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CHARACTERIZATION AND EVALUATION OF IRON NANO-EMULSION PREPARED BY HIGH SPEED HOMOGENIZATION

NAVEEN S AND KANUM F*

Biochemistry & Nutrition Discipline, Defence Food Research Laboratory, Siddharathanagar, Mysore-570 011

***Corresponding Author: E Mail: dr.farhathkhanum@gmail.com; Tel.: 011-0821-2473290; Fax: 011-0821-2473468**

ABSTRACT

Phospholipid / oil -in-water nano-emulsion of iron were prepared by high speed homogenization. The influence of emulsifying conditions including emulsifier type (phosphatidylcholine; cholesterol, cholesterol ester, soyabean oil, surfactant mix), pH and conductivity on the properties and stability of the nano-emulsions were investigated using a Zetasizer nanoseries (DLS) and Differential Scanning calorimetry (DSC). The nano-emulsions were used to fortify milk and were studied for the bio-availability of iron in rats. The nano-emulsions produced with phosphatidylcholine and cholesterol had the smallest particle sizes and smallest size distribution. The mean diameter (z-average) of the dispersed particles was 59 nm and the size distribution was unimodal and extended from 16 to 198 nm. The particle sizes increased with change of emulsifier type, and also with pH/conductivity. The physical stability of the nanoemulsions decreased with the elevation of temperature but increased with the emulsifier combination of phosphatidylcholine and cholesterol. During storage, iron in the nano-emulsions degraded after one month with loss of 22% at 25^{o}C. This promising emulsion had considerably high zeta-potential, suggesting the stability against particle aggregation. DSC studies showed two exothermic peaks at 120°C and 152°C. Its *in-vivo* bioavailability was confirmed to be greater than that of a standard control, where iron was directly mixed in milk. After feeding the fortified milk with the nano-emulsion; serum iron levels increased significantly compared to standard control.

Toxicity of the emulsion was nil when fed twice daily at 10ml/kg body weight for fifteen days. These studies suggest that the nano-emulsion can be used to fortify iron rich food products such as milk to provide additional nutrient supplementation.

Keywords: Iron Nano-Emulsion, Differential Scanning Calorimetry (DSC) and Zetasizer Nanoseries (Nano DLS)

INTRODUCTION

Poor bioavailability by the oral route can be due to poor solubility, degradation in gastrointestinal lumen, poor membrane permeation and presystemic elimination. Any of the approaches, which can alter these characteristics, should help in improving the bioavailability of iron. Iron is one of the most important micronutrient required day to day for normal functioning and circumventing the major problem of its poor bioavailability remains a bigger challenge for pharmaceutical scientists. Nanoemulsions being colloidal nanodispersions of oil in water (or water in oil), thermodynamically stabilized by an interfacial film of surfactant(s) and cosurfactant(s) have revealed tremendous potential in nanoengineering of various micronutrients **[1, 2]**. Droplet size in thermodynamically stable nanoemulsions is usually 10-100 nm. The homogeneous systems that can be prepared over a wide range of surfactant concentrations and oil to water ratios are all fluids of low viscosity. Nanoemulsions provide ultra low interfacial tensions and large o/w interfacial areas. Nanoemulsions have a higher solubilization capacity than simple micellar solutions and their thermodynamic stability offers advantages over unstable dispersions, such as emulsions and suspensions, because they can be manufactured with little energy input (Low energy emulsification techniques/heat or mixing) and have a long shelf life. The nanosized droplets leading to enormous interfacial areas associated with nanoemulsions would influence the transport properties of the micronutrient, an important factor in sustained and targeted delivery. The attraction of o/w nanoemulsion systems lies in their ability to incorporate hydrophobic drugs into the oil phase thereby enhancing their solubility. Nanoemulsions have been reported to make the plasma concentration profiles and bioavailability of drugs more reproducible **[3-6]**. The objective of the present study is to prepare nanoemulsion of iron to improve its solubility and bioavailability. Methods have been standardized for the preparation of nanosomes and iron incorporated nanosomes,

characterization of size using TEM and Nano DLS.

