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NANOEMULSIONS: A NOVEL APPROACH FOR INCREASING POSSIBILITIES IN DRUG DELIVERY

***Mohd Junaid and Dr. Rahul Shukla**

School of Pharmaceutical Science, Shri Venkateswar University, NH # 24, Delhi Kanpur Highway, Gajraula, Amroha, UP, India.
*Corresponding author E-mail:-pharmajunaid@gmail.com

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ABSTRACT

Nanotechnology, the science of nano sized particles, has rapidly gained the spotlight in diversified areas including pharmaceutical sector. With the blessings of accurate drug designing and better pharmacological action, it has entered the pharmaceutical arena bringing promising discoveries along with it. Nanoemulsion is one of the greatest and advantageous dosage forms with the application of nanotechnology in pharmaceutical formulations. Very small size droplets of the nanoemulsion favor better drug absorption and targeting. It not only improves the conventional emulsion systems but also opens new opportunities for other drugs to be designed more precisely with better bioavailability and accurate dosing rendering minimum side effects. This article depicts various advantageous features of nanoemulsions delineating different methods of preparation. The foci of this review also include the opportunities of other drugs to be formulated through nanoemulsification in order to ensure better therapeutic effect. The summary shows recent researches on nanoemulsion formulation from different classes of drugs as well as some formulations based on nanoemulsion templates. The methods of preparing nanoparticles, characterized by nanoemulsion templates, have also been discussed which, as a whole, presents the best possibilities of nanoemulsions.

INTRODUCTION

The world of biomedical science has recently changed significantly with the development of nanotechnology which has enabled the scientists to think more precisely in designing better therapies. Nanotechnology is the science that deals with the particles of nano scale sizes. Use of nanotechnology in pharmaceutical sciences has emerged at a great extent from the last couple of years [(1)]. Different sorts of pharmaceuticals currently being used or in the process of development by using nanoparticles include, nanoemulsions (NE) (submicron sized emulsions), nanosuspensions (submicron sized suspensions), nanospheres (drug nanoparticles in polymer matrix), nanocapsules (encapsulated drug nanoparticles), lipid nanoparticles (lipid monolayer enclosing a solid lipid core), dendrimers (nano sized three-dimensional branched molecules of polymer), nanotubes (sequence of nanoscale C60 atoms arranged in a long thin cylindrical structure), and nanoshells (concentric sphere consisting of a dielectric core and a metal shell) [(2)]. Nanoemulsion is an emulsion system having the droplet size in nanometer scale in which oil or water droplets are finely dispersed in the opposite phase with the help of a suitable surfactant to stabilize the system [(3, 4)]. The average droplet size usually ranges from 0.1 to 500 nm. The size of the droplets varies depending on the drug particles, mechanical energy, composition and relative amount of the surfactants (5). Nanoemulsions are also

known as miniemulsions, fine-dispersed emulsions, submicron emulsions etc., which can be either O/W (oil in water) or W/O (water in oil) emulsion. The amount of oil in O/W nanoemulsions may vary but generally is within 5%–20% w/w. Sometimes a mixture of oils may be used to improve drug solubilization in the oil phase. A co-surfactant or a co-solvent may be used in addition to the surfactant to facilitate the stabilization process [6, 7]. Few nanoemulsions have been manufactured commercially in oral, topical, ophthalmic and even in intravenous (IV) drug delivery system. IV administration of nanoemulsions requires biodegradable surfactants. Numerous researches are presently being carried out in order to manufacture nanoemulsions from different classes of drugs for a variety of purposes. The use of nanoemulsion spreads from antibiotic therapy, atherosclerosis treatment, transdermal drug delivery and ophthalmic application to as far as cancer therapy, vaccine delivery etc. (2, 9). It can bring a great revolution in cancer treatment since it was always a difficult measure to destroy the cancer cells completely with minimal interference to the normal body cells (10). This compilation, illustrating the manufacturing methods as well as the recent researches, portrays the rising possibilities of nanoemulsions to serve the human being accurately with minimum damage.

Classification

There are three types of nanoemulsions on the basis of composition of oil and water portions.

- **a)** Oil in water (O/W) nanoemulsions where oil droplets are dispersed in continuous aqueous phase
- **b)** Water in oil (W/O) nanoemulsions where water droplets are dispersed in continuous oil phase and
- **c)** Bi-continuous nanoemulsions where microdomains of oil and water are inter-dispersed within the system.

In all three types of nanoemulsions, the interface is stabilized by an appropriate combination of surfactants and/or co-surfactants (8). Based on the surfactants used, O/W nanoemulsions can be further classified into three types, which are neutral O/W nanoemulsion (neutral surfactants are used), cationic O/W nanoemulsion (cationic surfactants are used) and anionic O/W nanoemulsion (anionic surfactants are used).

Nanoemulsions offer various advantages over other dosage forms and these advantages are,

(1) Increased rate of absorption
(2) Reduced variability in absorption
(3) Protection from oxidation and hydrolysis in O/W nanoemulsions,
(4) Delivery of lipophilic drugs after solubilisation,
(5) Aqueous dosage form for water insoluble drugs,
(6) Enhanced bioavailability for many drugs
(7) Ability to incorporate both lipophilic and hydrophilic drugs,
(8) Delivery systems to enhance efficacy while reduce total dose and side effects,
(9) Non-toxic and non-irritant vehicles for skin and mucous membrane delivery.
(10) Release control by permeation of drug through liquid film, whose hydrophilicity or lipophilicity as well as thickness can be precisely controlled. 32

The disadvantages of nanoemulsion and the overcoming strategy

Nanoemulsion is relatively an attractive system for many industrial application due it their purity, simplicity and the ability to sterilize through filtration and the capacity of increase bioavailability of drug solubilized in them.[15] Nevertheless, the nanoemulsion is clearly constitute that nanoemulsion r < 100nm are thermodynamically not stable and leads the respective system to breakdown with various instability mechanisms in it.[16] Moreover, nanoemulsion has only kinetic stability, in long-term, it is sometimes stated to as 'nearing thermodynamic stability' because of the absence of flocculation.[17] Small droplet size of nanoemulsion also contributes to irreversible destabilization as a result of the mechanisms of Ostwald ripening or coalescence. When smaller droplets dissolved and redeposit on larger particles to influence decreasing droplet's radius.[17]

Theory of the Formation of Nanoemulsion:

In Nanoemulsion which is categorized as multiphase colloidal dispersion which is generally characterized by its stability and clarity. There is an application of high shear generally obtained by micro fluid or ultrasonic approach generally used to reduce the droplet

size to nanoscale. There is a marginal difference between the terms Nanoemulsion and microemulsion also known as micellar phase or mesophase. The microemulsion generally forms through thermodynamic self assembly whereas nanoemulsion requires external shear for rupturing the droplets.

In retrospect, the historical choice of the word "microemulsion" to describe the nanoscale is unfortunate since they are structurally between 1 to 100 nm as for Nanoemulsion. Micro emulsions are not the emulsions of micro scale droplets. They are formed by self assembled equation phase in which the surface tension dose not play a significant role. The Nanoemulsions underlines the basic principle in its formulation. They generally comprises of two immiscible phase with an interfacial tension between them reduced by addition of surfactant.

