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Development and Characterization of Pazopanib-Loaded Amino-Functionalized Mesoporous Silica Encapsulated As Oral Delivery System in Cancer Treatment

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Abstract

Objectives: The present study aimed to develop and optimize pazopanib-loaded amino-functionalized mesoporous silica nanoparticles (NH₂-MSNs) to overcome the inherent poor aqueous solubility and low oral bioavailability of pazopanib, a BCS Class II tyrosine kinase inhibitor, for improved therapeutic efficacy against renal cell carcinoma. **Methods:** Nanoparticles were fabricated using a modified sol-gel method and systematically optimized employing Box-Behnken design (BBD) with three independent variables: TEOS concentration, CTAB concentration, and reaction temperature, evaluating encapsulation efficiency and particle size as critical response variables. Post-synthesis amino-functionalization was performed using APTES grafting. Optimized nanoparticles were encapsulated into hard gelatin capsules (CF1–CF4) and evaluated for physicochemical characterization, in vitro drug release, accelerated stability per ICH Q1A(R2) guidelines, and cytotoxicity against 786-O renal cell carcinoma cells via MTT assay. **Results:** Formulation F16, selected as optimal based on maximum desirability score of 1.0, demonstrated encapsulation efficiency of $89.12 \pm 0.60\%$, particle size of 171.74 ± 4.42 nm, PDI of 0.136 ± 0.003 , and positive zeta potential of $+20.81 \pm 0.30$ mV confirming successful amino-functionalization. Capsule formulation CF1 exhibited highest dissolution of $72.46 \pm 0.40\%$ at 60 minutes with excellent drug content uniformity of $98.64 \pm 0.84\%$. Stability studies confirmed physicochemical integrity over three months. Pazopanib-loaded MSNPs demonstrated significantly superior cytotoxicity (IC_{50} 18.37 ± 0.72 μ g/mL) compared to pure pazopanib (IC_{50} 28.64 ± 0.84 μ g/mL). **Conclusion:** The developed NH₂-MSN platform successfully enhanced pazopanib delivery with superior cytotoxic efficacy, offering clinically significant potential for reduced dosing, minimized systemic toxicity, and improved patient compliance, warranting further in vivo pharmacokinetic evaluation and clinical translation.

Keywords: Pazopanib; Mesoporous silica nanoparticles; Amino-functionalization; Box-Behnken design; Renal cell carcinoma; Controlled drug release; Cytotoxicity.

1. INTRODUCTION

Cancer therapy is a very critical therapy and there are limitations in the conventional therapy that needs to be overcome by targeted drug delivery systems [1]. The challenges associated with current cancer chemotherapy are low bioavailability of the administered drugs, non-specific distribution of the drugs, high systemic toxicity, and fast clearance from target sites [2]. Such difficulties lead to sub-optimal therapeutic response, necessitating high levels of drug dosage which can have a detrimental effect on patient compliance and quality of life [3]. Cancer therapeutics is a global market estimated to be worth \$180 billion and is growing due to the rising incidence of cancers affecting more than 19 million people around the world per year. Treatment costs for cancer account for a large portion of health care spending and are over \$200 billion worldwide [4]. Epidemiological data from recent years suggest that there has been an increase in incidence of several types of cancer, such as renal cell carcinoma, lung cancer and hepatocellular carcinoma, especially in developing countries. Conventional chemotherapy formulations show low tumor selectivity and are rapidly cleared from the circulation, requiring frequent and high doses of chemotherapy. Current research priorities are focused on developing innovative drug delivery systems that not only target tumors more effectively, but also reduce systemic toxicity [5].

Pazopanib has proven to be a promising tyrosine kinase inhibitor for cancer treatment with particular potential benefits in the treatment of metastatic renal cell carcinoma and soft tissue sarcomas. Its broad-spectrum kinase inhibitory activity is most notable for its activity against VEGFR, PDGFR and c-Kit pathways, which play a major role in the growth and spread of tumors; it is usually taken orally at 800 mg per day [6]. Anti-angiogenic effects result in controlled inhibition of tumour growth without an excessive vascularization of normal tissues. Unlike traditional single-target drugs, Pazopanib has been seen to have potent anti-tumor activity by inhibiting multiple oncogenic pathways. Recent clinical studies have shown that pazopanib can significantly provide progression-free survival benefits in renal cell carcinoma patients [7]. The tolerable safety profile of pazopanib and manageable side effects such as fatigue and gastrointestinal side effects, help to achieve the best therapeutic efficacy and ensure patient compliance [8]. In addition, pazopanib has excellent temperature stability in physiological conditions and is capable of formulation with different drug delivery systems to obtain a higher bioavailability. The drug has been tested in combination treatments with other targeted drugs and immunomodulators in previous studies and has been found to significantly improve the therapeutic index when compared to monotherapy treatments [9].

Amino-functionalized mesoporous silica nanoparticles are novel materials to improve drug delivery to cancer cells via targeted nanocarriers [10]. Such smart delivery platforms possess high surface area and tunable pore structures, which enables efficient loading of the drugs in them along with controlled release at the tumor site [11]. The amino functionalization causes the surfaces to become positively charged which improves cell uptake and allows surface modification with targeting ligands. The technology provides enhanced protection of the drug and controlled drug diffusion mechanisms for achieving sustained therapeutic effect within the stable silica framework [12]. New possibilities for the synthesis of mesoporous silica, such as sol-gel and template-assisted techniques, have been achieved and have shown

excellent biocompatibility and negligible systemic toxicity. The nanoparticulate matrix is optimized for tumor penetration via the enhanced permeability and retention effect and rapid clearance via reticuloendothelial system uptake is prevented [13]. Moreover, these systems can prevent enzymatic degradation and provide highly accurate control of drug release profiles. The synergistic effects of pazopanib and amino-functionalized mesoporous silica nanoparticles (MSN) can be attributed to both passive targeting of tumor (passive targeting) and surface charge interactions with tumor cell (active targeting), which is a major improvement of cancer nanomedicine technology [14].

The goal of the present study is to design and optimize the drug delivery system of pazopanib loaded amino-functionalized mesoporous silica nanoparticles for the enhancement of oral bioavailability in cancer therapy. Formulation optimization by Box–Behnken design, physicochemical characterization and in vitro drug release are primary objectives. Secondary objectives include evaluation of cytotoxicity on the renal cell carcinoma lines 786-O, stability evaluation according to ICH guidelines and the definition of therapeutic superiority over the conventional pazopanib formulations.

