



## In-vitro diffusion studies and anticancer activity of cytarabine loaded nanocochleates against MCF-7 cell lines

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### ABSTRACT

Cochleates are a relatively new type of crystalline particles having a large and continuous lipid bilayer sheets rolled up in a spiral structure of less internal aqueous phases. Cytarabine is used in the treatment of the leukemia. It can bind with the cross-linking agent like calcium chloride to form the coiled structure. The In-vitro diffusion studies was performed by altering the various concentrations of cross-linking agent, drug, cholesterol: the formulation has its effect by change in the release pattern and the entrapment efficiency. In this study the concentration of the crosslinking agent is related to the release pattern.

The Nanocochleates is used to increase bioavailability, to achieve the maximum therapeutic efficacy, to reduce side effects and adverse effects. Nanocochleates was prepared by ionic crosslinking method. The *in-vitro* release was studied at different pH conditions 7.4 and acidic pH 5.3. The formulation shows maximum entrapment efficiency of  $86.22 \pm 3.41\%$  and good stability. Prolonged drug release was observed in pH 5.3 when compared to pH 7.4. Particle size was found in the range of 0.94 to 1.8  $\mu\text{m}$  and the zeta potential was  $-7.3 \pm 3.3\text{mV}$ . Moreover, the stability studies of the prepared formulation show excellent stability. The *in-vitro* MTT assay was performed using the MCF-7 breast cancer cell lines. CYT- NCs were more cytotoxic than CY solution at 24hrs. These results suggest that CY-NCs can be explored for treatment of leukemia owing to their ability of providing sustained drug release, stability and improved cytotoxicity in MCF-7 cell line.

**Keywords:** Cytarabine, Nanocochleates, In-vitro cell line- MCF-7, Cross-linkers.

### INTRODUCTION

Cochleates exhibit efficient incorporation of hydrophobic or even hydrophilic drugs into the lipid layers or internal phases, and they were reported to have a potential for slow release for drugs. Better clinical efficacy activity is attributed mainly to the sustained release properties of cochleates. This enhanced activity may also be

attributed to the membrane fusion capability of cochleates and an increased contact by increasing the number of particles due to their size and the high tension at the bilayer edges of cochleates. This effect can also create a kind of driving force for the molecule to penetrate efficiently [1, 2, 3]. The interaction with the tissue or cell membrane can be also important; the contact time between cochleates

and cell membrane can be also increased because of structure or composition similarities. Penetration into the tumor or enhancing activity at the site of action may also be high because of Enhanced Permeability and Retention (EPR) effect. The effect was found to be high with cochleates than liposomes, and this was attributed to the shape difference [4, 5, 6].

Many factors were found in nanocochleates formulation to represent a better activity including slow drug release, longer release time, penetration enhancing effect of cochleates, similarities of cochleate composition with cell membranes, alteration of distributions, eliminations and half-life. To further advancement of the therapeutic utility of cytarabine, the present study investigates the potential of nanocochleates as vehicle for systemic delivery of cytarabine. Such formulation has not been reported previously [7, 8].

When calcium ions bind with phosphatidylcholine head groups, nanocochleate internal water space is replaced, resulting in a restructuring of the lipid bilayer and formation of membrane discs that fuse into lipid-based supramolecular assemblies composed of rolled sheets of lipid bilayers and calcium. When drugs are incorporated into nanocochleates, the unique multilayered cylindrical structures provide protection from drug degradation in hazardous environmental conditions [9, 10].

It is of interest to consider what properties of calcium chloride with a positive charge are important to give some of these systems the unique property of promoting nanocochleate structures. One feature to consider is the charge on the lipid. Because the negative charge on the lipid appears to play a key role in the formation of nanocochleates, but it has also been shown to influence dehydration of the lipid membrane interface and facilitate coiling of the lipid bilayer when bridged by cationic molecules [11, 12, 13].

Nanocochleate formulation technology is particularly applicable to macromolecules as well as small molecule drugs that are hydrophobic/hydrophilic, positively charged, negatively charged, and that possess poor oral bioavailability. Cytarabine is colourless and odourless crystalline powder substance. It has the molecular weight of 243.2g/mol. It is freely soluble in water and DMSO, insoluble in ethanol. It has the LogP value of -2.8. which indicates poor

permeability in membrane, which helps in the preparation of the nanocochleate formulation an ideal one [14]. The release study of the nanocochleate was done by using the buffer solution with neutral pH and the acidic pH, to mimic the biological environment. The drug release is monitored for the 24 hrs. The release rate is proportional directly to the entrapment efficiency. Cytarabine administered orally will turn into biologically inactive metabolite, so mode of administration is better for parenteral. For parenteral administration: Sterile isotonic aqueous or non-aqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use [15].

