A synopsis on Ph.D. work entitled

"FABRICATION OF TARGETED FORMULATIONS TO IMPROVE EFFICACY OF THERAPY IN BREAST CANCER TREATMENT"

Submitted by:

Mr. Parth J. Thakkar (FOPH/22)

M.Pharm (Pharmaceutics)

Submitted for

Award of degree of Doctor of Philosophy in Pharmacy

Under the guidance of

Prof. (Dr.) Krutika K. Sawant,

(Professor of Pharmaceutics)

The Maharaja Sayajirao University of Baroda

Certificate

Name of the PhD Research Scholar	Mr. Parth J. Thakkar
Enrolment number	FOPH/22
Enrolment date	10th July 2019
Title	"FABRICATION OF TARGETED FORMULATIONS TO IMPROVE EFFICACY OF THERAPY IN BREAST CANCER TREATMENT"
Name and Designation of Research Guide	Prof. Krutika K. Sawant Head, Pharmacy Department Dean, Faculty of Pharmacy
Department	Pharmacy Department
Faculty	Faculty of Pharmacy
Institute	The Maharaja Sayajirao University of Baroda.

Mr. Parth Thakkar
Research Scholar
M.Pharm (Pharmaceutics)

Prof. (Dr.) Krutika K. Sawant
Research Guide
Professor, Department of Pharmaceutics
Faculty of Pharmacy
The M.S. University of Baroda

Prof. (Dr.) Krutika K. Sawant
Head
Pharmacy Department
The M.S. University of Baroda

Prof. (Dr.) Krutika K. Sawant
Dean
Faculty of Pharmacy
The M.S. University of Baroda

Synopsis

INTRODUCTION

Out of all cancers, lung cancer was leading type of cancer until 2020, but latest review and statistics show that breast cancer has been the topmost of all cancers with occurrence in 1 out of 8 women(1). In 2021, 2.4 million people were affected with breast cancer leading to 6,54,368 deaths. Out of total patients, 80% were hormone receptor positive patients, specifically estrogen receptor positive(2). The vast majority of these tumors are initially dependent upon activation of ERαand Erβ nuclear receptors promotes proliferation and survival of both normal and cancerous breast tissues through transcription of pro-survival genes and activation of cellular signalling(3). Owing to the strong dependency of breast tumorigenesis on the estrogen-ER axis, estrogen suppression and ER antagonists have remained main stay of ER^+ breast cancer treatment for several years.

Endocrine therapies such as selective ER modulators (SERMs), selective estrogen down regulators (SERDs) and aromatase inhibitors are approved as an adjunct therapy for patients with ER^+ breast cancer(4). Aromatase Inhibitors deplete the levels of systemic estrogen by blocking the conversion of androgens to estrogen. SERMs compete with estrogen for binding to ER and have mixed agonist/antagonist capacities and are first line of treatment for pre-menopausal women. SERDs such as fulvestrant are said to work by suppressing ER activity by impairing intra-nuclear ER mobility(3, 5).

ROLE OF NANOCARRIERS IN CANCER THERAPY.

Nanotechnology provides an innovative and promising alternative to small molecule chemotherapeutics circumventing MDR by encapsulating, attaching and conjugating drugs or therapeutics biological products to nanocarriers(6). Nanocarriers can include small molecules such as lipids or polymer nanoparticles that target the therapeutic payload to tumors or tumor cells(7). Simultaneously multifunctional drug loaded nanocarriers can also enhance particle penetration of physiological barriers and protect the labile drugs or therapeutic biological products(8).

Polymer Lipid hybrid nanocarriers (PLHNCs)

Variety of nanocarriers have been studied i.e., Liposomes, polymeric nanoparticles, micellar solutions for better delivery of anticancer therapeutics with surface modifications with specific ligands(9). Among all the nanocarriers, liposomes and polymeric nanoparticles have been most researched as novel strategy for delivery of variety of therapeutics(10). Polymeric nanoparticles possess key attributes such as long-term stability and tunability but generally

lacks inherent biocompatibility and potential toxicity of long-term accumulation of synthetic molecules in the body(11). On contrary, liposomes are biocompatible, non-denaturing interface, but unfortunately lacks long term stability. PLHNCs have advantages of both liposomes and polymeric nanoparticles. Some extra-ordinary advantages provided by PLHNCs are: the solid core made up of polymer act as a cytoskeleton that provides mechanical stability, controlled release morphology narrow size distribution and higher availability of surface area. The outer lipid coat that encapsulates the polymeric core is biocompatible in nature and mimics the characteristics of cellular membrane(12). The lipid shell can interact with a huge variety of drugs and indigenous molecules and surface can be modified for efficient targeting(13).

