocapsule (LNC).

In NLC, the lipid phase contains solid fats and liquid oils (at room temperature). NLC contains fat droplets that are crystallized to a small extent and have a lower order crystalline structure or amorphous solid form (formless). This system was introduced, in late 1990 to improve the features of the SLN system. The purpose of NLC production is to produce particles in which oil is trapped in the center of solid fat, in order to increase the loading capacity and the release of the drug is more controlled [2]. That way, the drug is dissolved in liquid oil and encapsulated along with it in solid fat.

The main components of NLC include water, fatty phase (in the case of NLC, solid and liquid fats), and surface-active compounds (surfactants). In NLC, a large part of the oil contained in the O/W emulsion is replaced with a solid fat, which makes the solid matrix of this carrier at the temperature of the body. To provide this matrix, solid fats must be mixed with oil.

The most important features required to describe the NLC include: Particle size, polydispersity index and Zeta potential by particle size analyzer, Particle shape by TEM and SEM, Crystal properties by using the DSC device, the stability of the particles during storage and the process (either alone or as an additive), the encapsulation efficiency and loading rate, determination of the amount by HPLC and release method, by using the method of dialysis bag.
Emulsions are a dispersed colloidal system with at least two non-intermolecular liquids that do not spontaneously form. The emulsion structure consists of a dispersed phase (discrete phase or internal phase) in another liquid (continuous phase or external phase). The emulsions are divided into two groups of water-in-oil emulsion (W/O) and oil-in-water emulsion (O/W), depending on which phase is continuous phase and which phase is dispersed phase. The emulsions are divided according to the size of the emulsion droplets into micro emulsion, nanoemulsion and macro emulsion [3]. Many features of emulsion, including stability, rheology, appearance, color and texture depend on the size of the emulsion droplets and the particle size distribution. Basically, nanoemulsions have a clear or semi-transparent appearance due to their specific size, and due to the distribution of droplet size, they have low viscosity and high stability against the phenomena of creaming together, precipitating and clotting.

To produce the emulsion, water, oil, surfactant and energy are required. Like the NLC, the selection of a suitable surfactant plays an important role in the preparation of a stable nanoemulsion.

Vitamin D is a fat soluble vitamin. Its importance and its role in the health of the community are expressed in various studies. Vitamin D is known as a component of food, which the main source of supply is sunlight and diet. Vitamin D deficiency has become a global problem today. The type of diet as well as the inadequate or ineffective exposure to direct sunlight has led to a significant increase in the prevalence of this vitamin deficiency in modern societies. Vitamin D is activated twice by hydroxylation. This metabolite is the dominant form of vitamin D in the bloodstream. The second hydroxylation by the α-1-hydroxylase enzyme in the kidney produce 1 and 25-dihydroxyvitamin D, which is the most active form of vitamin D. Its main function is to react with cell membrane receptors and vitamin D receptor proteins in the nucleus and affect the genetic coding in many tissues.

Vitamin D is naturally found in animal products, the richest of them is fish oil. There is also a small and highly variable amount in butter, cream, egg yolk and liver. One of the most important effects of vitamin D is its beneficial effects on bone health,cardiovascular disease, lipid profiles, insulin sensitivity, inflammatory factors, blood pressure, Changes in muscle strength and function, memory and mental function, depression, liver function, menstrual disorders.

## MATERIALS AND METHODS

### Materials

Vitamin D was purchased from Zahravi Company (Iran). Precirol® ATO 5 (glyceryl palmitoestearate) was offered by Gattefosse, Lyon, France. Oleic acid was presented by Auxin Chemistry Company (Iran). Poloxamer 188 and Tween 80 from Uniqema (USA). Olive oil and grape oil and olive oil have been purchased from OLITALIA (Italy). Ethanol 96% is produced by the World of Alcohol medicine (Arak, Iran). Methanol (grade HPLC) was purchased from Scharlau (Spain).