## MATERIALS AND METHODS

Cytarabine, cholesterol and dialysis bag (molecular weight cut off 12,000 D) were purchased from Sigma Aldrich Chemical Private Ltd, Bangalore, India. Soya Phosphatidyl Choline (SPC) was gifted from Lipoid GmbH Ludwigshafen, Germany. Ethanol, Ethylene Diamine Tetra Acetic Acid (EDTA) and calcium chloride were of analytical grade. Water were of HPLC grade and procured from Merck, Mumbai, India. All other solvents used for study were of analytical grade.

### Preparation

Cytarabine-loaded small unilamellar vesicles were prepared by Ethanol Injection method. Briefly, specific amount of cholesterol and Soya Phosphatidyl Choline (SPC) were dissolved in 2 ml of ethanol and heated up to the phase transition temperature (40°C) of SPC. This ethanolic solution was rapidly injected into 10 ml of the phosphate buffer (pH 7.4) containing cytarabine, which was kept under stirring at 500 rpm using Teflon coated beads and it was continued until complete evaporation of ethanol under vacuum. Further, phosphate buffer was added to adjust the volume of final lipid vesicles suspension to 10 ml. Now the vesicles were passed through a membrane filter of 0.22 µm to obtain a cytarabine-loaded small unilamellar vesicle suspension with uniform size.

With this CYT-SUV, by trapping method 20µl of calcium chloride solution (0.1 M) were added drop-wise to get NCs, which were under vortex.

### Particle size analysis

The particle size was determined by laser diffraction technique by particle size analyser (Malvern 2000 SM; Malvern Instruments, Malvern, UK). To determine the particle size, the samples must be prepared by taking CYT-NCs formulation and it was diluted with the distilled water. 10 $\mu$ l of the sample is taken in a 2ml of the standard volumetric flask and make up the volume. The sample is then placed inside the plastic cuvette for analysis.

### Zeta potential

The Zeta Potential determination was based on particle electrophoretic mobility in aqueous medium. The Zeta Potential characterizes the surface charge and gives the information about long term stability. At higher Zeta Potential, the particle aggregation is less likely due to electric repulsion. Similarly, the zeta potential of the NCs was also found to know its surface potential charge. Zeta potential was determined using Zetasizer HSA 3000 (Malvern Instrument Ltd., UK). Samples are placed in clear disposable zeta cells and results are recorded.

### Entrapment efficiency for CYT entrapped NC<sub>s</sub>

Entrapment efficiency is determined by using the centrifuge apparatus of Hermle (Inkarp), Germany. 1ml of cytarabine NCs were introduced into polypropylene centrifuge tube. The tube was centrifuged at 15000 rpm for 30 min at 4°C and the supernatant and pellets were separated; 50 $\mu$ l of pH 9.5 EDTA was added to the cochleate pellet to allow the opening of the cochleates into vesicles and the release of CYT. The resulting solution was suitably diluted with phosphate buffer (pH 7.4) and vortexed completely. Then the absorbance was determined at 272 nm. The free CYT concentration in the supernatant was also measured and percentage of encapsulation efficiency was calculated using following Equation (1).

**Equation: 1= Encapsulation efficiency (%) = Amount of drug entrapped in the cochleates / Total amount of the drug  $\times$  100**

### In-vitro diffusion study

The *In-vitro* release of CYT was studied at physiological temperature of 37°C and CYT-NCs at

pH of 7.4 (the physiological pH) and pH of 5.3 (the endosomal pH of cancer cells) using dialysis bag diffusion technique. Briefly, formulation equivalent to 10 mg of CYT-NCs was introduced into a dialysis bag (cellulose membrane, molecular weight cut off 120,000Da), hermetically sealed and immersed into 50ml of Na<sub>2</sub>HPO<sub>4</sub>-KH<sub>2</sub>PO<sub>4</sub> buffer solution with pH of 7.4 and 5.3. The entire system was kept at 37 $\pm$ 0.5°C with continuous magnetic stirring at 100 rpm/min. At selected time intervals, sample was removed and replaced with the buffer solution. The amount of CYT in the buffer solution was quantified using UV-VIS absorption spectrophotometer at 272nm.