MATERIALS AND METHODS

Materials

All the chemicals used in the present investigation were of analytical grade and purchased from Sigma Chemicals Co, (St. Louis Mo. USA), Sisco Research Laboratory, Hi Media and Merck, India. Fresh, pasteurized milk was purchased from a local dairy and used without further treatment. Milli Q water was used throughout the experiment.

Animals

Healthy, male albino Wister rats weighing 150-200g, which were inbred in the Animal House, Defense Food Research Laboratory, Mysore, were used for the present study. They were maintained under standard environmental conditions and were fed with standard pellet diet and water *adlibitum*. The experiments were performed after the approval from the institutional animal ethical committee.

Nano-emulsions were formed in water / iron system with different surfactants and water contents above 75 wt%. The nano-emulsion with Phosphotidyl choline and cholesterol (70/30) and 90 wt% of water was chosen as per **Table 1**.

Solubility of Nanoemulsions

The solubility of emulsions with various surfactants and co-surfactants selected as per the list in **Table 1** was determined by dissolving an excess amount of the iron in 2 mL of each of the selected surfactants and co-surfactants in 5-mL stoppered vials and mixed using a vortex mixer. The vials were then kept at room temperature for 72 hours to get to equilibrium. The equilibrated samples were centrifuged at 3000 rpm for 15 min. The supernatant was taken and filtered through a 0.45μm membrane filter. The concentration of iron was determined in each formulation **(Table 3)**.

Nano-Emulsion Droplet Size Analysis

The average size of the nano-emulsion droplet was determined by photon correlation spectroscopy (PCS) with a commercial lightscattering setup, 4700C, Malvern Instruments, Malvern, UK, with an argon laser of wavelength $\lambda 0 = 488$ nm. PCS makes it possible to calculate the average diffusion coefficient (D) of the particles. Assuming that the particles are solid, the mean diameter (d) can be easily calculated by the Stokes–Einstein equation,

$$d = kT\,(1) \div 3\pi\eta D$$

Where k is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of the medium. It should be noted that sizes given in the figures of this paper are slightly

overestimated, as soft particles possess lower D than hard ones due to frictional reasons. Previously, to carry out the measurements, the best experimental conditions were investigated and set at the following values: laser power = 33 mW; particle concentration per volume unit = 10^{-3}; and measurement angle = 90◦. Volume phase transitions induced by adding salt were simultaneously studied by static light scattering (SLS) and PCS using the same optical instrument.

The number and size of the prepared nanoemulsions was carried out using MALVERN Zetasizer nanoseries (173^0 orientation) which performs size measurements using a process called Dynamic Light Scattering (DLS) which measures Brownian motion and relates this to the size of the prepared emulsions and also the stability of the prepared nano-emulsions were determined by measuring their Zeta potential.

Transmission Electron Microscopy (TEM)

Morphology and structure of the nano-emulsion were studied using transmission electron microscopy TOPCON 002B operating at 200 KV (Topcon, USA) and 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 nano-emulsion droplets. In order to perform the TEM observations, a drop of the nano-emulsion was directly deposited on the holey film grid and observed after drying.

Stability Studies

A stability study on optimized nano-emulsion was performed by keeping the sample at refrigerator temperature (4°C) and room temperature (25°C). These studies were performed for the period of 3 months. The droplet size and dispensability were determined using methods described above during storage.

Food Fortification with the Prepared Nanoemulsions

The samples, nano-emulsions were prepared using 100 mL of fresh pasteurized milk instead of using water to hydrate the thin film obtained in the round bottom flask after evaporating chloroform as done earlier, after that they were homogenized, sonicated and centrifuged at 12000 rpm for 45 min and supernatant was taken.