### Preparation of nanostructured lipid carrier (NLC) by high-shear homogenization and ultrasound methods

Preparation of NLC by high-shear homogenization and ultrasound method was performed. This method requires two phases of lipid and aqueous to prepare NLC. The lipid phase of the formulation is acid oleic and Precirol. Vitamin D also enters this phase because vitamin D is soluble in fat. The water phase contains Poloxamer 188 plus deionized water. After weighing the formulation materials, the lipid and water phases were separated into two separate falcons and were placed in Ben marie. The amount of 1250 micrograms of vitamin D was 0.01% w/v for each formulation. The Ben marie was set to about 76 °C until the fat phase melted and the two phases were allowed to heat. The mixing of the two phases should be performed in the Ben marie. At this stage, a white emulsion is formed (each sample volume was 10 cc) and then homogenized with DIAX for 8 minutes with round 4. After homogenization, the compound was transferred to the Probe sonicator. The Sonication was adjusted to 6 cycles. After sonication, the mixture was placed at ambient temperature until it was completely cooled to form lipid carriers with a nanostructure.

## Preparation of nanoemulsion by high-pressure homogenization

The purpose of producing nanoemulsion by high-pressure homogenization (HPH) is achieving a method that does not have the defect of the ultrasound method, which is the release of metal particles in the formulation. HPH is also more suitable for industrial production. HPH requires lipid and water phases. The lipid phase of the formulation includes a type of liquid vegetable oil (sesame oil or olive oil or grape seed oil), that vitamin D also enters the same phase. The aqueous phase contains tween 80 and deionized water. To prepare nanomulsion, surfactant was first dissolved in deionized water and mixed with oil and vitamin D and then homogenized with DIAX for 8 minutes with round 4. At this stage, a white emulsion was formed (a volume of 20 cc sample was prepared). After homogenization, the combination was transferred to the HPH machine. The homogenization operation was performed on this device in 7 cycles and a pressure of 1000 bar (equivalent to 15000 psi). After the HPH operation, the mixture was placed at ambient temperature until completely cooled to form nanoemulsion. To prepare nanoemulsions used 3 types of liquid oil with a concentration of 5% and surfactant with different concentration.

## Determination of the melting point of components and investigation of the network of nanostructured lipid carriers by Differential Scanning Calorimetry (DSC)

To do this study, Differential Scanning Calorimetry (DSC) is equipped with the Stare software was used. After the program was given to the device and samples and reference were placed in a special aluminum pan and placed on the device, according to the plan, device will transfer thermal energy to both sides to produce the same temperature in both. The suitable program of this test is to be able to include the melting point of the main components of the formulation to show the existence of all the main components in the final formulation. In this way, the samples and reference are compared at a temperature range of -10 to 150 degrees Celsius and at a rate of 5 degrees Celsius per minute and under nitrogen gas with a flow rate of 20 mL/min. For this test one of the final formulations of NLC is formed as a freeze-dried powder (the liquid formulation was placed in a freezer-dried for 24 hours after being placed in a freezer with temperature of -20°C for 24 hours and completely dried). It was also taken from Precirol powder.

## Microscopic morphology of Nanostructured lipid Carriers and Nanoemulsions

Transient electron microscopy (TEM) imaging was used to investigate the morphology of lipid carriers with nanostructure. Then, 20 microliters of the diluted sample was covered with carbon coated grids and dried by paper filter. 20 µl of uranyl acetate was placed in water on the grids. After drying, the sample was observed under an electron microscope. For the SEM before observation, a drop of NLC and diluted nanoemulsion (10 times) was poured on a lame and allowed to dry, then placed under vacuum in a medium containing argon with a gold spray and placed inside the device.

## Release of vitamin D from nanostructure lipid carriers and nanoemulsions using dialysis bag

The dialysis bag method is used to examine the release behavior of lipid nanoparticles. For this purpose, 1 cc of NLC sample and 1 cc of nanoemulsion containing 125 micrograms of vitamin D (125 µg/ml concentrations), also 2500 micrograms of vitamin D, was isolated from the Zahravi sample in three separate dialysis bags. The dialysis bag was also selected for use with vitamin D, 12 kilo Daltons. Then the dialysis bag was inserted into 45 cc of the

stomach buffer (pH=1.2) at 37°C and a speed of 100 rpm for two hours. After two hours, the dialysis bag was removed and placed within 45 cc of the intestinal buffer (pH=6.8) at 37°C and 100 rpm for 24 hours. The stomach area with pH=1.2 was made from 2 g of sodium chloride and 7cc of hydrochloric acid according to the USP method, which volume was reached to 1000 cc. The intestinal environment was also prepared with pH=6.8 according to the USP method. First, 6.8 g monobasic potassium phosphate was dissolved in 250 cc distilled water. Then, 77 cc of sodium hydroxide 0.2 N was added and the pH was set at 6.8 and volume was reached to 1000 cc. At a specified interval (for the gastric environment every 20 minutes and for the bowel environment every hour), 1 cc was removed from the buffer, and 1 cc of the new environment was replaced to maintain the sink condition. The collected samples were also determined by HPLC.

