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### PREPARATION OF PLGA NANOPARTICLES FOR ENCAPSULATING HYDROPHILIC DRUG: MODIFICATIONS OF STANDARD METHODS AND IT'S *IN VITRO* BIOLOGICAL EVALUATION

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#### ABSTRACT

The effective encapsulation of the drugs that are highly soluble in both aqueous and organic solvent are intricate to attain using standard nanoparticle preparation methods, such as nanoprecipitation (NPC) and double emulsion solvent evaporation method (DESE) due to the rapid partitioning of drug to the external aqueous phase. Modifications of standard methods are required to enhance the encapsulation efficiency. The present work focused on enhancing the encapsulation of highly aqueous and solvent soluble model drug rivastigmine tartrate. The prepared NP was evaluated for its physicochemical properties. The change of aqueous phase pH from 6-9 in NPC method showed encapsulation efficiency from 15 to 35% with the size of 125±12nm and potential ranged from -31±2 to -40±3mv. In DESE method the use of DCM: EA (50:50) as an organic phase resulted in 1 fold increase of encapsulation with the size of 175±15nm. When EA used as an organic phase it results in 2.5 folds increase in encapsulation efficiency. The NP prepared using Pluronic F-127 showed the zeta value (-13±2mv) while DMAB showed the zeta value (+ 40±1mv) with smaller size (105±10nm). *In vitro* release studies for NPC and DESE method for 24hr was 45.4 ±2.7 and 51.2±3.2 %. The cytotoxicity study using SH-SY-5Y cell line evidenced no toxicity of prepared NP.

#### KEYWORDS

Hydrophilic drug, Nanoparticle, Encapsulation, Stabilizer, Nanoprecipitation and Emulsification.

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#### INTRODUCTION

Developing strategies for the delivery of hydrophilic drugs and macro molecules such as proteins and peptides is emerging as an important in research field as several new synthesized molecules are hydrophilic in nature<sup>1</sup>. Some of the major problem incurred by the small hydrophilic molecules is low encapsulation, low permeability across barriers, shorter half-life in the circulatory systems, toxicity and poor distribution to the target site<sup>2</sup>. And in order

to overcome these critical problems nanocarriers have been used to encapsulate the small hydrophilic drugs. Encapsulation of drugs in the nano carrier system provides various advantages like (i) enhancing the encapsulation efficiency (ii) protection of a drug against *in vivo* degradation, (iii) the reduction of potentially toxic side effects, and (iv) the achievement of better drug pharmacokinetics<sup>3</sup>.

Some available various nanocarriers are liposome's<sup>4</sup>, magnetic nanoparticles<sup>5</sup>, solid lipid nanoparticles<sup>6</sup> and polymeric nanoparticles. Among these nanocarriers polymeric nanoparticles have emerged as a potential carrier system. Nanoparticles are solid drug carriers of natural, semi synthetic or synthetic polymer systems in nanometre range<sup>7</sup>. The commonly used standard techniques for preparation of nanoparticles are nanoprecipitation<sup>8</sup>. Emulsification solvent evaporation (ESE)<sup>9</sup> and double emulsion solvent evaporation (DESE),<sup>10-11</sup> But, the major problem encountered by these techniques is low encapsulation values, caused due to rapid partitioning of the drug to the external aqueous phase.

An attempt was made to improve the encapsulation efficiency using rivastigmine tartrate (RT) a small hydrophilic molecule with poor penetration across blood brain barrier used for treating Alzheimer's disease., Further the particle size of <200 nm is required to cross the blood brain barrier. Owing to the solubility of RT in both organic and aqueous phase preparation of nanoparticles by standard preparation techniques still a challenging task.

The main objective of the present work was to modify the standard preparation technique to improve encapsulation efficiency in poly (lactic –co-glycolic acid) nanoparticles (PLGA) Two different nanoparticle preparation methods nanoprecipitation (NPC) and double emulsion solvent evaporation (DESE) are attempted .PLGA was selected as the polymeric carrier as it is biodegradable and biocompatible. In addition to encapsulation efficiency, the nanoparticles were evaluated in terms of their size, zeta potential and drug release profile. A particle size of <200 is desirable to facilitate an effective permeation across blood brain barrier, and

a sustained release drug profile is favourable for reducing the dosing frequency. Further the cytotoxicity of the nanoparticles was examined in SH-SY-5Y human neuroblastoma cell lines.

## EXPERIMENTAL

### MATERIALS

PLGA resomer 502 H was purchased from Sigma-Aldrich, Rivastigmine tartrate was received as a gift sample from Alembic Pharmaceutical (Vododra Gujarat), Pluronic F-127, didodecyl dimethyl ammonium bromide (DMAB) were purchased from sigma (U.S.A) Potassium dihydrogen phosphate, acetone, dichloromethane, ethyl acetate sodium hydroxide, D-mannitol, acetonitrile were of analytical grade.

### Nanoparticle preparations by standard methods

#### RT loaded PLGA nanoparticles by nanoprecipitation (NPC)

PLGA and RT are dissolved in acetone to form the organic phase. The organic phase was added slowly to 10 ml of aqueous phase containing 1% (v/w) of Pluronic F-127 following which the organic solvent was allowed to evaporate for 4 hours with continuous stirring (50 rpm) on magnetic stirrer (Remi). The NP suspension was then centrifuged at 13,000 rpm for 1hr at 4° C using high speed centrifuge (Eppendorf) and the sediment is comprising NPs was freeze dried for 24 hours (Lyodel, India) using 2% D-mannitol as a cryoprotectant.