MATERIALS AND METHODS

MATERIALS

Pazopanib (USP grade, 99.5%). Tetraethyl orthosilicate (TEOS), 3-aminopropyltriethoxysilane (APTES), and potassium bromide. Cetyltrimethylammonium bromide (CTAB), microcrystalline cellulose, magnesium stearate, and talc were sourced from Neeta Chemicals, Pune, India. Anhydrous toluene, methanol, ethanol, sodium hydroxide, potassium dihydrogen phosphate, disodium hydrogen phosphate, and aerosil were purchased from Research Lab Fine Chem Industries, Mumbai, India. Hard gelatin capsules (size 0) and dialysis membrane (MWCO 12,000-14,000 Da). All other chemicals and reagents used were of analytical grade.

METHODS

Calibration Curve of Pazopanib

Calibration curve was developed to quantify pazopanib by the UV-Visible spectrophotometry (UV-1800, Shimadzu Scientific Instruments, Mumbai, India). A PSS (primary stock solution) (1000 µg/mL) of pazopanib was prepared by dissolving 10 mg pazopanib in methanol and serial dilutions were done in phosphate buffer pH 7.4, to obtain the concentrations of 2-20 µg/mL. Phosphate buffer blank was used to determine Absorbance against at 273 nm (n=3). Linear regression analysis was used to calculate correlation coefficient (R^2) and the equation ($y = mx + c$), where y is absorbance and x is concentration (µg/mL). According to the ICH Q2(R1) guidelines, the R^2 value should be ≥ 0.999 , which was achieved [15].

Solubility Study of Pazopanib

The shake flask procedure with excess drug was used to determine the solubility of pazopanib in water, methanol, ethanol and phosphate buffer, at pH 6.8 and pH 7.4. The sample was shaken on an orbital shaker (RSO-2D, Remi Elektrotechnik Ltd., Mumbai, India) at 100 rpm,

25 ± 0.5°C for 24 hours, filtered through 0.45 µm membrane, diluted to appropriate level and analysed using UV spectrophotometry at 273 nm. The values of solubility were obtained from the calibration curve and expressed as mg/mL ± SD (n=3). One-way ANOVA analysis was carried out using significant level of $p < 0.05$ [16].

Differential Scanning Calorimetry Analysis

The differential scanning calorimeter (DSC-60 Plus, Shimadzu Scientific Instruments, Mumbai, India) was used for DSC analysis to assess the thermal behavior and drug-excipient compatibility. Accurately weighed samples (3-5 mg) of pure pazopanib and physical mixture were crimped in aluminum pans with non-hermetic lids against the empty aluminum reference pan. The samples were heated from 30°C to 300°C at 10°C/min under constant nitrogen flow (50 mL/min) to avoid oxidative degradation. DSC thermograms were obtained and examined for characteristic endothermic/exothermic peaks, shifts of the peaks and/or formation of new peaks as a result of drug-excipient interaction. Data was collected in triplicate (n=3) and analyzed with the help of TA Universal Analysis software [17].

Fourier Transform Infrared Spectroscopy Analysis

An FTIR spectrophotometer (IR Affinity-1S, Shimadzu Scientific Instruments, Mumbai, India) was used for FTIR analysis for identification of functional groups and drug-excipient compatibility. The samples were prepared using the KBr pellet method, by mixing 1–2 mg of sample, with the KBr and compressing the mixture into transparent pellets by applying a hydraulic pressure of 10 tonnes. The measurements were recorded between 4000–400 cm^{-1} , at a resolution of 4 cm^{-1} and 16 scans per sample. The drug and physical mixture spectra were compared to each other for the presence of any characteristic peak shift, broadening or disappearance that may be associated with chemical interaction. Three replicates (n=3) were used for analysis at ambient conditions [18].

Experimental Design

Design Expert software (version 12.0, Stat-Ease Inc.) was used with Box-Behnken design to systematically optimize. The experimental factors studied were the concentration of TEOS ($X_1=3, 5, 7\text{mL}$), the concentration of CTAB ($X_2=0.05, 0.10, 0.20\text{M}$) and the reaction temperature ($X_3=70, 80, 90^\circ\text{C}$), with each factor having 3 levels, coded as -1, 0 and +1. Two dependent variables namely, encapsulation efficiency (Y_1) and particle size (Y_2) were assessed. The experimental design consisted of 17 experimental runs with 12 factorial points and 5 center points (Table 1), the runs were tested in randomized order to avoid the experimental bias. Multiple regression analysis and ANOVA were used to assess the significance of the model for the responses. The correlation coefficient (R^2), adjusted R^2 , predicted R^2 , and lack of fit test were used to conduct model adequacy analysis. The p value < 0.05 and $R^2 > 0.9$ values were set as the criteria for the models to be considered statistically significant and validated [19].

Table 1: Independent Variables and Their Levels in Box-Behnken Design

Independent Variables	Symbol	Low Level (-)	Medium Level	High Level
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		1)	(0)	(+1)
TEOS Concentration (mL)	X ₁	3	5	7
CTAB Concentration (M)	X ₂	0.05	0.10	0.20
Reaction Temperature (°C)	X ₃	70	80	90
Dependent variables				Goals
Encapsulation efficiency (%)				Maximize
Particle size (nm)				Minimize

Formulation of Pazopanib-Loaded Mesoporous Silica Nanoparticles

The Pazopanib loaded mesoporous silica nanoparticles were prepared by a modified sol-gel method with magnetic stirring (Remi 2MLH, Remi Elektrotechnik Ltd, Mumbai, India). Pazopanib (10 mg) was weighed and completely dissolved in HPLC grade methanol. CTAB was dissolved in 50 mL deionized water and stirred constantly at specific reaction temperatures 70°C, 80°C and 90°C. To prepare alkaline conditions for condensation of silica, solution pH was adjusted to 11 with 2M sodium hydroxide. The pazopanib-methanol solution was then dropwise added into the CTAB solution with continuous stirring at 500 rpm and then TEOS (3, 5 or 7 mL) as the silica precursor was slowly added dropwise. The reaction mixture was stirred continuously for 2 hours at the respective temperatures so as to allow the complete hydrolysis and condensation to be carried out (Table 2). This gave a white precipitate that was filtered on Whatman filter paper No. 41, washed with deionized water and methanol several times in order to wash away the unreacted materials, and dried in a hot air oven at 60°C for 24 hours. Dried nanoparticles were gathered and kept in airtight amber containers at room temperature. The seventeen formulations were prepared using Box-Behnken design parameters, all in random order (n=3) [20].