### Stability testing

The formulated CYT entrapped nanococheleates were studied for the stability using the stability chamber of Thermolab scientific by following the ICH guidelines. The CYT-NCs was separated into three portions and kept well closed in glass container. One portion was kept at room temperature, the second at 37°C, and the third at 4°C for 6 months in a stability chamber. At monthly intervals for the maximum period of six months, these portions were then evaluated for their physical stability, drug content and release characteristics to know the storage stability of the formulation.

### In-vitro anticancer activity

The CYT-NC formulations was dissolved in different concentration (12.5 to 200  $\mu$ g/ml) in 10% Dimethyl Sulfoxide (DMSO) to give a final concentration of DMSO not more than 0.5% and did not affect cell survival. The cell viability test was done using the cells, was assessed by MTT assay (Mosmann, 1983) using MCF-7 cell lines. MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide): 0.5 mg MTT/ml of serum-free DMEM medium was used and the Solubilizing solution was Dimethyl sulfoxide, using the Phosphate buffered saline (PBS) (pH 7.4). The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. Only live cells can take up the tetrazolium salt. The enzyme (succinate dehydrogenase) present in the mitochondria of the live cells can convert internalized tetrazolium salt to formazan crystals, which are purple in colour. Then the cells are lysed

and dissolved in DMSO solution. The colour developed is then determined in an ELISA reader at 570 nm.

### Procedure

The MCF-7 cells were plated separately in 96 well plates at a concentration of  $1 \times 10^5$  cells/well. After 24 h, cells were washed twice with 100  $\mu$ l of serum-free medium and starved for an hour at 37°C. After starvation, cells were treated with different concentrations of test compound (25-500  $\mu$ g/ml) for 24 h. At the end of the treatment period the medium was aspirated and serum free medium containing MTT (0.5 mg/ml) was added and incubated for 4 h at 37°C in a CO<sub>2</sub> incubator. The 50% inhibitory concentration value (IC<sub>50</sub>) of the NCs was identified for normal fibroblast cell line. The MTT containing medium was then discarded and the cells were washed with PBS (200  $\mu$ l). The crystals were then dissolved by adding 100  $\mu$ l of DMSO and this was mixed properly by pipetting up and down. Spectrophotometrically absorbance of the purple blue formazan dye was measured in a microplate reader at 570 nm (Biorad 680). Cytotoxicity was determined using Graph pad prism5 software. The cell growth was measured using the Equation (2).

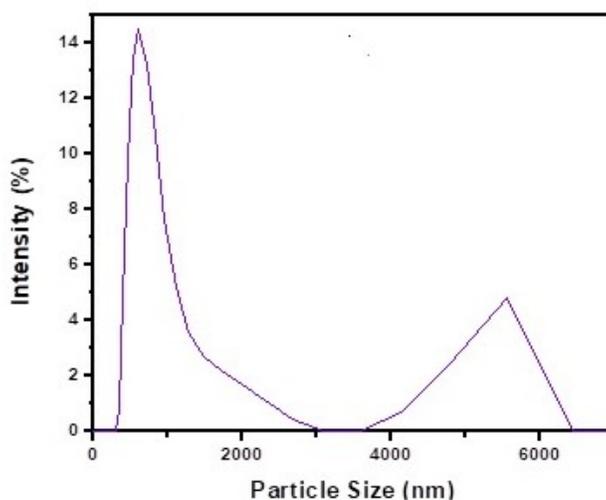
**Equation: 2 = Cell growth (%) = Average absorbance of the test well / Average absorbance of the control wells  $\times$  100**

Using the six absorbance measurements (time zero (Tz), control growth (C) and test growth in the presence of drug at various concentration levels (Ti), the percentage growth was calculated at each of the drug concentration levels. Percentage growth inhibition was calculated as:  $[(Ti - Tz) / (C - Tz)] \times 100$  for concentrations where Ti -Tz or (Ti - Tz) is positive or zero; as  $[(Ti - Tz) / Tz] \times 100$  for concentrations where Ti -Tz or (Ti - Tz) is negative. Growth inhibition of 50 % (GI50) was calculated from equation  $[(Ti - Tz) / (C - Tz)] \times 100 = 50$  as the drug concentration resulting in a 50% reduction in the net protein increase (as measured by MTT staining) in control cells during the drug incubation.