In order to mitigate certain drawbacks associated with liposomes and polymeric nanoparticles, novel integrated structures known as polymer lipid hybrid nanocarriers (PLHNCs) were introduced. Briefly, to create a potentially superior delivery system, the biomimetic properties of lipids and the architectural benefits of polymer structure are combined(14). PLHNCs are solid, submicron sized particles composed of minimum two components; the polymer and the lipid. In the developed hybrid system(15), various bioactive molecules such as drugs, genes, proteins and targeting ligands may be entangled, absorbed or covalently bond(16). Poly lactic-co glycolic acid (PLGA), polycaprolactone (PCL), dextran or albumin are likely choices for biodegradable polymers because of their biocompatibility, biodegradability, non-toxicity and prior use in licensed product(17). Zwitter ionic, cationic, anionic and neutral phospholipids such as lecithin, hydrogenated soyabean phosphatidyl choline (HSPC), soyabean phosphatidyl choline (SPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-3-trimethylammonium-propane (DOTAP) are commonly used lipids(18).

Mesoporous Silica nanoparticles (MSNPs)

Over the last two decades, a large number of nanoparticles delivery systems have been developed for cancer therapy, including organic and inorganic materials(19, 20). Among inorganic materials the functionalization of mesoporous silica nanoparticles (MSNPs) with molecular, supramolecular or polymer moieties, gives them a great versatility(21). While performing drug delivery tasks, which makes the delivery process highly targeted and controllable(22). Mesoporous silica nanoparticles are highly biodegradable and mostly eliminated through renal clearance(23). The unique mesoporous structure of silica facilitates effective loading of drugs and their subsequent release of the target site. Other features such as

tuneable pore size and uniform pore size leading to uniform release, zero premature release and good biocompatibility and biodegradability are an added feather to their cap(24-26).

Due to strong Si-O bond, silica- based mesoporous nanoparticles are also more stable to external response such as degradation and mechanical stress. Hence, potential drugs which were earlier ignored because of poor pharmacokinetics can be re-evaluated(27, 28). The surface of the nano-carriers can be modified to increase blood circulation half-life and enhanced biodistribution, while attachment of targeting ligand to the surface of nanocarrier can lead to increase in their cellular uptake(29, 30). Among a variety of nano drug delivery system, mesoporous silica materials have several attractive features for use in the delivery of water-insoluble drugs(31, 32). These particles have porous interiors and large surface areas which can be used as a reservoir for storing hydrophobic drugs. Textual properties of MSNs provide the possibility to load high percentage of drugs within MSNs carriers(33, 34). The pore size is tuneable and can be tailored to selectively store different molecules of interests, while the size and shape can be altered to maximize cellular uptake. Unlike polymer-based nanoparticles, this sturdy inorganic material can withstand many organic solvents(35). Moreover, silica is endogenous substance, especially abundant in bone cartilage and other supportive tissues. It is 'generally regarded as safe (GRAS)' by USFDA(36).

Surface modifications and attachment of various moieties on MSNPs can be done as per requirement of drugs release(37). The surface modification is needed to load proper type of drug molecules (hydrophobic/hydrophilic or positive/ negative charged), specific actions can also have a natural quality or characteristics by the functionalization through chemical links with other materials such as stimuli-responsive, luminescent or capping material, leading to smart and multifunctional properties(38). The platform can easily be further functionalized, for instance by attachment of fluorophores for imaging and ligands for cell specific targeting. Active surface enables functionalization to changed surface properties and link therapeutic molecules(23, 39). The biggest challenges in cancer treatment are not able to achieve targeted or selected delivery of drug molecules. Various targeting molecules such as folate, aptamer antibodies, hyaluronate and others can be attached on surface functionalized MSNs(40). They are also suitable for multidrug delivery. On a very general level, surface functionalized MSNs seen to reduce the observed harmful effects as compared with pristine mesoporous silica(41, 42). As nanocarriers, mesoporous silica nanoparticles with unique mesoporous structure have

been explored as effective drug delivery systems for a variety of therapeutic agents to fight against various kinds of diseases including diabetes, inflammation and cancer.

