Research Article

DEVELOPMENT AND *IN VITRO* CHARACTERIZATION OF NANOEMULSION AS ADJUVANT FOR THE ADMINISTRATION OF VACCINE USING A MODEL ANTIGEN

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ABSTRACT

The present study has investigated the development of oil in water type nanoemulsion (NE) for the incorporation of vaccine. NE act as adjuvant and potentiate the immunogenicity of antigen. The NE was formulated using plant essential oil, surfactant tween 80 and co surfactant ethanol by high energy emulsification method. The formulations were optimized for surfactant and cosurfactant ratio, S_{mix} concentration and a ternary phase diagram was constructed to identify the area of NE formation. Formulations of NE1 to NE5 were prepared by sonication which are subjected to varying number of sonication cycles to get NE in 100nm – 200nm range and NE was subjected to short term and long term stability assessment. The NE was characterized in vitro for transparency, droplet size, zeta potential, pH, viscosity, conductivity, surface tension, fourier transform infrared spectroscopy. Transparent stable nanoemulsion (NE5) having mean droplet diameter of 84.23nm was obtained at 3:2 ratio of S_{mix} (10%) and the oil and surfactant ratio of 1:5 (v/v). No significant change in droplet diameter was observed after one week of storage at different temperatures. Surfactant concentration has been found to play a vital role in obtaining a stable nanosized emulsion. Further the NE5 was incorporated with pertussis toxin (PTAg-NE5) as a model vaccine and analysed for compatibility, size and stability. The PTAg-NE5 showed the incorporation of antigen with a slight increase in size of 99.42nm. The developed NE was found to be suitable for the incorporation of vaccine and thus could potentiate the immunogenicity of antigen.

Keywords: Nanoemulsion, Formulation, *In vitro* characterization, Stability, Vaccine adjuvant, Antigen.

INTRODUCTION

Vaccines are long been effectively and economically used against many infectious diseases. Most of which are administered topically via injection into intramuscular, subcutaneous & intradermal site in early childhood stage which produces pain and other side effects that leave the people with mental agony and stress. Needle less jet injections are available as an alternate to this problem. However the cost of such injection is very high & is not affordable for economic countries. Hence formulation development approaches have been made to improve the safety, efficacy and convenient means of its administration. In the formulation of vaccine, adjuvant plays a significant role in inducing the immunogenicity of antigen. Among the several adjuvants employed, emulsion type of adjuvant like freund's complete adjuvant are most widely used because of their effective interaction with the antigen and their potential to form granulomas at the injection site which are well recognized by the immune cells present in our human body.

With the advent of nanotechnology, vaccine formulations has gained lot of benefits. Nano particulates offers in large surface area, surface free energy, sustained & targeted delivery of therapeutics that provides protective & effective immunity. Further these nanocarriers can be charged and have hydrophobic (or) receptor interacting properties that leads to better interaction with antigen presenting cells. Further nano vaccines are non-invasive, allowing painless delivery. Scientists started developing numerous therapeutic vaccines using nanotechnology against all kinds of antigens. One such novel formulation have been shown significant vaccine adjuvant activity is nanoemulsion (NE). In addition to its vaccine

adjuvant effect it acts as a versatile delivery carrier, skin penetration enhancer, biocompatible formulation, protective for the antigen from degradation, targeted carrier, reduced dose of drug, thermodynamically stable, etc. The NE act as an effective adjuvant for vaccines by binding with the cell membranes of microbes and potentiate their action without affecting normal healthy cells. They have been reported to produce a strong and long lasting immunity through the induction of mucosal, systemic and cellular immunity. They help uptake by antigen presenting cells present in the skin, mucosa¹ etc. As they have low surfactant concentration, they are non skin irritating with lots of energy. Further their long term stability does not necessitate the use of cold chain storage for vaccine formulation². These NE's have been successfully adopted for mucosal and transdermal immunization³. However the immunogenicity greatly rely on the development of NE formulation. Many of the developed NE's have focused on mucosal immunity rather than the topical/transdermal immunization⁴. Human skin contains abundant dendritic and Langerhans cells which acts as antigen presenting cells in the immunization process. NE are found to penetrate effectively across the skin and through the hair follicles. Hence an attempt has been made for the development of o/w type NE suitable for the topical/transdermal delivery of antigen. Here, pertussis toxin (*Bordetella pertussis*) has been used as a model antigen. Pertussis vaccine is a subunit vaccine & administered intramuscularly along with adjuvant as part of DTap combined immunization to prevent the occurrence of whooping cough in children. Generally, the intramuscular administration of adjuvanted vaccines are done to reduce the local adverse effects due to hyper activation of immune system. Here the development of NE as adjuvant for topical administration may reduce the risk of

local adverse effects by their diffusion across the skin layers allowing slow release of antigen which could be processed effectively by the APC'S such as Langerhans cells & dendritic cells present in skin layers.

