CHAPTER 8: SUMMARY

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In the present research work, we have designed a mesoporous platform based delivery system for efficient and targeted delivery of two widely used anticancer drugs namely ETO and BIC. Though various other Nano delivery platforms have been researched upon for both these drugs, this was the first instance of exploring mesoporous silica nanoparticles both bare MCM-41 and surface functionalized PAA-MSNs and FA-MSNs as delivery agents for ETO and BIC. Their role for the purpose of solubility and bioavailability enhancement was extensively researched upon via oral route. Additionally intravenous administration and targeting along with the biosafety parameters were also studied for drug loaded surface functionalised MSNs.

ETO and BIC belong to BCS class IV and II respectively. Their efficacy is limited by solubility and permeability constraints. This in turn might also have an effect on the bioavailability of these drugs. Furthermore, though both the drugs have been used for cancer treatment since a long time, their efficacy is still limited. The major reasons being lack of targeted treatment. This leads to adverse and lethal effects on healthy organs also along with the affected organs. Thus other vital functions are affected. Thus, the required amount of drug also fails to reach the target organ in turn limiting the therapeutic efficacy. To find answers to all these problems the role of mesoporous silica nanoparticles in improvement of therapeutic efficacy was studied.

Cancer cells are distinguished from healthy cells in various aspects. The pH of healthy cells is 7.4. Cancer cells have acidic environment with pH of extracellular tumours being 6.8 and endosomal pH of 5.5 is one of them. The other major one being overexpression of certain receptors like folate in cancer cells. Based on these facts a pH responsive drug delivery system and receptor targeted drug delivery system was prepared for efficient delivery of anticancer drugs. Polyacrylic acid (PAA) was used as a pH responsive polymer and coated onto the MSNs via aminated layer. PAA was selected due to various advantages and features like good pH responsiveness, excellent biocompatibility, minimal toxicity, and its ability to impart additional

hydrophilicity to MSNs. Further, there is known to be an overexpression of folate receptors in different cancers. Exploiting this fact folic acid was used as a ligand and grafted onto the surface of MSNs. The synthesized FA-MSNs were further characterized and evaluated for their anticancer efficacy and biosafety.

8.1 Summary for ETO

First of all basic skeletal of mesoporous silica nanoparticles was fabricated based on modified Stober template based synthesis method. Thereafter, the surfactant template was removed by calcination at a high temperature. The calcinated mesoporous silica nanoparticles were further used for post synthetic surface modification. Amination was done on the MCM-41 MSNs by by using APTES. Functionalization offered advantages like achieving a sustained release for ETO, which would allow its slow and steady drug release. Additionally, the release study was performed in presence of enzymes to investigate interaction of amine moiety with gelatin shell and its effect on in vitro drug release. The amination also served as a strong platform for further functionalization with PAA. The success of surface functionalization with PAA and FA was ascertained in qualitative and quantitative manner by FT-IR and TGA analysis respectively. The TGA data exhibited 4% amine, 20.19% PAA and 23.75% FA grafting respectively on the external surface of MCM-41 nanocarriers. Thus, a healthy grafting percentage was obtained for both PAA and FA groups. This ensured the success of facile strategy employed for functionalization of MSNs. A final confirmation was given by the change of zeta potential on successive functionalization. The original zeta potential of MCM-41 was -36.86 mV, on surface grafting with positively charged amine groups it increased to +33.92mV for MCM-41-A, again on top of it grafting with negatively charged PAA groups led to reduction in zeta potential with -31.15 mV value. An increment in the potential value for FA-MSN to +36.04 mV was observed in comparison to the negative potential of MCM-41. The elemental detection data also supported this conclusion. The z average diameter was useful in determining the size of MSNs

and it was observed that as coating layers increased the size of MSNs increased further with z average for MCM-41, MCM-41-A, PAA-MSN and FA-MSN being 110.2, 124.7, 142.85 and 135.38 nm respectively.