Selection of Drug Candidate

1. Fulvestrant (Selective Estrogen Receptor Degrader)

Fulvestrant, a newer anticancer agent is utilized in management of progressive breast cancer. The action of drug includes binding, blocking and degradation of estrogen receptors, leading to cessation of estrogen signalling through receptors in body(43). It acts as complete antagonist unlike SERMs like tamoxifen, it doesn't show any agonist activity, which in turn leads to blockage of estrogen activity completely(4). The formulation is available in pre-filled intramuscular injection which is well tolerable. But due to being non-targeted, it presents various side effects, that are related to estrogen deficiencies such as weight gain, thromboembolic problems(44), neutropenia, leukaemia, anaemia. Some fewer common side-effects observed are vulvovaginal dryness, pelvic pain and vaginitis(45).

2. Exemestane (Aromatase Inhibitor)

Exemestane, an orally available aromatase inhibitor and available as 25 mg once a day oral tablet, is well tolerated dosage form(46). Exemestane acts by behaving as a false substrate for aromatase enzyme, which causes enzyme inactivation by binding to active site, also termed as suicide inhibition. The formulation suffers from two major drawbacks of low solubility and rapid first pass effects. The drug related effects lead to liver dysfunction and sometimes liver failure(47).

Aim & Objectives

Aim:

The present work is aimed to develop targeted delivery systems namely Polymer Lipid Hybrid Nanocarriers and Mesoporous Silica Nanoparticles with surface modifications in order to target the formulation to breast cancer cells. The developed nanoparticles will be able to release the loaded drugs within tumor microenvironment and thereby reduce the toxicity associated with conventional anticancer therapy.

Synopsis

Objectives:

For PLHNCs

1. Selection of suitable polymers for synthesis of PLHNCs.
2. Development of PLHNCs.
3. Optimization of prepared formulation using Box Behnken Design.
4. Evaluation of the prepared PLHNCs for their physicochemical properties.
5. Selection of suitable ligand for surface modification.
6. Attachment of ligand and evaluation of the product for cell cycle and cytotoxicity studies.

For MSNs

1. Selection of excipients and method of synthesis of MSN using OFAT analysis.
2. Synthesis of MSN with required characteristics such as surface area pore size and pore volume.
3. Optimization of prepared MSN using Response Surface Methodology.
4. Functionalization of prepared MSN surface.
5. Loading of FLV and EXE into functionalized MSN.

Overall objectives:

1. Preparation and optimization of drug loaded nanocarriers for FLV and EXE.
2. Attachment of suitable ligand for active targeting of drugs to cancer cells.
3. Evaluation of prepared nano-carriers for their safety and efficacy profile by in-vitro and in-vivo studies.
4. To carry out cell uptake and cell cytotoxicity studies using suitable cell lines.
5. To carry out pharmacokinetic profile studies of developed nanocarriers.
6. To carry out tumor regression study using chemically induced rat tumor model.

Hypothesis, Rationale & Expected Outcomes

Hypothesis:

It is hypothesized that prepared nanoparticulate formulation in association with attached ligands will achieve targeting of the nanocarriers to tumor cells which will facilitate enhanced cellular uptake and hence greater drug localization in cancer cells.

Synopsis

Rationale:

The rationale of the present work is to develop novel drug delivery systems for anti-neoplastic drugs for targeted delivery and improvement of both cellular uptake and efficacy of drug for the treatment of breast cancer. The existing formulation of **Fulvestrant** is depot formulation, which has some significant problems such as its inability to reach steady state plasma concentration, its route of administration, its termination of therapy and its metabolism. Similarly, for **Exemestane** its low solubility and bioavailability is matter of concern. Nanocarriers are the most widely researched as novel strategy for delivery of variety of therapeutic agents for their efficient delivery of drugs and biocompatibility. Targeted, biodegradable polymer-based nanoparticles in the treatment of cancer will show selective accumulation of drug at tumor site and increase the drug concentration due to their nano dimension and thus will lead to a reduction in the incidence of side effects of the anti-cancer agents. The addition of **targeting moieties** such as Hyaluronic acid and Folic acid will improve the specificity of the nanoparticles towards target, resulting in increase in intratumoral concentration of drug and decreasing its side effects.

