3. MATERIALS AND PROTOCOLS

3.1 Materials

Fulvestrant (FLV) and Exemestane (EXE) were kind gift sample from Sun Pharmaceuticals Industries Limited, Vadodara, India. The materials used for formulation development are portrayed in Table 3.1. Chemicals and reagents used for the preparation of buffers, analytical solutions and other general experimental purposes are shown in Table 3.2. Reagents and autoclavable plasticwares used for cell line study are displayed in Table 3.3. Equipments used at various stages are listed in Table 3.4. Double distilled water and HPLC grade water were prepared in lab.

Table 3.1 List of Excipients for Formulation Development

Sr. No.	Excipients	Suppliers and Place
1.	Poly lactic co- glycolide (PLGA) (75:25)	Gift sample from PURAC England
2.	Poly lactic co- glycolide (PLGA) (50:50)	Gift sample from PURAC England
3.	Polycaprolactone (PCL)	Sigma Aldrich, India
4.	PEG: Polycaprolactone (PEG: PCL)	Sigma Aldrich, India
5.	Poly Lactic acid (PLA)	Sigma Aldrich, India
6.	Soyabean Phosphatidylcholine (SPC -3)	Gift Sample from Lipoid GmBH, Germany
7.	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [amino (*polyethylene glycol*)-*2000*	
8.	Phospholipon 90G	
9.	Egg Phosphatidylcholine	
10.	Didioleoyl trimethylammonium propane (DOTAP)	
11.	1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)	
12.	Dioleoyl phosphatidyl ethanolamine (DOPE)	
13.	Sodium silicate	Sigma Aldrich, India

14.	Cetyltrimethylammonium bromide (CTAB)	Sisco Research Labs, Mumbai
15.	Isopropyl alcohol (IPA)	Rankem Chemicals, India
16.	Ethyl Acetate	Rankem Chemicals, India
17.	Poloxamer 407/Pluronic F127	BASF India, Mumbai
18.	Hydrochloric acid	Rankem Chemicals, India
19.	Ethanol	Avantor, India
20.	Poloxamer 188/Pluronic F68	BASF India, Mumbai
21.	Cremophor EL	Sigma Aldrich, India
22.	Tocopherol Polyethylene Glycol Succinate	Sigma Aldrich, India
23.	Polyethylene Oxide	Sigma Aldrich, India

Table 3.2 List of Chemicals, Reagents and Filters used for Analysis

Sr. No.	Excipients	Suppliers and Place
1.	Methanol HPLC grade	Rankem Chemicals, India
2.	Acetonitrile HPLC grade	Rankem Chemicals, India
3.	Formic acid HPLC grade	Rankem Chemicals, India
4.	Hydrochloric acid HPLC grade	Rankem Chemicals, India
5.	Orthophosphoric acid HPLC grade	Rankem Chemicals, India
6.	Tetrahydrofuran HPLC grade	Rankem Chemicals, India
7.	Triethylamine AR grade	Rankem Chemicals, India
8.	Milli Q water HPLC grade	Sigma Aldrich, India
9.	Potassium Hydroxide HPLC grade	Rankem Chemicals, India
10.	Potassium dihydrogen orthophosphate HPLC grade	Sigma Aldrich, India
11.	Disodium hydrogen phosphate HPLC grade	Rankem Chemicals, India
12.	Sodium Hydroxide Pellets AR grade	Sigma Aldrich, India
13.	25 mm, 0.22µ nylon filter	Pall Lifesciences, Paraguay
14.	25mm, 0.22µ PTFE filter	
15.	47mm, 0.22µ nylon filter	
16.	47mm, 0.45µ nylon filter	
18.	47mm, 0.45µ PTFE filter	

