# Chapter 5

# Summary and Conclusions

## 5.1. Summary

The idea to work for the present thesis was conceived from drug and dose-related problems associated with the available anti-neoplastic drugs. Anti-cancer drugs usually suffer from low solubility, rapid *in-vivo* degradation, poor pharmacokinetics, undesirable biodistribution and poor permeability across biological barriers. Docetaxel is a second-generation taxane derived from the needles of the European yew tree. Unlike paclitaxel, docetaxel exhibits linear pharmacokinetics and, due to differences in drug efflux, is retained intracellularly for a longer period. During chemotherapy, large doses are recommended for treatment, which may induce adverse effects on normal cells and the surrounding healthy organs. Thus, the objective of this study was to design and develop targeted delivery systems with the aim of restricting high dose administration and reducing the dose-related adverse side effects and also the frequency of dosing. Chitosan is a nontoxic, semicrystalline, biodegradable and biocompatible linear polysaccharide of randomly distributed N-acetyl glucosamine and glucosamine units. Chitosan can be modified to chitosan nanoparticles (NP) by emulsion cross-linking, spray-drying, reverse micellar method, template polymerization, polyelectrolyte complex, precipitation and ionotropic gelation methods. TPGS is a surfactant used for pharmaceutical dosage form preparations. TPGS can be used as an absorption enhancer, emulsifier, solubilizer, additive, permeation enhancer and stabilizer. The redox sensitive nanomedicine has high efficacy, specificity and sensitivity and facilitates *in-vivo* imaging in lung cancer applications when loaded with an imaging material. The high levels (>20 mM) of glutathione (GSH, a cysteine-containing tri-peptide) in cancer cell microenvironment, compared to that of in blood circulation (2–20 μM), facilitates for quicker release of the anti-neoplastics from redox-responsive NP that are composed of redox-sensitive disulfide

(S–S) bonds. These S–S bonds will be cleaved to trigger the drug delivery from NP in the vicinity of cancer cells. The novelty of this work thus lies in the development of low-dose, bioadhesive and EGFR targeted chitosan nanosystem and redox sensitive nanosystem for the advanced therapy of non-small cell lung cancer.

The present study was divided into formulation of different NP, which include 1) Docetaxel (DTX) loaded and cetuximab (CTX) surface conjugated bioadhesive chitosan NP, 2) DTX loaded and CTX surface conjugated redox sensitive NP. The design, development, and optimization of nanoformulations were done by employing systematic design of experiments (DoE). DoE Involves stepwise assessment of critical quality attributes, factor screening, experimental design and optimization. The effect of independent variables on the responses was illustrated by 3D response surface methodology. A graphical and numerical optimization procedure was carried out to obtain the predicted value of various factors and responses. The final optimized batch of the nanoformulation was evaluated and validated. The optimization process comprises, a) identification of critical quality attributes (CQA) by Ishikawa fishbone diagram, b) selection of critical quality attributes (CQAs), c) factor screening study by Placket-Burman design (PBD) to evaluate the effect of independent factors on the dependent responses and use of Pareto charts to select the most important factors that highly influence the selected responses, and d) response surface methodology by factorial design (FD). Further, the optimized nanoformulation was subjected to different characterizations which consist of particle size, polydispersity index, zeta potential, % entrapment efficiency, *in-vitro* drug release, cellular uptake, cytotoxicity, migration and apoptosis studies on A549 cells, *in-vivo* pharmacokinetic, histopathology studies on Wistar rats and anti-cancer efficacy studies on albino mice.

Preliminary evaluation like particle size, zetapotential measurements revealed that DTX loaded chitosan nanoparticles were in acceptable and uniform size range of 180-210 nm with optimum surface charge to ensure proper stability during long-term storage. Also, DTX loaded redox sensitive TPGS-SH nanoparticles were found to have size in the range of 170-190 nm. Surface conjugation of EGFR targeting antibody CTX resulted in slight reduction of zetapotential which may be due to inherent negative surface charge of the antibody.

The morphology, surface texture and shape were evaluated by performing various electron microscopic studies such as TEM, SEM and AFM and the results reveled that both the NP are spherical shape, uniform sized with no obvious surface defects such as pits and crevices. XRD evaluation was performed to check the drug's crystalline properties within the formulation and the results revealed that drug was present in complete amorphous form in the NP which was evident from the absence of various peaks that were present in the spectrum of pure drug. Also, XPS studies were performed to evaluate the surface composition and the results showed that targeted NP have higher nitrogen content on their surface as compared with non-targeted NP which may be due to surface conjugation of CTX.

Both the formulations were evaluated for entrapment efficiency and revealed superior entrapment efficiency in the range of 64-75 % w/w. *In-vitro* release studies in buffers with pH 5.5 and 7.4 revealed that chitosan NP displayed pH dependent drug release with almost 90% cumulative drug release in 72 h. In case of redox sensitive NP also, drug release was pH and redox dependent in that, drug release was faster in buffer containing higher GSH concentration at lower pH. Particle size was actually decreased at lower pH and higher GSH levels which may be due to increased cleavage of disulphide bonds at

higher GSH concentrations. But at higher pH, particle size was actually increased which may be due to diffusion of solvent into the particles.

*In-vitro* bioadhesive evaluation studies on chitosan NP have shown that both the non-targeted and targeted NP adhered in huge numbers to the surface of A549 cells, which may be due to the bioadhesive property of chitosan in addition to its positive surface charge, which binds to the negatively charged cell surface quite efficiently.