## RESULTS AND DISCUSSION

### Particle size

The average particle size of Cytarabine entrapped NCs was found between 0.94 to 1.8  $\mu$ m. Average diameter (Z-Average) of cochleates was found out to be 1009 nm with peak obtained at 522.6 nm, this difference in the average diameter and peak can be attributed to the elongated shaped structure of cochleate particle. Size distribution report of the formulation also shows maximum particle intensity in the range of 450 -620 nm. The graph is shown below in Figure No.1.



**Fig. No.1 The particle size of the cytarabine entrapped NCs.**

### Zeta potential

Zeta potential for NCs was determined using Zeta sizer HSA 3000 (Malvern Instrument Ltd.,

UK). Charge on droplets and their mean Zeta potential values ( $\pm$ SD) were obtained from the instruments. Zeta Potential of the formulation was

found to be negative charge. The measurement was carried out at 633 nm using a  $173^\circ$  scattering angle at a temperature of  $25^\circ\text{C}$ . Poly dispersity index (PDI) values ranging from 0 to 1 reflects the polydispersity of the suspension with the lower value indicating a more monodispersed

suspension. Before putting the fresh sample, cuvettes are washed with the distilled water and rinsed using the sample to be measured before each experiment. The CYT-NC showed negative zeta potential of  $-7.3 \pm 3.3\text{mv}$  due to the anionic nature of the employed lipids as seen in Figure No.2(b).

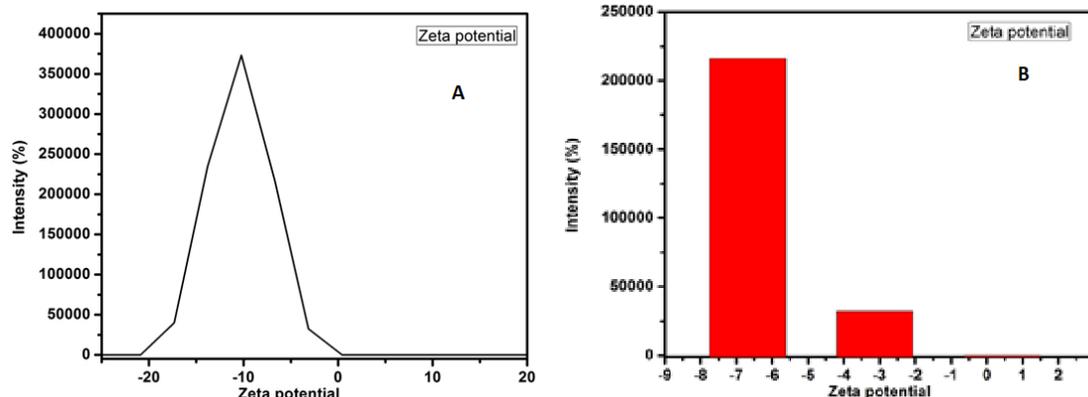


Fig. No.2 The zeta potential of the cytarabine NCs (a) zeta potential graph, (b) Histogram of the NCs.

### Entrapment efficiency (E.E.)

CYT-NCs formulations showed good entrapment efficiency (E.E) of  $86.22 \pm 3.41\%$ . The Cytarabine encapsulation efficiency of calcium induced nanocochleates was evaluated by adding various amounts of CYT at 5, 10 and 15(% w/w). A lipid to drug ratio of 10% gave the highest encapsulation efficiency. The vesicles size increased when CYT concentration increased from 5 to 15mg. However, further increase up to 10mg resulted in low encapsulation efficiency. CYT, a hydrophilic bioactive gets easily solubilised into the aqueous phase of vesicles. The cohesive forces between the vesicles reduced due to insertion of CYT, thus increasing the vesicle size. At still higher concentration of CYT, however, the vesicles size increased but with lower encapsulation efficiency due to exclusion of components from the lipid vesicles as shown in Table No.1. The formulation CYT-NC presented good encapsulation efficiency; hence the maximum amount that can be loaded into the phospholipid vesicles was fixed at 10 mg. Drug concentration is inversely proportional

to the E.E. CYT-loaded vesicles (CYT-SUV) obtained by ethanol injection were converted into nanocochleates using calcium chloride. The addition of calcium ions to SUV induces fusion of lipid membrane and the formation of planar sheets, which eventually coil around an initial point of folding to form rod shape nanocochleates.