MATERIALS AND METHODS

Soybean oil was obtained from Fortune refined oil, India. Tween 80 and ethanol were purchased from SDFCL, Mumbai- Sodium Chloride and Potassium Chloride were bought from Loba chemie, Mumbai. Disodium hydrogen phosphate, Potassium dihydrogen phosphate and Sodium Hydroxide were obtained from Merck specialties pvt ltd, Mumbai.

Compatibility determination

Compatibility between the formulation ingredients was tested by FTIR (Fourier Transform Infrared Spectroscopy (Jasco 6300 series, Jasco). For this the FTIR spectra for soya bean oil, Tween80, formulation NE5 & 5% PTA_g-NE5 were taken. Spectra were recorded from 4000 to 650 cm^{-1} scanning range and the observed spectrum were compared to determine the compatibility.

Formulation optimization of nanoemulsion

NE formulation optimization was focused with respect to the selection of oil, surfactant, co-surfactant and their ratio/concentration. Among the various oils such as soybean oil, castor oil, clove oil and eucalyptus oil found from the literature regarding the formulation of NE, soya bean oil having a viscosity of 1.572 mPas was chosen as the oil phase⁵. It has been said that the droplet size of emulsion gets decreased when emulsion viscosity is increased⁶. Based on the HLB (Hydrophilic Lipophilic Balance) value tween 80 and ethanol were used as surfactant and co-surfactant. Surfactant and co-surfactant ratio was optimized for various ratios i.e. 1:1, 1:2, 1:3, 1:4, 4:1, 3:1, 2:1, 3:2, 3:1. The ratio of 3:2 and 10%v/v concentration of Smix (mixture of surfactant & co-surfactant) was concluded to be ideal for the formulation of transparent stable soybean oil NE. Less than 10%v/v concentration of Smix (i.e. 5% and 8%) has resulted in the formation of unstable NE.

A pseudo ternary phase diagram was constructed using 3:2 ratio of S_{mix} by mixing different proportions of soya bean oil and aqueous phase. The mixture was gently stirred in a vortex mixer (Himedia, India) and kept at ambient temperature (25°C) to attain equilibrium. The equilibrated samples were visually examined and determined as being clear and transparent nanoemulsion, or crude emulsions or gels. The physical state of clarity was represented on a pseudo-ternary phase diagram with one axis representing water, one representing oil and the third representing the Smix. The influence of weight ratio of surfactant to co-surfactant on the area of o/w nanoemulsion region was investigated on the pseudo-ternary phase diagram using online free version of (Figure 1) prism ternary diagram software.

Preparation of Nanoemulsion

The NE was prepared by high energy emulsification technique^{7, 8, 9} using a bath sonicator (Citizen pvt. Ltd., Chennai). The composition of NE is given in table.1.

Primary emulsion was formed by agitating soybean oil and S_{mix} (3:2) ratio thoroughly in a magnetic stirrer (Remi equipment Pvt Ltd, Mumbai) followed by water in drop wise manner with a help of a insulin syringe. The emulsion was maintained under

magnetic stirring at 2000rpm for 45min in order to get a stable preparation. It was then placed in a bath sonicator (Citizen Pvt ltd, Chennai) to size reduce the globules. Sonication was done for 8min which is considered as 1pass (or cycle). Based on number of sonication cycle different formulations such as NE1 to NE5 were prepared. NE1=1pass, NE2-2 passes, NE3-3passes, NE4-4passes, NE5-4 passes

Addition of antigen in nanoemulsion

2ml (84 $\mu\text{g}/\text{ml}$) of pertusis toxin as a model antigen was incorporated into the prepared stable nanoemulsion (5%- PTA_g-NE5) in the presence of phosphate buffer saline to maintain the pH in order to find its suitability. The composition of this formulation PTA_g-NE5 is given in table 2.

