# *Summary & Conclusion*

# SUMMARY AND CONCLUSION

## 8.1 Summary

Etoposide and Cytarabine are two of the most commonly used drugs in chemotherapy of leukaemia. Both these drugs are usually required to be administered intravenously and are available as multidose vials. The conventional parenteral therapy with these drugs is painful to the patients even with as little as effective concentration and cause severe side effects. These side effects are mainly caused by the lack of specificity of these anticancer drugs, that is, the anticancer drugs not only kill cancer cells but also inhibit normal cell growth and eventually lead to necrosis of normal cells. Moreover, the conventional drugs kill the leukaemia cells in the blood but are not effective in penetrating into the spinal chord or brain. Leukaemia cells flourish into these central nervous systems hideouts, eventually causing fatal complications. Other crucial problem in conventional drug delivery systems is that drugs cannot be released in a sustained manner.

Thus improvement of treatment modalities for leukaemia requires a drug delivery system which is long circulating in blood and can provide sustained release of the drug. The polymeric materials used for the carrier have to be biodegradable and biocompatible. Colloidal drug carriers such as polymeric nanoparticles have recently gained attention for targeting and sustaining the release of the drug. The most widely used polymer for biodegradable nanoparticles has been PLGA, known for its biocompatibility and resorbability through natural pathways. Polyethyleneglycol (PEG) modified biodegradable polymer is one of the most popular materials to prepare stealth nanoparticles which could avoid, or at least reduce the uptake by phagocytes and prolong the time of drug in effective concentration in blood circulation. Pluronic is a triblock copolymer composed of polyethyleneoxide and propyleneoxide (PEO-PPO-PEO). Surface modification of PLGA by Pluronics can make the particles hydrophilic and avoid rapid recognition by MPS and makes them long circulating in the blood. Pluronic block copolymers have good biocompatibility and can be used for sustaining the release of the drug. Nanoparticle preparations by intravenous route are employed for the sustained release of drugs, for the passive targeting of anticancer drugs and increasing the circulation time of labile or rapidly eliminated drugs.

The present investigation was aimed at developing etoposide and cytarabine loaded PLGA based biodegradable nanoparticles which would have sustained release, have steric barrier for longer blood circulation time, provide distribution of the drug to brain and bones and increase the half life of the drugs. The nanoparticulate formulation would reduce the side effects and dosing frequency associated with conventional treatment thereby improving the therapy of leukaemia.

**Etoposide loaded PLGA Nanoparticles**

Etoposide loaded PLGA nanoparticles were prepared by oil-in-water single-emulsion solvent evaporation method using high pressure homogenization. $3^2$ factorial design was used to optimize homogenization pressure (10000psi) and number of homogenization (4). The solvent ratio of internal to external phase of the oil in water emulsion was optimized to be 1:5 based on formulation of PLGA nanoparticles with least mean particle size (MPS). Etoposide loaded PLGA NP were prepared by a $3^2$ factorial design to study the effect of two independent variables, ratio of drug and polymer (X1), surfactant concentration (X2) on the two responses, percentage entrapment efficiency (Y1) and mean particle size (Y2) of the PLGA Nanoparticles. The % EE of ETO in PLGA 50:50 NP varied from 43.64±5.51% to 79.05±4.39%. The results showed that % EE was greatly dependent on the drug to polymer ratio. Increase in drug to polymer ratio from 1:4 to 1:10 increased the %EE. The concentration of the surfactant did not have significant effect on the EE as the P value obtained was more than 0.05 in all the X2 terms. It was concluded from the contour and surface plots that the % EE of 80% could be achieved with X1 range from -1.2 to -0.5 level and X2 range at -0.6 level to 1.2 level. The mean particle size of NP ranged from 105±5.4 to 182±5.5 nm. It was concluded from the contour and the response surface curves that the MPS of 109 nm could be obtained with X1 range from -1 level (1:10) to 0.0 level (1:6) and X2 range from 0.2 (1.25%) to 1.0 (1.5).

The zeta potential values ranged between -23.0 to -34.2mV. The surfactant concentration affected the charge on the particle. It was seen that as the surfactant concentration was increased from 0.5 to 1.5%, there was a decrease in the zeta potential value. The optimized batch of ETO loaded PLGA nanoparticle (ENP5) was found to have Zeta potential of -32.7±1.68 mV, which was good for well-stabilized nanoparticles. A rapid

reversal of surface charge of NP was observed from anionic to cationic with the change in pH of the dispersion medium; such NPs would escape into the cytosol, which would prevent their degradation in the cell. Hence the NP would not degrade in the cells in vivo.