#### RT-loaded PLGA nanoparticles by double-emulsification solvent- evaporation (DESE)

Briefly, RT (10mg) was dissolved in 1ml water and added to 6ml of Dichloromethane containing PLGA and the solution was emulsified under high shear homogenizer (10000 rpm) to form primary w/o nano emulsion which was subsequently transferred into the aqueous phase containing (1%v/w) Pluronic F-127. The mixture was emulsified under high shear homogenizer at 24000 rpm to form w/o/w nano emulsion. The nano emulsion was stirred overnight at room temperature in order to evaporate the organic solvent. The resulting nanoparticles suspension was separated by high speed

centrifugation (13000 for 1 hour) and the sediment was dried (Lyodel, India) using 2% D-mannitol as a cryoprotectant.

### **Preparation of fluorescent loaded PLGA nanoparticles**

To prepare fluorescent loaded PLGA nanoparticles, rhodamine-B (Sigma-Aldrich) was dissolved in water and emulsified with EA containing PLGA. The primary emulsion was added to stabilizer containing solution and homogenised to form secondary emulsion. The secondary emulsion was stirred over night at room temperature for the removal of organic solvent. The resulting suspension was centrifuged (13000 for 45 minutes) and the sediment containing nanoparticles was freeze-dried and it reconstituted with distilled water used for cellular uptake study.

### **Nanoparticle characterization**

#### **Particle size and Zeta potential**

Particle size distribution, zeta potential and polydispersity index (PDI) of the formulated nanoparticles were determined by dynamic light scattering (DLS) analysis using the Zetasizer Nano ZS90 (Malvern Instruments Ltd, UK), the samples were placed in disposable cuvettes for particle size measurements and zeta dip cell was used to find the potential. Each experiment was conducted in triplicate<sup>12</sup>.

#### **Morphological studies**

The surface morphology of the prepared NPs was determined by using high resolution transmission electron microscopy (HRTEM) and atomic force microscopy (AFM). A drop of nanosuspension was placed on a carbon film coated copper grid for TEM and the studies were performed at 80kv using JOEL JEM 2100, Japan equipped with a selected area electron diffraction pattern (SAED)<sup>13</sup>. AFM images were captured using (Multimode Scanning probe microscope (NTMDT, NTEGRA prima, Russia)<sup>14</sup>.

#### **Drug entrapment**

Freeze-dried nanoparticles 50mg were dissolved in acetonitrile and the drug concentration was measured by ultraviolet spectroscopy at 220nm<sup>15</sup>.

Drug entrapment efficiency was estimated using the following equation:-

Drug entrapment (%) =  $\frac{\text{Mass of drug in nanoparticles}}{\text{Mass of drug used in formulation}} \times 100$

#### **In vitro drug release studies**

*In vitro* release studies were performed using dialysis sac method<sup>16</sup>. The freeze dried nanoparticles drug equivalent to (2mg) were suspended in phosphate buffer pH 7.4 and sealed in a dialysis membrane (molecular weight cut off 10,000 - 12,000Da) membrane clips. The sealed dialysis membrane was then placed in a beaker containing 50ml of 0.1 M phosphate buffer (pH 7.4), and maintained at 37 °C with continuous magnetic stirring. At specified time intervals of 1, 2, 4, 8, 12 and 24 hours 2ml of aliquots were withdrawn from the medium and replaced with equal volume of phosphate buffer. The concentration of RT was assayed spectrophotometrically at 220 nm.

#### **In vitro cytotoxicity Assays**

Human SH-SY5Y cells were obtained from National Centre for Cell Science (NCCS), Pune. Cells were cultured in MEM supplemented with non-essential amino acid, ham F-12, 10% fetal bovine serum, 2 mmol/L L-glutamine, penicillin (100 U/mL) and streptomycin (100 µg/mL), and maintained at 37 °C and 5% CO<sub>2</sub> in a humid environment. The medium was replaced twice each week. Cellular viability was assessed using MTT assay. SH-SY5Y cells were seeded at a density of  $1 \times 10^4$  cells per well in 96-well plates containing 10% for 24 h. Gradual reduction of serum (5%) was done in consecutive day and finally cells were maintained serum free for 16 hrs. The wells were divided in triplicate as normal control group, placebo and R-sol and RNP at a dose of 10 µg and 100 µg. Formulations were incubated with cells for 24 hrs. MTT at a concentration of 5 mg/ml was prepared and 20 µl of MTT solution was added to each well and incubated for 4 hrs. Followed by MTT incubation, medium containing MTT was discarded and 50 µl of DMSO was added to each well to dissolve formazan crystals. Optical density was measured at 570 nm. Percentage viability was measured against control.

## RESULTS AND DISCUSSION

### Physical characterizations of NP prepared by standard Methods.

RT loaded PLGA nanoparticles were prepared by standard preparation techniques (NPC and DESE) and characterized for particle size, encapsulation efficiency and zeta potential it showed in Table No.1. The drug: polymer ratio 1:1 was used and the particle size of the nanoparticles prepared by standard nanoprecipitation techniques was considerably small ( $125\pm 12\text{nm}$ ) due to the presence of acetone it diffused out quickly in to aqueous phase to forms droplets which leads to formation of smaller particle compared to that of the nanoparticles prepared by DESE method ( $192\pm 8\text{nm}$ ).