The surface area and porosity estimation from the nitrogen sorption studies gave a clear trend of maximum surface are and pore size obtained for MCM-41 nanocarriers. As the surface decoration proceeded further there was an evident decrease in the surface area, pore volume and pore size of the MSNs. Same trend was observed post drug loading when compared to bare carriers. The BET and BJH surface areas were highest for bare MCM-41 with value of 1089.97 m^2/g and 1441.97 m^2/g respectively. The trend was same for BET and BJH both the surface areas. The SEM and TEM images of the fabricated MSNs were proof of spherical and uniform morphology and intact hexagonal structure of MSNs.After complete characterization of the synthesized MSNs, drug loading was performed by rotary solvent immersion method. The solvent used was methanol which was easily evaporated giving a facile way of obtaining drug loaded MSNs. The complete entrapment of drug into the mesoporous network was ascertained by the DSC and WXRD techniques. Absence of sharp crystalline ETO peak in the DSC and WXRD spectra indicated that drug was completed loaded into the MSNs and might have been converted to amorphous form post encapsulation. A supportive evidence of the same fact was also exhibited by FT-IR spectra of the drug loaded MSNs when compared with that of drug. The characteristic IR peaks were found to be absent in the spectra of drug loaded MSNs. Moreover, complete absence of ETO peak in the DSC thermogram ruled out any possibility of drug remaining on external pore surface. The internal structure of the nanocarriers was intact and well preserved even after the drug loading as evident from the LXRD spectra where three characteristic diffractions observed at 100, 110 and 200 in MCM-41 MSNs as also also seen in functionalized and drug loaded MSNs.

The MSNs were evaluated for drug release in vitro by dissolution and diffusion for oral and intravenous formulations respectively. The dissolution study was also carried out in simulated and biorelevant media and effect of food and enzymes on the in vitro drug release was studied. The release of ETO loaded MCM-41 was much faster than the ETO-MCM-41-A and ETO itself in all media. Though amination gave a slightly extended and controlled release pattern and hindered the ETO release to some extent. Bare MCM-41 showed a typical burst release of drug in the initial phase. The food or enzyme presence did not have any effect on drug release and hence it was concluded that formulation could be taken either before or after meal without any adverse effects on its release and absorption. A dissolution enhancement of 5.1 times and 1.16 times was obtained as compared to ETO alone for ETO-MCM-41. The drug release was also faster from MCM-41 carriers with 92.86% of drug released within 30 minutes. In the same time period the drug release from MCM-41-A and Marketed formulation was 47.82% and 75.54% respectively.

The diffusion study data showed a higher drug release at PBS of acidic pH than pH7.4. This was more prolonged and sustained in case of PAA-MSN and FA-MSNs as compared to MCM-41 and MCM-41-A. No difference in release pattern was observed for ETO loaded MCM-41 MSNs in different pH conditions, although drug loaded MCM-41-A MSNs exhibited a slight pH controlled behaviour. For ETO loaded PAA-MSNs. At the end of 72h 87.08% drug was released at pH 5.6 whereas only 36.21% drug was released at pH 7.4 and 70.72% being released at pH 6.8. Clearly a strong pH responsive behaviour was observed by PAA coated MSNs. Even in case of ETO-FA-MSNs difference in release percentage was evident from the data which served as an added advantage for this formulation along with the receptor based targeting. The release mechanism was found to be anomalous from drug loaded surface coated MSNs. In vitro cell cytotoxicity was performed by MTT assay. PAA-MSNs and FA-MSNs were relatively non- toxic on both LNCaP and PC-3 cells with more than 90% viability obtained even after

72h. In MTT assay of drug loaded MSNs, significant cytotoxicity was observed on both the prostate carcinoma cell lines with IC_{50} values as low as 3.56 and 4.28 µg/mL obtained for ETO-PAA-MSNs. For ETO-FA-MSNs the IC_{50} value was 3.18±0.43 and 2.92±0.21 µg/mL for LNCaP and PC-3 cells respectively at the end of 72h. A concentration dependent increase in cell cytotoxicity was seen in both ETO-PAA-MSNs and ETO-FA-MSNs. In case of Caco-2 permeability study the increment in permeability was found to be 4.66 times with respect to ETO alone for ETO-MCM-41 . Moving further, cellular uptake study was an important part of MSN evaluation. PAA-MSNs and FA-MSNs exhibited a significantly higher cellular uptake than MCM-41-A MSNs. Cellular uptake by LNCaP cells was 23.74 %, 84.22% and 87.72% for FITC#MCM-41-A, FITC#PAA-MSN and FITC#FA-MSN respectively. Cellular uptake by PC-3 cells was 24.83%, 80.86% and 91.87% for FITC#MCM-41-A, FITC#PAA-MSN and FITC#FA-MSN respectively. Cell death mechanism was studied by apoptosis assay on both LNCaP and PC-3 cells. Notably, necrosis was higher in ETO-MCM-41 treated cells whereas apoptotic cells both early and late were 55.62%, 72.13% and 80.26% for ETO-MCM-41-A, ETO-PAA-MSN and ETO-FA-MSN respectively for LNCaP cells. For PC-3 cells was 16.78%, 55.88%, 77.05% and 73.94% respectively for ETO-MCM-41, ETO-MCM-41-A, ETO-PAA-MSN and ETO-FA-MSNs. Thus, it could be inferred that both ETO-PAA-MSN and ETO-FA-MSN induced a programmed apoptotic cell death and negligible necrosis.