## RESULTS AND DISCUSSION

### Statistical methods

The NLC formulations were designed using the Design expert software and the CCD (Central Composite Design) software, according to the unknowns given to the software and analyzed by the software for the results of manufacture. Prism software also evaluated the data obtained from quantitative studies of particle stability results over time and at different temperatures [4]. Comparisons were performed based on one-way ANOVA and Tukeys multiple comparison test. Significant differences were observed only in the results of particle storage at 37°C for the selected NLC sample ($P<0.05$).

According to the results of the Design expert software analysis, all the NLC samples prepared had Desirability=1 and there was no difference in the choice of formulations for the tests. On the other hand, according to the results of the stability of all the prepared nanoemulsion formulations mentioned in the results section, we could choose any of the formulations for the other tests.

### Investigating of particle size and zeta potential and stability of nanostructure lipid carriers and nanoemulsions

Particle size and Polydispersity Index (PDI) play an important role in stability, release rate and solubility. Also, the bioavailability of encapsulated compounds increases with decreasing particle size. Particle size and PDI depend on production condition, ratio of components and lipid phase viscosity.
Zeta potential determines the electrical charge on the surface of nanoparticles and provides information about the repulsive force between nanoparticles.it is an important factor to provide physical stability.

Polydispersity index shows the particle size distribution that is between 0-1. If the PDI value is between 0.1-0.25 indicates a relatively narrow distribution and if it is higher than 0.5 indicates a broad distribution.

At first, NLC formulations were produced by high-shear homogenization and ultrasound method. Easy access to the device and easy operation are the advantages of this method. In optimal conditions, the particle size that is prepared by this method is 100-200 nm. In this study, the particle size of all formulations was found in this range.
The nanoemulsion formulation was produced by high pressure homogenization, which is suitable for scale up and has a good repeatability. In a study, the pressure of 1000 bar showed a good reduction in particle size, but with increasing number of homogenization cycles, some formulations showed an increase in size compared to pressure of 800 bar.

Size and zeta potential stability studies in this case confirm this point that nanoparticles were stable within 3 months and after 6 months of this study. This stability is probably due to the appropriate zeta potential at the surface of the NLC, since the same surface charge in these particles causes a repulsive force between them and prevents the accumulation of particles and the particle size remains constant for a long time.

All prepared NLCs have an average size of 126.3 ± 10.81 to 148.1 ± 13.05 nm. Precirol content has a significant effect on the average size of NLC particles, and it also increases the size with increasing of the Precirol to the acid oleic proportions. This phenomenon can also be due to a reduction in the amount of surfactant in the formulation by increasing the concentration of the oil. Reducing particle size protects drops against accumulation.

By increasing the amount of surfactant, size is reduced first, but when the amount of surfactant is increased by a greater amount, the size increases. It can be justified in this way when the size becomes small, the surface-to-volume ratio increases and surface energy increases and the particles stick together.

The ratio of Precirol to acid oleic and surfactant has no effect on the zeta potential. According to this study, surfactant level did not effect on Polydispersity Index (PDI), but as the size, PDI increases by increasing the ratio of Precirol to acid oleic.

Formulations, maintained at 4, 25, and 37°C, were constructed with relative strength after 1, 3 and 6 months, taking into account the size and PDI and Zeta potential. only In the case of NLCs maintained at 37°C, an increase of about 5 times was observed for both the size and PDI and the decrease in zeta potential, which could be due to the instability of particles at 37°C and the accumulation and bonding of particles.