Analytical Method

Iron gives a purple coloured complex with ferrozine solution which has the absorption maxima at 620nm. The intensity of this purple colour can be taken as a measure of iron. Determination of total iron concentration in each sample was carried out using a modification of the ferrozine method

proposed by Reddy, Chidambaram, Fonseca, and Bates (1986).

***In Vivo* Bioavailability Test [2]**

All rats were housed in plastic cages and fed with iron free diet for 15 days. On the 16th day, food was removed and fasted overnight. On the 17th day blood was collected from all the three groups and mixed with anti-coagulant (Heparin). Blood was drawn before and after force feeding of the nano-emulsion in which the micronutrients were estimated.

Toxicity Studies [7-10]

10ml per kg body weight of the emulsions were force fed to rats and mice (both male and females) for a period of one month twice daily. Blood was collected from the retro-orbital puncture for haematological analysis; haemoglobin and packed cell volume (haematology analyzer, Sysmex Kx-21, JAPAN) and also for liver and kidney function tests using Erba kits in Autoanalyser EM200 from M/s Transsisia. The results indicated that the emulsions were non toxic **(Table 6 and 7)**.

RESULTS AND DISCUSSION

Light Scattering

Nano-emulsions were characterized by light scattering; the correlation function at 90^0 was fitted by means of a cumulants analysis. From the fitting it can be inferred that samples have a monomodal size distribution and low polydispersity. They may be formed by spherical entities. Nano-emulsions showed hydrodynamic radius of 140nm **(Table 2)**.

Stability of a dosage form refers to the chemical and physical integrity of the dosage unit and when appropriate, the ability of the dosage unit to maintain protection against microbiological contamination. An ideal emulsion formulation must be thoroughly characterized physically, chemically and microbiologically at the start of study and throughout the intended shelf life period Therefore, optimized nano-emulsion formulation was characterized for droplet size for the period of three months. During stability studies droplet size were determined at temperatures of $4^{\circ}C$ and $25^{\circ}C$. These parameters were determined at 0, 1, 2 and 3 months **(Table 3)**. These parameters were compared for statistical significance by one-way analysis of variance (ANOVA) using origin software. The changes in these parameters were not significant ($p \geq 0.05$). These results indicated that optimized formulation is stable.

Differential Scanning Calorimetry

Thermal behavior of the liposomes was studied quantitatively and qualitatively by DSC. The samples (8-9mg) were accurately weighed into standard aluminum pans and sealed. An empty pan was used as reference. Thermograms were recorded during heating

and cooling runs at a rule of 5°C/min between 50 to 180°C. Data was analyzed using the universal analysis 2000 software. The heat flow was related to the amount of sample analyzed. Upon heating the prepared formulation, two exothermic peaks around 120 and 152°C indicated re-crystallization of the lipid molecule. This shows the stability of the formulation to with stand 121°C (autoclavable temperature) w.r.t thermal changes **(Figure 2)**.

Nano-particle Characterization Using TEM (Figure 3)

TEM micrographs of nanoparticles obtained for the size characterization (negative staining with uranyl acetate).

Surface Charge Determination

Zeta potential measurements were seen using Nano DLS. The surface charge of the nano-particle dispersion helps the emulsion to be stable without agglomeration. Surface charges of the nano-particle dispersion are positive and depend on the pH of the medium. The lowest surface charge was at pH 1.36 **(Table 4)**.

Micronutrient Analysis in the Prepared Micro-Emulsions and Food Fortified with these Emulsions

Optimized nanoemulsion (sample-1) contains 465 µg of iron eq in one ml, which is a good concentration in the formulation, and can be used along with any type of diet such as milk, curd, yougurt, etc **(Table 5)**. Food fortified with optimized nanoemulsion (sample-1), which can provide additional nutrient supplementation and thus help in reducing the minimum requirement of micronutrient per day.