Expected outcomes:

This research is expected to result in development of novel nanocarrier system consisting of targeted moieties for efficacious therapy.

1. Targeting drugs using ligand-based system will enhance the efficacy of chemotherapy by decreasing the resistance of cancer cells to chemotherapeutic agents and will bridge gap in the treatment of chemotherapy resistant breast cancer.
2. It will enhance the efficiency of chemotherapy to a level that cannot be achieved by applying its components separately in treatment of breast cancer. Moreover, highly selective and targeted MSNs and PLHNCs for clinical evaluation as a novel alternative to currently researched chemotherapeutic strategies is anticipated.
3. It also emphasizes that targeted therapy will be the future drugs or future therapeutics for either increasing efficacy of available treatment or providing superior treatment for otherwise untreatable diseases such as cancer.

Part A

1. **Polymer Lipid Hybrid Nanocarriers (PLHNCs)**
   a. Two step method

b. One step method
   i. Modified solvent evaporation method
   ii. Single step nanoprecipitation

Selection of method of preparation

The method of preparation was selected on the basis of One Variable at a time (OVAT) method utilized for screening. The optimization parameters were divided into two groups: i) Process Parameters, ii) Formulation Parameters.

i) Process Parameters

a) Stirring speed
b) Stirring time
c) Rate of addition of organic solution
d) Stirring temperature

ii) Formulation Parameters

a) Lipid concentration
b) Polymer concentration
c) Aqueous to organic phase ratio
d) Ethanol concentration in aqueous phase
e) Lipid to polymer ration
f) Total drug concentration

Single Step Nanoprecipitation

EXE and FLV loaded PLHNCs were prepared by single step nanoprecipitation method. Briefly, FLV and PLGA were dissolved in acetonitrile to constitute organic phase. Phospholipid was dissolved in 4% ethanolic aqueous solution to constitute aqueous phase. The organic phase was injected using syringe at rate of 1ml/min slowly. Solution was kept for stirring at 1800 rpm for 4h. The excess organic solvent was removed on rotary flask evaporator(48). The nanoparticle free of organic phase were centrifuged at 12000 rpm for 15 mins to separate free drug and nanoparticles. The supernant contains free drug and nanoparticles settles down at bottom. The evaluation of free drug is carried out using HPLC analysis. The nanoparticles were then freeze dried using Virtis Lyophilizer at specified

lyophilization cycle using Trehelose as a cryoprotectant in optimized ratio to improve storage stability.

Evaluation of prepared PLHNCs

The characterization of EXE-PLHNCs and FLV-PLHNCs was done with respect to size, PDI, zeta potential, surface morphology and drug entrapment efficiency.

a) Particle Size and Zeta potential

Size and PDI of NPs were determined by dynamic light scattering, while zeta potential was determined on the basis of electrophoretic mobility under an electric field by using zeta sizer (Nano ZS, Malvern, U.K.)

b) Surface Morphology

The surface morphology of PLHNCs was evaluated by transmission electron microscopy (TEM). A drop of diluted PLHNCs suspension was placed on a membrane coated grid surface and immediately stained with a drop of 1% phosphotungstic acid. After 1 min excess fluid was removed and the grid was air dried and was examined under High Resolution Transmission Electron Microscope (HRTEM; Fei, Electron Optics)(49).

c) Fourier Transform Infrared Spectroscopy

Fourier transform Infrared Spectroscopy measurement were carried out on a Shimadzu FTIR Spectroscopy Instrument. The machine was operated at a resolution of 4 cm^{-1} in the range of 450-4000 cm^{-1}.

