CHAPTER 5: FORMULATION DEVELOPMENT AND CHARACTERISATION

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5.1 Materials

Pure anhydrous active pharmaceutical ingredients (API) Etoposide (ETO) and Bicalutamide (BIC) were obtained as a sample gratis from Intas Pharmaceuticals Ltd, (Ahmedabad, Gujarat, India). Other ingredients utilized in synthesis of MSNs like Fumed silica, tetra methyl ammonium hydroxide pentahydrate (TMAOH; ≥98%), (3-Aminopropyl) triethoxysilane (99% purity) (APTES) were purchased from Sigma Aldrich (St. Louis, USA). Surfactant template Cetyl trimethyl ammonium bromide (CTAB) was supplied by Loba Chemie (Mumbai, India).

Chemicals required for preparation of dissolution media viz, hydrochloric acid, sodium acetate trihydrate, acetic acid, monobasic potassium phosphate, sodium chloride, ammonium acetate and potassium dihydrogen phosphate were purchased from S.D. Fine Chem Ltd, Mumbai. Vital ingredients like pancreatin, pepsin, sodium taurocholate and Lecithin were obtained from Sigma Aldrich. For diffusion study, dialysis tubes having cut-off Molecular weights (Mw) of 7000 g/mol and 3500 g/mol were purchased from Himedia laboratories, Mumbai.

Chemicals used in functionalization of MSNs like poly acrylic acid (PAA) (average molecular weight=1800) and pure folic acid were purchased from Sigma Aldrich and Himedia laboratories respectively. 1-(3-Dimethylaminopropyl)-3-Ethyl carbodiimide hydrochloride (EDC.HCl), N-HydroxySuccinimide (NHS), fluorescein isothiocyanate (FITC) and 4,6-Diamidino-2-Phenylindole Dihydrochloride (DAPI) staining dyes were purchased from SRL Chemicals (Mumbai).

Analytical and HPLC grade reagents methanol (MeOH) and AR grade DMSO, acetone and dimethyl Formamide (DMF) were procured from Fischer Scientific, India. De-ionized water was used for the synthesis of NPs through the entire work. Toluene was purchased from Loba Chemie, Mumbai. All the chemicals were used without any further purification.

Human epithelial colorectal adenocarcinoma Caco-2 cells and Human prostate cancer cell lines PC-3 and LNCaP were procured from National Center for Cell Science (NCCS), Pune (Maharashtra, India). Chemicals for cell culture and cytotoxicity study *viz;* Roswell Park Memorial Institute (RPMI) -1640 media, Dulbecco's modified eagle medium (DMEM), antibiotic mixture containing penicillin and streptomycin solutions of concentration 1% and Fetal bovine serum (FBS) and other materials used in cell line study were purchased from Hi media Laboratories (Mumbai, India). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich (St Louis, MO, USA). The blood was collected from a healthy human volunteer from blood bank for carrying out hemolysis study. Molecular biology grade DMSO was purchased from SRL ltd. Annexin V-FITC apoptosis kit was obtained from BD Biosciences. Cell culture flasks, well plates and trans well inserts were purchased from HI media Laboratories. Lucifer yellow dye used in Caco-2 cell permeability study was procured from Thermo scientific India.

Four months old healthy male Swiss Albino Mice (SAM) weighing 22-28g were provided by Zydus Research Centre, Ahmedabad, India for carrying out the pharmacokinetic and biodistribution studies. The experimental protocol was approved by the *Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Institutional Animal Ethics Committee (IAEC)* having protocol number MSU/IAEC/2017-18/1724. Anticoagulant EDTA disodium salt was procured from Loba Chemie, Mumbai. All the animals were kept under standard laboratory conditions with free access to food and water and acclimatized to the animal facility for at least 7 days before starting the experimental procedures.

5.2 Methods

5.2.1 Synthesis of bare MCM-41 type of MSNs

MCM-41 mesoporous silica nanoparticles were synthesized based on template synthesis method with few alterations (1). For the fabrication of MSNs, 2.21 grams (g) of CTAB playing a role of structural template was dissolved in de-ionized water and blended for 5 minutes (min). 3.24 milli liters (mL) of TMAOH was added to the reaction blend dropwise under continuous magnetic stirring. Later, 3g fumed silica was added after a time interval of 10 min. Finally, this was subjected to hydrothermal treatment for 48 h at 383K. The slurry obtained was filtered and dried appropriately. The asynthesized MSNs obtained in the form of dry powder were subjected to calcination at 823K for 6h to completely evacuate the template.