CHARACTERIZATION

Transparency, pH, viscosity and conductivity determination¹⁰

The transparency of the NE formulation was tested visually for clarity against a dark background. The apparent pH was measured using a calibrated pH meter (Sartorius, Germany) at 25°C. The pH was determined by dipping a glass electrode in to the formulation. Capillary viscometer (Sartorius, Germany) was used to assess the NE viscosity. For this 10 ml of nanoemulsion was poured into the filling tube and transferred into the capillary tube by gentle suction. The time was recorded in seconds for the flow of liquid from the upper to lower mark in the capillary tube. It was generally expressed in cp. Electrical conductivity was measured using a conductivity meter (Borosil ltd, Mumbai) having a cell constant of 0.11 cm^{-1} at the frequency of 94Hz and the measurements were carried out at 25 $\pm$ 1°C. The surface tension was determined using a stalagmometer (Borosil ltd, Mumbai) by drop count method. In this method, the formulation was sucked into the drop pipette up to a mark. Keeping the pipette vertically, the number of drops formed when the liquid level falls was noted. The relative surface tension of the samples were calculated using the formula Surface tension = $\frac{dl \cdot nw}{dw \cdot nl}$, dl = Density of formulation, nw= Viscosity of water, dw = Density of water, nl = Viscosity of formulation.

Droplet size and zeta potential

Size and zeta potential of NE was measured using a Malvern zeta sizer (Malvern Ver. 20, Germany). 1ml of the formulation was taken in a glass cuvette and placed in the instrument for particle size analysis and zeta potential determination.

The electrophoretic mobility was obtained by Malvern zetsizer instrument after diluting suitable quantity of sample with 5ml of filtered water followed by injection in to electrophoretic cell. The zeta potential value was calculated using the instrument software with the help of smoluchosky equation. The magnitude of the zeta potential values gives an indication about the stability of NE formulation.

Transmission Electron Microscopy analysis

The morphology of the prepared vaccine of (PTA_g-NE5) was studied using Transmission Electron Microscopy (TEM) (TECNAI 10 (Philips), Japan) operated at 70-80kv at 54700 x magnification. To perform the TEM observations, the formulation was very well diluted with distilled water. A drop of the diluted sample was directly deposited on a carbon-coated grid, allowed to dry and observed. In addition, droplet sizes

were measured using TEM by point-to-point resolution technique.

STABILITY STUDIES

Stability studies are highly required for emulsion kind of formulation, as because the changes in environmental stress conditions particularly temperature may influence the solubility of NE components that renders the formulation become unstable or less stable. The stability findings are helpful in determining the shelf storage of formulation. In order to hasten the process of stability testing, few of the methods have been reported in the literature. The methods of centrifugation, accelerated stability testing have been adopted to assess the long term stability of pharmaceutical formulations⁷.

Centrifugation

This method has been adopted by scientist to assess the short term stability of formulations in a laboratory scale. The optimized NE of NE5 was subjected to short term stability testing by centrifugation (REMI Sales & Engineering Ltd, New Delhi) at 10000rpm for 30min at 27°C. The stability was assessed in terms of phase separation in NE after centrifugation.

Accelerated stability testing

The long term stability of pharmaceutical formulations are usually tested by accelerated testing which involves acceleration of various stress conditions such as temperature & humidity according to ICH guidelines. Here, the samples were kept at accelerated temperature conditions of 25±2, 40±2 and 5±2 maintained in a stability testing chamber (Wadegati lab equipment's Pvt. Ltd., India). The samples were analyzed for droplet size, pH and electrical conductivity after a storage period of 24h, 3days and 7days.

STATISTICAL ANALYSIS

The experimental values represent the average of six determinations. The calculated standard deviation values were subjected to ANOVA to find the statistical difference among the formulations. The significance value of $P < 0.05$ was kept for all the determinations.

RESULTS AND DISCUSSION

The FTIR spectra for soya bean oil, tween 80, NE5 and PTA-NE5 are given in Figure 2. In the case of soyabean oil, a triglyceride that are an ester derivative ensures two strong absorption peaks at 1745cm⁻¹ and 1163cm⁻¹ due to C=O and C-O-C stretching and 2925cm⁻¹ due to CH stretching vibration. Tween 80 absorption bands appears at 2922cm⁻¹, 1105cm⁻¹ and 1735cm⁻¹ are related to -CH₂, C-O-C and C=O respectively. The PTA-NE5 spectra shows peaks at 3440cm⁻¹ owing to OH stretching vibration and peak at 1636cm⁻¹ confirms the C=O stretching vibration. In case of FTIR spectra of NE5 & PTA-NE5, no additional peaks were observed which indicates the compatibility of antigen with the ingredients of NE5 formulation. The FTIR of NE5 clearly displayed the interaction between tween80 & soybean oil that have resulted in the formation of NE. The absorption peaks for C=O, C-O-C stretching vibrations present in NE5 shows the internalization of soyabean oil by tween80 & the merging of polar and non polar regions with each other.