During the process of lyophilization addition of sucrose in a 20% w/w concentration was found to be optimum as a cryoprotectant as the lyophilized nanoparticles had good redispersibility and least increase in the mean particle size after lyophilization.

The DSC thermograms of etoposide, PLGA and etoposide loaded NP depicted endothermic peaks and it was it was seen that in the prepared PLGA NP the peak of pure drug was lost indicating that the drug was present in the amorphous phase and may have been homogeneously dispersed in the PLGA matrix. The X-ray diffraction studies showed that crystal peaks of ETO were absent in etoposide loaded NP confirming that ETO existed in the amorphous state in the polymeric nanoparticles and there was no crystalline drug present on the surface of the NPs. The electron micrographs showed spherical, discrete and homogenous particles in the nanometer size range.

The drug release studies showed that free ETO was released in four hours, whereas, ENP5 nanoparticles showed a sustained release up to 36h and ENP2 showed sustained release up to 72h. The difference in the release of the two NP was attributed to their different sizes, as other wise they were similar in composition. ENP5 (112nm) was of smaller size compared to ENP2 (160nm). Smaller nanoparticles lead to a shorter average diffusion path of the matrix entrapped and lead to faster release of the entrapped drug compared to bigger size NP. The other factor responsible for the different release rate was attributed to the amount of drug loading in each NP. ENP5 had higher drug loading (14%) than ENP2 (9%). An increase in the amount of drug in the nanoparticles not only increases the porosity of the system as the drug dissolves, but also, reduces the relative amount of polymeric material acting as a diffusional barrier and hence NPs with a greater amount of drug content (ENP5) released drug more quickly. The data obtained from the drug release was fitted to different kinetic models to understand the drug release mechanism and kinetics. The release from ENP2 followed Higuchi diffusion. Both ENP5 and ENP2 had high First order $R^2$ value (0.9910 and 0.9904 respectively) indicating release by first order kinetics. The diffusion exponent (n values) were obtained by fitting

the drug release data to Korsmeyer-Peppas model showed that both the nanoparticles had n values less than 0.43 indicating Fickian diffusion kinetics.

Stability studies of polymeric nanoparticles were carried out to evaluate the change in particle size and drug content of the drug over a period of 3 months (3M) at different storage conditions. It was concluded from the stability studies that Etoposide loaded PLGA NP were stable in terms of mean particle size at 2-8°C up to three months in the (freeze dried) FD state, one month in the aqueous dispersion (AD) state at 2-8°C and for 1M in both the FD and AD state at 25°C. The mean particle size of the nanoparticles increased at higher temperature over a period of time due to aggregation and degradation of polymer. It was observed that both the NP formulations were stable in terms of drug content upto 3 months in FD state and for 1month in AD state at 2-8°C. It was concluded that it was best to store nanoparticle formations in the freeze dried state at 2-8°C where they remained stable in terms of both MPS and drug content.

**Etoposide Loaded PLGA-MPEG and PLGA-Pluronic Nanoparticles**

In order to be useful as controlled drug delivery the nanoparticulate drug carriers must show persistence in systemic circulation after intravenous administration. But these carriers, when given intravenously are rapidly cleared by the cells of the mononuclear phagocytes system. Polyethyleneglycol (PEG) modified biodegradable polymer is one of the most popular materials to prepare stealth nanoparticles. The PEG layer provides a steric barrier to the particle and its opsonization is reduced. Pluronic coated PLGA nanoparticles avoided rapid recognition by reticulo endothelial system (RES) and are long circulating. PLGA-mPEG and PLGA-PLURONIC copolymers were synthesised for preparation of drug loaded NPs.

Poly(lactide-co-glycolide)–monomethoxy(polyethyleneglycol) copolymer (PLGA-mPEG) was synthesized by the ring opening polymerization by melting PLGA and mPEG and using stannous octoate as catalyst and the copolymer was analyzed and confirmed by $^{1}$H-NMR and FTIR spectras. PLGA and Pluronic F-68 were blended together in a 2:1 w/w ratio and dissolved in minimum amount of chloroform in a flask. The solvent was removed under vacuum overnight and the resultant copolymer was analyzed and confirmed by FTIR.