5.2.2 Surface functionalization of MCM-41 NPs

5.3.2.1 Amine functionalization of MCM-41

Aminated MCM-41 NPs were obtained by reaction of MCM-41 NPs with APTES as per a reported method in the literature (2). Typically, 250 mg of MCM-41 was accurately weighed and transferred to a round bottom flask (RBF). The MCM-41 MSNs were dispersed in 30 mL toluene. Thereafter, 3.43 mL of APTES was added. The reaction was kept under vigorous stirring at 70°C for 12 h. Thereafter, RBF was allowed to cool slowly at RT. The reaction mixture obtained was filtered and washed with methanol. The product obtained was dried and labelled as MCM-41-A. Successful functionalization with APTES laid a strong foundation for further functionalization with Polyacrylic acid (PAA).

5.3.2.2 Surface functionalization with Polyacrylic acid

PAA groups were grafted onto MCM-41-A as per the literature reported method with slight modifications (3). To a 100 mL RBF containing 30 mL dimethyl formamide 392 mg of MCM-41-A NPs were added. Subsequently, 400 mg PAA was added to the reaction mixture. This

was further subjected to vigorous stirring for 2 h at an elevated temperature of 140℃. The product was obtained by filtration and washed with anhydrous methanol. The powder obtained was dried for 4h at 45℃. The success of grafting was ascertained using FT-IR spectroscopy. Obtained NPs were labelled as PAA-MSN.

5.3.2.3 Surface functionalization: FA grafting on Bare MSNs

A facile post-synthetic grafting strategy was adopted for attachment of FA molecules onto the surface of MCM-41 NPs as reported earlier with minor moderation (4). 50 mL Round bottom flask (RBF) containing reaction mixture comprising of 10mL DMSO, FA (300 milligrams (mg)), NHS (90 mg), EDC.HCl (150 mg), and APTS 200µL was taken. After vigorous stirring for 7 h, 12 mL toluene and 150 mg of MCM-41MSNs were added. The aluminum foil covered RBF was kept under continuous stirring for 48 h at room temperature (RT). Further, the reaction mixture obtained was filtered and washed properly with various solvents in the order toluene (30mL), DMSO (30 mL), water (50mL) and acetone (40mL) to remove any free FA remaining. Final product was dried and named FA-MSNs. All the MSNs were sterilized using 0.22u PVDF sterile filters prior to intravenous administration.

5.2.3 FITC Labelling of MSNs

The NPs were labelled with FITC as per the procedures adopted earlier with slight modifications(5, 6). For FITC labelling, a methanolic solution of FITC (0.3 mg/mL) was used. In which, the 10 mg aminated MSNs, PAA-MSNs and FA-MSNs were kept for 12h, under continuous stirring in dark. Later, the solution was centrifuged and washed with methanol for complete removal of unconjugated FITC until the supernatants obtained were rendered colorless. The final product was tagged as FITC#A@MSNs, FITC#PAA-MSNs and FITC#FA@MSNs. An overview of entire synthesis scheme is given in Fig 1.