All NLC formulations have a negative charge in the range of -17.2 ± 1.65 mv to -23.9 ± 1.93 mv, which is a negative charge due to the ionization of Precirol fatty acids. All NLCs containing vitamin D had a PDI value of 0.107 ± 0.002 to 0.209 ± 0.017, that indicating a narrow distribution of particle size.

In the case of nanoemulsions, the average particle size was observed at the range of 137.0 ± 11.7 to 171.6 ± 13.67 nm and also decreased with the increase in the percentage of Tween 80. The PDI and zeta potential of particles were also in the range of 0.130 ± 0.015 to 0.226 ± 0.024 and -13.2 ± 2.01 to -23.2 ± 1.6. Also the results showed that vitamin D concentration did not have any significant effect ($P<0.05$) on particle size, PDI and zeta potential.

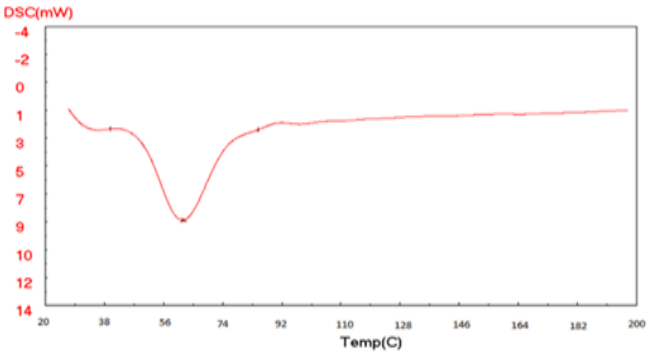
## Determination of the melting point of components and the investigation of the network of nanostructured lipid carriers and nanoemulsions by Differential Scanning Calorimetry (DSC)

The melting temperature of NLC produced from Precirol was reported 53.9°C that to be nearly of the melting temperature of Precirol powder. The reason for the decrease of NLC melting temperature compared to Precirol powder can be that due to the formation of NLC, the particle size decreases in relation to mass, and the reduction of particle size leads to an increase in the surface area and a decrease in the size of the crystals as well as a change in the crystalline properties due to Re-Crystallization, which itself leads to a reduction in the melting temperature. On the other hand, this reduction in melting temperature can be due to the placement of drug in the parts of the fatty crystal network, which leads to a change in the crystalline structure of NLC containing vitamin D.

The lack of peak of vitamin D in the DSC chart and the encapsulation of more than 86% of vitamin D in the NLC are corroborated. Because if vitamin D was not in the lipid network, it should be applied to the surface of the nanoparticles during the lyophilization, and since the freeze-dried powder was used for DSC, it should have peaked at -1.6°C. When not done, it indicates that vitamin D is fully contained in the lipid network.

DSC has not been tested for nanoemulsion because fluids such as vegetable oils cannot be described under the temperature and analytical conditions.

**Figure 1.** Precirol thermal analysis with a melting point of 61.7°C.



## Microscopic Morphology of Nanostructured Lipid Carriers and Nanoemulsions by SEM and TEM

The results of the TEM and SEM electron microscopy confirm that the particle size of nanoparticles is about 100 nanometers and that the vitamin D-containing lipid nanoparticles have a smooth, uniform, spherical surface. The spherical lipid nanoparticles makes these particles have the most ability to control releasing and protect the retention drug. Because the spherical shape has the longest pathway for moving the drug in the nanoparticle and also the lowest contact with the dispersed aqueous environment, compared to other forms of nanoparticles.

## Determination the amount of vitamin D by HPLC and the efficiency of encapsulation

The efficiency of encapsulation in nanocarriers depends on several factors such as the nature of the active substance (hydrophilicity or hydrophobicity and the degree of tendency to interact with the double layer membrane), the chemical structure of the lipid used, the lipid-to-active substance ratio, the production method and the physical properties of the membrane and the conditions of environment (temperature and pH) .The encapsulation efficiency (EE%) value in the NLC was 86.24% ± 4.631 and in the nanoemulsion was 92.18% ± 4.027, it shows high amount of vitamin D is imported in the nanoparticles [5]. One of the important features of lipid nanocarriers is their high ability to encapsulate high active hydrophobic substances. NLC from a mixture of liquid and solid lipids by melting and emulsification, and reported that EE has been improved by increasing the amount of liquid lipids in the NLC formulation.