Factors affecting the Formulation of Nanoemulsion :

- Appropriate composition is required to avoid Oswald ripening the dispersed phase should be highly insoluble in the dispersed medium.
- The surfactant is an essential part of the Nanoemulsion. They should not form lyotropic
- liquid crystalline "microemulsion" phases. Systems containing short chain alkanes, alcohols, water, and surfactants form the phases which are generally used with the co surfactant.
- The presence of excess surfactants enables new surface area of nanoscale to be rapidly coated during emulsification there by inhibiting induced coalescence.
- Extreme share must be applied to rupture microscale droplets to nanoscale by providing the stress level to reach above the Laplace pressure of the droplets with a pressure of 10- 100 atm. Out of various methods ultrasonication is widely used in laboratory.

Limitation of nanoemulsion

Although this formulation provide great advantages as a delivery system for the consumers but sometimes the re-duced size of droplets are responsible for the limited use of nanoemulsion formulation. Some limitations of nanoemul-sion are as follows [31].

- The manufacturing of nanoemulsion formulation is an expensive process because size reduction of droplets is very difficult as it required a special kind of instru-ments and process methods. For example, homogenizer (instrument required for the nanoemulsion formulation) arrangement is an expensive process. Again micro-fludization and ultrasonication (manufacturing process) require high amount of financial support.
- Stability of nanoemulsion is quite unacceptable and creates a big problem during the storage of formulation for the longer time period. Ostwald ripening is the main factor associated with unacceptability of nanoemulsion formulations. This is due to the high rate of curvature of small droplet show greater solubility as compared to large drop with a low radius of curvature.
- Less availability of surfactant and cosurfactant required for the manufacturing of nanoemulsion is another factor which marks as a limitation to nanoemulsion manufac-turing.

Formulation ingredients of Nanoemulsion: A typical nanoemulsion consists of a water phase, an oil phase and an emulsifier [5]. When present in small amounts, an emulsifier facilitates the formation of emulsions by decreasing the interfacial tension between the oil and water phases [5]. Additionally, emulsifiers aid the stabilization of nanoemulsions [11]. Formation and stabilization of nanoemulsions depend largely on the physico-chemical properties of the three aforementioned constituents. O/W nanoemulsions have the greatest application in commercial products [9]. The particles in O/W nanoemulsion have a core-shell-type structure with a shell of surface-active amphiphilic material covers a core made of lipophilic material.

2.1. Oil phase: The oil phase used to prepare food-grade nanoemulsions can be formulated from a variety of nonpolar molecules, such as free fatty acids (FFA), monoacylglycerols (MAG), diacylglycerols (DAG), triacylglycerols (TAG), waxes, mineral oils or

various lipophilic nutraceuticals [9]. TAG oils extracted from soybean, safflower, corn, flaxseed, sunflower, olive, algae or fish are the most commonly used in nanoemulsions primarily due to their low cost and nutritional value [9]. Physical and chemical characteristics of the oil phase such as viscosity, water solu-bility, density, polarity, refractive index and interfacial tension and chemical stability greatly influence the properties of nanoemulsions [1, 3, 5–8].

2.2. Aqueous phase: The aqueous phase used to prepare food-grade nanoemulsions can be formulated from water with a variety of polar molecules, carbohydrates, proteins, acids, minerals or alcoholic cosolvents [9]. The selection of the aqueous phase has a great impact on the physicochemical properties of the produced nanoemulsion.

2.3. Stabilizers: Stabilizers influence the long-term stability of nanoemulsions; therefore, the selection of the appropriate stabilizer is one of the most important factors to consider for the proper pro-duction of nanoemulsions. Various kinds of stabilizers are added to improve the long-term stability of nanoemulsions, and they are summarized in Table 1 [1, 3, 5–9]. Stabilizers can be emulsifiers, ripening retarders, texture modifiers and weighting agents.

Solubilizing, surfactants, emulsifying agents adsorption enhancers.

- Capryol 90
- Gelucire 44/14,50/13
- Cremophor RH 40
- Imwitor 191,308(1),380,742,780 K,928,988
- Labrafil M 1944 CS,M 2125 CS
- Lauroglycol 90
- PEG MW > 400
- Plurol oleique CC 497
- Poloxamer 124 & 188
- Softigen 701, 767
- Tagat TO
- Tween 8

Construction of pseudo-ternary phase diagrams 15-16

Surfactant and co-surfactant (Smix) in each group were mixed in different volume ratios (1:0, 1:1, 1:2, 1:3, 2:1, 3:1, 4:1) and the stock of 100 mL of each groups is prepare. These Smix ratios were chosen in increasing concentration of cosurfactant with respect to surfactant and increasing concentration of surfactant with respect to cosurfactant for detailed study of the phase diagrams for the nanoemulsions formation. For each phase diagram, oil and specific Smix ratio was mixed thoroughly in different volume ratios from 1:9 to 9:1 in different small glass test tubes. Sixteen different combinations of oil and each Smix, 1:9, 1:8, 1:7, 1:6, 1:5, 2:8 (1:4), 1:3.5, 1:3, 3:7 (1:2.3), 1:2, 4:6 (1:1.5), 5:5 (1:1), 6:4 (1:0.7), 7:3 (1:0.43), 8:2(1:0.25), 9:1 (1:0.1) were made so that maximum ratios were covered for the study to delineate the boundaries of phases precisely formed in the phase diagrams. For the determination of existence zone of microemulsion, pseudoternary phase diagrams were constructed using water titration method [17-19]. To construct pseudoternary phase diagrams, the oil phase mixed with different ratio of surfactant and cosurfactant and mixture was titrated with distilled water until it turned turbid. Examine each and every point I detailed and note it down. Pseudo ternary phase diagrams were drawn by using data obtained in aqueous titration method. The amount of water added to give water concentration in the range of 5-95% of total volume at 5% intervals. After every 5% addition of the water to the oil and Smix mixture, visual observations.

Solubility determination in the various oils, surfactants and co- surfactants: 2 ml of different oils was taken in small vials separately and excess amount of the drug was added to each vial. The vials were tightly stopper and were continuously stirred for 72 hrs in mechanical shaker at 250C and after that, oils were centrifuged. The supernatant was separated and dissolved in methanol and solubility was quantified by UV-Spectroscopy method at specific nm after appropriate dilution with methanol or other solvent.

Formulation procedures: There are mainly four methods for the preparation of nanoemulsion. Each method differs significantly from others and the objective of each method is to gain the particle size within 500 nm with a stable emulsion system. Selection of any method depends on the type

of drug and the dosage form. For producing nanoemulsions, surfactants must be chosen accurately so that an ultra low interfacial tension ($<10^{-3}$ mN/m) can be obtained (8).