The particle size distribution of the nanoparticles produced by NPC and DESE presented in Figure No.1 and Figure No.1A shows uniform distribution with a narrow size range. The zeta potential of nanoparticle obtained by NPC method is  $-31\pm 2\text{mv}$  which indicates a good colloidal stability whereas the zeta potential of nanoparticles obtained by DESE method showed  $-13\text{mv}$  indicating incipient instability of the nanoparticles. Apart from obtaining small sized nanoparticles by NPC it also resulted in lower encapsulation efficiency. Due to high aqueous solubility of RT, the encapsulation efficiency of RT in nanoparticles prepared by standards methods is low it caused by escaping of drug in to aqueous phase together with the water miscible acetone and later precipitation of PLGA, RT might get adsorbed over the surface of nanoparticles consequently during the centrifugation process, RT washed off easily resulting in low drug encapsulation in the polymer. The NPC method is mostly employed to encapsulate hydrophobic drugs where the low drug solubility in the aqueous phase leads to high encapsulation. In case of hydrophilic drugs, like RT encapsulated using standard NPC method results in low encapsulation.

The use of water immiscible solvent DCM in DESE method, it prevents RT adsorbed on the surface it resulted in slight increase in encapsulation efficiency as mentioned in Table No.1. The DESE method is commonly used to encapsulate hydrophilic drugs,

but not solvent soluble drugs. This method has advantage that the drug dissolved in the inner aqueous phase acts as a reservoir, whereas organic phase with polymer serves as a dissolution barrier preventing the drug leakage from the inner aqueous phase. As RT is highly soluble in both aqueous and organic phase, standard NPC and DESE methods have been incapable of producing high % encapsulation efficiency. So a modified approach by NPC and DESE was attempted to enhance the encapsulation efficiency of hydrophilic drugs.

### RT loaded PLGA nanoparticles prepared by modified NPC and DESE method

A novel approach for preparing RT loaded PLGA nanoparticles was attempted by making the following modifications 1) Using alternative aqueous phase consisting of buffer for NPC method 2) Using mixture of solvent DCM and EA for DESE method. Firstly by using phosphate buffer pH ranging from 6-9 as inner aqueous phase, the encapsulation efficiency increased which ranges from 15 to 35%. The affinity of RT towards the organic phase increased with an increase with the pH from 6-9, showing strong argumentation in pH 9. This increased drug entrapment can be related to the existence of RT in ionic state in the aqueous phases leading to a decrease in the solubility of drug in aqueous phase and an increase in the drugs affinity toward the organic phase. The zeta potential ranges from  $-31\pm 2$  to  $-40\pm 3\text{mv}$  due to the change in pH of the aqueous phase. The particle size remains unchanged.

Secondly, the modification was performed in the organic phase by replacing 100% DCM in standard DESE with 50% (v/v) mixture of DCM and EA and 100% EA. In this case when mixture of water immiscible solvent (DCM) and partially water miscible solvent (EA) was used the particle size slightly decreased. However solubility of DCM in the water is low, but vapour pressure is high, so DCM rapidly diffused in to water and evaporated leading to the fast precipitation of polymer without partitioning of drug to the aqueous phase resulting in increased encapsulation efficiency from 20 to 30% Recent studies reported that DCM is more toxic than

EA (According to class III and II according to the ICH specifications)<sup>17</sup>. When 100% EA a partially water miscible solvent was used it diffused freely through the aqueous phase creating phase transformations and the aggregation of polymer takes place in the region of each emulsion droplets to forms several NP. The encapsulation efficiency of RT in NPs formulated using EA showed the encapsulation of 50% which was 1.5 folds higher when compared with 50% (v/v) DCM/EA. Probably due to the partial miscibility of EA in water enabled a slight mutual solubility of the organic and aqueous phase and the temporary phase was created in which the polymer and RT were dissolved in EA. The particle size, zeta and encapsulation efficiency were showed in Table No.2.

The effect of stabilizer in DESE method was also studied. The Pluronic F-127 and DMAB were used to study the effect of zeta potential. DMAB is a double tail cationic surfactant and has much lower critical micelle concentration than Pluronic f-127 leading to formation aggregates at low concentration and better solubilisation of organic solvent when compared to Pluronic F-127. DMAB is more effective in lowering the interfacial tension resulting in smaller particle size of  $105 \pm 10\text{nm}$  when compared to nanoparticles obtained by DESE with Pluronic F-127  $175 \pm 15\text{nm}$ . Further it also produced a higher positive zeta value of  $+40 \pm 1\text{mV}$  in comparison with Pluronic F-127  $-13 \pm 3\text{mV}$  shown in Table No.3. Recent literature suggests that cationic charged nanoparticles enhance the brain endothelial uptake due to the presence of anionic charged luminal surface<sup>18</sup>. Therefore DMAB has dual role (particle size reduction and positive zeta value) in formulation of nanoparticles which will enhance the drug targeting efficiency to brain.

#### ***In vitro* drug release**

*In vitro* release profiles of RT from nanoparticles prepared by NPC and DESE are shown in Figure No.2. The drug release pattern from nanoparticles was biphasic with an immediate release was  $17 \pm 1.2$  and  $21 \pm 2.8$  % within 60 min. This was followed by the slower release of the remaining drug. However only  $45.4 \pm 2.7$  and  $51.2 \pm 3.2$  % of drug was release

after 24 hrs it may be due to the high molecular of the polymer which leads to slower drug release. The rapid initial release of drug from nanoparticles is due to the adsorbed drug on the surface of the nanoparticle and which is due to the water soluble nature of RT. Upon addition of nanosuspension to the dissolution medium RT partitioned rapidly to the dissolution medium leading to the burst effect. The sustained release of RT may be attributed to the slower diffusion of dissolved drug within the PLGA core of the nanoparticle in to the dissolution medium.