Table 2: Formulation Composition of Pazopanib-Loaded Mesoporous Silica Nanoparticles (F1–F17)

Formulation	Drug (mg)	Factor 1: TEOS (mL)	Factor 2: CTAB (M)	Factor 3: Reaction Temperature (°C)
F1	10	7	0.05	70
F2	10	3	0.125	70
F3	10	7	0.125	70
F4	10	5	0.05	90
F5	10	5	0.125	80
F6	10	3	0.125	90
F7	10	5	0.2	70
F8	10	3	0.2	80
F9	10	5	0.2	90

F10	10	5	0.125	80
F11	10	5	0.05	70
F12	10	5	0.125	80
F13	10	5	0.125	80
F14	10	7	0.125	90
F15	10	7	0.125	90
F16	10	7	0.2	80
F17	10	3	0.05	80

Amino Functionalization of Mesoporous Silica Nanoparticles

The post-synthesis grafting was carried out with APTES as a functionalizing agent to create amino functionalization. Dried MSNs (100 mg) were dispersed in anhydrous toluene (50 mL) in a round bottom flask and sonicated for 15 minutes using probe sonicator (Vibra-Cell VCX 130, Oscar Ultrasonics Pvt. Ltd., Mumbai, India) for uniform dispersion. APTES was then added dropwise while the mixture was stirred and refluxed for 24 hours at a temperature of 110°C and under anhydrous conditions, in a drying tube containing calcium chloride, to exclude moisture from entering the reaction mixture. The amino-functionalized nanoparticles were recovered on centrifugation at 10,000 rpm for 15 minutes in a cooling centrifuge (R-8C BL, Remi Elektrotechnik Ltd., Mumbai, India), washed three times with anhydrous toluene and then with dry ethanol to remove the unreacted APTES. The dried nanoparticles were stored at room-temperature in airtight bottles for 24 hours under vacuum at 60°C. It was carried out three times (n=3) to ensure consistency [21].

Capsule Formulation Development

The optimized pazopanib loaded amino functionalized mesoporous silica nanoparticles were encapsulated in hard gelatin capsules (size 0) by manual filling technique. To achieve an encapsulation efficiency of 80%, the capsule formulation was a 250 mg of the nanoparticle formulation for each capsule, which corresponds to 200 mg pazopanib equivalent. The formulation was prepared using a geometric dilution method in polyethylene bags, 10 minutes with accurately weighed nanoparticle to ensure homogeneity, with the excipients microcrystalline cellulose (diluent), magnesium stearate (lubricant), talc (glidant) and aerosil (flow enhancer). The blend was filled in the capsule bodies with the help of manual capsule filling machine (Karnavati Engineering Ltd., Gandhinagar, India) and capsule caps were tightly locked. For this purpose four batches of formulation (CF1-CF4) were formulated with different proportions of MCC and Mg-stearate to optimize the blend property (Table 3). The 100 capsules were prepared under controlled conditions for each batch (n=3) [22].

Table 3: Formulation Composition of Pazopanib-Loaded Amino-Functionalized Mesoporous Silica Nanoparticles Capsule Formulations (CF1–CF4)

Ingredient	CF1	CF2	CF3	CF4
Pazopanib-loaded NH ₂ -MSNs (mg)	250	250	250	250
Microcrystalline Cellulose (mg)	120	100	80	60

Magnesium Stearate (mg)	2	4	6	8
Talc (mg)	5	5	5	5
Aerosil (mg)	3	3	3	3
Total Fill Weight (mg)	380	362	344	326
Capsule Size	0	0	0	0

Characterization of amino functionalized mesoporous silica nanoparticles

Particle Size and Polydispersity Index

The particle size and polydispersity index (PDI) was measured using dynamic light scattering technique by Zetasizer Nano ZS (Malvern Panalytical India Pvt. Ltd., Pune, India). The nanoparticle dispersions were then serially diluted as needed to give an optimum concentration for measurement and placed in disposable polystyrene cuvettes. The measurements were carried out at a scattering angle of 173° and temperature of 25°C and with an automatic measurement time. All samples were measured three times (n=3) and the mean particle size (Z-average) and PDI were obtained. Before analysis, the instrument was calibrated with latex beads and the data were processed with Zetasizer software version 7.11 [23].

Zeta Potential

The same Zetasizer Nano ZS instrument was then used to perform measurements of zeta potential using the laser Doppler electrophoretic technique. Nanoparticle dispersions were diluted with deionized water and placed in folded capillary zeta potential cells (DTS1070). The measurements were made at 25°C, and the duration of the measurement and the automatic selection of the voltage were set. Surface charge and colloidal stability of each sample were measured three times (n=3). The instrument was calibrated with the zeta potential transfer standard (50 ± 5 mV). The data were analyzed with Zetasizer software in which the Smoluchowski approximation was used to calculate the zeta potential [24].

Encapsulation Efficiency

Indirect method was used to determine the encapsulation efficiency by calculating the amount of untrapped drug present in the supernatant. The free drug was separated from the known amount of nanoparticle dispersion by centrifugation for 30 minutes at 4°C at 15000 rpm in a cooling centrifuge (R-8C BL, Remi Elektrotechnik Ltd, Mumbai, India). Supernatant was collected and diluted with phosphate buffer (pH 7.4) and analysed by UV spectrophotometry at 273 nm. The free drug concentration was determined from the calibration curve and the encapsulation efficiency was determined as follows:

$$\text{Encapsulation Efficiency (\%)} = \left[\frac{(\text{Total drug added} - \text{Free drug in supernatant})}{\text{Total drug added}} \right] \times 100$$

All determinations were performed in triplicate (n=3) and results expressed as mean ± SD [25].

Drug Loading Capacity

The capacity of the drug loading was estimated with complete extraction of the drug from the dried nanoparticles. Accurately weighed nanoparticles (10mg) were dispersed in HPLC grade methanol and then probe sonicated for 30 minutes on a probe sonicator (Vibra-Cell VCX 130, Oscar Ultrasonics Pvt. Ltd., Mumbai, India) for complete drug extraction. The sample was filtered through 0.45 µm membrane filter, diluted to an appropriate level according to the phosphate buffer pH 7.4 and analyzed by UV spectrophotometry at 273 nm. The amount of drug present in the nanoparticles (drug content) was determined from the calibration curve and drug loading calculated by the following formula:

$$\text{Drug Loading} = \left(\frac{\text{Weight of drug in nanoparticles}}{\text{Weight of nanoparticles}} \right) \times 100$$

$$\text{DL\%} = (\text{Weight of drug in nanoparticles} / \text{Weight of nanoparticles}) \times 100$$

All measurements were made in triplicate (n=3) and the results are presented as mean ± SD [26].