MICROFLUIDIZATION: It is a patented manufacturing technology where a device called microfluidizer is used (Figure 2). There is a high pressure displacement pump (500–20,000 psi) in this device which gets the materials pass through a chamber containing many microchannels. The materials move to an impingement area flowing through the microchannels and convert into very fine particles. The liquid phases (oil phase and aqueous phase) are processed in an inline homogenizer to give a coarse emulsion. Then the coarse emulsion is passed through the microfluidizer to obtain the fine nanoemulsion. This process is continued repeatedly until the desired particle size is obtained. The nanoemulsion is then filtered under nitrogen to remove the larger particles (2, 7).

HIGH PRESSURE HOMOGENIZATION

Oil in water nanoemulsions, containing oil portion <20%, can be prepared by this method. Anne Desrumaux and her team showed that average droplet diameters increase with increasing oil content because of the limitation on surface-active agents in the most oil concentrated emulsions due to the strong increase of the interfacial area created by the homogenising process. Moreover, the shear-thinning behavior of most oil-concentrated emulsions can be attributed to the formation of clusters or aggregates of droplets which eventually increases droplet size. As a result, producing nano sized droplets will not be possible in case of preparing W/O nanoemulsions by high pressure homogenization method (18). Here, very high pressure is applied on the system containing oil phase, aqueous phase and the surfactant. This process is done with the help of a high pressure homogenizer (Figure 3) or piston homogenizer (2). First, the micro particles enter the valve at a relatively low velocity. The pressure is then generated by the positive-displacement pump which provides a relatively constant rate of flow. The liquid, containing micro particles, flows between the valve and seat at high velocity. As the velocity increases, the pressure decreases at the same time. The fluid is finally discharged as homogenized nanoemulsion (18).

EIP AND PIT METHOD: The emulsion inversion point (EIP) method is a low-energy and spontaneous emulsification method. At a constant temperature, it results in diverting the intrinsic features of thermodynamically stable microemulsions or liquid crystals to be nano-structured by a progressive dilution with water or oil, respectively, for creating thermodynamically unstable but kinetically stable direct or inverse nanoemulsions. A small change in the water or oil proportion within the established microemulsion system will change the pattern of surfactant hydration and their affinity for the aqueous phase. As a result, instabilities are created in the microemulsion system which results in its breaking up into nano-emulsion (6, 19–23). In the phase inversion temperature (PIT) method, temperature of the emulsion system is increased to change the solubilizing pattern of the surfactant (hydrophilic to lipophilic) which forms bicontinuous microemulsions followed by emulsion inversion. The process involves four steps (Figure 4). (a) Temperature is below the PIT, it presents a macro-emulsion and the nonionic surfactants, mostly hydrophilic. (b) Temperature is increased; the surfactants gradually become lipophilic and are solubilized by the oil phase. (c) Temperature is at the PIT, bicontinuous microemulsions form. (d) Temperature is brought above the PIT, the emulsion is inverted and water is dispersed into the mixture of oil and lipophilic surfactant. The system is then cooled rapidly using water dilution, making the surfactant hydrophilic instantaneously and inducing spontaneous and rapid migration to the aqueous phase. This turbulent displacement induces the generation of nanoemulsions.

SONICATION: Sonication is referred to as applying ultrasound energy to agitate particles in a sample. Emulsification by ultrasonic technology mainly occurs through two mechanisms. Firstly, the use of an acoustic field produces interfacial waves which become unstable, eventually resulting in the eruption of the oil phase into the water medium in the form of droplets. Secondly, the application of low frequency ultrasound causes acoustic cavitation, that is, the formation and subsequent collapse of microbubbles by the pressure fluctuations of a simple sound wave.

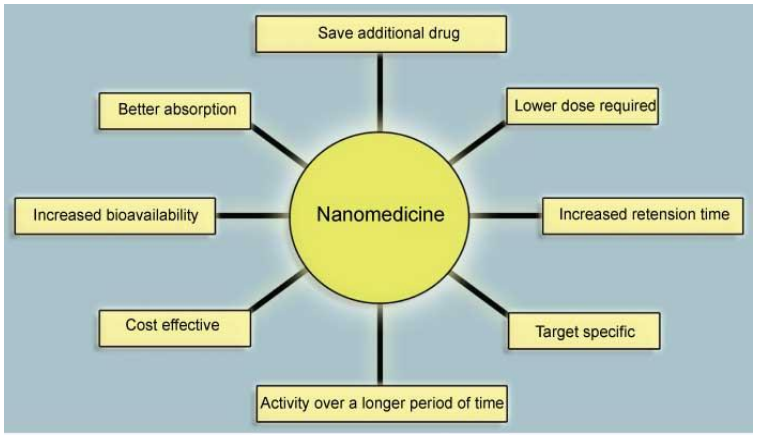

Figure 1: **Key advantages of nanoemulsion based nanomedicines.**

The Characterization of Emulsion Type Based on Colour[30]

Type of Emulsion	Colour	Size	Characterization
Maroemulsion	Milik White	1 to 100 µm	Phase interfaces scatter light as it passes through the emulsion
Emulsion	White	1 to 100 µm	Phase interfaces scatter light as it passes through the emulsion Emulsion is dilute enough, higher-
Microemulsion	Translucent bluish	10-100nm	Frequency and low-wavelength light will be scattered more, and the emulsion will appear bluer Emulsion is dilute enough, higher-
Nanoemulsion	Translucent bluish	20-200nm	Frequency and low-wavelength light will b scattered more, and the emulsion will appear blue

Components	Examples
Oils	Castor oil, Corn oil, Coconut oil, Evening primrose oil, linseed oil, Mineral oil, olive oil, peanut oil
Emulgent	Natural lecithins from plant or animal source, phospholipids, castor oil. Derivatives, polysorbates, sterylamine
Surfactant	Polysorbate20, Polysorbate80, Polyoxy-60, castor oil, Sorbitan mono oleate, PEG300, Caprylic glyceride
Co- Surfactant	Ethanol, glycerine, PEG300, PEG400, Polyene glycol, Poloxamer
Tonicity modifiers	Glycerol, Sorbitol and xylitol
Additives	Lower alcohol (ethanol), propylene glycol, 1, 3-butylenes glycol, sugars such as butylenes glycol, sugars such as glucose, sucrose, fructose, and maltose
Antioxidants	Ascorbic acid and tocopherol