*In-vitro* cellular uptake studies established that targeted NP of both the formulations displayed highest cellular uptake compared to non-targeted NP and C6 control, which was revealed by the quantitative assessment of fluorescence intensity by using ImageJ software. This superior uptake may be due to the receptor mediated endocytosis and synergistic bioadhesion conferred by chitosan.

*In-vitro* cytotoxicity studies by performing MTT assay on A549 cells have proved that EGFR targeted NP showed superior cytotoxicity and required less dose as compared with non-targeted NP and DTX control which was also evident from their respective $IC_{50}$ values. But redox sensitive NP displayed cytotoxicity which was a bit lower as compared to chitosan NP.

*In-vitro* wound healing studies showed that the prepared formulations effectively prevented the cell migration as compared to solvent control where the acellular space was completely covered within 48h. in case of NP, the space was actually increased which may be due to the cell death induced by DTX containing NP.

The same fact was established further by *in-vitro* apoptosis (morphology) studies, where the cells treated with targeted NP showed highest apoptosis which was evident from the chromosomal condensation and nuclear fragmentation with in the cells. And they lost their normal polygonal morphology of healthy cells.

*In-vivo* pharmacokinetics studies were performed on CF rats by injecting the prepared formulation through tail vein and collecting the blood samples at predetermined time intervals. The collected blood samples were evaluated for DTX concentration by reverse phase HPLC. The plasma concentration-time profile was constructed and by using kinetica software, various pharmacokinetic parameters were evaluated. The results showed that targeted NP displayed superior bioavailability, MRT, $V_d$ etc.as compared to non targeted NP as well as DTX control due to receptor mediated endocytosis in addition to bioadhesive nature of chitosan. Non-targeted NP also shoed better pharmacokinetic profile as compared to DTX control which may be due to bioadhesive properties of chitosan with entrapped DTX within the matrix.

*In-vivo* histopathological evaluation on the vital organs such as heart, lungs, liver and kidney of CF rats showed that prepared formulations produced insignificant toxicity to these vital organs as compared with DTX control. As both the formulations were formulated with the biocompatible, biodegradable and safe polymers, they were indeed proved to be safe for systemic administration.

*In-vivo* anticancer studies were performed on benzopyrene induced mice lung cancer model to establish the efficacy of prepared formulations. Here, the non-targeted and targeted NP were injected into mice after administration of first dose of benzopyrene. After the specified time, the lungs were extracted and the corresponding sections were subjected to H&E staining. The microscopic images were processed with ImageJ software to separate the nucleus from cytoplasm by using a feature named colour deconvolution. By selecting three different regions, the cell number was counted and the numbers were compared for statistical significance. The results revealed that targeted NP significantly reduced the cell number as compared to non-targeted NP and drug control. Indeed,

bioadhesive chitosan NP portrayed better anticancer efficacy as compared with redox sensitive TPGS-SH NP.

## 5.2. Conclusion

- Conclusively, the present thesis embodies to design, develop, optimize and characterize polymeric nanosystems for targeted therapy of non-small cell lung cancer (NSCLC). Chitosan-based bioadhesive nanomedicine and redox sensitive nanosystem of docetaxel were successfully formulated by the quality by design (QbD) approach by using minimum material and resources. Preliminary evaluations like size, surface charge and polydispersity demonstrated promising results.
- For bioadhesive chitosan NP, XRD characterization and revealed the complete amorphous state of DTX within the formulations.
- The surface chemistry studies using XPS confirmed the conjugation of CTX onto the surface of the targeted NP.
- *In vitro* drug release studies indicated a pH-dependent drug release with a faster release at pH 5.5. SEM micrographs of treated A549 cells showed that NP have adhered in large numbers to the cell surface.
- For redox sensitive NP, the *in-vitro* drug release study in media containing different GSH concentrations showed that drug release was higher and faster at lower pH and higher GSH concentrations.
- Also, the pH and redox sensitivity studies established the same fact that these particles were more stable at higher pH and lower GSH levels.
- With both formulations, the *in-vitro* cell culture studies on A549 cells have confirmed that targeted NP displayed superior uptake and cytotoxicity than non-

targeted NP and drug control. Indeed, the bioadhesive chitosan nanoformulation showed much higher cytotoxicity with a low $IC_{50}$ value as compared with redox sensitive NP.

- The *in-vitro* apoptosis study demonstrated a superior effect with targeted NP. Moreover, EGFR targeted nanoformulations effectively prevented the migration of cells.
- All the pharmacokinetic parameters were found to be superior for targeted NP than non-targeted Ns and Docel™.
- Histopathological evaluation of vital organs has revealed minor lesions compared to that of Docel™, which confirmed their safety.
- *In-vivo* tumor inhibition studies stated that cell number was reduced significantly ($P<0.05$) by targeted bioadhesive chitosan NP than Docel™. The bioadhesive chitosan NP reduced the cell number and comparatively showed superior *in-vivo* anticancer efficacy than redox sensitive NP.
- The results obtained from in-vivo anticancer studies are in absolute agreement with those observed in in-vitro toxicity studies in that both showed significant reduction in the cell number which was measured in terms of reduction of fluorescent intensity in MTT assay and as reduction in cell number in case of in-vivo anticancer studies.

## 5.3. Future Perspective

We want to explore other possible novel molecular and cellular targets for active targeting of anticancer therapeutics. The focus of our future research is on further individualization of therapy, based on particular molecular profiles. We want to establish rodent xenograft models for the in-vivo evaluation of anticancer nanomedicine particularly in nude mice.

We also want to evaluate and establish the application of these targeted nanomedicine in other types of lung cancer such as squamous cell carcinoma, large cell lung carcinoma and small cell lung carcinoma after their successful development by chemical carcinogens in mice.