Table 3.3 Cell line study materials

Sr. No.	Excipients	Suppliers and Place
1.	Human breast carcinoma cells (MCF-7)	NCCS, Pune, India
2.	Human breast carcinoma cells (MDA MB 231)	NCCS, Pune, India
3.	RPMI 1640	Himedia Chemicals, India
4.	Dulbecco's Minimum Essential Media	Himedia Chemicals, India
5.	DAPI dye (4',6-diamidino-2-phenylindole)	Himedia Chemicals, India
6.	MTT dye (Methylthiazoyldiphenyl tetrazolium bromide)	Himedia Chemicals, India
7.	AnnexinV- FITC kit	Thermo Fisher Scientific, USA
8.	Penicillin/Streptomycin (10,000 U/mL/ 10,000 µg/mL)	Himedia Chemicals, India
9.	Fetal Bovine Serum	Himedia Chemicals, India
10.	Dimethyl Sulfoxide Sterile filtered	Himedia Chemicals, India
11.	Trypsin EDTA	Himedia Chemicals, India
12.	Trypan Blue	Himedia Chemicals, India
13.	Rhodamine 123	Sigma Aldrich, India
14.	Fluorescein isothiocyanate (FITC)	Sigma Aldrich, India
15.	Propidium Iodide	Himedia Chemicals, India
16.	Sterile Tissue culture flask T25	Corning, USA
17.	Sterile Tissue culture plates 6 wells	Himedia Chemicals, India
18.	Sterile Tissue culture plates 12 wells	Himedia Chemicals, India
19.	Sterile Tissue culture plates 24 wells	Himedia Chemicals, India
20.	Sterile Tissue culture plates 96 wells	Himedia Chemicals, India

Table 3.4 List of Instruments

Sr. No.	Name of Instrument	Company and Place
1.	BOD shaker incubator	Orbitek, Scigenics, India
2.	Centrifuge C24 Plus	Remi Elektrotechnik Ltd., India
3.	UV Visible spectrophotometer (UV 1900)	Shimadzu, Japan
4.	Laminar air flow cabinet	Weiber vertical laminar airflow, India
5.	BD FACS AriaIII	BD Biosciences, USA
6.	Confocal laser scanning microscope	CarlZeiss LSM 710, Germany
7.	Steri cycle i160 CO_2 incubator	Thermo Scientific, Germany
8.	Inverted Fluorescence microscope	Nikon Eclipse TS 100, Japan
9.	Deep Freezer (-40° C)	Biobase Refrigeration, India
10.	ELISA micro plate reader	Multiskan, Thermo Fisher, Germany
11.	RP – HPLC	Vanquish Core, Thermo Scientific, Germany
12.	Transmission Electron Microscope	Technai G2 (FEI, USA)
13.	Scanning Electron Microscope	JOEL JSM – 7600F, Japan
14.	Surface and porosity measurement	Micromeritics, ASAP 2010, USA
15.	Lyophilizer	Virtis Lyophilizer, Germany
16.	Particle Size Analyzer (DLS)	Nano ZS 90, Malvern Analytics, Switzerland
17.	Rotary Evaporator	RV 10, Rotavapor (IKA Instruments, India
18.	Magnetic stirrer	2 MLH, Remi Elektrotechnik Ltd., India
19.	X Ray Diffractometer	X'Pert (PANalytics, Singapore)
20.	Stability Chamber	NLHC7SI, Newtronic India

3.2 General Procedure for characterization technique

3.2.1 Particle size

Particle size and its distribution is one of the most important characteristics for nano-sized formulation as it governs permeation and uptake by cells. For size measurement, which is in nano range, scattering techniques are preferred. Scattering techniques can provide information about the structure as well as size of nanomaterials(1). Amongst the commonly used scattering techniques are DLS, SANS, small-angle X-ray scattering (SAXS) and static light scattering (SLS). SLS, SAXS and SANS can be used to find interactions of the particles, structure and size. DLS is based on diffusion of particles in solution, which is related to its hydrodynamic size(2).

The type of samples that can be studied by scattering techniques, the sample environment that can be applied, the actual length scale probed and the information that can be obtained, all depend on the nature of the radiation employed. For example, SANS with high penetration depth of monochromatic beam of neutrons can easily be applied to opaque samples which cannot be studied by DLS. SANS measures the actual size of the particle, whereas DLS measures the hydrodynamic size of the particle. As the LASER diffracts from the hydration layer around the particles, the hydrodynamic diameter is always larger than the actual. DLS results are biased towards larger length scale present in the system, whereas SANS provides more statistical results. Thus, to a large extent these techniques are complementary to each other, whilst sharing many similarities also**.**