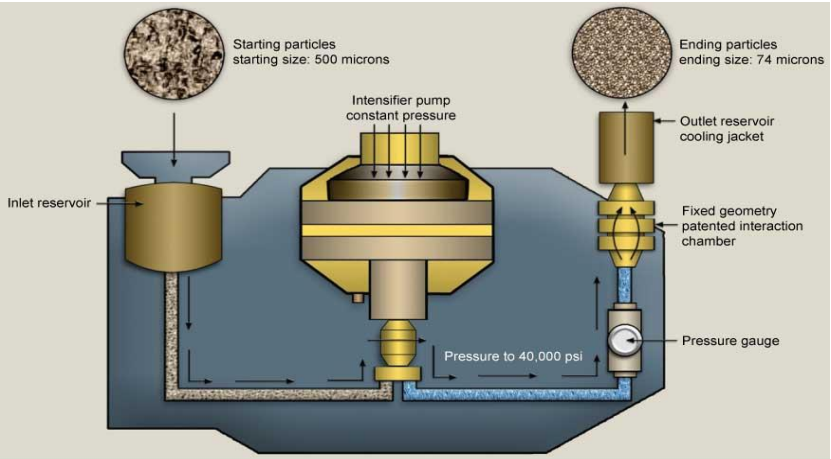

Figure 2: **Basic concept of a microfluidizer**

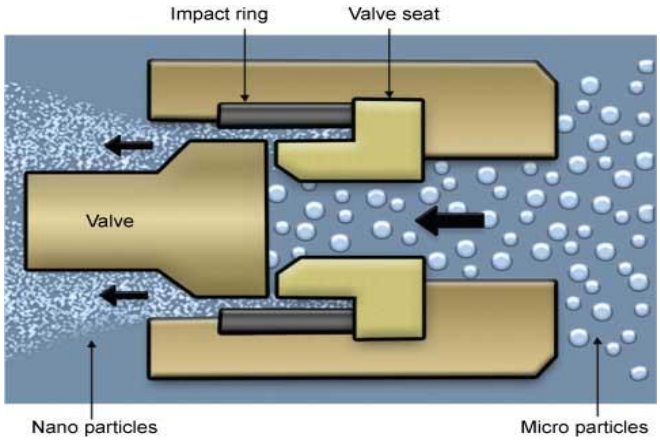

Figure 3: High-pressure homogenizer [Regenerated from (2)].

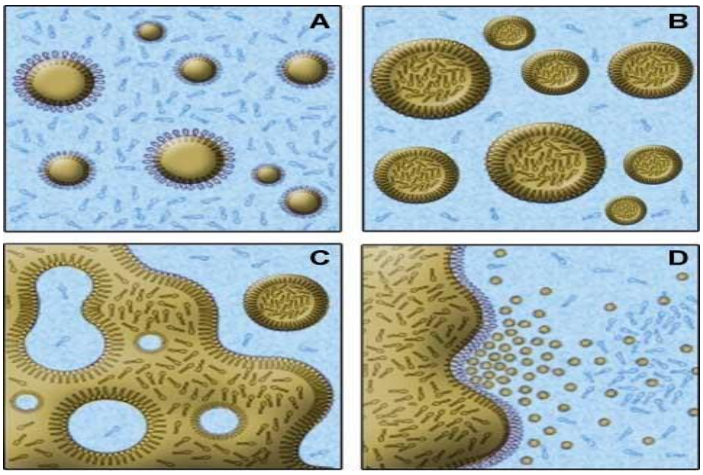

Figure 4: Generation of nanoemulsions using the PIT method. A water/nonionic surfactant/oil system undergoes a phase inversion

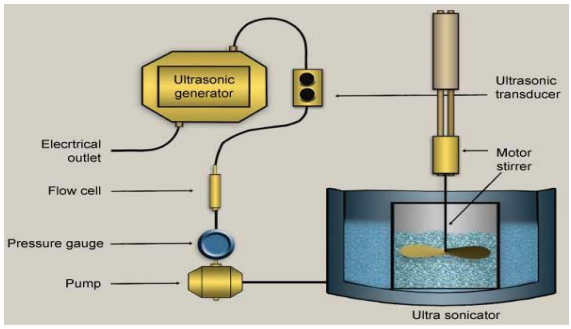

Figure 5 : Emulsification by ultrasonic technology Screening of formulations on the basis of thermodynamic stability studies

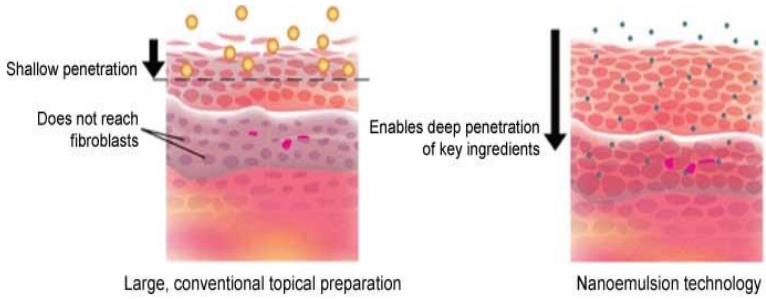

Figure 6: Comparison of nanoemulsion with the conventional transdermal formulations in case of crossing skin barrier.

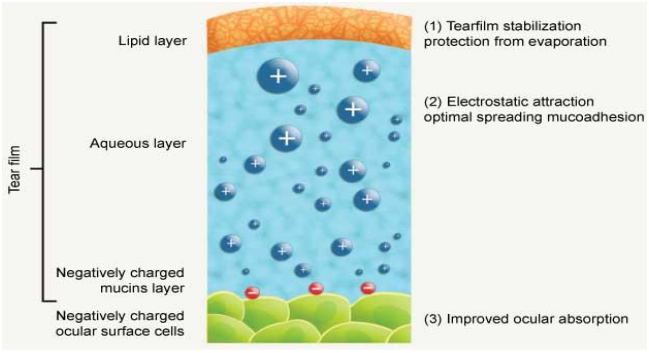

Figure 7: Three advantages of cationic nanoemulsions for ophthalmic delivery

Each bubble collapse (an implosion on a microscopic scale) event causes extreme levels of highly localized turbulence (Figure 5). The turbulent micro-implosions act as the very effective method of breaking up the primary droplets of dispersed oil into the droplets of sub-micron size (25, 26).

The thermodynamic stability studies were performed on the basis of following tests.

Centrifugation study: The selected formulations were centrifuged (REMI, India) at the 5000 rpm for 30 mins and observed for phase separation, creaming or cracking. The formulations which showed maximum stability (no creaming, cracking, phase seperation) were selected and studied for heating-cooling cycle, freeze-thaw cycles and Dispersibility tests.

Heating cooling cycles: It is used to see the stressed effect of heating and cooling on the nanoemulsion`s stability. In this study the formulations were kept at 450 c and at 0 0C temperature for not less then 48 hrs for each temperature cycle.

Freeeze –thaw cycles (Accelerated ageing): This test was performed for accelerated stability testing of nanoemulsion formulations. In this study the formulations were exposed at two different temperatures i.e -210C and 210C for each temperature cycles not than 24 hrs. For the better estimation of accelerated stability studies three such cycles should be run for each batch of formulation .The

formulations which showed the maximum stability were selected for further study.

Dispersibility tests: The efficiency of self-emulsification of oral nanoemulsion was assessed using a standard USP XXII dissolution apparatus 2. [20]. One ml of each formulation was added to 500 mL of distilled water and in 0.1N HCl respectively at 37 ± 0.5 oC. A standard stainless steel dissolution paddle rotating at 50 rpm provided gentle agitation. The in vitro performance of the formulations was visually assessed using the following grading system (Table 5). Those formulations that passed the thermodynamic stability and also dispersibility test in Grade A were taken for the further studies. Further from each Smix Group one formulation is selected, having the least Smix concentration irrespective of Smix ratio used, but passing dispersibility test in Grade A in distilled water as well as in 0.1N HCl.