Among the various nanoemulsions (NE1 to NE5) prepared, formulation NE5 was appeared clear and transparent & the

photograph is shown in Figure 3. The pertussis mixed NE i.e PTA-NE5 formulation is given in Table 2. Properties such as zeta size, pH, viscosity, conductivity & surface tension determined for the prepared nanoemulsions are presented in Table 3.

In the formulation of NE, co-surfactant plays a significant role in reducing the size of internal oil globules (o/w emulsion). This size reduction might be due to the rapid diffusion of co-surfactant¹¹. Another criteria is the right choice of surfactant & co-surfactant which determines the HLB value of the system, so that a stable NE is formed. Hence HLB value >10 has been chosen in the formation of o/w type NE. The HLB of tween 80 is 16.7 & that of ethanol is 4.2. Hence the HLB of Smix obtained was >10. Further, the co-surfactant ethanol has got good miscibility with the oil phase. Next, the concentration of surfactant was optimized to 10% w/v since, too low & too much surfactant influences NE stability. The ratio of Smix was found to be important in obtaining transparent NE. Here a ratio of 3:2 was fixed from the experiment. Further the oil & Smix ratio was optimized for different ratios ranging from 1:9 to 9:1. The 1:5 ratio of oil & Smix has led to the formation of stable transparent NE, whereas the 1:6 & 1:3 ratios of Smix had resulted in less stable NE formulation.

The smallest droplet size 84.45nm with a PDI value of 0.197 was obtained for the formulation NE5. This small size might have arrived due to the high energy emulsification preparation technique parameters particularly the sonication time. The time of sonication is important as the effectiveness of emulsifying agents increases with increasing the mixing time, however prolongation of time above certain period may decrease their effectiveness by dropping out from the liquid interface¹². A small size of NE is very much prerequisite for molecule delivery across skin as the oil droplets tend to provide a channel for antigen delivery¹³. A slight increase in size was obtained for the NE incorporated with antigen i.e (PTAg-NE5) which was determined as 99.42nm (Figure 4f). The size & its distribution chart obtained for the NE are given in Figure 4. The size of the nanoemulsions had ranged from 84.45nm to 231.4nm with narrow distribution as dictated from the value of polydispersity index (PDI) that was <0.2.

Emulsifiers, here Smix not only act as an interfacial barrier between oil & water but also through formation of surface charges called zeta potential, which can produce repulsive electrical forces among approaching oil droplets hinders coalescence¹⁴. Hence, the value of zeta potential is an indicator of NE physical stability. However, values greater than -10mV shows high degree of their stability. It could be said that the formulation NE5 was stable as found from its zeta potential value of -5.23mV (Figure 5) that controls the droplets interaction within a NE. The charges might have been arisen due to the adsorption of traces of ions from the aqueous phase & ethanol. The effect of tweens on charge may be less as because these are non-ionic type of emulsifying agents.

The estimated pH values were ranged from 5.92 to 6.83, a slightly acidic nature made the NE favorable for topical administration as it's a non skin irritating pH. Moreover this pH range matches with the reports of Hepatitis B antigen delivery through nasal mucosa using stabilized nanoemulsion². The viscosity values elicited by all the NE preparations were found to be less than one. This indicates the dilute nanoemulsion which could allow for thorough mixing of antigen with the NE before administration. Again the viscosity of oil, composition of NE and preparation technique may perhaps influences the NE viscosity. Further, the dilute nature of NE would help in its

conversion into topical gel state for application over skin. The reduced value of surface tension was found to be constant for all the NE formulations. This might be due to the action of added Smix in the developed nanoemulsion. The results are in accordance with the earlier reports of NE for antigen delivery and confirms the suitability of the presently developed formulation for the topical/transdermal delivery of incorporated antigen. A low value of electrical conductivity shown might be the reason for ions present in the dispersion medium and also the integrity of other components such as soybean oil after NE preparation. Generally conductivity measurements are made to identify the occurrence of any phase inversion in emulsion after subjecting to various process conditions.

TEM image of PTA_g-NE5 (Figure 6) showed the incorporation of pertussis antigen in NE5. The nanoemulsion PTA_g-NE5 appears dark and the surroundings are bright. A positive image was observed under TEM. Some droplet sizes were measured during TEM analysis, as it is capable of point-to-point resolution. The measured droplet size was in agreement with the results obtained from droplet size analysis using Zetasizer. The photograph specifies the nano globules and its morphology clearly.