Divalent cations  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ba}^{2+}$  and  $\text{Zn}^{2+}$  can be used for preparing nanocochleates. It has been reported that  $\text{Ca}^{2+}$  forms a more tightly packed, highly ordered and less hydrated structure than does  $\text{Mg}^{2+}$  with phospholipids. Also, it is required in much lower concentration than  $\text{Mg}^{2+}$ . Additionally, it is well documented that  $\text{Ca}^{2+}$  plays a vital role in natural membrane fusion phenomena while other cations listed above are ineffective in most such systems. Hence, it is most compatible with the body. Thus, calcium is the most suitable divalent cation for preparing Nanocochleates and hence used in the present work. The increase in particle size due to free drug encapsulation and demonstrated better encapsulation efficiency as compared to SUVs.

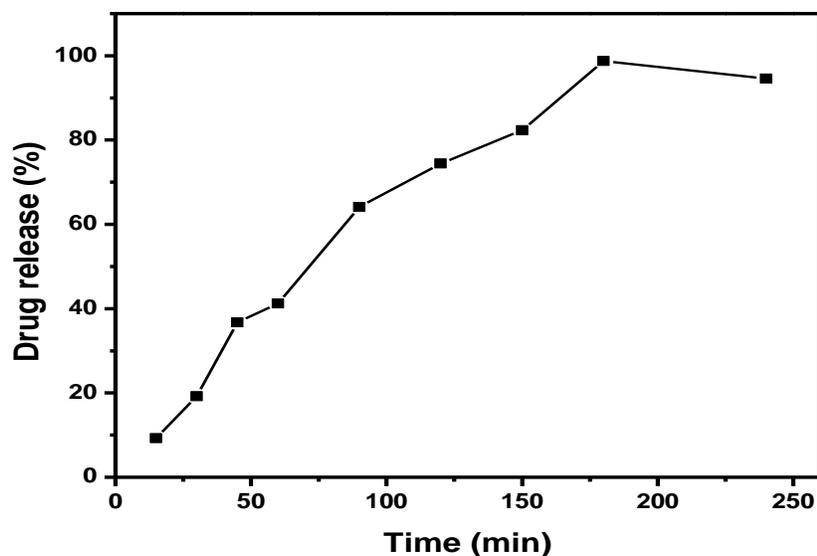
Table No. 1 Entrapment Efficiency and Size of the Nanocochleates

Type of Formulations	Drug Entrapment (%)	Size ( $\mu\text{m}$ )
F1 (5mg)	68.2	0.90
F2 (10mg)	86.6	0.98
F3 (15mg)	80.4	1.2

### ***In-vitro* diffusion study**

The results of release profile revealed that cytarabine solution could freely diffuse in the dispersion form causing 100% drug release within 5hr as shown in the Figure No 3. However, CYT release from nanocochleates showed a biphasic pattern with initial burst release (10%) within the first 1hr followed by controlled release up to 24hr. In this *in-vitro* release study, the initial burst release may be due to the dissolution of excess drug absorbed on the surface of the nanocochleates, while the further controlled release could be caused by diffusion of the drug. The drug encapsulated into the inner core compartment stayed firmly inside the nanocochleates showing a very slow release even at sink conditions with 10% of the initially incorporated drug still being associated with the nanocochleates even after 24 hr.

We found that at pH 7.4, CYT released in a slower and controlled manner from CYT-NC. It is described to the hydrogen-bonding interaction between CYT and nanocochleates, which is more prominent in the neutral condition, resulting drug release in a controlled manner upto 24 hr as shown in Figure No.4(a). On the other hand, the release pattern under acidic media pH 5.3 indicates that the amount of CYT released was much higher than at neutral conditions. It was found that around 88% of the total bound CYT was released from the CYT-NC after 24 hr as shown in Figure No.4(b). Under acidic conditions, the amine ( $-NH_2$ ) group of CYT get protonated resulting in the partial dissociation of hydrogen-bonding interaction, hence the amount of released CYT from nanocochleates is much higher. It is shown in the graph below. For the anti-cancer drug like cytarabine the release should be more for the better activity.