Droplet size analysis (Particle size distribution): Droplet size of the prepared nanoemulsion was determined by using photon correlation spectroscopy, which analyzes the fluctuations in light scattering due to Brownian movement of the particles . The formulation (0.1 mL) was dispersed in 50 mL (500 dilution) of distilled water in a volumetric flask and gently mixed by inverting the flask and measurement done using a Zetasizer (Nano ZS-90, UK). Light scattering was monitored at 25°C at a 90° angle Morphology and structure of the nanoemulsion were studied using transmission electron microscopy (TEM) TOPCON 002B operating at 200 KV and of a 0.18 nm capable of point to point resolution. Combination of bright field imaging at increasing magnification and of diffraction modes was used to reveal the form and size of the nanoemulsion. In order to perform the TEM an observation, the nanoemulsion formulation was diluted with distilled water (1/100). A drop of the diluted nanoemulsion was directly deposited on the Copper holey film grid and observed after putting fixing agent and drying it in the filtered air.

Characterization of the nanoemulsion

Nanoemulsion has been characterized using a wide variety of techniques. The characterization of nanoemulsion is a difficult task due to their complexity, variety of structures and components involved in these systems, as well as the limitation associated with each technique, but such knowledge is essential for their successful commercial exploitation. The rate of release of sodium salicylate from a lecithin-based nanoemulsion, is dependent upon their microstructure7. A complementarily of methods is generally required in order to fully characterize these systems. At the macroscopic level viscosity, conductivity and dielectric methods provide useful information.

Morphological study of nanoemulsion: The morphological study of nanoemulsion is carried by using transmission electron microscopy (TEM). In TEM, a beam of electron is incident on a thin foil specimen and passed through it. On interacting with the specimen, these incident electrons transform into unscattered electrons, elastically scattered electrons or inelastically scattered electrons. The distance among the objective lens and the specimen and among the objective lens and its image plane regulates the magnification. The electromagnetic lenses concerted the unscattered or scattered electrons and cast them onto a screen that produce amplitude-contrast picture, a phase-contrast image, electron diffraction, or a phantom picture of distinct darkness, which is dependent upon the density of unscattered electrons. Bright field imaging at increasing magnification in combination with diffraction modes used for disclosing the size and form of nanoemulsion droplets. For performing TEM, few drops of nanoemulsion or a suspension of lyophilized nanoparticles is prepared in double-distilled water and are placed onto holey film grid and immobilized. Excess solution has to be wicked off from the grid following immobilization and stained. The stained nanoparticles are then examined at particular voltage[31].

Phase Behavior studies: Phase diagram showing the nanoemulsion region, information about of different phases as a function of composition variables can be analyzed from vigorous study of the phase diagrams.

Viscosity measurements: The viscosity of the prepared nanoemulsion formulations were determined as such without dilution by Brookfield DV III ultra V6.0 RV cone and plate rheometer (Brookfield Engineering Laboratories, Inc, Middleboro, MA) using spindle # CPE40 at 25 ±0.5°C. The software

used for the viscosity calculations was Rheocalc V2.6.

Dielectric measurements: They are powerful means of probing both structural and dynamic features of nanoemulsions systems.

Electron microscopic studies: Morphology and structure of the nanoemulsion was studied using transmission electron microscopy (TEM) TOPCON 002B operating at 200 KV and of a 0.18 nm capable of point to point resolution. In order to perform the TEM, the nanoemulsion formulation was diluted with distilled water (1/100). A drop of the diluted nanoemulsion was directly deposited on the Copper holey film grid and observed after putting fixing agent and drying it in the filtered air.

Scattering techniques: Dynamic light scattering photon correlation spectroscopy (PCS) is used to analyze the fluctuations in the intensity of the scattering by the droplets due to Brownian motion.

Interfacial tension measurement: The formation and properties of nanoemulsion can be studied by measuring the interfacial tension. Spinning-drop apparatus can be used to measure the ultra low interfacial tension.

Droplet size distribution: Droplet size of the prepared nanoemulsion was determined by using photon correlation spectroscopy, which analyzes the fluctuations in light scattering due to Brownian movement of the particles8. The formulation (0.1 mL) was dispersed in 50 mL (500 dilution) of distilled water in a volumetric flask and gently mixed by inverting the flask and measurement done using a Zetasizer (Nano ZS-90, UK). Light scattering was monitored at 25°C at a 90° angle.

Isotropicity: Refractive index of nanoemulsions formulations was determined using an Abbe type refractrometer. It basically gives an idea about the isotropicity of the formulations. The isotropic nature of microemulsions and their optical clarity makes their study by spectroscopic techniques straightforward, particularly in comparison to conventional macroemulsions.

Determination of encapsulation efficiency:

For determining the amount of drug entrapped in the formulation, weighed amount of formulation is dispersed in organic solvent by ultrasonication and the drug is extracted into suitable buffer. Drug content is estimated by analysing the extract spectrophotometrically at λ_{max} of drug after making suitable dilutions against suitable blank. The entrapment efficiency (EE) and loading efficiency (LE) of the drug can be calculated by using the following Eqns. [27], drug EE = drug content in the product obtained (mg)/total amount of drug added (mg)×100 and drug LE = drug content in the product obtained (mg)/total product weight (mg)×100. Drug content could also be determined using reverse phase high-performance liquid chromatography (HPLC) techniques. Singh *et al.* employed this technique for finding primaquine concentration and reported 95 % encapsulation efficiency of formulated nanoemulsion[5].

Determination of particle size and polydispersity index (PDI):

The particle size and PDI of nanoemulsions are analysed employing photon correlation spectroscopy (PCS) using Malvern Zetasizer, which monitors the variation in light scattering because of Brownian motion of particles as function of time. PCS is based on the principle that the particles with small size travels with higher velocity as compared to particles with large size. The laser beam gets diffracted by sub-micron particles present in solution. Due to diffusion of particles, rapid fluctuations in laser scattering intensity occur around. A mean value at a fixed angle and this is dependent upon particle size. The calculated photoelectron time-correlation function generates a histogram of the line width distribution that can be related to the size of particle. For measuring particle size, weighed amount of formulation is dispersed in double-distilled water for obtaining homogenous dispersion and that has to be used instantly for measuring the particle size and PDI. The PDI can range from 0 to 1, where 0 (zero) stands for monodisperse system and 1 for a polydisperse particle dispersion[28]. Evaluated the particle size and PDI of risperidone nanoemulsion by using this method and reported mean particle size around 160 nm with mean size distribution less than 0.15. The same technique and reported particle size of primaquine nanoemulsion in the range of 20-200 nm[5].

Determination of zeta potential: The zeta potential is a method for measuring surface charge of particles when it is placed in liquid. Zeta potential is used for predicting dispersion stability and its value depends on physicochemical property of drug, polymer, vehicle, presence of electrolytes and their adsorption. It is measured by Malvern Zetasizer instrument. For measuring zeta potential, nanoemulsion is diluted and its value is estimated from the electrophoretic mobility of oil droplets. Zeta potential of ±30 mV is believed to be sufficient for ensuring physical stability of nanoemulsion. Đorđević *et al.* obtained zeta potential around –50 mV by using Malvern Zetasizer for risperidone nanoemulsion[29].