Fourier Transform Infrared Spectroscopy

To confirm the amino functionalization, FTIR analysis was carried out with FTIR spectrophotometer (IR Affinity-1S, Shimadzu Scientific Instruments, Mumbai, India). Plain mesoporous silica nanoparticles, APTES, amino-functionalized nanoparticles, pure pazopanib and drug loaded nanoparticles were prepared by KBr pellet method. Each sample was dried and mixed with dry KBr (1:100) and then pressed to form transparent pellets under 10 tonnes using a hydraulic press. Spectra were taken on 16 scans with a 4 cm⁻¹ resolution in the range 4000-400 cm⁻¹. The characteristic amine peaks (N-H stretching ~3300 cm⁻¹ and N-H bending ~1550 cm⁻¹) were analyzed to ensure that the grafting of APTES was achieved. Triplicate analyses (n=3) were carried out under ambient conditions [27].

Scanning Electron Microscopy

Scanning electron microscopy (SEM) (JSM-7100F, JEOL India Pvt. Ltd., Mumbai, India) was used to investigate the surface morphology. The dried nanoparticle samples were glued on the aluminum stubs with carbon adhesive tape and coated with gold for 120 s with the help of ion sputter coater (JFC-1600, JEOL India) using 20 mA as the coating current. High vacuum (8-10 mm and 10-20 kV) was used to examine the samples. The particle morphology, surface texture, particle size distribution and tendency to agglomerate were assessed by taking photomicrographs at magnifications ranging from 1000X to 50,000X. Morphological characterization was carried out by image processing software on SEM images [28].

In Vitro Drug Release Study

Membrane-constrained passive diffusion was adopted as the in vitro strategy to interrogate pazopanib liberation from the nanoparticulate matrix, using a dissolution testing unit (EDT-08Lx, Electrolab India Pvt. Ltd., Mumbai, India) as the hydrodynamic platform. Pre-hydrated dialysis membranes (MWCO: 12,000–14,000 Da) were charged with 5 mL of pH 7.4 phosphate buffer suspending nanoparticles equivalent to 10 mg of the drug, sealed, and submerged into 200 mL of identically buffered receptor fluid maintained under physiological

thermal conditions ($37 \pm 0.5^{\circ}\text{C}$) with sustained mechanical agitation at 100 rpm. Timed aliquots of 5 mL were collected at 0.5, 1, 2, 4, 6, 8, 12, 16, 20, and 24 h, with volumetric replenishment after each withdrawal to uphold uninterrupted sink conditions; each fraction was passed through a $0.45\ \mu\text{m}$ filter, appropriately diluted, and spectrophotometrically quantified at 273 nm against a pre-established calibration reference. The resulting cumulative permeation profiles were subjected to multi-model kinetic regression via DDSolver, encompassing zero-order, first-order, Higuchi, and Korsmeyer–Peppas frameworks to delineate the underlying release mechanism, with all assays performed in triplicate ($n = 3$) and data expressed as mean $\pm$ SD [29].

Characterization of Mesoporous silica nanoparticles loaded capsule

Appearance

The appearance of the capsules was evaluated in the appropriate conditions of white light in the capsules formulation (CF1, CF2, CF3 and CF4). Each capsule was individually evaluated for color, texture, deformation, denting, leakage and general grace. Physical acceptability and manufacturing consistency were evaluated for all batches ($n=20$) [30].

Average Weight Variation

Variation in weight was calculated based on Indian Pharmacopoeia guidelines by using analytical balance (AUW220D, Shimadzu India Pvt. Ltd., Mumbai, India). 20 capsules in each batch were weighed separately and their contents were poured out and weighed the following the capsules' shells were weighed. Shell weight was deducted from total weight to determine net fill weight. The mean fill weight was calculated as percentage deviation (20 capsules with maximum of 20% deviation) [31].

Disintegration Time

The disintegration time was determined by the disintegration test apparatus (ED-2AL, Electrolab India Pvt. Ltd. Mumbai, India) as per Indian Pharmacopoeia. Six capsules from each batch were transferred to basket tubes and kept in 900 mL phosphate buffer pH 6.8 for $37 \pm 0.5^{\circ}\text{C}$. The apparatus was run at 29-32 cycles per minute and the time of complete disintegration recorded. There were six determinations ($n=6$) and the mean disintegration time was calculated [32].

Dissolution Study

Dissolution studies were performed using USP Type II apparatus (TDT-08L, Electrolab India Pvt. Ltd, Mumbai, India) with phosphate buffer pH 6.8 (900 mL) at $37 \pm 0.5^{\circ}\text{C}$ and 75 rpm paddle rate. Five mL aliquots were removed at 5, 10, 15, 20, 30, 45 and 60 minutes and replaced with fresh medium and one capsule per vessel was tested. These were filtered through $0.45\ \mu\text{m}$ membrane, diluted with buffer and measured by UV spectrophotometry at 273 nm. The comparison of the calibration curve and dissolution profiles was used to determine cumulative drug release ($n=6$) [33].

Drug Content Uniformity

To assess for drug content uniformity, the uniformity of pazopanib distribution in capsules was evaluated. Ten capsules were taken in each batch and emptied and were sonicated for 30 min in methanol in a bath sonicator (PCI Analytics Pvt. Ltd., Mumbai, India) and contents were taken out. The solutions were filtered using a 0.45 μ m membrane filter, diluted in phosphate buffer pH 7.4 and measured by UV spectrophotometry at 273 nm. Drug content was determined by reading off the calibration curve and reported as % of label claim (200 mg). Individual capsules were required to be between 85-115% of labeled amount (n=10) for acceptance [34].

Accelerated Stability Study

Stability studies were carried out according to ICH Q1A (R2) guidelines in a stability chamber (TH-180, Thermolab Scientific Equipments Pvt. Ltd., Mumbai, India). Optimized formulations of capsules were packed in the aluminum foil pouches and placed at 40°C $\pm$ 2°C and 75% $\pm$ 5% relative humidity in a dark place for three months. Samples were collected at 0, 1, 2 and 3 months and tested for physical appearance, weight change, uniformity of drug content and dissolution profile. To check the physicochemical stability during the accelerated storage period, the parameters were compared with the initial values (n=3) [35].

In Vitro MTT Assay

The cytotoxicity was assessed in human renal cell carcinoma (786-O) cells by MTT assay in CO₂ incubator (Galaxy 170R, Eppendorf India Ltd., Mumbai, India). All cells were cultured at 37°C, 5% CO₂ in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were plated out at 10,000 cells per well into 96 well plates and incubated for 24 hours. The following five groups were tested: negative control, pure pazopanib, blank nanoparticles, pazopanib loaded at 5, 10, 25, 50 and 100 μ g/mL. After 48 h, MTT reagent (5mg/mL) was added and incubated for 4 h at 37 °C and absorbance was taken at 570 nm using micro plate reader (BioTek India Pvt. Ltd., Mumbai, India). The viability of cells and IC₅₀ values were determined by using GraphPad prism software. All experiments were repeated three times (n=3) and results reported as mean $\pm$ SD [36,37].