**Fig. No. 3 *In-vitro* release of the plain drug cytarabine**

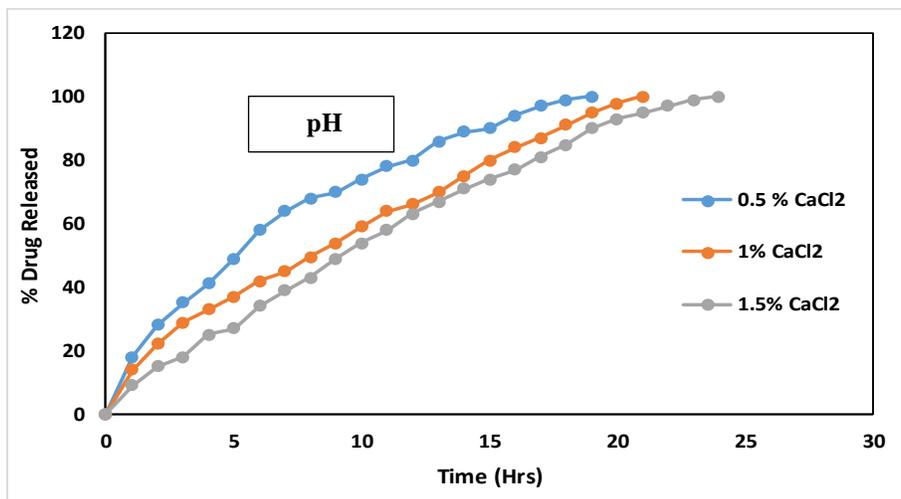


Fig.No.4(a) *In-vitro* release of CYT-NCs from the pH 7.4

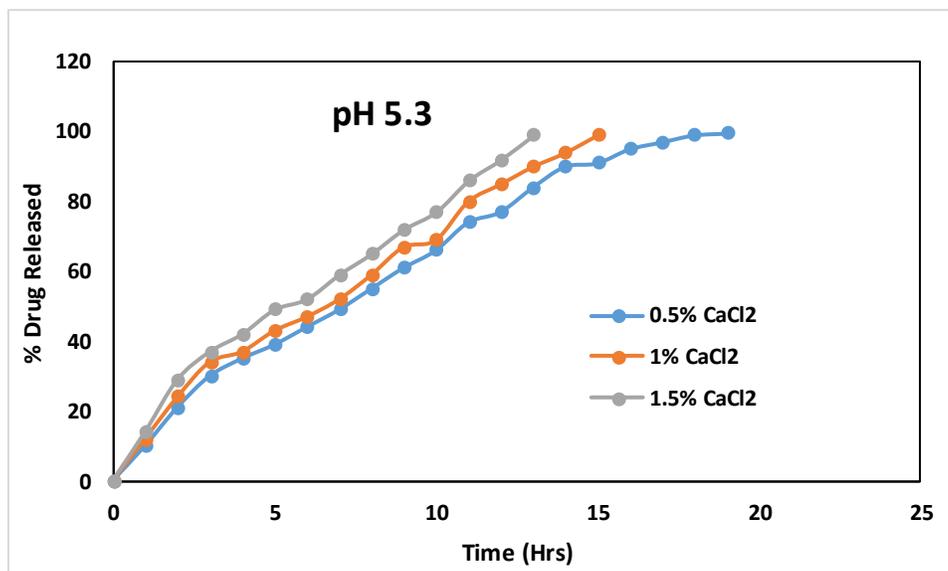


Fig. No. 4(b) *In-vitro* release of CYT-NCs from the pH 5.3

### Stability studies

Short term stability studies were conducted for the optimized formulations as per ICH guidelines. The storage conditions used for stability studies were at  $37^{\circ}\text{C}\pm 2^{\circ}\text{C}/75\%\pm 5\%$  RH and  $25^{\circ}\text{C}\pm 2^{\circ}\text{C}/60\%\pm 5\%$  RH and  $4^{\circ}\text{C}\pm 2^{\circ}\text{C}$ . Sample of CYT-NCs were analyzed after 0 and 6 months for

its physical characters, drug content and release properties were performed followed by *in-vitro* release. The NCs were analyzed and report was shown in Table No. 2. The results revealed that no considerable different in all the parameters were observed.