Fourier-transform infrared spectroscopy (FTIR)

Spectral Analysis:

FTIR analysis can be carried out for the assessment of drug excipient interaction, polymerization, crosslinking as well as drug loading in the formulation. It is also used for identifying the functional groups with their means of attachment and the fingerprint of the molecule. At low temperature a molecule exists in ground state and on absorbing the radiant energy, they get excited to higher energy states. IR spectroscopy is based on determining this energy difference (ΔE) between the excited and ground states of the molecule. For performing FTIR, sample can be prepared by employing suitable method such as potassium bromide pellet method, Nujol mulls and then sample is scanned in FTIR at moderate scanning speed between 4000-400 cm-1. Srilatha *et al*. conducted FTIR studies on pure drug and glipizide nanoemulsion and reported absence of drug excipient interactions (hence compatibility of drug and excipient) as all the characteristics peaks of drug appeared at same point in formulation[30].

Atomic force microscope (AFM):AFM is comparatively a new technique being used these days for exploring the surface morphology of nanoemulsion formulations. AFM is carried out by diluting nanoemulsions with water followed by drop coating of the diluted nanoemulsion on a glass slide. Further the coated drops are dried in oven and scanned at of 100 mV/s[32]

***In vitro* drug release study:** *In vitro* drug release studies help to estimate the *in vivo* performance of drug formulation. The *in vitro* release rate of a drug is usually studied on a USP dissolution apparatus. Nanoemulsion or dried nanoparticles containing drug equivalent to 10 mg were dispersed in buffer and then it is introduced into dialysis membrane pouches and placed in a flask containing buffer. This study is carried out at 37±0.5° and a stirring speed of 50 rpm. Sample are withdrawn at periodic intervals and each time replaced by the same volume of fresh dissolution medium. Samples are then diluted suitably and the absorbance of sample is measured spectrophotometrically at a particular wavelength. Absorbance of the collected sample is used for calculating % drug release at different time intervals using calibration curve.

***In vitro* skin permeation studies:**

Keshary Chien-diffusion cell is used for investigating *in vitro* and *ex vivo* permeation studies. For performing permeation studies, abdominal skin of adult male rats weighing 250±10 g is usually employed. The rat skin is positioned between the donor and the receiver chambers of diffusion cells. Temperature of receiver chambers containing fresh water with 20 % ethanol is fixed at 37° and the contents of the chamber are continuously stirred at 300 rpm. The formulations are kept in the donor chamber. At specific time intervals such as 2, 4, 6, 8 h, a certain amount (0.5 ml) of the solution from the receiver chamber was removed for performing gas chromatographic analysis and each time replaced with an equivalent volume of fresh solution immediately. Each sample is performed three times. Cumulative corrections are done for obtaining total amount of drug permeated through rat skins at each time interval and are plotted against function of time. Slope of plot is used for calculating the permeation rates of drug at a steady-state.

Stability studies:

Stability studies are performed for assessing stability of the drug substance under the influence of a various environmental factors like temperature, humidity and light. The stability studies of nanoemulsion are

carried out after storing the formulation for 24 mo in dispersed and freeze-dried state as per International Conference on Harmonisation guidelines. The storage conditions followed are ambient (25±2°/60±5 % RH), refrigeration (5±3°) and freeze (–20±5°). The requisite volume of nanoemulsion is stored in glass bottles and is tightly sealed. Samples are withdrawn at predefined time interval and analysed for the characteristics such as particle size, loading and EE and *in vitro* drug release profile[26]. Singh *et al*. performed stability studies on nanoemulsion and observed that no change in viscosity, drug content and particle size when the formulation was stored for 3 mo at 25°/60 % RH and 30°/65 % RH[5].

Shelf life determination: For determining shelf life of a nanoemulsion, accelerated stability studies are performed. The formulations are stored at three distinct temperatures and ambient humidity conditions (30°, 40° and 50±0.5°) for almost 3 mo. After a particular time interval (0, 30, 60 and 90 d) samples are withdrawn and analysed using HPLC at λ_{max} for estimating the remaining drug content. Samples withdrawn at zero time are used as controls. The order of the reaction is determined by this and after that the reaction rate constant (K) for the degradation is calculated from the slope of the lines by using following equation at each elevated temperature: slope = −K/2.303, the logarithm values of K are plotted at different elevated temperatures against the reciprocal of absolute temperature (Arrhenius plot). From this plot value of K at 25° is determined and it is further used for calculating shelf life by putting the value in following Eqn.: $t_{0.9}=0.1052/K_{25}$. Where $t_{0.9}$ stands for time required for 10 % degradation of the drug and it is termed as shelf life[31]. Ali *et al*. determined the shelf life of clobetasol propionate-loaded nanoemulsion around 2.18 y at room temperature (25°) and concluded that the stability of clobetasol propionate can be augmented by incorporating in a nanoemulsion[37]. Parveen *et al*. reported that the shelf life of a silymarin nanoemulsion to be around 3.8 y when stored in a refrigerator[38].

Thermodynamic stability studies:

Thermodynamic stability studies are usually carried out in three steps. Firstly heating-cooling cycle, which is performed for observing any effect on the stability of nanoemulsion by varying temperature conditions. Nanoemulsion is exposed to six cycles between 4° (refrigeration temperature) and 40° by storing the formulation at each temperature for not less than 48 h. The formulations which are stable at these temperatures are further chosen for centrifugation studies. Secondly, centrifugation study in which the formulated nanoemulsions are centrifuged at 5000 rpm for 30 min and observed for phase separation or creaming or cracking. Those which did not show any sign of instability are subjected to freeze thaw cycle. Thirdly, the freeze-thaw cycle, in which nanoemulsion formulations are exposed to three freeze-thaw cycles with temperature varying between –21° and +25°. Formulations that show no signs of instability pass this test and deemed to have good stability[6]. These formulations are then subjected to dispersibility studies for evaluating the efficiency of self-emulsification. Srilatha *et al*. performed thermodynamic studies on glipizide nanoemulsion by subjecting it to three cycles of stability and reported good physical stability of nanoemulsion with no appearance of phase separation, creaming or cracking[30].

Dispersibility studies:

Dispersibility studies for evaluating the efficiency of self-emulsification of nanoemulsion are carried out by using a standard USP XXII dissolution apparatus 2.1 ml of each formulation is incorporated into 500 ml of distilled water maintained at 37±0.5°. A standard stainless steel dissolution paddle rotates at 50 rpm for providing gentle agitation. *In vitro* performance of the nanoemulsion formulations is evaluated visually by using a grading system described below[6]. Grade A nanoemulsions form rapidly within 1 min and appear to be clear or bluish. Grade B nanoemulsions form rapidly but are slightly less clear emulsions appear to be bluish-white. Grade C nanoemulsions are fine milky emulsion that form within 2 min. Grade D are those dull, greyish-white emulsions that has a little oily appearance and are slower to form (>2 min). Grade E nanoemulsions display either poor or

negligible emulsification with large oil globules present on the surface.