Etoposide loaded PLGA-mPEG nanoparticles were prepared by oil-in-water single-emulsion solvent evaporation using high pressure homogenization as per $3^2$ Factorial Design. The mean particle size of NP ranged from 94.02 ±3.4 nm to 132.9 ±11.3nm. Response surface and contour plots demonstrated that there was a linear fall in particle size from 115nm to 95 nm when the surfactant concentration (X2) was between 1.0 to 0.0 and drug: polymer concentration (X1) was between -1.0 to 0.0. However, there was a linear rise in particle size from 111 nm to 130 nm when the surfactant concentration changed from 0.0 to -1.0 and drug: polymer concentration was between 0.0 and 1.0.The %EE of ETO varied from 62.09±3.13% to 71.22±1.1%. The results show that % EE greatly depend on the concentration of the surfactant used. An increase in the surfactant concentration from 0.5 to 1.5% markedly decreased the % EE. Based on the results of the multiple regression analysis and F statistics, it was concluded that the highest %EE could be obtained when X1 was kept at medium level (0) and X2 at the highest level (1). The predicted and observed values of response parameters showed low values of the relative error (< 5%) indicating a reasonable agreement of predicted values and experimental values. Three dimensional surface response plots and two dimension contour plots for the response of %EE were found to be non–linear with upward and downward segments which indicate non-linear relationship between X1 and X2 variables. An EE of 70% could be obtained by keeping the values of X1 (Drug: polymer ratio) between -0.3 to 0.4.

The zeta potential values ranged between -6.0 mV to -8.9 mV for PLGA-mPEG NP. The optimized batch (based on least MPS and highest EE) had zeta potential value of −6.9±1.3 mV (EPMPEG NP6) and the Zeta potential distribution showed a bell shaped curve with one sharp peak (-8.0 mV) signifying homogeneous distribution of surface charge on the particles. Zeta potential was decreased with increase in the concentration of surfactant. It is generally expected that greater is the zeta potential of the nanoparticle, it is more likely to be stable because the charged particles repel each other. However, this rule cannot be strictly applied to systems containing steric stabilizers, because adsorption of the steric stabilizers would decrease the zeta potential due to shift in the shear plane of the particle. It was concluded that the PLGA-mPEG NP would be stable due to presence of mPEG on the surface, which would create a shield between the NP surface and the

surrounding medium, thus masking the charged groups on the surface and preventing the particles from aggregation.

Etoposide loaded PLGA-Pluronic nanoparticles were prepared by oil-in-water single-emulsion solvent evaporation using high pressure homogenization. The NPs formed were uniform, discrete and less than 200 nanometers in size. Nine batches in triplicate were prepared by varying drug: polymer ratio and concentration of the surfactant. NPs had mean particle size between 148.0 ±2.1nm and 169.9 ±6.3nm. Highest entrapment efficiency of 73.12±2.7% could be obtained for batch no. EPPLUNP5. As the drug loading was increased from 9.0 to 14.2%, the entrapment efficiency increased, but at 20% drug loading the entrapment efficiency was decreased. This may be due to failure of the polymer to incorporate the excess of the drug in the nanoparticles. Size distribution of batch EPPLUNP5 with Mean particle size of 146.0nm showed a bell shaped showing homogenous distribution of particles around the mean. This was also supported by a very low PDI of 0.117 indicating homogeneous particles. Nanoparticles prepared from PLGA-Pluronic copolymers were found to have negative zeta potential values ranging from -17.1 ±1.3mV to -21.5 ±1.6mV. The zeta potential of PLGA-Pluronic NP was comparatively reduced with respect to PLGA NP (-32.7mV). This decrease in zeta potential was attributed to Pluronic F-68 on the surface of the particle. However, it was expected that the PLGA-Pluronic NP would be sterically stable.

During the process of lyophilization, sucrose was added as cryoprotectant. Use of sucrose in a 50%w/w concentration showed minimum increase in particle size for PLGA-mPEG NP and use of 20% w/w sucrose as cryoprotectant showed minimum increase in particle size of PLGA-Pluronic NP after lyophilization. These NP were also having good redispersibility. X-ray powder diffraction studies showed that in the prepared PLGA-mPEG and PLGA-Pluronic NP, the drug was present in the amorphous state and may have been homogeneously dispersed in the polymer matrix. The electron micrograph showed spherical discrete and homogenous particles in the nanometer size range.