d) X Ray Diffraction

X-ray Diffraction (XRD) pattern of the NPs was recorded on X'PertPRO-PANalytical (Netherlands) Advanced X-ray diffractometer. Free drug, blank PLHNCs and drug loaded PLHNCs were analysed. A known amount of each sample (10-15 mg) was loaded in a 25 mm polymethyl methacrylate (PMMA) holder. The diffractograms were analysed with X'Pert high score software(50).

e) Entrapment Efficiency

Drug entrapment efficiency was calculated by direct lysis method. Briefly, weighed amount of freeze dried PLHNCs was added to 5 mL acetonitrile (ACN) followed by brief sonication to

lyse the particles. The mixture was then filtered and diluted suitably analysed by HPLC (Agilent Infinity II 1260) method previously developed and validated. Reversed phase C18 column (5µm, 4.6 mm×250 mm, Agilent) was used for chromatographic separation. Percentage drug encapsulation efficiency was calculated by using following equation(51).

$$Entrapment\ Efficiency\ (\%) = \frac{Amount\ of\ drug\ entrappedinPLHNCs}{Total\ Amount\ of\ Drug} \times 100$$

In vitro drug release

The *in vitro* drug release study of optimized formulations was performed in phosphate buffer saline (pH= 7.4) and acetate buffer saline (pH 5.4); both containing a small amount of method (in 5:1 ratio) to mimic the physiological and lysosomal pH, respectively by using dialysis bag method. Briefly, a known amount of lyophilized PLHNCs (equivalent to 5 mg of drug) was dispersed in 2 mL of respective buffer) and put into dialysis bag. This dialysis bag was then immersed in 20 mL of release medium. Samples were withdrawn at regular time intervals and replaced with fresh medium to maintain sink conditions. Samples were analyzed by HPLC method and drug release was recorded(52).

In vitro drug release data were fitted to various kinetic models such as zero order, first order, Higuchi model, Hixon-Crowell model and Korsemeyer-Peppas model. The regression analysis was performed. The graphs of the respective models were plotted according to the need of each equation.

In vitro cell culture studies

MDA MB 231, human breast adenocarcinoma cells (ATCC, Manassas, VA, USA) were cultured and maintained as earlier reported. In brief, MDA MB 231 cells were grown in tissue culture flasks and maintained under 5% CO2 atmosphere at 37°C. The growth medium comprised of Minimum Essential Medium Eagle (MEM, Himedia) supplemented with Earle's salts, L-glutamine, nonessential amino acids, sodium bicarbonate, sodium pyruvate, 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (Himedia, India). The growth medium was changed on alternate day. The cultured salts were trypsinized once 90% confluent with 0.25% trypsin-EDTA solution (Himedia, India). MDA MB 231 cells were seeded at a density of 10,000 cell/well and 50,000 cells/well in 96-well and6-well culture plate (Costars, Corning Inc., NY, USA) for quantitative cell viability by MTT assay and qualitative cell uptake analysis by CLSM respectively.

Cell cytotoxicity

The cell suspension was added in 96 well tissue culture plates (0.2 mL/well) and incubated overnight for cell attachment. Following attachment, the growth medium was replaced with complete medium (0.2 mL) containing the free FLV or FLV-PLHNCs) to the different wells so as to achieve net concentrations of 0, 0.1, 1, 10 and 20 µg/mL (equivalent to free FLV) for 24, 48 and 72 h. Following treatment and completion of particular time, the cells were washed with PBS, pH 7.4 followed by addition of 150 µL of MTT solution (0.5mg/mL in PBS) to each well and reincubation for 3−4 h to facilitate formation of formazan crystals. The excess solution was then aspirated carefully, and MTT formazan crystals were dissolved in 200 µL of DMSO. The optical density (OD) of the resultant solution was then measured at 550 nm using an ELISA plate reader (BioTek, USA) and cell viability was assessed.