Determination of viscosity:

Viscosity assessment is an important parameter for physicochemical characterization of nanoemulsion. Various instruments are employed for measuring viscosity such as Ostwald viscometer, Hoeppler falling ball viscometer, Stormer viscometer, Brookfield viscometer and Ferranti-Shirley viscometer. Among all these viscometer, Brookfield is the preferred one for measuring the viscosity of nanoemulsion. Determination of viscosities affirms whether the system is O/W or W/O emulsion. Low viscosity of systems shows that it is O/W type and high viscosity shows that it is water in oil type system[28]. However, currently survismeter has been the most widely employed equipment as it measures surface tension, viscosity, interfacial tension, contact angle, dipole moment and particle size and hydrodynamic volumes of the nanoemulsions[39].

Refractive index: Refractive index tells how light propagates through the medium and transparency of nanoemulsion. Refractive index (n) of medium can be defined as ratio of speed of wave (c) in reference medium to the phase speed of wave (vp) in medium: n=c/vp. Refractive index of the nanoemulsion can be determined by Abbes type refractometer at 25±0.5° by placing a drop of nanoemulsion on slide and comparing it with refractive index of water (1.333). If refractive index of nanoemulsion has equal refractive index as that of water, then the nanoemulsion is considered to have transparent nature[2,28].

Percent transmittance: Percent transmittance of a formulated nanoemulsion is estimated using UV spectrophotometer at a particular wavelength with distilled water as a blank. If percent transmittance of a nanoemulsion is found to be greater than 99 %, then it is considered as transparent in nature[31].

pH and osmolarity measurements:

The pH meter is used for measuring the pH of a nanoemulsion and microosmometer is used for determining the osmolarity of emulsion, which is based upon freezing point method. For performing this, 100 µl of nanoemulsion is transferred in microtube and measurements are taken[41].

Dye solubilisation: A water soluble dye is dispersible in an O/W globule whereas it is soluble in the aqueous phase of the W/O globule. Similarly an oil soluble dye is dispersible in the W/O globule but soluble in the oily phase of the O/W globule[3]. On adding water soluble dye to O/W nanoemulsion, it will evenly takes up the colour whereas if it is a W/O emulsion, dye will remain in dispersed phase only and the colour will not spread evenly. This can be seen with microscopic examination of emulsion[4].

Dilutability test:

The rationale of dilution test is that continuous phase can be added in larger proportion into a nanoemulsion without causing any problem in its stability. Thus O/W nanoemulsions are dilutable with water but W/O nanoemulsions are not and go through a phase inversion into O/W nanoemulsion. The W/O nanoemulsion can be diluted with oil only[3,4].

Conductance measurement:

The O/W nanoemulsions are highly conducting because they have water in external phase whereas W/O nanoemulsions are not conducting as they have water in internal or dispersal phase. Electrical conductivity measurements are very much beneficial for determining the nature of the continuous phase and for detecting phase inversion phenomena. At low volume fractions, increase in conductivity of certain W/O nanoemulsion systems was observed and such kind of behaviour is deduced as an indicator of a percolative behaviour or ions exchange among droplets prior to the development of bicontinuous structures. Dielectric measurements are a great means of exploring the structural and dynamic features of nanoemulsion systems[3]. Conductometer is employed for determining the conductance of nanoemulsion. For carrying out conductance measurement, a pair of electrodes is attached to a lamp and an electric source is immersed into an emulsion. When the emulsion is O/W type then water will conduct the current and

lamp will glow because of passage of current among connecting electrodes. The lamp will not glow if it is water in oil emulsion as oil in external phase does not conduct the current.

Interfacial tension:

By measuring the interfacial tension, the formation and the properties of nanoemulsion can be investigated. Ultra low values of interfacial tension corresponds to phase behaviour, mainly the coexistence of surfactant phase or middle-phase nanoemulsions with aqueous and oil phases in equilibrium. For determining ultra-low interfacial tension spinning-drop apparatus is used. Interfacial tensions are obtained by measuring the shape of a drop of the low-density phase, rotating it in cylindrical capillary filled with high-density phase[3].

Fluorescence test:

There are numerous oils that show fluorescence under UV light. If a W/O nanoemulsion is subjected to a fluorescence light under a microscope, the whole field will fluoresces and if it is an O/W the fluorescence will be in spots[4].

APPLICATION OF NANOEMULSION

Applications in cosmetics

Recently importance of nanoemulsions have become increasing as good vehicles for the controlled delivery of cosmetics and for the optimized dispersion of active ingredients in particular skin layers. Due to their lipophilic interior, nanoemulsions are more suitable for the transport of lipophilic drug than liposomes Similar to liposomes, nanoemulsions supports the skin penetration of active ingredients and thus increases their concentration in the skin.

Antimicrobial nanoemulsions

Antimicrobial nanoemulsions are o/w droplets that range from 200-600 nm. They are made of oil and water and are stabilized by surfactants and alcohol. The nanoemulsions has a broad spectrum of activity against bacteria like E. coli, salmonella, S. aureus; enveloped viruses like HIV, herpes simplex; fungi like candida, dermatophytes, and spores like anthrax. The nanoemulsions particles are thermodynamically driven to fuse with lipid-containing organ- isms. This fusion is enhanced by the electrostatic attraction between the cationic charge of the emulsion and the anionic charge on the pathogen. When enough nanoparticles fuse with the pathogens, they release part of the energy trapped within the emulsion. Both the active ingredient and the energy released destabilize the pathogen lipid membrane, resulting in cell lysis and death. In the case of spores, additional germination enhancers are added into the emulsion. Once starting of germination takes place, the germinating spores become susceptible to the antimicrobial action of the nanoemulsions.

Prophylactic in bio-terrorism attack

Because of their antimicrobial activity, research has begun on use of nanoemulsions as a prophylactic medicated dosage form, a human protective treatment, to prevent the people exposed to bio-attack such as Anthrax and Ebola. The broad-spectrum nanoemulsions were checked on surfaces by the US Army (RestOps) in Dec 1999 for decontamination of Anthrax spore. It was checked again by RestOps in March 2001 as a chemical decontamination agent. This technology has been tested on gangrene and clostridium botulism spores, and can even be used on contaminated wounds to salvage limbs.

Nanoemulsions in vaccines delivery

This medication delivery system uses nanotechnology to vaccinate against human immuno- deficiency virus (HIV). There is recent evidence that HIV can infect the mucosal immune system. Therefore, developing mucosal immunity through the use of nanoemulsions may become very important in the future fight against HIV [50]. The oil-based emulsion is administered in the nose, as opposed to traditional vaccine routes. Recent research results indicate that genital mucosa immunity may be attained with vaccines that are administered into the nasal mucosa [56-58,115]. Nanoemulsions are being used to transport inactivated organisms to a mucosal surface to produce an immune response. The first applications as vaccine, an influenza vaccine and an HIV vaccine, can proceed to clinical trials. The nanoemulsion causes proteins applied to the mucosal surface to be adjuvant and it help uptake by antigen presenting cells. This results in the significant systemic and mucosal immune response due to

that the production of specific IgG and IgA antibody as well as cellular immunity.