RESULTS AND DISCUSSION

RESULTS

Calibration Curve determination

A calibration curve of pazopanib was obtained in pH 7.4 phosphate buffer in the range of 2-20 μ g/mL at 273 nm (Figure 1). The regression equation obtained was $y = 0.0127x + 0.0034$; excellent correlation coefficient ($R^2 = 0.9985$) was obtained which is within ICH Q2(R1) acceptance criteria ($R^2 \geq 0.999$). The results validated that the method was suitable for all subsequent quantitative estimations as it followed strict linearity, which followed Beer-Lambert's law.

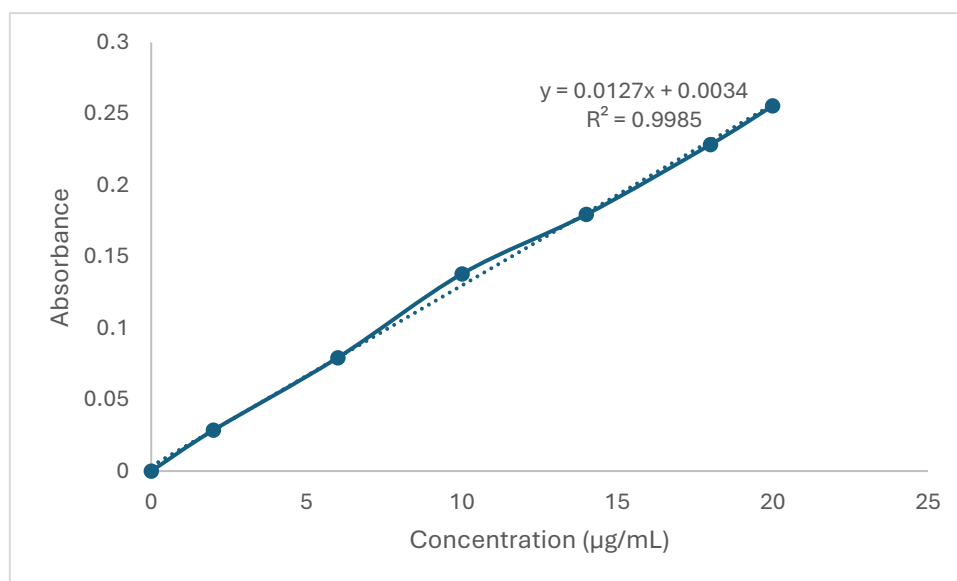


Figure 1: Calibration curve of Pazopanib in Phosphate Buffer pH 7.4

Results of solubility study

The results of the solubility study are presented in the following table. Table 4 shows the solubility of pazopanib in different solvents and buffer solutions. The practically negligible solubility of Pazopanib in water (0.042 ± 0.003 mg/mL), phosphate buffer pH 7.4 (0.038 ± 0.002 mg/mL) and phosphate buffer pH 6.8 (0.051 ± 0.004 mg/mL) confirmed the BCS Class II classification. The solubility results of methanol were the highest (18.64 ± 0.42 mg/mL) and ethanol practically showed insoluble behavior (0.087 ± 0.006 mg/mL) both of which shows the need for developing a nanocarrier-based delivery system as it has its own solubility limitations in water.

Table 4: Solubility Profile of Pazopanib in Various Solvents and Buffer Systems

Sr. No.	Solvent	Solubility (mg/mL)	Inference
1	Water	0.042 ± 0.003	Practically insoluble
2	Methanol	18.64 ± 0.42	Sparingly soluble
3	Ethanol	0.087 ± 0.006	Practically insoluble
4	Phosphate Buffer pH 7.4	0.038 ± 0.002	Practically insoluble
5	Phosphate Buffer pH 6.8	0.051 ± 0.004	Practically insoluble

All values are expressed as mean $\pm$ SD, n=3

DSC analysis

Figure 2 shows the DSC thermograms of pure pazopanib sample and physical mixture of pazopanib with excipients. Pure pazopanib (A) showed a sharp characteristic endothermic melting peak at 285.98°C , which is a sign of crystallinity. It was observed that in the physical mixture (B), the drug peak was also observed at 285.91°C with small shift and there was no

significant drug-excipient interaction or chemical incompatibility as an excipient peak was also observed at 302.12°C. The presence of the characteristic peak of the drug indicates the physicochemical compatibility of pazopanib with selected excipients and their compatibility for further formulation development.

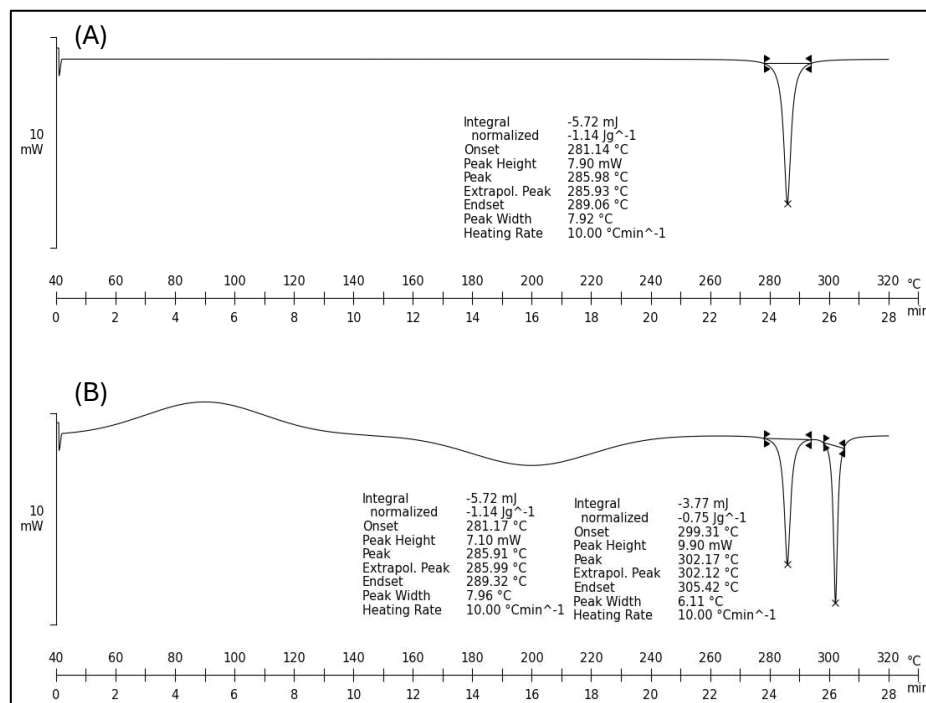


Figure 2: DSC Spectra of (A) Pure drug [285.98] and (B) Physical mixture [285.91 and 302.12]