Results and Discussion (PLHNCs)

a) Particle Size and Zeta Potential

The particle size for optimized formulation was found to be 134±2.6 d.nm and 163±5.4 d.m for FLV-PLHNCs and EXE-PLHNCs respectively. The particle size was found to be in range for intracellular perfusion and penetration into tumor microenvironment. The zeta potential for both the PLHNCs namely FLV and EXE were found to be -34 mV and -41.28 mV respectively. The negative zeta potential helps the formulation to be stable for a longer period of time during storage.

b) Surface Morphology

The particle size and shape were observed under the Transmission Electron Microscope (TEM). The shape was found to be spherical in nature and a thin ring of lipid coating was observed on the polymer surface. The particle size of blank nanoparticles was found to be 128 d. nm and the thickness of lipid layer was found to be 16.82 d. nm for FLV PLHNCs. For EXE PLHNCs the size was found to be 146 d. nm including the coat.

c) Differential Scanning Calorimetry (DSC)

The DSC curve of FLV shows the endothermic peak at 108°C, the curve of formulation doesn't show any endothermic peak deviation, indicating the matrix encapsulation of FLV in polymer lipid core. In case of EXE, there was an endothermic peak at 267°C, which diminished in case of nanoparticulate formulation, referring to engulfment of drug in polymeric matrix.

d) X Ray Diffraction pattern

The XRD pattern of FLV pure drug shows the characteristic crystalline peak at 2θ position of 12.47°, 16.36°, 18.02°, 19.78°, 22.51°. The XRD pattern of blank PLHNCs shows the crystalline peak at 2θ value of 19.26° and 23.32° respectively. But, in case of FLV loaded PLHNCs the XRD pattern shows slight shifting of XRD patterns to 2θ value of 19.32° and 23.63° respectively, indicating the encapsulation of drug into polymer matrix and conversion of crystalline form to amorphous form.

The XRD pattern of EXE pure drug shows the characteristic crystalline peak at 2θ position of 10.48°,14.18°, 15.63°, 17.80°, 21.81°. The XRD pattern of blank PLHNCs shows the crystalline peak at 2θ value of 19.25° and 23.42° respectively. But, in case of FLV loaded PLHNCs the XRD pattern shows slight shifting of XRD patterns to 2θ value of 19.45° and 23.84° respectively, indicating the encapsulation of drug into polymer matrix and conversion of crystalline form to amorphous form.

e) Fourier Transform Infrared Spectroscopy (FTIR)

The characteristic peaks of FLV were observed at 983.70 cm-1, 1197.79, 1446.21, 1608.63, 2926.01, and 2854.65, and characteristic peaks of blank PLHNCs are observed at 846.75, 943.13, 1456.26, 1759.48, and 2881.65. The characteristic peaks of FLV by FLV loaded PLHNCs were observed with slight shifting at 989.47, 1199.72, 1456.26, and 2850.79. These results were confirmatory of FLV loading into PLHNCs.

The characteristic peaks of EXE were observed at 821.68, 904.61, 1620.21, 1732.08, and 2956.87, and characteristic peaks of blank PLHNCs are observed at 844.82, 1454.33, 1757.15, and 2881.65. The characteristic peaks of EXE by EXE loaded PLHNCs were observed with slight shifting at 910.40, 1757.15, 2974.23. These results were confirmatory of EXE loading into PLHNCs.

f) Entrapment Efficiency

The entrapment efficiency for FLV loaded PLHNCs was found to be 79.63±2.32% and drug loading was found to be 7.23±1.13%. The entrapment efficiency for EXE loaded PLHNCs was found to be 82.04±1.89% and drug loading was found to be 7.45±1.64%.

Part B

2. Mesoporous Silica Nanoparticles (MSNs)

Synthesis of MSN (MCM-41)

MCM-41 was synthesized as per the literature reported method based on template-based synthesis with slight modifications. Concisely, around 0.5 g of CTAB was dispersed in 40 grams (g) deionized water at 90°C under continuous stirring. Thereafter, 2 g TEOS and 4g of ethanol was added to the mixture under continuous stirring for 2 hours (h) at 90°C. The thick slurry obtained was filtered and washed with de-ionized water and dried at RT yielding uncalcinated-MCM-41. The final step consisted of removal of the surfactant template. This was achieved by treating the powder with 10% HCl in methanol. Eventually, CTAB free MCM-41 type MSN was obtained.