**Table No. 2 Stability study profile of the NCs**

Form. Code	Conditions	Time (M)	Colour	Assay (%)	In vitro release (%)					
					1hr	2hr	6hr	10hr	18hr	24hr
CYT entrapped Nanocochleates	25±2°C/	0	White	99.45	19	39	64	85	93	95
	60±5% RH	6	White	98.33	18.4	39.0	62.3	85.0	92.7	94.1
	37±2°C/	0	White	99.45	19	39	64	85	93	95
	75±5% RH	6	White	98.21	18.0	37.3	62.1	82.8	91.6	93.9
	4±2°C/	0	White	99.10	22	46	65	85	95	98
		6	White	98.61	21.4	45.3	65.2	86.0	93.7	97.1

### ***In-vitro* anticancer activity**

The *In-vitro* anticancer activity of CYT-loaded nanocochleates was investigated and compared with free drug in dispersion and blank-nanocochleates against human breast cancer MCF-7 cells using *in-vitro* MTT assay. The results illustrated in the below Figure no. 5 indicated that CYT-loaded nanocochleates demonstrated superior anticancer activity than the free drug dispersion and blank nanocochleates. The GI50 of CYT-loaded nanocochleates was lower ( $8.42 \pm 0.43 \mu\text{g/ml}$ ) than that of free CYT dispersion ( $19.32 \pm 1.2 \mu\text{g/ml}$ ) and blank nanocochleates ( $> 95 \mu\text{g/ml}$ ). The CYT-NCs were shown significant cancer cell killing efficiency when compared to cytarabine solution (P value  $< 0.0001$ ).

The *in-vitro* anticancer study demonstrated that the GI50 concentration of CYT-NC is less than free CYT and blank NC. This can be attributed to higher accumulation of the drug via direct interaction or phagocytosis i.e. fusion between nanocochleates and the cancer cell membrane followed by controlled release of the drug. Many

natural membranes fusion processes involve calcium-induced perturbations of membranes containing negatively charged lipids. The Hypothesis is that contact of the calcium-rich, highly ordered membrane of a cochleate with a natural membrane causes a perturbation and reordering of the cell membrane. Subsequently, a fusion occurs between the outer layer of the cochleate and the cell membrane. As a result, a small amount of the encochleated material is transferred into the cytoplasm of the target cell. The nanocochleate could then break free of the cell and be available for another fusion event, either with this or another cell. Alternatively, particularly with active phagocytic cells, the nanocochleate may be taken up by endocytosis and fuse from within the endocytic vesicle. This tumour retention effect is apparently due to the cochleates' extravasation through the porous capillary endothelium of the tumour caused by enhanced permeability and retention effect. Therefore, CYT-loaded nanocochleates might serve as a potential carrier for CYT.