FTIR Analysis

FTIR spectra of pure pazopanib (A) and its physical mixture (B) are given in figure 3. Pure pazopanib displayed characteristic absorption peaks at 3365.36, 3296.36, 3178.36 cm^{-1} (N-H stretching), 3064.36, 3017.36, 2972.36, 2920.36, 2870.36 cm^{-1} (C-H stretching), 1523.36, 1447.36 cm^{-1} (aromatic C=C stretching), and 1015.36 cm^{-1} (S=O stretching). The peaks in the fingerprint region (1638.36, 1577.36, 1527.36, 1164.36 cm^{-1}) were intact and no peak was found to have completely disappeared or new peak formation occurred, indicating that there was no chemical interaction between pazopanib and excipients and their compatibility was concluded for formulation development of nanoparticles.

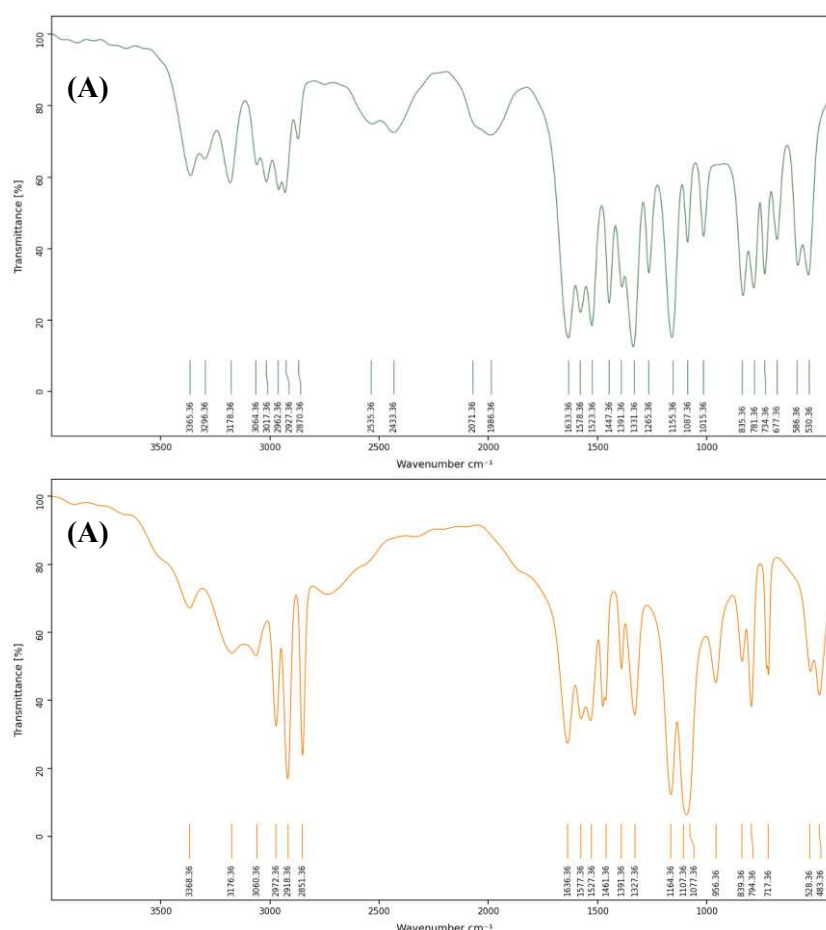


Figure 3: FTIR spectra of (A) Pure drug and (B) Physical mixture

Results of Characterization of pazopanib loaded amino functionalized mesoporous silica nanoparticles

Results of Particle Size, Polydispersity Index, Zeta Potential Encapsulation Efficiency and Drug Loading Capacity

All seventeen formulations have been chemically and physically characterized and the summary is presented in Table 5. Encapsulation efficiency ranged from $78.05 \pm 0.61\%$ (F17) to $89.12 \pm 0.60\%$ (F16), particle size from 171.74 ± 4.42 nm (F16) to 248.55 ± 4.09 nm (F17), and PDI from 0.136 to 0.240, confirming acceptable monodispersity. All formulations showed positive zeta potential values between +11.78 mV and +20.81 mV indicating amino functionalization was successful, with F16 showing the most desirable overall physicochemical profile of all the formulations prepared.

Table 5: Physicochemical Characterization of Pazopanib-Loaded Amino-Functionalized Mesoporous Silica Nanoparticles (F1–F17)

Formulation	Encapsulation Efficiency (%)	Particle Size (nm)	Polydispersity Index	Zeta Potential (mV)
F1	83.42 ± 0.83	228.64 ± 3.03	0.194 ± 0.004	$+16.25 \pm 0.30$
F2	78.36 ± 0.68	246.18 ± 1.91	0.240 ± 0.005	$+11.83 \pm 0.28$

F3	84.91 ± 0.49	214.92 ± 2.14	0.181 ± 0.005	+17.20 ± 0.36
F4	82.27 ± 0.32	232.75 ± 1.93	0.209 ± 0.007	+15.03 ± 0.23
F5	85.63 ± 0.48	205.36 ± 3.61	0.170 ± 0.004	+17.82 ± 0.45
F6	79.84 ± 0.49	241.28 ± 2.71	0.225 ± 0.006	+13.28 ± 0.21
F7	83.95 ± 0.75	220.84 ± 3.25	0.193 ± 0.006	+16.69 ± 0.52
F8	81.72 ± 0.69	236.91 ± 4.36	0.211 ± 0.003	+14.31 ± 0.44
F9	87.88 ± 0.85	189.67 ± 2.53	0.148 ± 0.006	+19.98 ± 0.25
F10	85.21 ± 0.58	207.43 ± 2.98	0.177 ± 0.004	+18.61 ± 0.18
F11	80.96 ± 0.36	239.12 ± 3.94	0.214 ± 0.003	+14.71 ± 0.46
F12	85.74 ± 0.74	206.58 ± 2.48	0.168 ± 0.008	+17.99 ± 0.42
F13	85.18 ± 0.77	208.14 ± 2.05	0.176 ± 0.008	+17.83 ± 0.43
F14	88.62 ± 0.64	178.92 ± 2.65	0.135 ± 0.007	+19.71 ± 0.44
F15	88.47 ± 0.77	179.36 ± 2.29	0.137 ± 0.005	+19.37 ± 0.21
F16	89.12 ± 0.60	171.74 ± 4.42	0.136 ± 0.003	+20.81 ± 0.30
F17	78.05 ± 0.61	248.55 ± 4.09	0.239 ± 0.006	+11.78 ± 0.22