To summarize, for NPC method modifications of aqueous phase pH from 6-9 showed a slight increase in the drug encapsulation efficiency up to 35% which is still low, and produced the negative zeta potential. The change in organic phase from DCM (100%) to DCM: EA (50:50) in DESE method showed one fold increase in drug encapsulation efficiency and slight decrease in particle size. When organic phase was replaced with partially miscible EA, it resulted in increased encapsulation up to 50% which was 2.5 fold higher when compared to standard method. Further usage of cationic stabilizer DMAB produced positive zeta value which is required to penetrate blood brain barrier through ionic interaction with the luminal membrane and it remains the particles size of  $>200$  nm. The nanoparticles prepared by DESE with EA and DMAB were superior 1) higher drug encapsulation than NPC method 2) low particle size. The prepared NP was subjected to selected area electron diffraction pattern to find out the physical state of drug in NP. Figure No.3 indicated that the physical state of encapsulated drug in the nanoparticle was in amorphous or molecular dispersed state. Figure No.4 shows the morphology of NP prepared by DESE method examined using TEM indicates both spherical and sub spherical shape. Figure No.5 and 5A showed the 2D and 3D image of prepared NP by AFM with spherical shape without aggregation. Hence it was selected as optimal formulation for further *in vitro* cytotoxicity assay and cellular uptake.

### In vitro cytotoxicity assay

The cytotoxicity of R-sol and R-NP was evaluated by MTT assay using SH-SY-5Y human neuroblastoma cell lines which is commonly used to test the neurotoxicity of the compounds. The cells were incubated with drug solution and rivastigmine nanoparticles at the concentration of 10 and 100µg/ml for 12 and 24 hr. The results of cell viability (%) are shown in Figure No.6 and 6A the data represents that the nanoparticles are not toxic on the cells even at higher concentration at 12 and 24 hrs. The percentage cell viability was above 90% in all assays.

### Cellular uptake of PLGA nanoparticles

To evaluate the internalisation, rhodamine loaded PLGA nanoparticles were incubated in SH-SY-5Y human neuroblastoma cells and analyzed using

fluorescence microscopy. Figure No.7A and 7B showed the uptake of Rho-sol at 1<sup>st</sup> and 4<sup>th</sup> hr. At 4<sup>th</sup> hr it showed very less internalisation of dye. Figure No.7C and 7D indicated the internalisation of Rho-NP at 1<sup>st</sup> and 4<sup>th</sup> hr. After prolonged incubation of Rho-NP up to 4hr the internalisation was seen throughout the entire cytoplasm which moved near to the nucleus forming a highly fluorescence region. The internalisation of rhodamine loaded PLGA nanoparticles was higher over a period of time for the encapsulated rhodamine when compared to the free rhodamine solution. The higher internalisation of nanoparticles was due to the smaller size and presence of DMAB which produces a positive charge which can enhance the cellular uptake due to anionic nature of luminal layer.

**Table No.1: Physicochemical characterization of RT loaded PLGA nanoparticles by standard methods**

| S.No | Method | Particle Size (nm) | Zeta Potential (mv) | Drug Encapsulation (%) |
|------|--------|--------------------|---------------------|------------------------|
| 1    | NPC    | 125±12             | -31±2               | 15                     |
| 2    | DESE   | 192±8              | -13±3               | 20                     |

**Table No.2: Physicochemical characterization of RT loaded PLGA nanoparticles prepared by modified DESE method**

| S.No | Modified Phase | DESE Modification | Size (nm) | Zeta Potential (mv) | Drug Encapsulation (%) |
|------|----------------|-------------------|-----------|---------------------|------------------------|
| 1    | Organic Phase  | 50% (v/v) DCM: EA | 175±15    | -13±2               | 30                     |
|      |                | 100% EA           | 150±10    | -19±1               | 50                     |

**Table No.3: Effect of stabilizer on particle size and zeta potential of RT loaded PLGA nanoparticles**

| S.No | Modified Phase | DESE Modification | Size (nm)    | Zeta potential (mv) | Drug Encapsulation (%) |
|------|----------------|-------------------|--------------|---------------------|------------------------|
| 1    | Stabilizer     | Pluronic          | F127, 175±15 | 13±230              | Pluronic               |
| 2    | ---            | DMAB +            | 105±10+      | + 40±1              | 50                     |

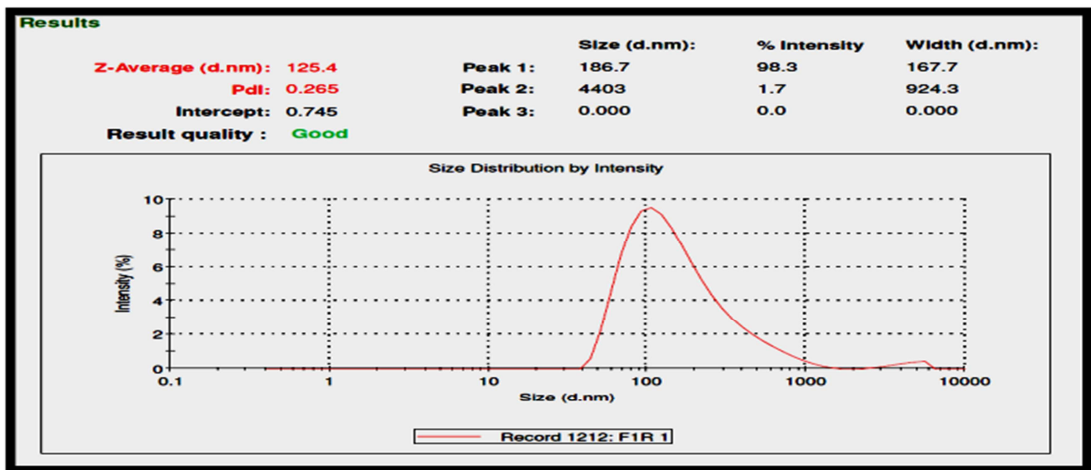


Figure No.1: Particle size distribution of RT loaded PLGA nanoparticles prepared by NPC method