3.2.1.1 Dynamic Light Scattering (DLS)

DLS also known as Photo-Correlation Spectroscopy (PCS) gives hydrodynamic size of the particulate system based on Brownian motion. The diffusion of particles is calculated by Stokes-Einstein equation. For DLS principle-based technique of size determination, zeta sizer Nano-ZS (Malvern Instruments, UK) equipped with 4.0 mW internal laser was used. 1 mL of suitably diluted sample was taken in disposable polystyrene sizing cuvette and measured at 4.65 mm position. Sufficiently diluted samples were used for all measurement to avoid multiple scattering. All measurements were performed at 25°C, at a scattering angle of 173°. The intensity-weighted mean diameter was obtained for each sample in triplicate(3).

3.2.1.2 Zeta Potential

Zeta potential is a physical property exhibited by colloidal system. It is an index representing the electrostatic stabilization. The liquid layer surrounding the particle exists as two parts; an inner region (Stern layer) where the ions are strongly bound and an outer (diffuse) region where they are less firmly associated. Within the diffuse layer there is a notional boundary inside

which the ions and particles form a stable entity. When a particle moves, ions within the boundary move it. Those ions beyond the boundary stay with the bulk dispersant. The potential at this boundary (surface of hydrodynamic shear) is the zeta potential. Zeta potential was measured using the folded capillary cell in Zetasizer (Nano ZS, Malvern Instruments Ltd., Malvern, UK). The measurement was carried out at 25°C in triplicate using multimodal analysis strategy. Smoluchowski approximation was used for zeta potential value determination(2).

3.2.2 Solid State characterization

3.2.2.1 Powder X-ray diffraction (PXRD)

XRD depends on the dual wave and particle nature of X-ray to obtain information about crystallinity of materials. The dominant effect that occurs when an incident beam of monochromatic X-rays interacts with a target material is scattering of those X-rays from atoms within the target material. In materials with crystalline structure, the scattered X-rays undergo constructive and destructive interference which is termed as diffraction. The diffraction of X-rays by crystals is described by Bragg's Law, n(lambda) = 2d sin(theta). The directions of possible diffractions depend on the size and shape of the unit cell of the material. The intensities of the diffracted waves depend on the kind and arrangement of atoms in the crystal structure(4). The crystal structure of drug and changes in crystallinity (correlated with intensity in XRD graph) were observed using powder X-ray diffractometer (X'Pert Pro, PANalyticals, Singapore) equipped with Ni-filtered Cu-Kα radiation, and voltage diffraction. Scan was performed for around 10-20 mg powder sample placed on sample holder at a 2 – theta range from 5-60° with a scan rate of 3°/min.

3.2.2.2 Fourier Transform Infrared Spectroscopy (FTIR)

When IR radiation is passed through a sample, some radiation is absorbed by the sample and some passes through (is transmitted). The resulting signal at the detector is a spectrum representing a molecular 'fingerprint' of the sample(5). The usefulness of infrared spectroscopy arises because different chemical structures produce different spectral fingerprints which are characteristic for each. The compatibility between components can be identified from change in wavenumber(6). Scanning of samples were performed in the range of 500–4000 cm^{-1} to obtain FT-IR spectra (FTIR, Bruker; Germany). All solid samples were compressed into KBr disks whereas liquid samples were placed in NaCl cell.

3.2.2.3 Differential Scanning Calorimetry (DSC)

The thermoanalytical technique, DSC measures difference in amount of heat required to increase the temperature of sample and reference as a function of temperature(7). The

instrument (DSC- 60) was calibrated with indium under nitrogen purging to avoid any kind of oxidative degradation. Thermal behavior of drug, its physical mixture with the excipients and formulation was studied(8). For this, around 5 mg sample was placed in aluminum pan which was heated from 25° to 300° under nitrogen atmosphere at a scanning rate of 10°/min.

3.2.3 Morphology study

3.2.3.1 Transmission Electron Microscope (TEM)

Max Knoll and Ernst Ruska of the Berlin Technische Hochschule demonstrated the first TEM in 1931, and in 1933 achieved a resolution greater than that of light with this microscope. Working at Siemens, Ruska helped to develop the first commercial TEM in 1939. TEM is used to reveal sub-micrometre, internal fine structure (the microstructure or ultrastructure)(9). TEM images of the nanoparticles were obtained on a Technai G2 transmission electron microscope (FEI Company Ltd., Hillsboro, USA) operating at 20-200kV voltage with resolution of 2.4 Ao. The samples for TEM measurements were made by casting one drop of the samples on copper grids coated with carbon(10).