In vitro drug release from the pure drug was complete within 4 hours, but was sustained up to 7 days from PLGA-mPEG nanoparticles and 5 days from PLGA-Pluronic nanoparticles. The release was first ordered and followed non-fickian diffusion kinetics in

both the cases. Data fitted to Higuchi model showed that EPMPEG NP had high $r^2$ value of 0.9929 indicated that it follows higuchi diffusion kinetics, where as EPPLU NP had a slighty low $r^2$ value of 0.945. Korsmeyer-Peppas model gave the value of n, which indicated the release mechanism, for EPMPEG NP the n value was found to be 0.53 and for EPPLU NP the n value was found to be 0.64., both the values indicate a non-Fickian release.

It was concluded from the stability studies that Etoposide loaded PLGA-mPEG NP (EPMPEG NP) and Etoposide loaded PLGA-Pluronic NP (EPPLU NP) were stable in terms of mean particle size at 2-8°C up to three months in the freeze dried (FD) state, one month in the aqueous dispersion (AD) state at 2-8°C and for 1M in both the FD and AD state at 25°C. At higher temperature the NPs were not stable as an increase in size was observed. It was seen from the stability studies that Etoposide loaded PLGA-mPEG NP (EPMPEG NP) and Etoposide loaded PLGA-Pluronic NP (EPPLU NP) were stable in terms of % drug content upto three months in the FD state, two months in the AD state at 2-8°C, for 1M in both the FD and AD state 25°C and 1M in FD state at 40°C. It was concluded that it is best to store nanoparticle formations in the freeze dried state at 2-8°C where it remains stable in terms of both MPS and drug content.

**Cytarabine loaded PLGA and PLGA-mPEG Nanoparticles**

Modified nanoprecipitation method was used for the preparation of Cytarabine loaded PLGA Nanoparticles. In this method, a double emulsion technique was used in which the aqueous solution of the hydrophilic compound (along with a co-solvent) was first emulsified in an organic solution (non solvent) of the polymer and then this primary emulsion was poured into a large volume of water with surfactant. This was done to achieve good drug loading. The method was first optimized for choice of co-solvent based on MPS. Three batches in triplicate were taken, first without a co-solvent, second with acetone and third with methanol. With acetone, the particle size achieved was higher compared with methanol because of the tendency of drug substance to precipitate in the presence of acetone. Based on the least MPS (138nm) obtained for batch No. CPNP3, methanol was chosen as the co-solvent. Then a $3^2$ factorial design was used for the optimization of volume of co-solvent and non solvent based on MPS of the nanoparticles

obtained. The effect of two independent variables, volume of the co-solvent (X1) and volume of non solvent (X2) on the response, mean particle size (Y1) of the Cyt-PLGA Nanoparticles was studied. The mean particle size of NP ranged from 127±3.1 to 148±5.6 nm. Contour plots and the response surface curves respectively drawn at -1 level to 1 level of X1 and X2. It was concluded from the non linear plots of contour and surface response that the MPS of 153 nm could be obtained with X1 range from 0.2 level (0.22ml) to -0.2 level (0.35ml) and X2 range from 0.2 (3ml) to -0.2 (3ml).

For the optimization of drug:polymer ratio and stirring time, nine batches were prepared in triplicate as per $3^2$ factorial design to study the effect of two independent variables, ratio of drug and polymer (X1), stirring time (X2) on the two responses, mean particle size (Y1) and percentage entrapment efficiency (Y2) of the Cyt-PLGA Nanoparticles. The mean particle size of NP ranged from 125±2.5 to 151±2.4. The contour plots and the response surface curves were drawn at -1 level to 1 level of X1. The plots were found to be linear; therefore linear relationship exists between X1 and X2 variables. It was concluded from the contour plots and the response surface curves that the MPS of 125 nm could be obtained with X1 range from -0.6 level (1:7) to -1.0 level (1:5) and X2 range from 0.2 (23min) to 1.0 (30min). The % EE of CYT in PLGA NP varied from 17±2.0% to 22.0±2.1%. The linear contour and surface response curves concluded that the % EE of 21% could be achieved with X1 range from 1.0to 0.1 level and X2 range from 0.7 to 1.0. CYT loaded PLGA NP had a zeta potential of -29.7mV.