Bioavailability Studies

10ml per kg body weight of the emulsions were force fed to rats and mice (both male and females) for a period of one month twice daily. Blood was collected from the retro-orbital puncture for haemoglobin and packed cell volume analysis (haematology analyzer, Sysmex Kx-21, JAPAN) and also for liver function test and kidney function test parameters using Erba kits in Autoanalyser EM200 from M/s Erba. The results were normal and indicated the emulsions were non toxic **(Table 6 and 7)**. In-vivo bio-availability of micronutrients encapsulated nano-emulsion was found to be appreciable.

CONCLUSION

Emulsions were having mean droplet radius of about 140nm as determined by DLS, significantly smaller than that of the template nano-emulsion. Nano-emulsions showed negative zeta potential values which were dependent on the pH of the diluting medium. Nano-emulsion stability assessment by its zeta potential and size as determined by DLS

revealed that nano-emulsion with PC and Cholesterol were stable and could be used to fortify with milk for better absorption and assimilation. Bioavailability and toxicity studies of the fortified milk also showed no toxic and were better absorbed as compared to control. Bioavailability of the products prepared based on this formulation will help in reducing the requirement micronutrients per day i.e., RDA levels. Iron deficiency anemias problems of the developing countries could be solved.

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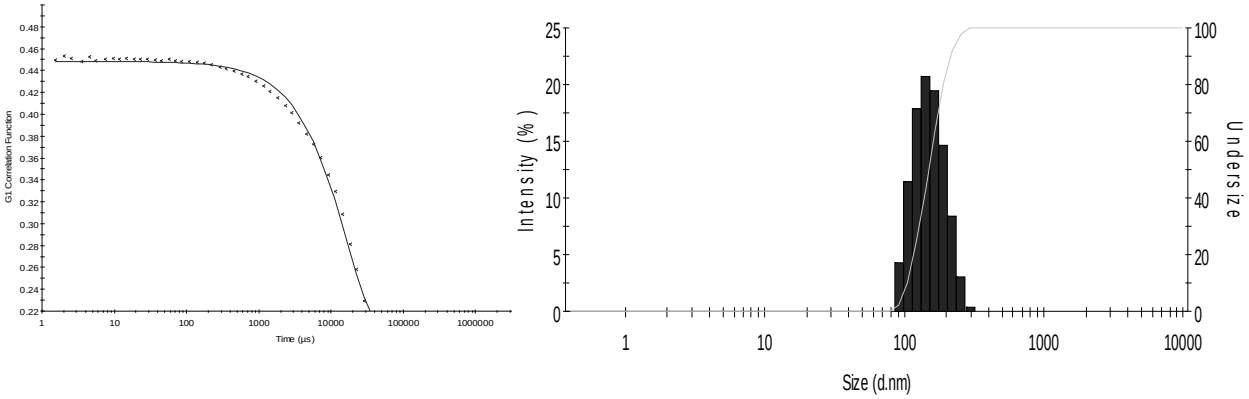

Figure 1: Correlation Function and Fitting Based on Cumulant Analysis for the Selected Nano-Emulsion

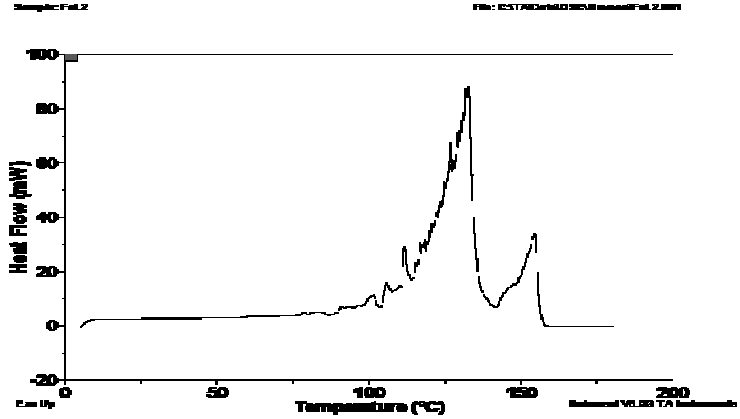

Figure 2: Stability of the Formulation to With Stand 121ºC (Autoclavable Temperature) w.r.t Thermal Changes