Functionalization with Amine groups

The amine functionalization was done using a methodology adapted (42) with trivial modifications. Precisely weighed 500 mg MCM-41 was transferred to a round bottom flask (RBF). 50 mL of toluene was added to the RBF. Followed by addition of 6.87 ml of APTES. The reaction was kept at a higher temperature of 70 °C for 12 h. After completion of reaction, RBF was carefully removed and allowed to cool to room temperature. The reaction mixture obtained was filtered and washed with Methanol. The formation of aminated carrier was confirmed by FT-IR spectroscopy. They were tagged as MCM-41-AMN. The functionalization of MCM-41 with amine groups offered positive charged groups and imparted a base functional group to MSN for further decoration with COOH.

Preparation of Carboxyl functionalized MSNs

The preparation of carboxyl-functionalized MSNs followed previous procedures. In brief, an aliquot of MSN-NH2 (0.2 g) was dissolved in acetone (30 mL) and the solution was stirred at room temperature for 4 h. After that, 0.015 g of succinic anhydride was added into above solution. After incubation for 24 h, the synthesized MSNs-COOH was obtained by centrifugation, and washed three times with deionized water and methanol, respectively.

Synthesis of Hydrazine functionalised MSNs

The preparation of hydrazine-functionalized MSNs followed a previous procedure. In brief, an aliquot of MSNs-COOH (0.8 g) was well dispersed in a beaker containing 200 mL of PBS

buffer (0.1 M, pH 5.0), after that, EDC·HCl (2.88 g) and NHS (1.73 g) were added into the above solution. The mixture was stirred at 4°C for 4 h to activate the carboxyl group. Then, 1.32 g of tert-butyl carbazate were added dropwise to the solution, and MSNs-NH-NH-BOC were obtained after vigorously stirred for 24 h. Subsequently, the obtained products dispersed in 50 mL CH_2Cl_2 containing 10 mL TFA were then stirred for another 4 h. After that, the resulting products were obtained through centrifugation, washed three times with deionized water and ethanol, respectively.

Preparation and optimization of drug loaded MSNs

The screening of process and formulation parameters for preparation of MSN was carried out by OVAT analysis. The method of preparation was selected on the basis of trial-and-error analysis wherein, the nanoparticles were synthesized by different methods reported for preparation. Out of all methods, modified stober method was found to meet the quality criteria required for drug delivery.

i) Process Parameters

a) Processing Temperature
b) Stirring time
c) Stirring speed
d) Rate of addition

ii) Formulation Parameters

a) Type of surfactant
b) Surfactant concentration
c) Co solvent volume
d) Amount of pH modulator
e) pH for synthesis

The drug was loaded into the MSN carriers by active loading method, wherein, a specified quantity of hydrazine functionalised MSN was dispersed in Methanol and sonicated for 10 minutes to disperse the MSNs uniformly. The methanolic drug solution (15mg/ml) was added to the above solution and was subjected to sonication for 5 minutes. This mixture was kept for stirring at room temperature in closed condition for 24h to load the completely into the core of MSN particles by pore opening mechanism. The prepared nanoparticles were subjected to centrifugation at 8000 rpm for 15mins to separate free drug from formulation. The supernant contains free drug as silica nanoparticles are dense in nature and particles settle at bottom. The encapsulation efficiency was found out by following formula:

$$Entrapment\ Efficiency\ (\%) = \frac{Amount\ of\ drug\ entrapped\ in\ MSNs}{Total\ Amount\ of\ Drug\ added} \times 100$$

Results and Discussion for MSN

a) Particle Size and Zeta Potential

The particle size for optimized formulation was found to be 54±1.36 d. nm and 68±3.62 d. nm for FLV-MSNs and EXE-MSNs respectively. The particle size was found to be in range for intracellular perfusion and penetration into tumor microenvironment. The zeta potential for both the MSNs namely FLV and EXE were found to be -16.13 mV and -21.28 mV respectively. The negative zeta potential helps the formulation to be stable for a longer period of time during storage.

b) Differential Scanning Calorimetry (DSC)

The DSC curve of FLV shows the endothermic peak at 108°C, the curve of formulation doesn't show any endothermic peak deviation, indicating the matrix encapsulation of FLV in polymer lipid core. In case of EXE, there was an endothermic peak at 267°C, which diminished in case of nanoparticulate formulation, referring to engulfment of drug in polymeric matrix.

c) X Ray Diffraction pattern

The XRD pattern of FLV pure drug shows the characteristic crystalline peak at 2θ position of 12.47°, 16.36°, 18.02°, 19.78°, 22.51°. The XRD pattern of blank MSNs shows the crystalline peak at 2θ value of 18.48° and 22.82° respectively. But, in case of FLV loaded MSNs the XRD pattern shows slight shifting of XRD patterns to 2θ value of 18.92° and 23.32° respectively, indicating the encapsulation of drug into polymer matrix and conversion of crystalline form to amorphous form.