$3^2$ Factorial Design was used for formulation of Cyt-PLGA-MPEG NP by the modified nanoprecipitation method. The effect of two independent variables, ratio of drug and polymer (X1) and Volume of the non solvent (ml) (X2) was studied on the two responses, mean particle size (Y1) and percentage entrapment efficiency (Y2) of the Cyt-PLGA-MPEG Nanoparticles. The mean particle size of NP ranged from 152±6 to 198±3nm. It was concluded from contour plots and the response surface curves that the MPS of 152 nm could be obtained with X1 range from -1 level (1:5) to -0.7 level (1:6) and X2 range from 0.6 (2.5ml) to 1.0 (8ml). The % EE of CYT in PLGA-MPEG NP varied from 29.4±0.3% to 41.1±0.8%. It was concluded from the linear contour plots and the response surface curves that the % EE of 41% could be achieved with X1 in two different levels of 0.2 to 1.0 as well as -0.2 to -1.0 and X2 range at 0.6 level to 1.0 level.

Lyophilization was carried out using sucrose in five different concentrations of 10, 20, 50, 75 and 100%w/w to act as both a cryoprotectant and a redispersant. An increase in size of the NPs was seen following freeze-drying with the use of sucrose as cryoprotectant. Optimization of the cryoprotectant was based on its ability to give minimum increase in size and dispersibility. Use of sucrose in a 50%w/w concentration showed minimum increase in particle size of the Cyt-PLGA NP and 20% w/w sucrose was optimum for Cyt-PLGA-MPEG NP as after lyophilization it had minimum increase in MPS. The freeze dried NPs were redispersible and they showed minimum increase in the mean particle size after lyophilization.

The endothermic curves of Differential Scanning Calorimetry showed that the drug peak was absent in both the nanoparticle formulations, indicating cytarabine was dispersed as an amorphous state in the Cyt-PLGA NP and Cyt-MPEG NP. X-ray powder diffraction studies concluded that in the prepared Cyt-PLGA NP and Cyt-PLGA-MPEG NP, the drug was present in the amorphous phase and may have been homogeneously dispersed in the polymer matrix. The SEM of Cyt-PLGA NP and Cyt-MPEG NP showed spherical and discrete particles in the nanometer size range.

In vitro drug release of the pure drug was complete within 2 hours, but the PLGA nanoparticles sustained the release of CYT for 24h and PLGA-mPEG nanoparticles sustained the release of CYT up to 2 days. The sustained release of the drug was attributed to the formation of a homogeneous matrix with the drug randomly distributed throughout the polymer particle. $R^2$ value of first order was higher (> 0.99) in both cases than the zero order, indicating that the release followed first ordered kinetics in both the cases. Data fitted to Higuchi model showed that CPM NP had higher $R^2$ value (> 0.95) than CPNP indicated that CPM NP follows Higuchi diffusion kinetics. It was concluded that mechanisms of drug release from the particles follows diffusion of drug from the matrices. The release data were fitted to Korsmeyer and Peppas equation and Diffusion exponent (n value) was obtained from the slope and was found to be 0.3754 for CPNP and 0.411 for CPM NP. In both the cases, the n value was less than 0.43 indicating a Fickian release mechanism from both the NP.

Stability studies of polymeric nanoparticles were carried out to evaluate the change in mean particle size and drug content over a period of three months storage at 2-8, 25 and

40°C. It was observed that cytarabine loaded PLGA-mPEG NP and cytarabine loaded PLGA NP were stable at 2-8°C for 2M and at 25°C for 1M as there was no significant change in the mean particle size and in the drug content. Nanoparticles were not stable at higher temperatures (> 25°C) due to aggregation of particles and degradation of the polymer. It was concluded that the developed PLGA NPs should be stored in the freeze dried state at 2-8°C where they would remain stable in terms of both MPS and drug content.

**Cytotoxicity Studies**

Cytotoxicity and cellular uptake of the formulated PLGA nanoparticles of the two drugs etoposide and Cytarabine were studied on two cancer cell lines, L1210 and DU145. Cytotoxicity was determined by the MTT assay and the concentration of drug or NP formulation required to inhibit cell proliferation by 50% ($IC_{50}$) was determined by plotting the percentage of cell growth inhibition versus the concentration of pure drug or NP formulation. PLGA based NP formulations played an important role in enhancing the cytotoxic effect of ETO, which was probably due to increase in their intracellular uptake. The $IC_{50}$ values for L1210 cells were 18.0, 6.2, 4.8 and 5.4 μM for ETO, ETO-PLGA NP, ETO-PLGA-MPEG NP and ETO-PLGA-PLU NP respectively. The order of cytotoxicity was ETO-PLGA-PLU NP> ETO-PLGA-MPEG NP > ETO-PLGA NP >ETO. The $IC_{50}$ values for DU145 cells were 98.4, 75.1, 60.1 and 71.3 μM for ETO, ETO-PLGA NP, ETO-PLGA-MPEG NP and ETO-PLGA-PLU NP respectively. The order of cytotoxicity was ETO-PLGA-MPEG NP >ETO-PLGA-PLU NP>ETO-PLGA NP >ETO. It was concluded from the cytotoxicity studies that the nanoparticulate formations of ETO had lower $IC_{50}$ values than the pure drug and among the three NP formulations; Eto-PLGA-Pluronic NP had the highest cytotoxicity effect on L1210 and ETO-PLGA-MPEG NP on DU145 cells.