All values are expressed as mean ± SD, n=3

Optimization by Box-Behnken Design

Encapsulation Efficiency (Y_1)

The quadratic model presented a very good fit with an Adjusted $R^2 = 0.8924$, Predicted $R^2 = 0.8666$ and a very significant $F = 15.74$ at a significance level of 0.0007 (Table 6). ANOVA analysis revealed TEOS concentration (A) as the most significant factor ($F = 74.77$, $p < 0.0001$), followed by CTAB concentration (B, $F = 30.91$, $p = 0.0009$) and reaction temperature (C, $F = 10.12$, $p = 0.0155$). Quadratic terms were significant ($F = 12.06$, $p = 0.0104$) while interaction terms were not significant ($p > 0.05$).

$$Y_1 = +86.08 + 3.49A + 2.25B + 1.29C + 0.5075AB + 0.5200AC + 0.6550BC - 1.93A^2 - 1.06B^2 - 1.25C^2$$

Positive linear coefficients suggested that the factors had a positive effect on the encapsulation efficiency, and negative quadratic coefficients verified the presence of curvilinear relationships with optimal points. The encapsulation efficiency was further plotted as response surface and contour plot (Figures 4 and 5), which showed curved response surfaces having clear maximums, indicating synergy between TEOS and CTAB concentrations.

Particle Size (Y_2)

The results of the quadratic model were presented in Table 6, showing Adjusted $R^2 = 0.8485$, Predicted $R^2 = 0.8732$ and significance ($F = 10.96$, $p = 0.0023$); it had good predictability. TEOS concentration (A) was the most influential factor ($F = 43.09$, $p = 0.0003$), followed by CTAB concentration (B, $F = 22.88$, $p = 0.0020$) and reaction temperature (C, $F = 8.25$, $p = 0.0239$). AB was marginally significant ($F = 5.56$, $p = 0.0506$).

$$Y_2 = +201.29 - 22.28A - 16.24B - 9.75C - 11.32AB - 7.66AC - 6.20BC + 10.01A^2 + 10.17B^2 + 9.14C^2$$

Positive linear coefficients suggested that particle size decreased as factors increased, and positive quadratic coefficients suggested parabolic relationships between particle size and the factors. The surfaces in the response surface plots (Figures 4 and 5) showed a bowl-shaped surface with minima of the particle sizes at intermediate-to-high factor levels, which were the optimal conditions for the smallest particle size achievement.

Table 6: Model Fit Summary for Response Surface Methodology Optimization

Source	Sequential p-value	Lack of Fit p-value	Adjusted R ²	Predicted R ²	Suggested Model
Encapsulation Efficiency (Y₁)					
Quadratic	0.0130	0.9381	0.8924	0.8666	Suggested
Particle Size (Y₂)					
Quadratic	0.0387	0.9906	0.8485	0.8732	Suggested

Table 7: ANOVA Summary for Quadratic Model of Response Variables

Source	Sum of Squares	df	Mean Square	F-value	p-value	Significance
Encapsulation Efficiency (Y₁)						
Model	185.01	9	20.56	15.74	0.0007	Significant
A-TEOS	97.65	1	97.65	74.77	< 0.0001	Significant
B-CTAB	40.37	1	40.37	30.91	0.0009	Significant
C-Temperature	13.21	1	13.21	10.12	0.0155	Significant
AB	1.03	1	1.03	0.7889	0.4039	Not significant
AC	1.08	1	1.08	0.8282	0.3930	Not significant
BC	1.72	1	1.72	1.31	0.2893	Not significant
A ²	15.75	1	15.75	12.06	0.0104	Significant
B ²	4.77	1	4.77	3.65	0.0976	Not significant
C ²	6.54	1	6.54	5.01	0.0602	Not significant
Residual	9.14	7	1.31	-	-	-
Lack of Fit	0.8051	3	0.2684	0.1288	0.9381	Not significant
Pure Error	8.34	4	2.08	-	-	-

Cor Total	194.15	16	-	-	-	-
Particle Size (Y₂)						
Model	9093.27	9	1010.36	10.96	0.0023	Significant
A-TEOS	3972.08	1	3972.08	43.09	0.0003	Significant
B-CTAB	2109.25	1	2109.25	22.88	0.0020	Significant
C-Temperature	760.50	1	760.50	8.25	0.0239	Significant
AB	512.12	1	512.12	5.56	0.0506	Not significant
AC	235.01	1	235.01	2.55	0.1544	Not significant
BC	153.76	1	153.76	1.67	0.2375	Not significant
A ²	421.64	1	421.64	4.57	0.0698	Not significant
B ²	435.23	1	435.23	4.72	0.0663	Not significant
C ²	351.90	1	351.90	3.82	0.0917	Not significant
Residual	645.30	7	92.19	-	-	-
Lack of Fit	15.71	3	5.24	0.0333	0.9906	Not significant
Pure Error	629.59	4	157.40	-	-	-
Cor Total	9738.57	16	-	-	-	-