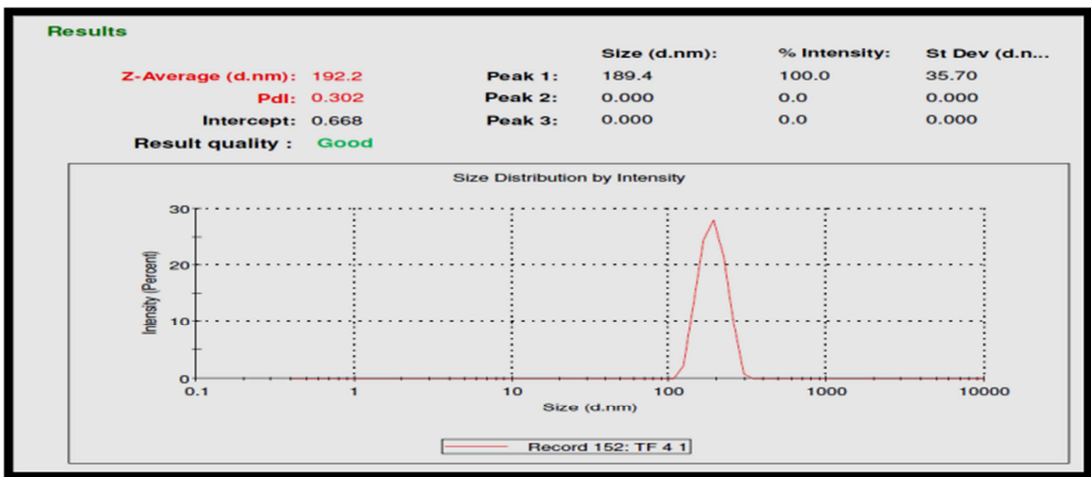


Figure No.1A: Particle size distribution of RT loaded PLGA nanoparticles prepared by DESE method

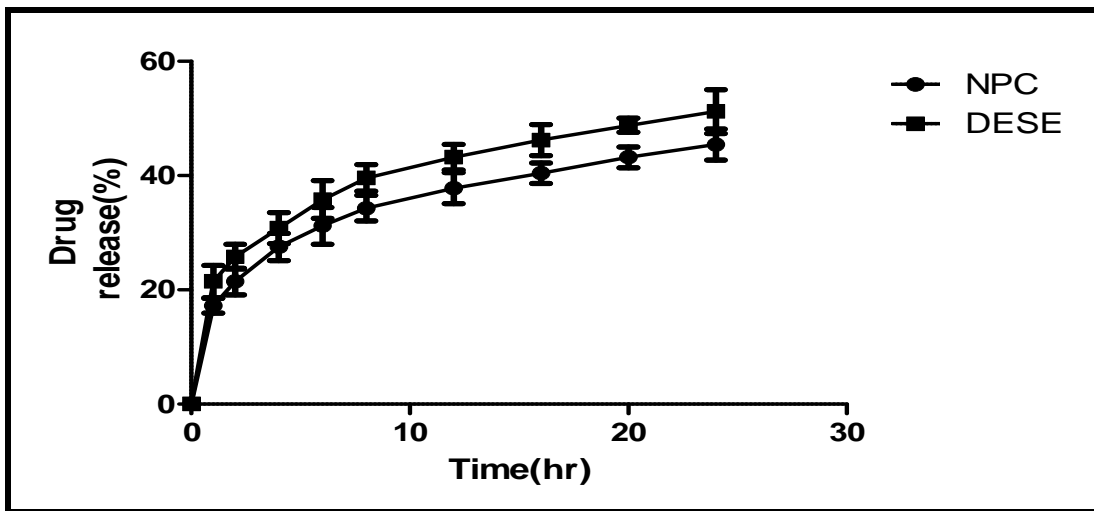


Figure No.2: *In vitro* drug releases from RT loaded PLGA nanoparticles prepared by modified methods

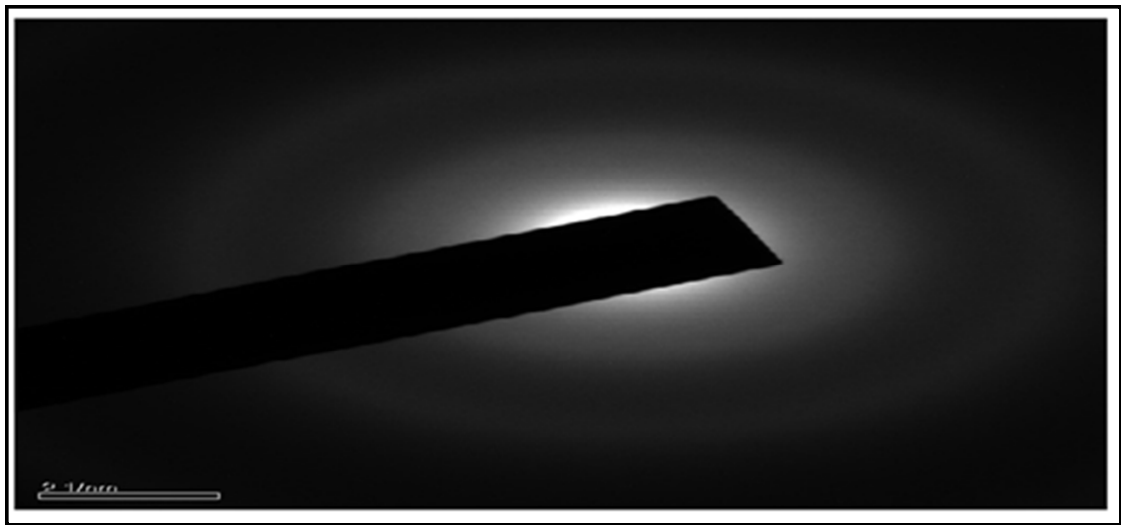


Figure No.3: Selected area diffraction pattern of RT loaded PLGA nanoparticle prepared by DESE Method