The synthesised MSNs were also found to exhibit excellent hem compatibility with lysis in all the formulations way less than acceptable criteria of 5%. Oral Pharmacokinetic study was carried out for ETO, ETO-MCM-41, ETO-MCM-41-A and MF of ETO in healthy male swiss albino mice. The $t_{1/2}$ and AUC were enhanced in MCM-41 and MCM-41-A as compared to ETO and MF. The enhancement in bioavailability was 4.35times and 2.47 times as compared to ETO alone for ETO-MCM-41 and ETO-MCM-41-A formulation respectively. Pharmacokinetic study was also carried out for parenteral formulations ETO-MCM-41-A,

ETO-PAA-MSN and ETO-FA-MSN along with ETO drug alone. The AUC of ETO-PAA-MSN and ETO-FA-MSN was also increased as compared to free ETO along with a significantly higher $t_{1/2}$ for both. Hence having a slower plasma elimination rate and increased circulation time. From biodistribution study and histological examination established the biosafety of ETO-MCM-41-A, ETO-PAA-MSNs and ETO-FA-MSNs as compared to ETO. Lastly, SXRD and DSC of all the formulations was done to determine their stability at 40±2 ºC and 75±5 %RH and they were found to be completely stable at the end of 6 months analysis.

8.2 Summary for BIC

A similar pH responsive and folate coated ligand based approach was used for developing BIC formulation. Bicalutamide was successfully encapsulated into MCM-41, MCM-41-A, PAA-MSNs and FA-MSNs. The completion of drug loading was adjudged by DSC and WXRD spectra. The melting point of BIC was found to be 192.54℃. The % loading of BIC was 38.95%,36.15%, 33.28% and 35.02% for MCM-41, MCM-41-A, PAA-MSNs and FA-MSNs. The entrapment percentage was 96.48%, 91.72%, 82.00% and 87.99% for MCM-41, MCM-41-A, PAA-MSNs and FA-MSNs respectively. The zeta potential values for BIC were -24.74mV, +36.98mV, -20.86 mV and +28.56mV for BIC-MCM-41, BIC-MCM-41-A, BIC-PAA-MSN and BIC-FA-MSNs respectively. The change in nature of charges and values were consistent with those of charges present on bare and functionalised MSNs. The BET surface areas for BIC-MCM-41, BIC-MCM-41-A, BIC-PAA-MSN and BIC-FA-MSN was 813.28, 762.12, 501.38, and 530.25 m^2/g and decreased post drug loading as compared to respective carriers and also as functionalisation proceeded further.

The developed BIC-MSNs were evaluated for their release properties and mechanism by in vitro dissolution and in vitro diffusion study. The dissolution data for biorelevant and simulated media revealed no definitive distinction and hence it could be concluded that the formulation intake was independent of presence or absence of meal and could be taken without these

consideration either before or after meal without affecting its release ability. The dissolution rate of BIC-MCM-41 and BIC-MCM-41-A was found to be enhanced 3.12 and 2.61 times as compared to BIC. The release model followed by BIC-MCM-41 was Weibull and the one which defined release from BIC-MCM-41-A was Higuchi. The increment in permeability was 4.66 times and 2.71 with respect to BIC for BIC-MCM-41 and BIC-MCM-41-A. The in vitro diffusion study was carried out for parenteral formulations. Drug release from PAA-MSNs was found to varying inversely and decreased with increase in pH. Maximum BIC release at 72 h was observed at pH 5.6 with percentage cumulative release being 89.92±0.65%. The release at pH 6.8 and 7.4 was found to be 68.85±0.98 % and 30.72±0.72 % respectively. For BIC-FA-MSN after 72 h the release was 89.92%, 81.25% and 76.74% at pH 5.6, 6.8 and 7.4 respectively. The anomalous transport mechanism was observed in these cases.