A relatively short incubation period used in the MTT-based cytotoxicity assay (24 or 48 h) is not enough to determine long-term cytotoxicity of the nanoparticulate formulations, and a more prolonged incubation time would be required to fully exert the cytotoxicity effect of nanoparticles. Hence, time based cytotoxicity on L1210 cell lines were carried out for 1, 3 and 5 days. The study showed that the cytotoxicity of ETO pure drug solution on L1210 cells did not show any significant change in the $IC_{50}$ values on the 3$^{rd}$

and 5th day. But the $IC_{50}$ values had a significant decrease ($P>0.05$) in the $IC_{50}$ values on 3rd and 5th day for all the three nanoparticulate formulations. Among the three NP formulations, ETO-MPEG-PLGA NP had a comparatively lower $IC_{50}$ value on the 5th day. The study concluded that the drug loaded PLGA nanoparticulate formulations were efficient in decreasing the viability of the L1210 cells over a period of five days, whereas the pure drug did not show any significant change in the $IC_{50}$ value after 1 day.

The three different polymers PLGA, PLGA-MPEG and PLGA-PLURONIC used in the NP formulations were also tested for their cytotoxicities at two concentrations, AC (actual concentration used in the NP formulation) and DC (double concentration used in the NP formulation). It was seen that viability was more than 99% in AC and DC for PLGA and more than 97% for PLGA-MPEG and PLGA-PLURONIC. The study concluded that what ever cytotoxicity was observed for the drug loaded NP in L1210 and DU145 cells was due to the release of the entrapped drug from the NP and not because of the polymers used.

Cytotoxicity of CYT, CYT -PLGA NP and CYT -PLGA-MPEG NP were studied on two cell lines, L1210 and DU145. The $IC_{50}$ values for L1210 cells were 6.5, 5.3, and 2.2 μM for CYT, CYT -PLGA NP and CYT -PLGA-MPEG NP respectively. The order of cytotoxicity was CYT-PLGA-MPEG NP > CYT-PLGA NP >CYT. The $IC_{50}$ values for DU145 cells were 62.5, 20.2, and 14.3 μM respectively for CYT, CYT -PLGA NP and CYT -PLGA-MPEG NP respectively. The order of cytotoxicity was CYT-PLGA-MPEG NP > CYT-PLGA NP >CYT. Comparing the results of the two cell lines, it was seen that CYT had lower $IC_{50}$ value for L1210 cells (leukaemic cells) than the DU145 cells (prostate cancer cells) as expected because CYT is mostly recommended in leukaemia therapy rather than treatment of prostate cancer.

Long term cytotoxicity study showed that there was no significant change in the reduction of $IC_{50}$ values of pure drug solution (CYT) on the 3rd and 5th day. A significant difference was observed in reduction of $IC_{50}$ values in both CYT-PLGA NP and CYT-MPEG-PLGA NP. The nanoparticles released the entrapped drug for a longer duration and showed significant change in the reduction of $IC_{50}$ values upto 5 days. Hence it was

concluded that CYT loaded nanoparticles had long term cytotoxic affect on the L1210 cells.

**Cellular Uptake Studies**

To investigate the in vitro cellular uptake of NP, fluorescent NPs were prepared by substituting drug with 6- coumarin (0.01 % w/w), using solvent evaporation technique. Confocal microscopy and Flow cytometry were used to visualize cellular uptake of the nanoparticles by cells L1210 and DU145. The time and concentration dependent cellular uptake of the NPs was determined by quantifying the percentage of fluorescence. It was concluded that the uptake was both time and concentration dependent and was highest at a concentration of 100 μg/ml and at 4 hours duration for both the cell lines.