3.2.3.2 Scanning Electron Microscopy (SEM)

Although first developed in the early 1930's and perfected to a high degree in the late 1950's, the scanning electron microscope have been slow to find their proper fields of application. In 1965 the scanning electron microscope became commercially available, and since then there has been a great spurt in use of this equipment as a research tool(11). SEM is a characterization technique that is used widely for all types of samples, from hard materials such as metals and ceramics to soft materials such as polymers and biological tissues(12). Morphology of the synthesized nanoparticles was characterized using Field Emission Gun-Scanning Electron microscope (JSM 7600F, JEOL, Japan) operated at a voltage of 0.1 to 30.0 kV.

3.2.3.3 Cryo- Transmission Electron Microscopy (Cryo-TEM)

Cryo-TEM was used to study internal structure of ultra-thin cut samples. Appropriately diluted sample was applied on perforated carbon film supported by copper grid, the surface of which was modified for proper adhesion of sample to the grid surface. A thin film of the sample formed when it was put in liquid ethane followed by liquid nitrogen(13). Blotting parameters such as film thickness and vitrification were kept similar between observed samples. Subsequently, the grid was transferred to cryo holder already maintained at cryo temperature of -170°C and samples were observed for morphology and size using Tecnai G2 cryo-TEM (FEI Company Ltd., Hillsboro, USA).

3.2.4 Nitrogen Sorption 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(14). 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(15).

The surface area was evaluated with nitrogen adsorption-desorption isotherm measurements on a 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.

3.2.5 In-vitro drug release study and drug release kinetics

3.2.5.1 Polymer Lipid Hybrid Nanocarriers (PLHNCs)

Solutions:

- ✓ **Sodium Sulphide solution 0.3%:** 0.3 g of accurately weighed sodium sulphide was dissolved in 100 ml distilled water to produce 0.3% Sodium Sulphide solution.
- ✓ **Sulphuric acid solution 0.2%:** 0.2 mL of concentrated sulphuric acid was dissolved in 100 mL of distilled water to produce 0.2 % sulphuric acid solution.
- ✓ **Potassium Dihydrogen phosphate, 0.2M:** 27.218 g of potassium dihydrogen phosphate was dissolved in 1000 mL of distilled water to produce 0.2M Potassium Dihydrogen Phosphate solution.
- ✓ **Sodium Hydroxide, 0.2M:** 8.0 g of sodium hydroxide was dissolved in 1000 mL of distilled water to produce 0.2M Sodium Hydroxide solution.
- ✓ **Phosphate Buffer:** 50.0 mL of the 0.2M potassium dihydrogen phosphate, 0.2M was taken in a 200-mL volumetric flask and specified volume of 0.2M sodium hydroxide, 0.2M and then water was added to make the final volume up to 200-mL. The pH of the buffer was checked using pH meter and adjusted if needed using sodium hydroxide solution or hydrochloric acid solution as necessary.
- ✓ **Amount of 0.2M NaOH to be added to 0.2M KH_2PO_4 solution to get buffer of required pH.**

Table 5.21 Amount of 0.2M NaOH required

pH	0.2M NaOH (mL)

6.6	16.4
7.4	39.1

- ✓ **Acetic acid solution 2N:** 116 mL of glacial acetic acid was dissolved in sufficient quantity of distilled water and after cooling of solution to room temperature final volume was made up to 1000 mL.
- ✓ **Acetate Buffer pH 5.5:** 5.98 g of sodium acetate trihydrate was weighed and sampled in a 1000 mL volumetric flask. To this 3.0 mL of the acetic acid solution was added and volume was made up to 1000 mL. The pH of the buffer was checked using pH meter and adjusted if needed using acetic acid solution or sodium hydroxide solution as necessary.

Dialysis membrane Set-up/activation

Before using dialysis membrane in drug release study, it should be activated be below mentioned process.