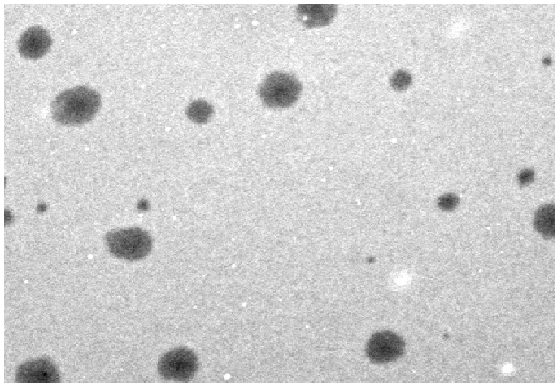

Figure 3: Nano-particle Obtenation and Characterization Using TEM

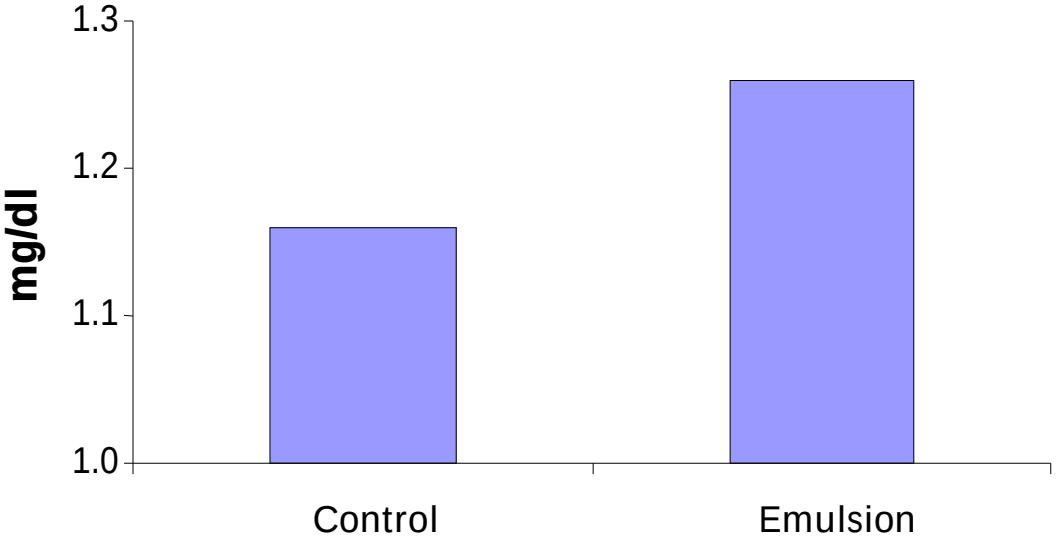

Figure 4: Bioavailability Studies; Iron Estimated After 3h

Table 1: Preparation of Micronutrient Incorporated Nanoemulsions

Sample 1	**Phosphotidyl choline, cholesterol, iron**
Sample 2	**Phosphotidyl choline, cholesterol acetate, iron**
Sample 3	**Phosphotidyl choline, iron**
Sample 4	**Groundnut oil, Surfactant mix (glyceryl triacetate, cremophor-EL, diethylene glycol monoethyl ether and triethanol amine hydrochloride), iron**
Sample 5	**Tween 80, iron**
Sample 6	**Iron**

Table 2: Number and Size of the Particles

Fortified food	Z-Avg (nm)
PC + Chol	98.52
PC + Chol Ac	140.7
PC	123.1
Oil + S- mix	104.6