Confocal microscopy of the cells exposed to PLGA nanoparticles showed fluorescence activity in the cells within 30 min which increased with time for both the cell lines L1210 and DU145. The control experiment performed by incubating cells with 6-coumarin solution showed that intracellular fluorescence was insignificant compared to that of cells incubated with nanoparticles. Hence, it was concluded that the fluorescence observed inside the cells was only due to the presence of nanoparticles. In the DU145 cells, the NPs were seen particularly inside the cytoplasm of the cells in 1h. In the 2h, the NPs were seen migrating towards the nucleus and were visible in both cytoplasm and nucleus. At 4h, the NPs were prominently seen in the nucleus where as only a small fraction of NPs were seen in the cytoplasm. Z-stack Confocal images of NP showing uptake in DU 145 and L1210 cells were taken and each section demonstrated fluorescence activity indicating that the nanoparticles were internalized by the cells and not simply bound to their surface.

Flow cytometry study was carried out on the L1210 cell lines using 6-coumarin as the fluorescent marker in the NPs and for each cell sample, 10000 events were collected, M1 was marked as the population of cells having fluorescence under control and M2 was marked as the population of cells with fluorescence intensity after the uptake of the NPs. Incubation of the L1210 cells with NPs caused substantial accumulation of cells in the M2 phase. Quantitative analysis of the events distribution revealed that only 16.81% of the cells were in the M2 phase for control cells, whereas the M2 population was increased

to 42.80%, 47.81 and 51.60% for the PLGA-NP, PLGA-MPEG NP and PLGA-PLURONIC NP treated cell samples. Similarly in the DU145 cells quantitative analysis of the events distribution revealed that only 11.21% of the cells were in the M2 phase for control cells, M2 population was increased to 28.80%, 42.23 and 40.01% for the PLGA-NP, PLGA-MPEG NP and PLGA-PLURONIC NP treated cell samples, respectively These data suggest that the three formulations were effective in increasing the cell population in the M2 phase, indicating uptake of the NPs.

**Radiolabeling, biodistribution and Blood clearance studies**

Etoposide, ETO-PLGA NP, ETO-PLGA-mPEG NP, Cytarabine, CYT-PLGA NP were radiolabeled with Technetium-99m ($^{99m}$Tc). High labeling efficiency of 97% in serum was obtained for all the formulations indicating their good stability for animal experiments. Balb/c mice having body weight between 20–25 g were used in group of three for biodistribution studies. Radiolabeled formulations were injected intravenously (100 µl) in the tail vein of the mice. Radioactivity was determined as the percentage injected dose/gram of organ or tissue after 1, 4 and 24h post injection.

From the results of the radiolabeled ETO and radiolabeld ETO loaded PLGA NPs of two sizes, 105 and 160nm, it was concluded that PLGA NP of size 105 ($^{99m}$Tc-Eto-PLGA $NP_{105}$) were present in the blood at a higher concentration and at lower concentration in liver, spleen and lungs as compared to $^{99m}$ Tc-Eto (Pure drug ETO) and $^{99m}$ Tc-Eto-PLGA $NP_{160}$ (PLGA NP of size 160nm). This was probably due to the fact that nanoparticles of size less than 100 nm diameter evaded the RES. It was concluded that PLGA-NP of size ~100nm were present in the blood at higher concentrations upto 24h and were able to reduce their uptake by the MPS as compared to the pure drug.

$^{99m}$Tc-Cyt-PLGA NP was available at a very low concentration in blood compared to $^{99m}$Tc-Cyt-PLGA-mPEG NP and was not able to avoid MPS uptake. PLGA-NP had more of splenic than hepatic uptake. It was seen that $^{99m}$Tc-Cyt-PLGA-mPEG NP was long circulating and its blood concentration was increased to 91.0 times in 24h compared to free drug. Comparatively, $^{99m}$Tc-Cyt-PLGA NP was available in blood at a very low concentration and was almost negligible after 4h, this was probably due to its higher uptake by the liver and spleen. The pure drug ($^{99m}$Tc-Cyt) had negligible uptake by in the

brain. $^{99m}$Tc-Cyt-PLGA-mPEG NP was seen in a concentration 25 times that of $^{99m}$Tc-Cyt-PLGA NP. It was concluded that $^{99m}$Tc-Cyt-PLGA-mPEG NP was present in blood in higher concentrations upto 24h, avoided MPS uptake and was available in brain and bones.

Sprague-Dawley rats weighing 200 to 250 g were selected for the blood clearance studies and nanoparticles containing 200 μCi of $^{99m}$Tc were intravenously injected in their tail. The blood samples were collected at different time points and analyzed for the radioactivity as percentage injected dose/gram of blood.