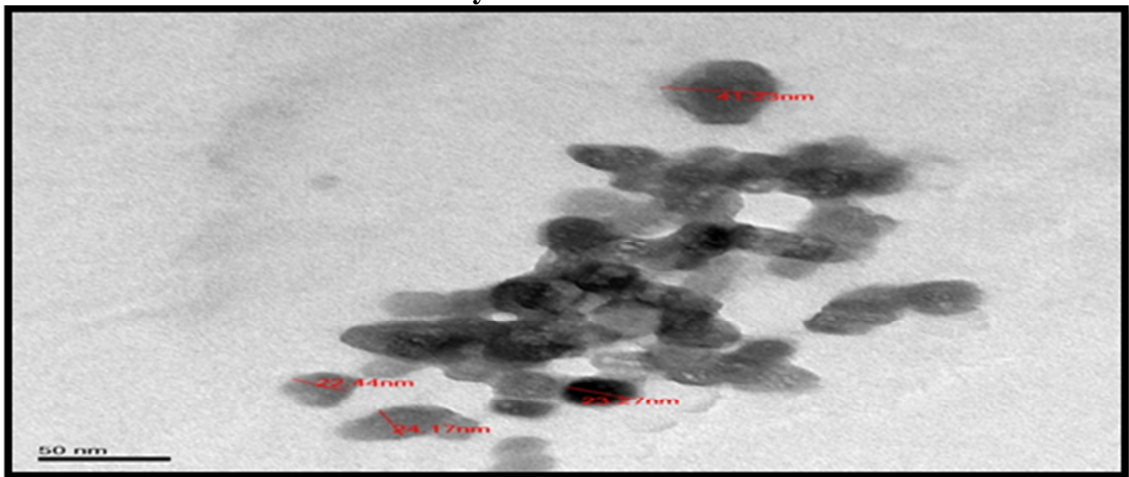
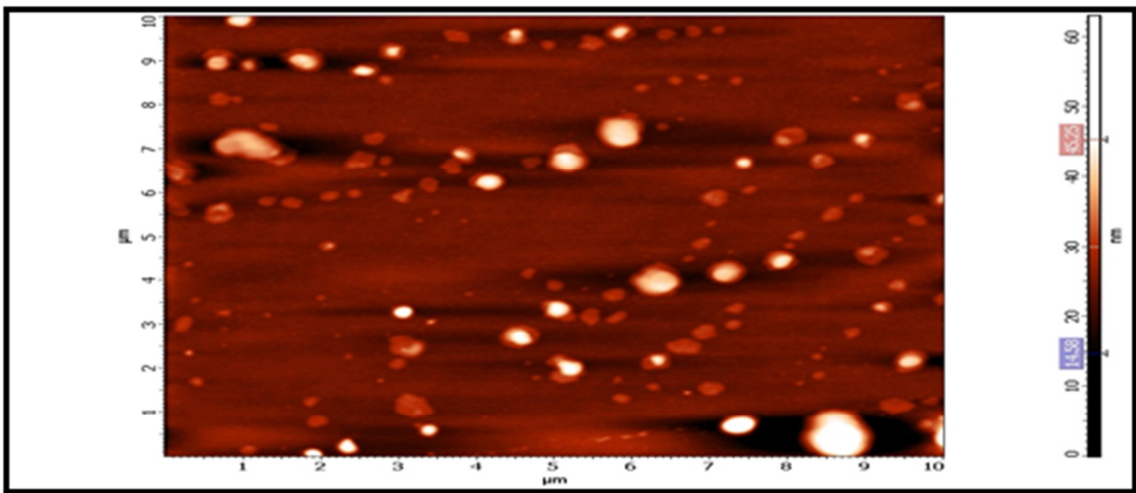