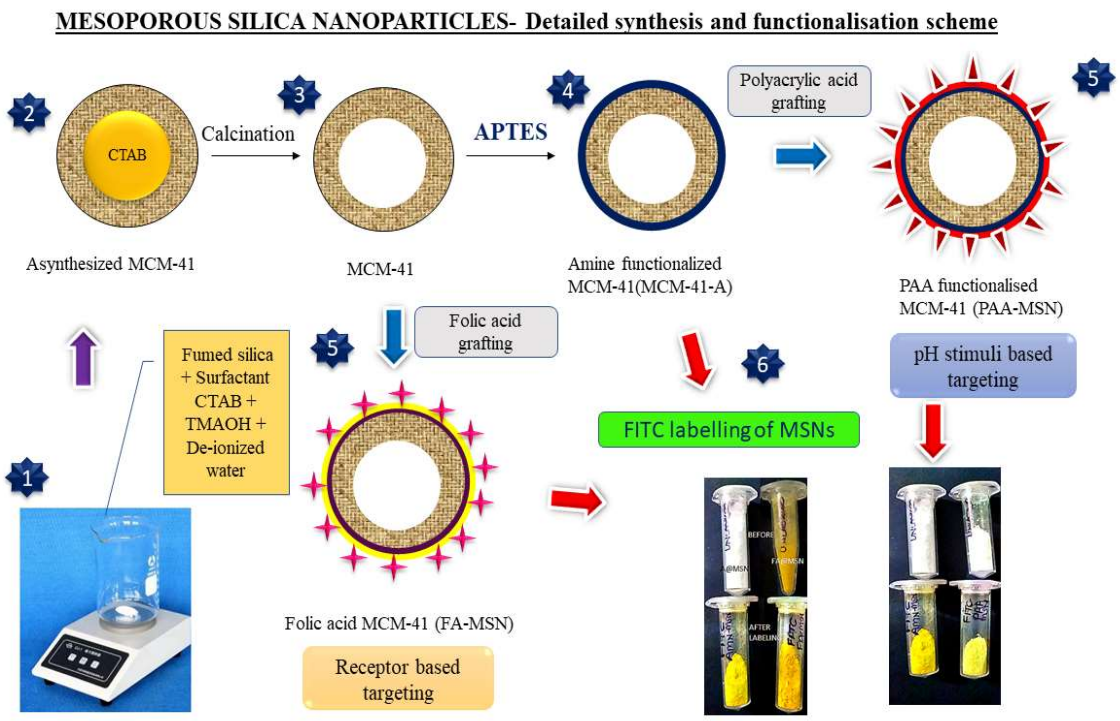

Figure 5.1. Overview of the synthesis, functionalization and FITC labelling of MSNs

5.4. Solid state evaluation of bare and surface functionalized MSNs.

Identical methods and techniques were used for characterization of bare and surface functionalized drug loaded as well as drug free MSNs. The synthesized MSNs were characterized for adjudging the mesoporous skeleton integrity, presence of functional groups for confirming the success of surface functionalization, surface properties and porosity, internal structure and morphology *etc* by employing various characterization techniques.

5.4.1 Fourier Transform Infra-red (FT-IR) analysis

FT-IR is useful in qualitative estimation for identification of functional groups present. Structural changes and absence of crystalline nature after loading can lead to alteration in the bonding between chemical groups, which can be detected by FT-IR (7). The characteristic functional groups from silica and functionalizing agents were characterized by FT-IR spectra taken in potassium bromide pellets were taken on a Bruker ALPHA-T instrument scanned from 600-4000 cm^{-1}. FT-IR spectra also served as a proof for successful synthesis and

functionalization of MCM-41 mesoporous silica nanoparticles step by step. This was ascertained in the FTIR spectra of MCM-41. MCM-41-A, PAA-MSN and FA-MSN.

To ensure complete entrapment of drug into the mesopores FT-IR spectra of bare mesoporous carriers asynthesized MCM-41, MCM-41, MCM-41-A, PAA-MSN, FA-MSN and drug loaded; ETO-MCM-41, ETO-MCM-41-A. ETO-PAA-MSN, ETO-FA-MSN, BIC-MCM-41, BIC-MCM-41-A, BIC-PAA-MSN and BIC-FA-MSN NPs were compared.

5.4.2 Differential scanning calorimetry (DSC) analysis:

Melting point and loading were determined by DSC Shimadzu-TA 60 thermal analyzer equipped with the TA 60-WS software. The heating rate was kept at 10°C/min. The existence of ETO and BIC on silica matrix in amorphous or crystalline form and complete encapsulation was further confirmed by DSC. Absence of any melting point depression is indicative of presence of drug in non-crystalline state in pores (8). Melting points of ETO and BIC were determined individually. DSC analysis of ETO-MCM-41, ETO-MCM-41-A, ETO-PAA-MSN, ETO-FA-MSN, BIC-MCM-41, BIC-MCM-41-A, BIC-PAA-MSN, BIC-FA-MSN was done and absence of any crystalline sharp drug peaks was ensured.

5.4.3 Thermogravimetric analysis

TGA analysis was performed to determine weight loss of samples by Shimadzu thermogravimetric analyzer (TGA) 50 WS nearly 5 mg from 25 to 700°C, at a temperature rate of 10°C/min.

5.4.4 Wide angle X-Ray diffraction analysis (W-XRD)

Absence of crystalline ETO and BIC on exterior pore walls and crystalline nature of drugs was confirmed by Wide angle XRD (WXD) measurements taken on a Bruker AXS instrument (Germany) fit with D8 Focus software. The scanning range was 10-50°.

5.4.5 Low angle X-Ray diffraction analysis (L-XRD)

EMPYREAN, PANalytical model equipped with Cu K radiation beam operating at 40 kV and 40 mA was used to determine low angle X-Ray powder diffraction (LXD) pattern. The structure of pores was ascertained by low-angle XRD measurements. The spectra was an indication of the intactness of the mesoporous structure. LXRD measurements were taken for MCM-41, ETO-MCM-41, MCM-41-A, ETO-MCM-41-A, PAA-MSN, ETO-PAA-MSN, FA-MSN and BIC-MCM-41, BIC-MCM-41-A, BIC-PAA-MSN and BIC-FA-MSN.