When $^{99m}$Tc-Eto was injected in rat, only 2.35 %ID was seen in 15min, which was further reduced to only 0.05% ID in 24h, indicating rapid elimination of ETO from the blood circulation. Among the three NPs, $^{99m}$ Tc- Eto-PLGA $NP_{160}$ concentration was reduced from 3.20% ID (15min) to 1.01% in 1h and 0.42% ID in 24h. Uncoated nanoparticles were rapidly removed from the circulation due to their uptake by the macrophages in the liver, spleen and lungs. $^{99m}$Tc-Eto-PLGA $NP_{105}$ was present in higher concentrations (15.38% of ID) in the blood upto 24h. This was probably due to the fact that nanoparticles of size less than 100 nm diameter can evade the RES and have a long circulation time in the blood. The two coated NPs, $^{99m}$ Tc- Eto-PLGA-PLU NP and $^{99m}$ Tc- Eto-PLGA-mPEG NP were available in higher concentrations in the circulation compared to the pure drug and among the two, $^{99m}$Tc-Eto-PLGA-mPEG NP had the higher value of 18.30% ID at 24h. This was probably due to the fact that Pluronic coated NP are faster eliminated compared to MPEG coated NP. Hence it was concluded that $^{99m}$Tc-Eto-PLGA-mPEG NP was present in the blood at higher concentrations upto 24h.

When $^{99m}$Tc-Cyt were injected in rat, only 2.35 %ID was seen in 15min, which was further reduced to 0.07% ID was seen in 24h. Similarly $^{99m}$ Tc- Cyt-PLGA NP was also only seen in small concentration of 2.15% in 1h and was further reduced to 0.21% ID in 24h. Whereas, $^{99m}$Tc-Cyt-PLGA-MPEG was present in higher concentrations in blood upto 24h. The blood concentration of $^{99m}$ Tc- Cyt-PLGA-MPEG NP was 32.25% ID in 15 min and 12.27 in 24h. The results indicated that only PEGylated NP of CYT could sustain the release in higher concentrations in the rats, whereas pure drug and PLGA NP were almost eliminated after the 4$^{th}$ hour. The study concluded that PEGylated

nanoparticles of CYT, $^{99m}$Tc-Cyt-PLGA-mPEG NP was present at higher concentrations in rat circulation upto 24h.

## 8.2 Conclusion

In the present investigation we developed etoposide loaded PLGA based biodegradable nanoparticles which had sustained release of the drug for a period of 3, 5 and 7 days for ETO-PLGA NP, ETO-PLGA-Pluronic NP and ETO-PLGA-mPEG NP respectively. Cytarabine loaded PLGA nanoparticles had a sustained release of the drug for 1 day and CYT loaded PLGA-PEG NP had a sustained release for 2 days. The developed nanoparticles of both the drugs had good steric stability and were stable at 2-8°C for 3 months in freeze dried state in terms of both size and drug content.

Cytotoxicity studies of the NP on L1210 and DU 145 cells showed that developed NP had increased cytotoxicities due to their better uptake by the cells. Moreover the PLGA-MPEG NP of ETO and CYT and PLGA-PLURONIC NP of ETO showed a sustained cytotoxic effect till 5 days on both the cell lines. The uptake of the NP by the cells was also confirmed by confocal and flow cytometry studies.

The biodistribution and blood clearance studies showed that PLGA-MPEG NP of ETO and CYT and PLGA-PLURONIC NP of ETO has reduced uptake by the RES due to their steric barrier and were therefore present in the circulation for a longer time. Moreover the NP had greater uptake in bones and brain in which the free drugs concentration (ETO and CYT) was negligible.

Hence the developed nanoparticulate formulations of ETO and CYT can be potentially useful clinically in treatment of leukaemia because side effects associated with the usual conventional dose of the drugs could be greatly reduced. Thus, the results of the present investigations conclusively indicate sustained release formulations of etoposide and cytarabine for intravenous administration. Moreover, the surface modified formulations (ETO-PLGA-MPEG NP, ETO-PLGA-PLURONIC NP and CYT-PLGA-MPEG NP) were present in blood in higher concentrations in animals and avoided RES uptake for a longer duration.

The drug delivery from these NP could be given as a single shot injection by IV route that would release the drug for a number of days, which would be beneficial in better control of the leukaemia therapy.

Hence, the developed PLGA based nanoparticulate formulations of ETO and CYT hold promise as better alternative to the conventional formulations. However, further investigations in human beings under clinical conditions are necessary before they can be fully exploited.