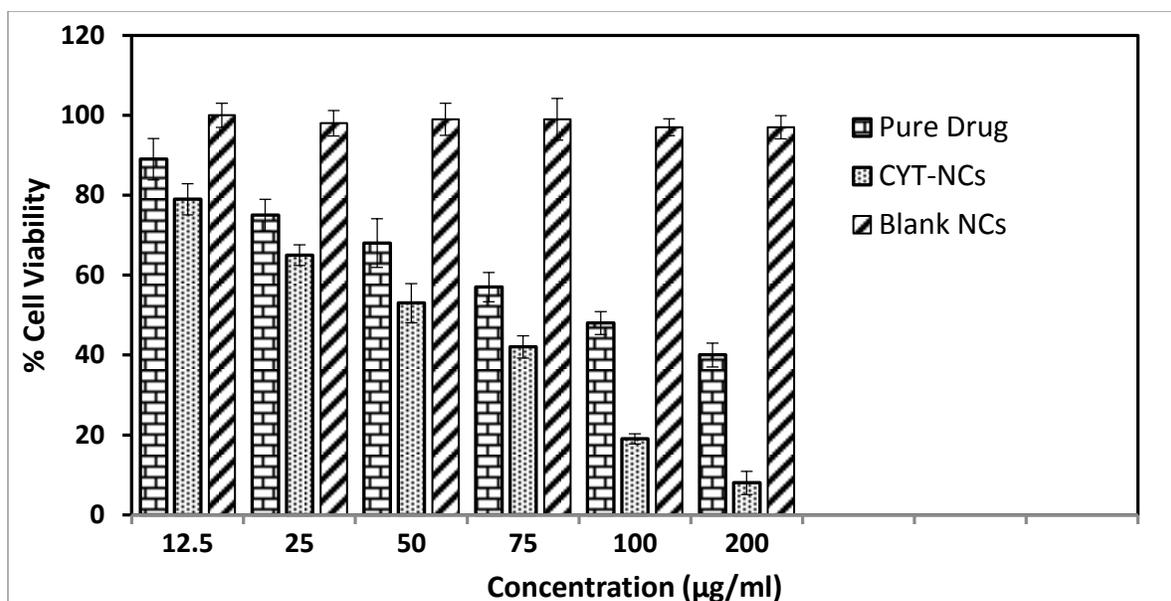


Fig. No. 5 MTT cell lines assay for pure drug, blank and CYT-NCs.

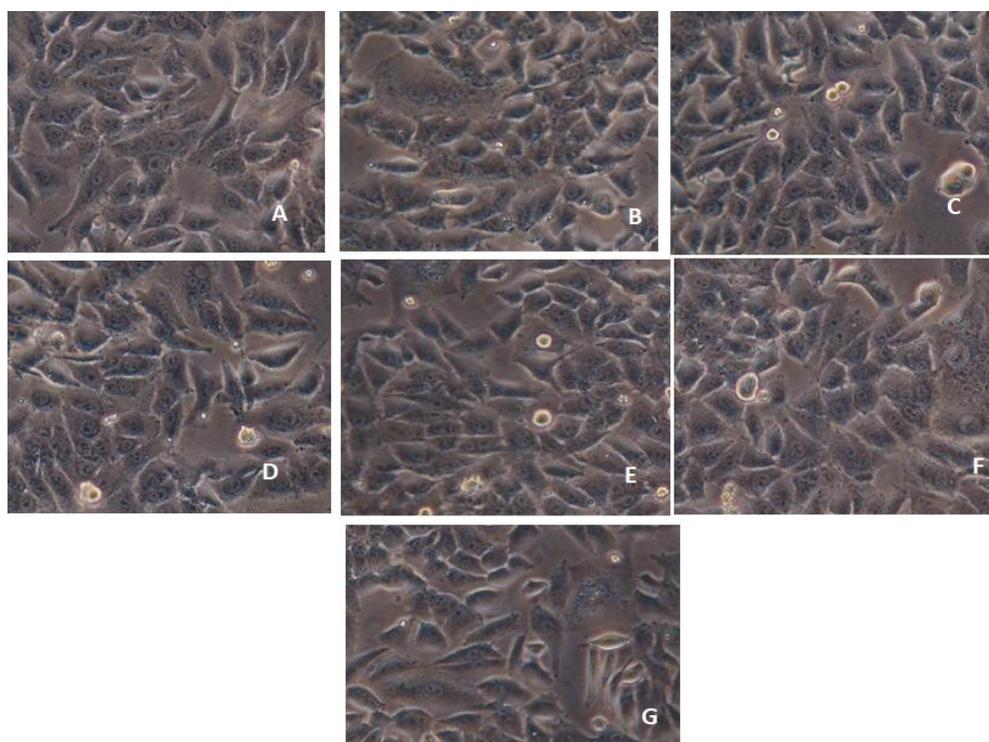


Fig. No. 6 The MCF-7 cell lines of the breast cancer cell lines by using MTT assay, (a) Control cells, (b) 12.5 ug/ml treated cells, (c) 25 ug/ml treated cells, (d) 50 ug/ml treated cells, (e) 75 ug/ml treated cells, (f) 100 ug/ml treated cells, (g) 200 ug/ml treated cells.

## CONCLUSION

The cytarabine nanococheate was prepared by altering the concentration of the calcium chloride. The particle is uniform in size and shape. The

surface charge of the particles from the zeta potential was found to be  $-7.3 \pm 3.3$ Mv. The *in-vitro* diffusion study showed the better release of drug takes place in the pH of 5.3 of nearly 86% of the

drug is released in to the site. The entrapment efficiency of the NCs was found 80%. *In vitro* drug release pattern showed initial fast release (15.99 ± 1.2 % in 1 h) followed by sustained release up to 24 h. stability report of the NCs revealed that no

change in the assay, physical appearance and drug release. From the above results it can be concluded that the developed NCs might have potential to target cancer cells.

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