Table 3: Droplet Size of Optimized Nano-Emulsion Formulation During Storage

Time (months)	Temp (^{0}C)	Droplet size(nm)
0	4.0 ± 0.5	98.52 ± 13.11
1	4.0 ± 0.5	115.12 ± 14.07
2	4.0 ± 0.5	115.34 ± 14.08
3	4.0 ± 0.5	125.58 ± 14.32
0	25 ± 0.5	98.52 ± 13.11
1	25 ± 0.5	119.49 ± 14.24
2	25 ± 0.5	128.12 ± 14.26
3	25 ± 0.5	129.33 ± 14.84

Table 4: Zeta Potential Measurements Were Seen Using Nano DLS

Record No	Sample	ZP [mV]
1	PC + Chol acetate	-51.3
2	PC + Chol	-46.5
3	PC	-55.6

Table-5: Concentration of Micronutrient in Emulsions and Fortified Food*

SAMPLES	COMPOSITION	IRON (µg of iron eq.)
SAMPLE-1	Iron (100mg); Phosphotidyl Choline (3g); Cholesterol (1g); - Distilled water (200ml)	465
SAMPLE-2 (control-1)	Iron (100mg); Distilled water (200ml)	507
SAMPLE-3	Iron (100mg); Groundnut oil (7.5ml); Glyceryl triacetate (7.5ml); Cremophor-EL (17.5ml); Diethylene glycol Monoethyl ether (17.5ml); Triethanol amine Hydrochloride (0.25g); Distilled water (200ml)	470
SAMPLE-4 (control-2)	Milk + Distilled water (3 : 1)	Not found
SAMPLE-5	Milk + Sample-1 (3 :1)	151
SAMPLE-6	Milk + Sample-2 (3 :1)	162
SAMPLE-7	Milk + Sample-3 (3 :1)	184

Table 6: Haematological Studies

Fortified food	PC + chol acetate	S-mix	PC + Chol	PC	PC + Chol	Normal Control
WBC ($x10^3$/mL)	8.1 ± 2.3	12.0 ± 2.1	5.9 ± 1.5	7.1 ± 1.3	10.0 ± 2.6	6.2 ± 1.5
RBC ($x10^6$/mL)	9.29 ± 1.0	8.45 ± 1.9	19.14 ± 3.1	9.29 ± 1.0	9.75 ± 2.1	10.57 ± 2.1
Hb (g/dL)	14.3 ± 1.5	18.5 ± 2.1	19.2 ± 3.2	15.3 ± 2.5	16.5 ± 1.9	17.7 ± 2.2
PLT ($x10^3$/mL)	719 ± 111	957 ± 121	624 ± 140	869 ± 101	755 ± 112	728 ± 121
LYM ($x10^3$/mL)	4.3 ± 1.21	10.4 ± 2.0	5.2 ± 0.82	5.3 ± 1.1	7.4 ± 2.0	3.1 ± 1.2

Table 7: Kidney and Liver Function Test Studies

Fortified food	Alb (mg/dl)	SGPT (IU/dl)	SGOT (IU/dl)	ALP (IU/dl)	Dir Bil (g/dl)	Total Bil (g/dl)	Cre (mg/dl)
PC + chol acetate	3.2±0.8	92±09	28±14	248±55	0.2±0.15	1.1±0.2	0.79±0.10
S-mix	2.5±1.1	102±21	56±16	555±86	0.8±0.3	2.0±0.4	3.22±0.30
PC + Chol	4.6±1.2	24±12	50±12	138±21	0.35±0.1	0.95±0.12	0.81±0.04
PC	5.1±1.2	45±20	55±12	28±11	0.13±0.09	1.1±0.2	0.65±0.11
PC + Chol	3.9±1.2	48±15	38±9	30±09	0.24±0.08	1.12±0.3	0.82±0.25
Normal Control	4.5±1.7	49±12	46±10	29±06	0.4±0.09	1.2±0.24	0.79±0.10