The XRD pattern of EXE pure drug shows the characteristic crystalline peak at 2θ position of 10.48°,14.18°, 15.63°, 17.80°, 21.81°. The XRD pattern of blank MSNs shows the crystalline peak at 2θ value of 18.25° and 22.42° respectively. But, in case of FLV loaded MSNs the XRD pattern shows slight shifting of XRD patterns to 2θ value of 18.45° and 22.84° respectively, indicating the encapsulation of drug into polymer matrix and conversion of crystalline form to amorphous form.

d) Fourier Transform Infrared Spectroscopy (FTIR)

The characteristic peaks of FLV were observed at 983.70 cm-1, 1197.79, 1446.21, 1608.63, 2926.01, and 2854.65, and characteristic peaks of blank MSNs are observed at 943.13, 1065.84, 1171.63, and 1472.28. The characteristic peaks of FLV by FLV loaded MSNs were observed with slight shifting at 933.55, 1070.49, 1141.86, 1492.90 and 1600.92. These results were confirmatory of FLV loading into MSNs.

The characteristic peaks of EXE were observed at 821.68, 904.61, 1620.21, 1732.08, and 2956.87, and characteristic peaks of blank MSNs are observed at 708.41, 1170.63, 1436.94 and 1714.31. The characteristic peaks of EXE by EXE loaded MSNs were observed with slight shifting at 719.45, 1178.51, 1469.76, and 1734.01. These results were confirmatory of EXE loading into MSNs.

e) BET analysis

The surface area is one of the most important quantities for characterizing novel porous materials. The BET analysis is the standard method for determining surface areas from nitrogen adsorption isotherms. It was originally derived for multilayer gas adsorption onto flat surfaces. The BET analysis assumes that adsorption occurs by multilayer formation and that the number of adsorbed layers is infinite at the saturation pressure, i.e., adsorption occurs as if on a free surface. The surface area was evaluated with nitrogen adsorption-desorption isotherm measurements on ASAP 2020 V4.01 Surface Area Analyzer (Micromeritics Corp., USA) t −1 5.8 °C. The nanoparticles were degassed at 50 °C overnight before analysis. The surface areas were calculated with BET theory using isotherm adsorption data at P/P0 from 0.01 to 0.99. The surface area was found to be in the range of 870 m^2/g to 1011m^2/g and pore volume was found to be in the range of 2.78 nm to 6.54 nm.

f) Cytotoxicity studies

The concentration range for MTT assay was 0.1-70 μg/mL. Cellular uptake studies by confocal microscopy and flowcytometry were conducted as per the procedure descried earlier for FLV and EXE. Also, biodistribution and apoptotic study to determine the cell death mechanism were carried out in a similar way as per the aforementioned procedure.

g) Elemental analysis

Synopsis

Energy dispersive X-ray spectrometry (EDX/EDS) is a widely applied elemental microanalysis method capable of identifying and quantifying all elements in the periodic table except H, He, and Li. EDS is a spectroscopic technique that determines the presence and relative abundance of the elements that compose the surface of the specimen under study. The X-ray photons that are produced when an energetic electron beam reaches the surface are detected and their energy depends on which atom they came from. SEM coupled with EDS are two analysis techniques that are widely used to study all kinds of solid samples, from inorganic to biological. They are used to determine morphological features of interest at a micron and sub-micron level as well as to study the chemical composition of the samples in terms of the amount of each element present. Morphology of the synthesized nanoparticles along with their chemical composition was characterized using FEG-SEM-EDS (JSM 7600F, JEOL, Japan) operated at a voltage of 0.1 to 30.0 kV.

Ongoing Work

- In vivo Pharmacokinetic studies
- In vivo tumor regression studies
- Thesis writing
- Publication

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