Figure No.4: TEM image of RT loaded PLGA nanoparticles prepared by DESE Method





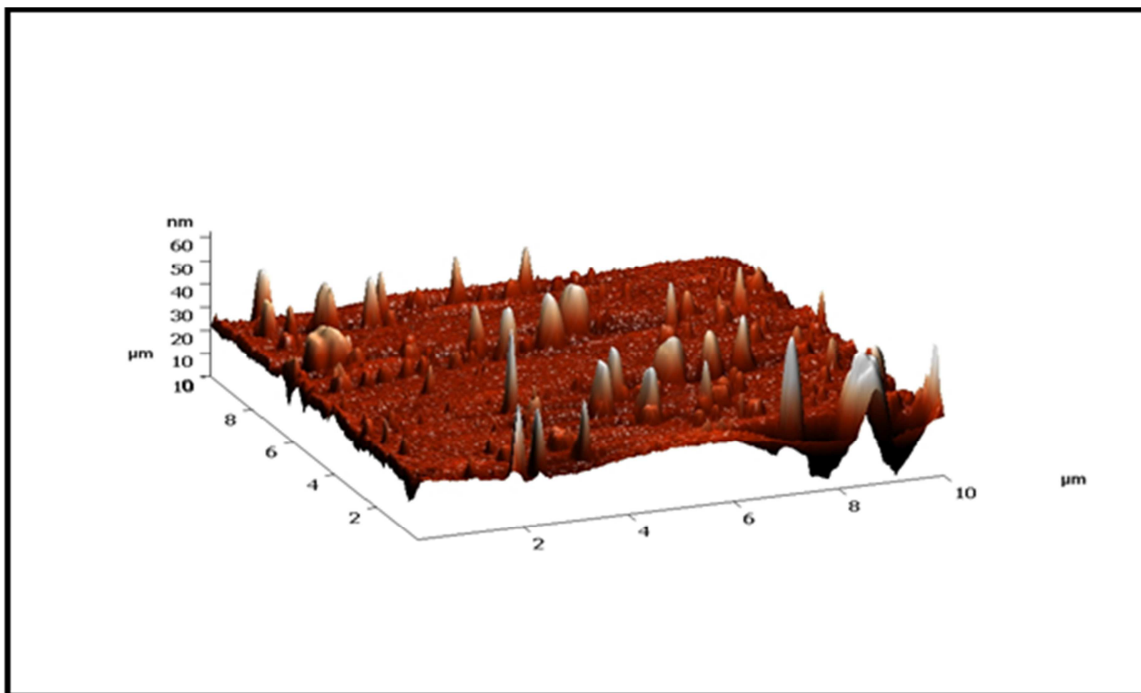
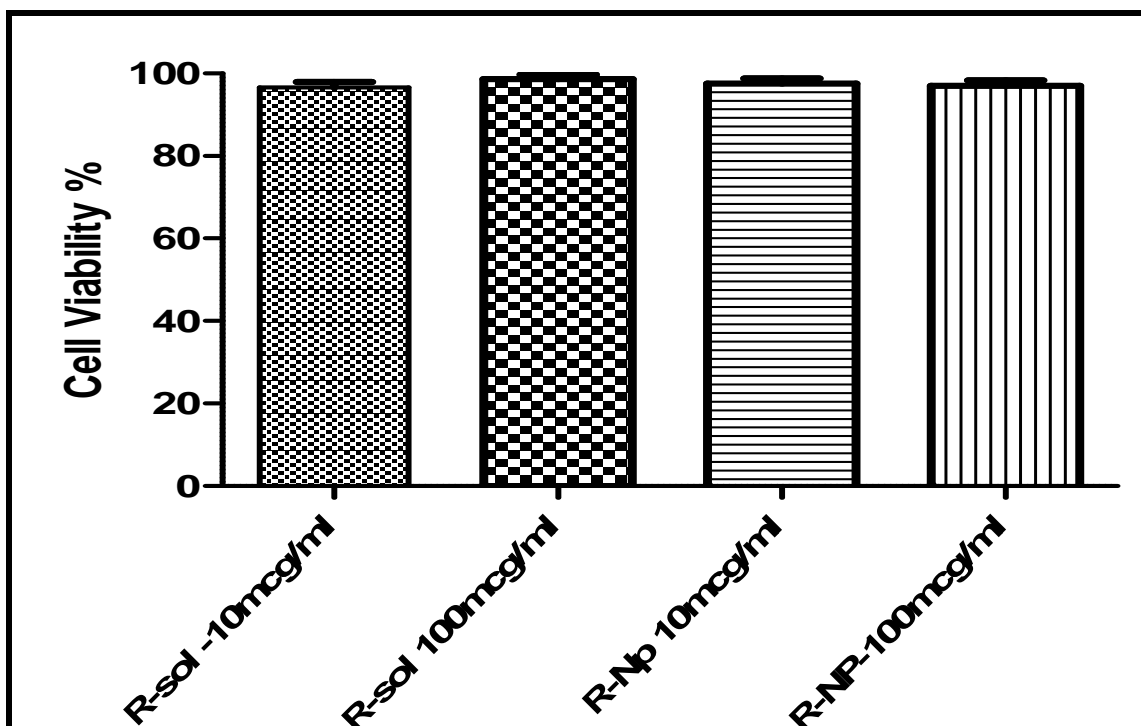


Figure No.5 (A and B): AFM image of RT loaded PLGA Nanoparticles (2D) A) 3D image of RT loaded PLGA nanoparticles