The formulation NE5 was found to be physically stable up to a study period 7days. The accelerated stability results are tabulated in table.3. There was no phase separation took place after centrifugal stability testing. Centrifugation was adopted to find out the short term stability of NE before going for a long term stability analysis. This also helps in the selection of more stable preparation for further development and characterization.

In case of long term stability analysis, the samples were monitored for any significant change with respect to their pH value as because pH changes point out the incidence of any

chemical reactions that may disturb the quality of the preparation. Mostly emulsions formed with vegetable oils may encompass a decrease in pH due to the hydrolysis of fatty acid esters into free fatty acid degradation products¹⁵. Here the pH values of NE5 remained around 6.0 and there was no much difference noted at all temperature conditions. This is an acceptable, non-skin irritating pH value suitable for vaccine incorporation. The small proportion of oil and its protection as internal phase in the NE might have sheltered its degradation during storage.

The electrical conductivity of NE5 remained unchanged at all storage conditions indicates that there was no phase inversion taken place. Conductivity rest on the concentration of ions in the media and o/w emulsion displays conductivity than w/o emulsion.

The droplet size and zeta potential values showed no significant difference at the end of 7days reflects the physically stable NE. The PDI value of the stored samples were found to be <0.2. This reveals that the emulsion with a fairly uniform size distribution is more stable. However a slight increase in size of the internal oil globules might have resulted from the disturbances in electrical double layer by aqueous ions & the double layer formation by non-ionic emulsifying agents may not be strong enough when compared to the double layers made up of ionic emulsifying agents¹².

However the formulation stored at 5±2 was much more stable with respect to size analysis compared with other storage conditions. The zeta potential value did not get altered significantly (P<0.05) when stored at different storage conditions indicates the formation of stable NE.

Table 1: Formulation composition of nano emulsion (NE)

Ingredients	%
Soybean oil	2
S _{mix}	10
Water	88

Table.2: Formulation composition of nano emulsion containing antigen (PTAg-NE5)

Ingredients	Quantity for 20ml
Antigen (ml)	2
Soybean Oil (ml)	0.4
Tween 80 (ml)	1.6
Ethanol (ml)	0.4
Water (ml)	14.6
Phosphate Buffer Saline (ml)	Quantity sufficient

Table 3: Properties of nanoemulsion formulations NE1, NE2, NE3, NE4 AND NE5

Property	NE1	NE2	NE3	NE4	NE5
Droplet size (nm)	231.4±3.2	211.8±2.8	117.1±2.5	94.12±3.0	84.45±2.1
pH	6.51±0.02	6.21±0.01	6.83±0.09	5.92±0.16	6.50±0.05
Viscosity (Poise)	0.829±0.042	0.865±0.031	0.873±0.06	0.861±0.04	0.852±0.056
Conductivity (ohm)	0.09±0.005	0.10±0.006	0.20±0.004	0.11±0.004	0.15±0.002
Surface Tension (dyne/cm)	23.43±1.5	23.97±2.0	25.01±1.7	24.08±0.09	23.01±1.02

Values are mean ± S.D, n=6.

Table 4: Accelerated stability determination of NE5 after storage at various temperature and at various time periods

Sl.no	Storage condition/ property	25±2	40±2	5±2
1	Day 1			
	pH	6.51±0.04	6.72±0.08	6.45±0.01
2	Day 3			
	Conductivity (ohm)	0.11±0.008	0.20±0.006	0.15±0.004
3	Day 7			
	pH	6.50±0.05	6.70±0.06	6.42±0.02
	Conductivity (ohm)	0.10±0.005	0.19±0.004	0.16±0.006
	pH	6.21±0.06	6.65±0.05	6.40±0.02
	Conductivity (ohm)	0.11±0.003	0.23±0.007	0.10±0.004
	Droplet size (nm)	121.3±2.30	143.3±1.94	112.7±2.06
	Zeta potential (mV)	-8.46±0.02	-9.59±0.04	-8.01±0.03

Values are mean ± S.D, n=6

Figure 1: Ternary diagram showing the region of nanoemulsion formation

Figure 2: Fourier Transform Infrared spectra of (a) Soybean oil, (b) Tween 80, (c) NE5, (d) PTAg-NE5

Figure 4: Size of nano emulsion formulations (a) NE1, (b) NE2, (c) NE3, (d) NE4, (e) NE5, (f) PTAg-NE5

CONCLUSION

An o/w type nanoemulsion containing nano sized droplets was prepared successfully by high energy emulsification technique and are found to be physically stable at the conditions tested. The NE was loaded effectively with the pertussis toxin and thus suitable for efficient topical immunization.

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