## Investigation the release of vitamin D from nanostructure lipid carriers and nanoemulsions with dialysis bag method

Vitamin D *in vitro* release test was performed in the same stomach environment (pH=1.2) and intestinal medium (pH=6.8). The results of the release of vitamin D encapsulated in the NLC indicate that 9.4% ± 0.83 vitamin D have been released in the stomach environment at 37°C for two hours, which, after exposure to the intestinal environment, 62.57 ± 1.76% of vitamin D Also released. The solubility of the bioactive material in the release medium and in the NLC center and also the dispersion between them plays a key role in the vitamin D release behavior. The NLC core is a mixture of liquid and solid so it guarantees homogeneous dispersion of the bioactive compound throughout the particles. Therefore, slow or fast release can be achieved by changing the oil used in the NLC core material.

The low levels of vitamin D intake from the NLC can be due to the presence of vitamin D lipophilic properties, which is why vitamin D in the fat core is greater than the particle surface. Another possibility is the rigid NLC outer shell that is resistant to digestion and acts as a barrier to the release of vitamins during the release test. Generally, it is assumed that the released compounds are released by several processes, such as emission through the particle matrix, degradation and propagation through the micro channels formed by erosion. It has also been reported in previous studies that NLC can improve the encapsulation capacity of bioactive substances, and also make slow and controlled release at the site.

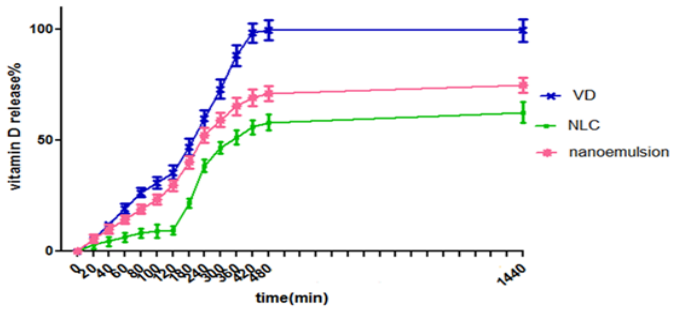
It can also be concluded that NLC has been able to maintain vitamin D against gastric acid and bring it to its intestinal absorption site and increase the bioavailability and absorption of vitamin D. In the case of nanoemulsion, 29.6% ± 1.47% vitamin D was released in the stomach area during the first two hours and then released in the intestinal environment after 24 hours 74.8% ± 2.27.

For the free form of vitamin D, is placed alone in the dialysis bag, at about eight hours, 99.57% of the drug was released, which is a quick release due to the lack of control over the release of vitamin D. These results indicate nanoparticles are a good option for protecting materials against the acidic and enzymatic conditions of the stomach, thus increasing absorption and bioavailability, an opportunity to enhance the absorption of vitamin D from food.

The core of NLC is liquid so there is a homogeneous dispersion of vitamin D in the entire particle. The low amount of vitamin D released from NLC can be attributed to the properties of vitamin D that soluble in fat, and also points out that vitamin D is more in the nucleus than the NLC surface. According to the results, it is predictable that the use of NLC carriers and nanoemulsion for vitamin D while protecting against oxidation, degradation by microorganisms and increasing bioavailability of vitamin D can also slow down the release of vitamin D and

controlled and prevented the formation of toxicity due to the uniform and rapid release of vitamin D emulsion. It is also expected to increase bioavailability.

**Figure 2.** Total comparison of vitamin D release from NLC, nanoemulsion containing vitamin D and free form of vitamin D in the simulated gastrointestinal tract.




## CONCLUSION

NLC was produced by high-shear homogenization and ultrasound method and nanoemulsion by high-pressure homogenization and vitamin D loaded them were produced in a small size. The results of TEM and SEM imaging showed that nanoparticles had a spherical shape. In general, the results showed that nanostructure lipid carriers and nanoemulsions containing vitamin D with high encapsulation rate are available and have a good stability. These nanocarriers can slowly release vitamin D in the stomach and intestinal simulant. The result of this paper showed that NLC and nanoemulsion could give a new hope for the efficient delivery of bioactive components in different food.

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