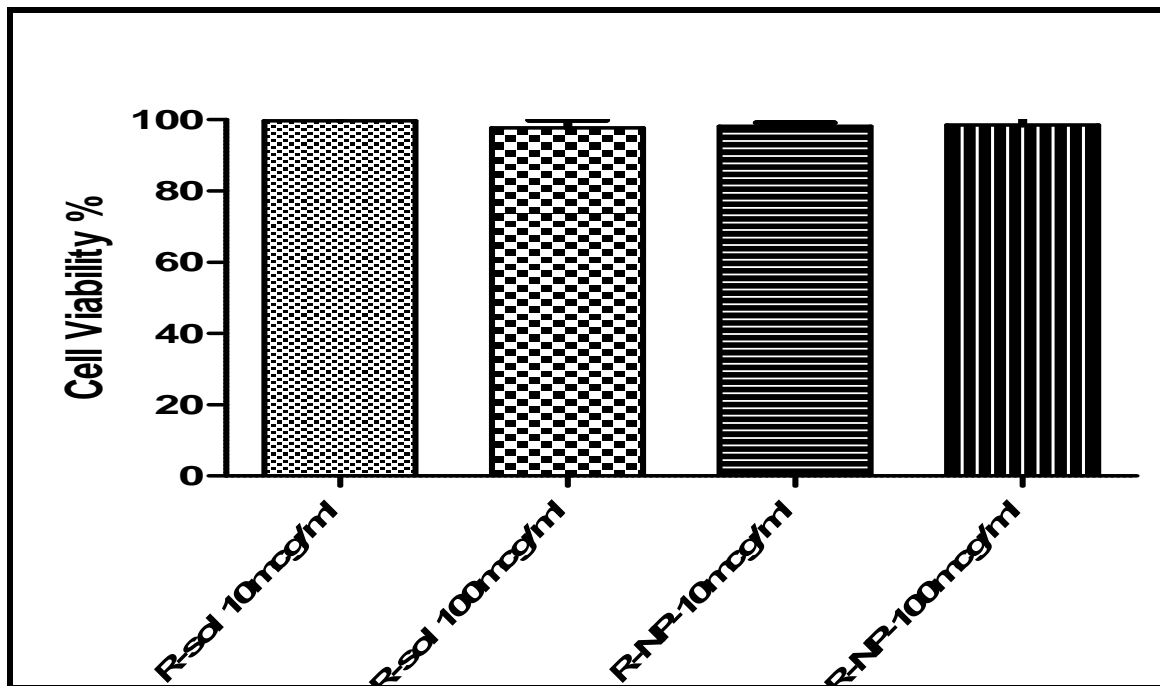


Figure No.6: Cell viability of SH-SY-5Y cells incubated for 12 hr A) 24 hr with free drug solution and rivastigmine nanoparticles

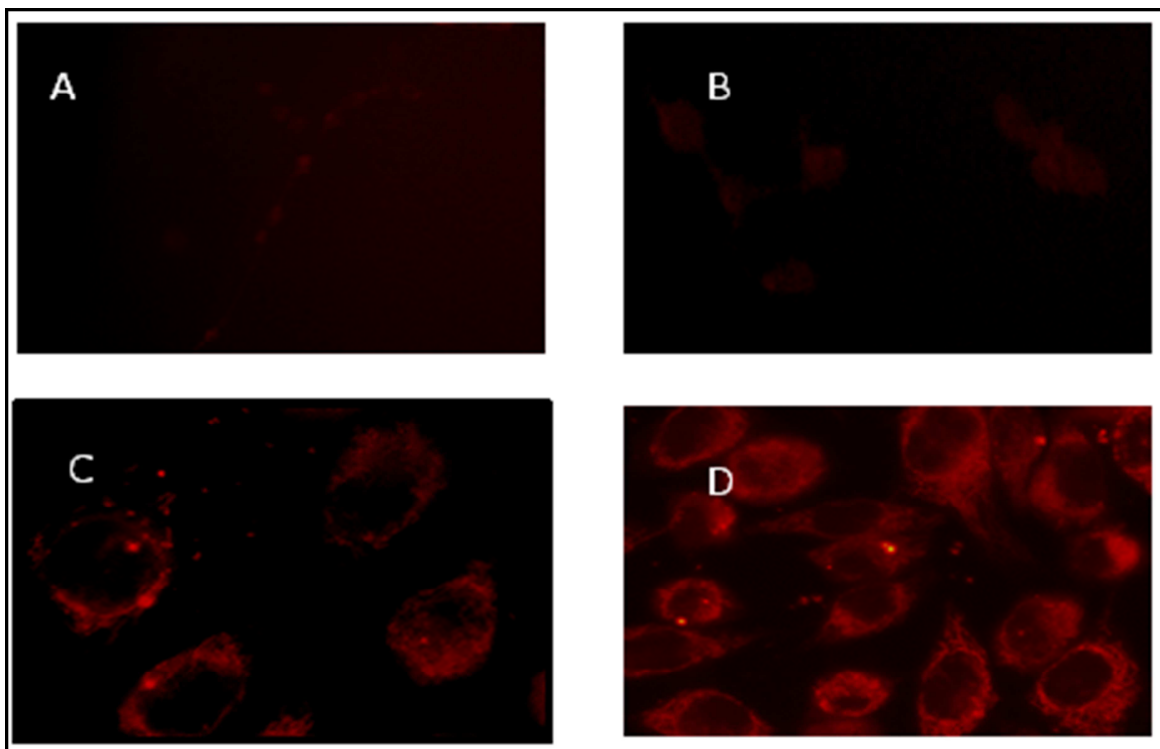


Figure No.7: Fluorescence microscopy images using SH-SY-5Y cells .Time course of binding and internalisation of Rho-sol (A, B) at 1<sup>st</sup>hr and 4<sup>th</sup> hr, Rho-NP (C, D) at 1<sup>st</sup> hr and 4<sup>th</sup> hr

## CONCLUSION

The present investigation suggests that organic solvents play a significant role in nanoparticle formation. Physical properties of the organic solvent effect the particle size formation and encapsulation. The particle size has directly correlated with the *in vivo* circulation time. Smaller particles have prolonged circulation time. The modified NPC and DESE showed enhanced encapsulation efficiency when compared to the standard methods i) the aqueous phase modified NPC method showed 2.5 folds increase with negative zeta value. ii) In DESE method using alternative solvent results in 1.5 and 2.5 fold increase in encapsulation. The optimal formulation was evaluated in terms of particle size and encapsulation, has been identified as DESE method containing EA as an organic phase and DMAB as a stabilizer. Cytotoxicity study indicates absence of toxicity with the formulations. Cellular uptake of NP was good in SH-SY-5Y cell lines. Further *in vivo* studies will conclude the potential of prepared NP for oral administration to target brain.

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## CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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