Nanoemulsions as non-toxic disinfectant cleaner

Nanemulsions have been employed as a disinfectant cleaner. A nontoxic disinfectant cleaner for use in routine markets that include healthcare, travel, food processing and military applications. They have been found to kill tuberculosis and a large spectrum of viruses, bacteria and fungi within 5 to 10 min without any of the hazards posed by other categories of disinfectants. The product requires no warning labels. It does not irritate eyes and can be absorbed through the skin, inhaled or swallowed with harmless effects. The disinfectant formulation is made up of nanospheres of oil droplets less than 100 μm which are suspended in water to produce a nanoemulsions requiring only small amounts of the active ingredient, parachlorometaxylenol.

Nanoemulsions in cell culture technology

Cell cultures are used for in vitro assays or to produce biological compounds like an antibodies or recombinant proteins. For optimization of cell growth, the culture medium can be supple- mented with a large number of molecules or with blood serum. It has been very difficult to provide the media with oil-soluble substances that are available to the cells, and only few amounts of the lipophilic compounds could be absorbed by the cells. Nanoemulsions are a new method for the delivery of oil-soluble substances to human cell cultures. The system is based on a nanoemulsions that is stabilized by phospholipids. This nanoemulsions is trans- parent and can be passed through 0.1 mm filters for sterilization. Nanoemulsions oil droplets are very easily taken up by the cells. The encapsulated oil-soluble substances therefore have a high bioavailability to cells in culture.

The advantages of using nanoemulsions in cell culture technology include:

- Better uptake of oil-soluble supplements in cell cultures.
- Improve growth and vitality of cultured cells.
- Allows toxicity studies of oil-soluble drugs in cell cultures

Nanoemulsion formulations for improved oral delivery of poorly soluble drugs

Nanoemulsions formulation was developed to increase oral bioavailability of hydrophobic drugs. Paclitaxel was selected as a model hydrophobic drug. The o/w nanoemulsions were made with pine nut oil as the internal oil phase, water as the external phase and egg lecithin as the primary emulsifier. Stearylamine and deoxycholic acid were used to give positive and negative charge to the emulsions, respectively. The formulated nanoemulsions had a particle size range of 100-120 nm and zeta potential ranging from 34 mV to 245 mV.

Nanoemulsions in ocular and otic drug delivery

Ophthalmic drug delivery is one of the most interesting and challenging endeavors facing the pharmaceutical scientist [117]. It is a common knowledge that the application of eye drops as conventional ophthalmic delivery systems results in poor bioavailability and therapeutic response because of lacrimal secretion and nasolacrimal drainage in the eye [118,119]. Most of the drug is drained away from the precorneal area in few minutes. As a result, frequent instillation of concentrated solutions is needed to achieve the desired therapeutic effects [120]. But, by the tear drainage, the main part of the administered drug is transported via the nasolacrimal duct to the gastric intestinal tract where it may be absorbed, sometimes causing side effects [121]. In order to increase the effectiveness of the drug, a dosage form should be chosen which increases the contact time of the drug in the eye. This may then increase the bioavailability, reduce systemic absorption, and reduce the need for frequent administration leading to improved patient compliance

Nanoemulsions as a vehicle for transdermal delivery

Drug delivery through the skin to the systemic circulation is convenient for a number of clinical conditions due to which there has been a considerable interest in this area. It offers the advantage of steady state controlled drug delivery over extended period of time, with self administration also being possible, which may not be the case with parenteral route. The drug input can be eliminated at any time by the patient just by removing the transdermal patch. Their transparent nature and fluidity, confers

on nanoemulsions a pleasant skin feel. An extra advantage is the total absence of gastrointestinal side effects like irritation and bowel ulcers which are invariably associated with oral delivery. Nanoemulsions have improved transdermal permeation of many drugs over the conventional topical formulations such as emulsions and gels.

Nanoemulsion in cancer therapy and in targeted drug delivery

Another interesting application, which is experiencing an active development, is the use of nanoemulsion formulations, for controlled drug delivery and targeting. Because of their submicron size, they can easily be targeted to the tumor area. Although nanoemulsions are chiefly seen as vehicles for administering aqueous insoluble drugs, they have more recently received increasing attention as colloidal carriers for targeted delivery of various anticancer drugs, photosensitizers, neutron capture therapy agents, or diagnostic agents. The develop- ment of magnetic nanoemulsions is an innovative approach for cancer therapy. This methodology can be used for the treatment of cancer in the form of photodynamic therapy.

Nanoemulsions and intranasal drug delivery

Intranasal drug delivery system has now been recognized as a reliable route for the adminis-tration of drugs next to parenteral and oral routes. This route is also painless, non-invasive and well tolerated. The nasal cavity is one of the most efficient sites because of its reduced enzymatic activity, high availability of immunoactive sites and its moderately permeable epithelium. There are several problems associated with targeting drugs to brain, especially the hydrophilic ones and those of high molecular weight. This is because of the impervious nature of the endothelium, which divides the systemic circulation and barrier between the blood and brain. The olfactory region of the nasal mucosa provides a direct connection between the nose and brain, and by the use of nanoe-mulsions loaded with drugs, conditions such as Alzheimer's disease, migraine, depression, schizophrenia, Parkinson's diseases, meningitis, etc. can be treated. It is inferred that this emulsion is more effective through the nasal rather than intravenous route. Another application of intranasal drug delivery system in therapeutics is their use in development of vaccines. Immunity is achieved by the administration of mucosal antigen.

Nanoemulsions and parenteral drug delivery

This is one of the most common and effective routes of drug administration usually adopted for actives with low bioavailability and narrow therapeutic index. Their capacity to dissolve large quantities of hydrophobics, together with their mutual compatibility and ability to protect the drugs from hydrolysis and enzymatic degradation make nanoemulsions ideal vehicles for the purpose of parenteral transport. Further, the frequency and dosage of injections can be reduced throughout the drug therapy period as these emulsions guarantee the release of drugs in a sustained and controlled mode over long periods of time.

Nanoemulsions and pulmonary drug delivery

The lung is an attractive target for drug delivery due to noninvasive administration via inhalation aerosols, avoidance of first-pass metabolism, direct delivery to the site of action for the treatment of respiratory diseases, and the availability of a huge surface area for local drug action and systemic absorption of drug. Colloidal carriers (ie, nanocarrier systems) in pulmonary drug delivery offer many advantages such as the potential to achieve relatively uniform distribution of drug dose among the alveoli, achievement of improved solubility of the drug from its own aqueous solubility, a sustained drug release which consequently reduces dosing frequency, improves patient compliance, decreases incidence of side effects, and the potential of drug internalization by cells.

Nanoemulsions as gene delivery vector

Emulsion systems have been introduced as alternative gene transfer vectors to liposomes [194]. Other emulsion studies for gene delivery (non-pulmonary route) have shown that binding of the emulsion/DNA complex was stronger than liposomal carriers. This stable emulsion system delivered genes more efficiently than liposomes.

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