1. The 5 cm long dialysis membrane was cut and kept for 3 hours in running water for glycerine elimination.
2. After that the dialysis membrane was dipped in 0.3 percent w/v solution of sodium sulphide solution for 1 min at 80°C.
3. The dialysis membrane treated with sodium sulphide was then dipped at 60°C for 1 min in warm water to extract sodium sulphide.
4. The dialysis membrane was then soaked in 0.2% H_2SO_4 for 1 min.
5. The it was dipped again in the warm water for H_2SO_4 elimination.
6. This activated dialysis membrane was then kept in Phosphate buffer (pH 7.4) for 24 h prior to drug release studies.

To simulate the physiological environment of tumor cells, interstitium of tumors and blood or normal cell, phosphate buffer with pH 5.5, 6.6, and 7.4 were investigated for in-vitro drug release study (9). A drug release study was performed using a dialysis bag with molecular mass cut-off of 3000 Da for 15 days. 2 mL of the formulation was filled in a dialysis bag and dipped in receptor media comprising 50 mL phosphate buffer at 37°C. At 2, 6, 12, 24, 48h and subsequent days up to 15^{th} day, 1 mL of sample was withdrawn periodically and fresh media was replaced to maintain sink condition. These samples were analysed using HPLC and the % drug released was calculated and plotted against the time to obtain the release curve. Data of

drug release are fitted in zero order, first order, Higuchi, Korsmeyer – Peppas and Hixon – Crowell models determine release kinetic pattern from PLHNCs (16).

1. Zero order release

For fulvestrant release that follows zero order kinetics following equation can be applied.

$Mt = Kt$

Where, Mt = amount of drug released at time t

K = zero order release rate constant

t = time

2. First order release

For fulvestrant release that follows first order kinetics following equation can be applied.

$\ln [1-(Mt/M_0)] = -Kt$

Where, Mt = amount of drug released at time t

M_0 = initial amount of drug present

K = First order release rate constant

t = time

3. Higuchi's model

Following equation can be for fulvestrant release that follows Higuchi's kinetics model.

$Mt = Kt^{½}$

Where, Mt = amount of drug released at time t

K = Higuchi's release rate constant

t = time

4. Korsmeyer – Peppas model

Following equation can be for fulvestrant release that follows Korsmeyer – Peppas kinetics model

$\ln Mt/M_0 = \ln K + n \ln t$

Where, Mt = amount of drug release at time t

M_0 = initial amount of drug present

K = Korsmeyer – Peppas release rate constant

t = time

n = Diffusional exponent that characterizes the mechanism of drug release

The value of diffusional exponent 'n' will help to understand mechanism of drug release from the dosage forms of different geometry like slab, cylinder, sphere etc.

- ✓ n = 0.5 to 1 (0.5< n <1) indicates non Fickian release
- ✓ n = 0.5 indicates Higuchi's Kinetics
- ✓ n = 1 indicates the first order release or case 2 transport
- ✓ n < 0.5 indicates Fickian release
- ✓ n > 1 indicates the Super case 2 transport

5. Hixon Crowell model

Following equation can be applied for fulvestrant release that follows Hixon Crowell kinetics model.

$$\sqrt[3]{M0} - \sqrt[3]{Mt} = Kt$$

Where, Mt = Mt = amount of drug release at time t

M_0 = initial amount of drug present

K = Hixon Crowell release rate constant; t = time.

3.2.5.2 For Mesoporous Silica Nanoparticles (MSNs)

To simulate the physiological environment of tumor cells, interstitium of tumors and blood or normal cell, phosphate buffer with pH 5.5, 6.6, and 7.4 were investigated for in-vitro drug release study. A drug release study was performed using a dialysis bag with molecular mass cut-off of 3000 Da for 7 days. 2 mL of the formulation was filled in a dialysis bag and dipped in receptor media comprising 50 mL phosphate buffer at 37°C. At 2, 6, 12, 24, 48h and subsequent days up to 7th day, 1 mL of sample was withdrawn periodically and fresh media was replaced to maintain sink condition. These samples were analysed using HPLC and the %

drug released was calculated and plotted against the time to obtain the release curve. Data of drug release are fitted in zero order, first order, Higuchi, Korsmeyer – Peppas and Hixon – Crowell models determine release kinetic pattern from MSNs (16).

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