5.4.6 Zeta potential and size determination:

Particle Z-average size and charge were measured using dynamic light scattering (DLS) and electrophoretic mobility measurements respectively, using the Malvern Zetasizer Nano ZS (Malvern instruments, Malvern, UK). Zeta potential indicates the surface residual charge of the particles. The zeta potential measurements were done for MCM-41, MCM-41-A, PAA-MSN, FA-MSN and drug loaded MCM-41, MCM-41-A, PAA-MSN and FA-MSN.

5.4.7 Nitrogen sorption analysis:

The *Brunauer–Emmett–Teller* (BET) surface area, Barrett-Joyner-Halenda (BJH) surface area, BJH pore size and pore volume information was gathered from the Nitrogen sorption studies. Porosity and surface area were determined by Nitrogen adsorption-desorption isotherms measured at -196°C using an ASAP 2020 volumetric adsorption apparatus from Micromeritics (Norcross, GA). Before the analysis, the samples were degassed under vacuum for 5 h at 70°C in the Degas port of the adsorption analyser. The specific surface area of the samples was obtained on the basis of the standard BET method. The porosity measurements were done for MCM-41, MCM-41-A, PAA-MSN, FA-MSN and ETO-MCM-41, ETO-MCM-41-A, ETO-

PAA-MSN, ETO-FA-MSN, BIC-MCM-41, BIC-MCM-41-A, BIC-PAA-MSN and BIC-FA-MSN.

5.4.8 SEM and TEM analysis:

Morphological characterization was performed using scanning electron microscopy (SEM) (FEI-Quanta 200 operating at 20 kv) (Thermo Scientific, USA). The samples were coated with gold to make them conducting before imaging. TEM images were resolved over a photographic film taken on a TEM CM-200 (Philips, India) model operated at 200 kV voltage with a resolution of 2.4 A. The images were resolved over a photographic film. SEM and TEM analysis was done to determine morphological and internal structure of bare MCM-41 and functionalized PAA-MSN and FA-MSNs respectively.

5.4.9 Elemental detection and quantification of surface moiety

An elemental detection for pristine and surface decorated nanoparticles was performed in order to investigate the presence surface elements for bare and surface functionalized nanoparticles adopting SEM-EDX (scanning electron microscopy-energy-dispersive X-ray spectroscopy) analysis conducted on FEI-quanta 200 model (Thermo Scientific, USA). EDS data clearly gave the qualitative and quantitative data of various elements present pre and post surface functionalization .

Further, the presence of carbon and nitrogen moiety in MCM-41 and after amine, poly acrylic acid and Folic acid acid grafting was studied by SEM-EDX analysis of respective nanoparticles. The quantitative data for surface functionalization was also obtained from TGA analysis.

5.5 References:

1. Wouters BH, Chen T, Dewilde M, Grobet PJ. Reactivity of the surface hydroxyl groups of MCM-41 towards silylation with trimethylchlorosilane. Microporous and mesoporous materials. 2001;44:453-7.

2. Yoncheva K, Popova M, Szegedi A, Mihály J, Tzankov B, Lambov N, et al. Functionalized mesoporous silica nanoparticles for oral delivery of budesonide. Journal of Solid State Chemistry. 2014;211:154-61.

3. Yuan L, Tang Q, Yang D, Zhang JZ, Zhang F, Hu J. Preparation of pH-responsive mesoporous silica nanoparticles and their application in controlled drug delivery. The Journal of Physical Chemistry C. 2011;115(20):9926-32.

4. de Oliveira Freitas LB, Bravo IJG, de Almeida Macedo WA, de Sousa EMB. Mesoporous silica materials functionalized with folic acid: preparation, characterization and release profile study with methotrexate. Journal of Sol-Gel Science and Technology. 2016;77(1):186-204.

5. Salis A, Fanti M, Medda L, Nairi V, Cugia F, Piludu M, et al. Mesoporous silica nanoparticles functionalized with hyaluronic acid and chitosan biopolymers. Effect of functionalization on cell internalization. ACS Biomaterials Science & Engineering. 2016;2(5):741-51.

6. Huo J, Aguilera-Sigalat J, El-Hankari S, Bradshaw D. Magnetic MOF microreactors for recyclable size-selective biocatalysis. Chemical science. 2015;6(3):1938-43.

7. ALOthman ZA. A review: fundamental aspects of silicate mesoporous materials. Materials. 2012;5(12):2874-902.

8. Heikkilä T, Salonen J, Tuura J, Hamdy M, Mul G, Kumar N, et al. Mesoporous silica material TUD-1 as a drug delivery system. International journal of pharmaceutics. 2007;331(1):133-8.