BIC formulations BIC-MCM-41, BIC-MCM-41-A, BIC-PAA-MSN and BIC-FA-MSNs were evaluated for in vitro cytotoxicity by MTT assay. The IC_{50} value of BIC was found to be 16.24 ± 0.37 and 25.72 ± 0.84 mg/mL for LNCaP and PC-3 respectively. They were significantly reduced for BIC-PAA-MSNs as compared to BIC to 8.21 ± 0.72 and 17.4 ± 0.99 mg/mL for LNCaP and PC-3 cells respectively. The percentage viability of cells decreased as the concentration of formulation increased. mechanisms were adjudged by FACS protocol using Annexin V-FITC apoptosis detection kit. The cells were treated with BIC,BIC-MCM-41, BIC-MCM-41-A, BIC-PAA-MSN,BIC-FA-MSN NPs and free BIC for 24h. For LNCaP cells, total apoptosis was observed as 32.22%, 64.07%, 78.34% and 87.48% in cells treated with BIC-MCM-41, BIC-MCM-41-A, BIC-PAA-MSN and BIC-FA-MSN respectively. For PC-3 cells total apoptosis including early and late was 30.32%, 61.53%,82.24 % and 92.51% respectively for BIC-MCM-41, BIC-MCM-41-A, BIC-PAA-MSN and BIC-FA-MSNs. The hemolysis exhibited was 2.1 %, and 1.08 , 1.25 and 0.96 %, 0.85% for BIC-MCM-41, BIC-MCM-41-A, BIC-FA-MSN and BIC-PAA-MSNs respectively. All the results obtained were meeting

the safety criteria of <5%. Poor oral bioavailability of BIC is attributed to its limited solubility. To determine the oral bioavailability of BIC oral pharmacokinetic study of BIC, BIC-MCM-41 and BIC-MCM-41-A was carried out in male swiss albino mice. All the blood samples collected were adequately processed and analysed by a well-developed and validated RP-HPLC-FL method. . The bioavailability of BIC-MCM-41 MSNs was 2.61 and 1.38 times more as compared to BIC and MF respectively. Whereas, the value was 1.71 in case of functionalized matrix MCM-41-A. The intravenous pharmacokinetic parameters of all the 5 different samples administered were quite different from each other. Free BIC exhibited a more rapid clearance from blood with $t_{1/2}$ 9.2 h and AUC 89.09 µg/mL h. BIC loaded MCM-41 NPs also exhibited a half-life of 12.25 h, Whereas, BIC-PAA-MSN exhibited a slow and steady clearance with longer $t_{1/2}$ 29.61 h and higher AUC 423.14 µg/mL h (3.21 and 4.74 times) as compared to free BIC. Higher $t_{1/2}$ indicates the ability of nanocarriers to accumulate at tumour site and give enhanced therapeutic effect. BIC-FA-MSN also exhibited a half-life of 16.22 h and AUC of 186.19 µg/mL h. BIC-MCM-41-A showed t1/2 and AUC of 11.93 min 178.81 µg/mL h respectively. The biodistribution study and histological examination established the safe nature of MSN nullifying any chances of toxicity to healthy organs.The DSC and SXRD data revealed that the synthesized MSNs were stable at 40±2 ºC and 75±5 %RH for the tested duration of 6 months. There was absence of any drug leakage and identical LXRD pattern at 0^{th} month and after 6^{th} month indicated the intactness of mesoporous skeleton and stability.

Thus, a successful entrapment of both the drugs ETO and BIC was achieved along with a high loading percentage. Encouraging results obtained for the mesoporous formulation of these drugs give a ray of hope for future developments in the field of targeted treatment of cancer. They can play a major role in finding a sure shot treatment for this deadly disease. However, more research and human clinical trials need to be undertaken to further determine their safety and effectiveness before they enter the commercial formulation market.