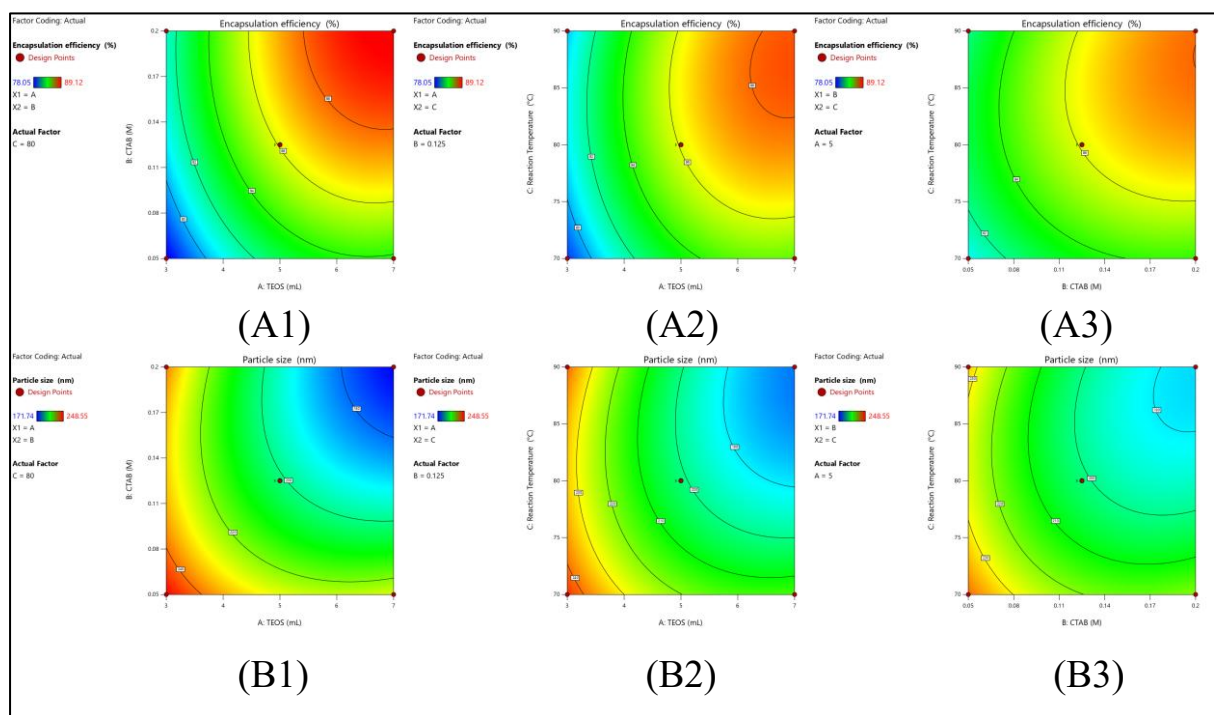


Figure 4. Two-dimensional contour plots illustrating the interactive effects of formulation variables on critical quality attributes of pazopanib-loaded amino-functionalized mesoporous silica nanoparticles. (A1, B1) Combined effects of TEOS concentration and CTAB concentration on encapsulation efficiency and particle size respectively; (A2, B2) Interactive influence of TEOS concentration and reaction temperature on encapsulation efficiency and particle size respectively; (A3, B3) Combined effects of CTAB concentration and reaction temperature on encapsulation efficiency and particle size respectively.

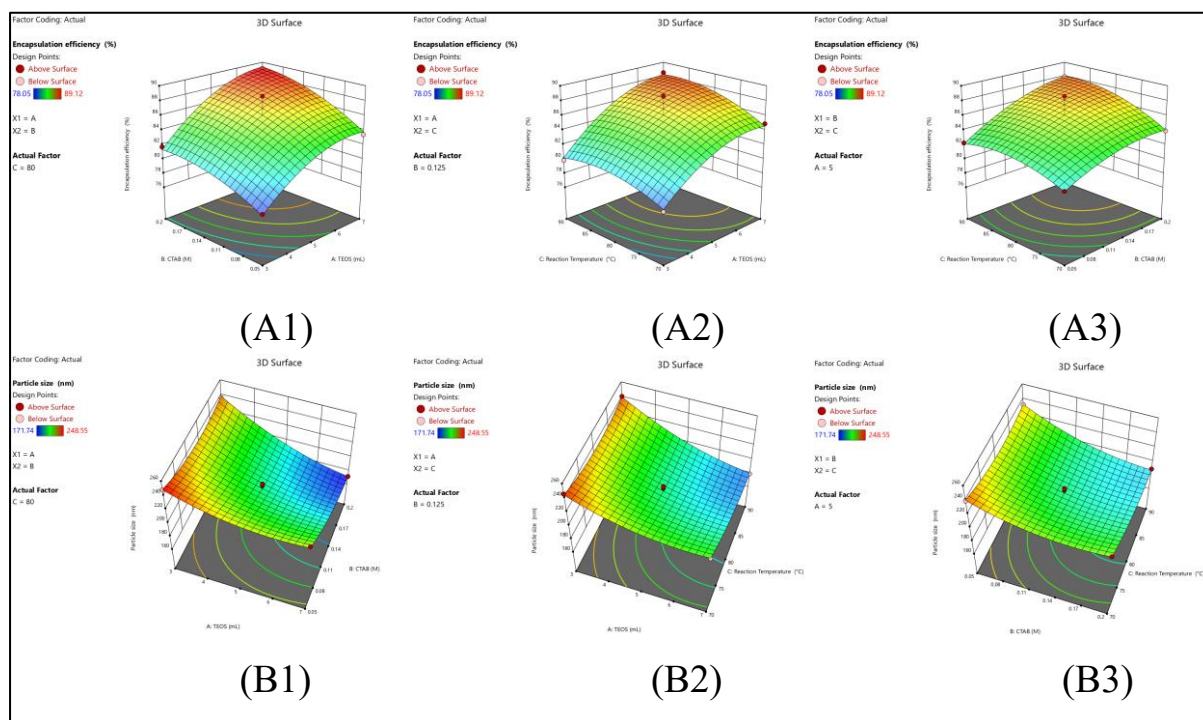


Figure 5. Three-dimensional response surface plots depicting the interactive effects of formulation variables on critical quality attributes of pazopanib-loaded amino-functionalized mesoporous silica nanoparticles. (A1, B1) Combined effects of TEOS concentration and CTAB concentration on encapsulation efficiency and particle size respectively; (A2, B2) Interactive influence of TEOS concentration and reaction temperature on encapsulation efficiency and particle size respectively; (A3, B3) Combined effects of CTAB concentration and reaction temperature on encapsulation efficiency and particle size respectively.

Validation of statistical model

The statistical model validation was performed by comparing predicted and experimentally observed values for the optimized formulation F16, as presented in Table 8. The predicted encapsulation efficiency of 89.325% closely corresponded to the experimental value of 89.12%, yielding a negligible relative error of 0.23%. Similarly, predicted particle size of 171.625 nm was in excellent agreement with the experimental value of 171.74 nm, with a minimal relative error of 0.07%. An overall desirability score of 1 confirmed the robustness and excellent predictability of the developed Box-Behnken design statistical model.

Table 8: Validation of Optimized Formulation Predicted vs. Experimental Values with Desirability

Batch	Response	Predicted value	Experimental value	% relative error	Desirability
F16	Encapsulation efficiency	89.325	89.12	0.23	1
	Particle size	171.625	171.74	0.07	

Results of in vitro drug release

The drug release profiles of all seventeen formulations are plotted in vitro for the duration of 24 hours as shown in Figure 6. A typical biphasic release pattern was observed in all formulations with an initial burst release (18.77% to 21.31%) within the first 0.5 hour and then a sustained controlled release for the next 24 hours. The formulation variables had a direct effect on the drug release behavior with formulation F16 exhibiting the highest cumulative drug release of 91.87% at 24 hours and formulation F17 exhibiting the lowest cumulative drug release of 73.04% at 24 hours. The sustained release profiles seen in all formulations further validate the ability to achieve controlled drug delivery with the amino-functionalized mesoporous silica nanoparticle system.

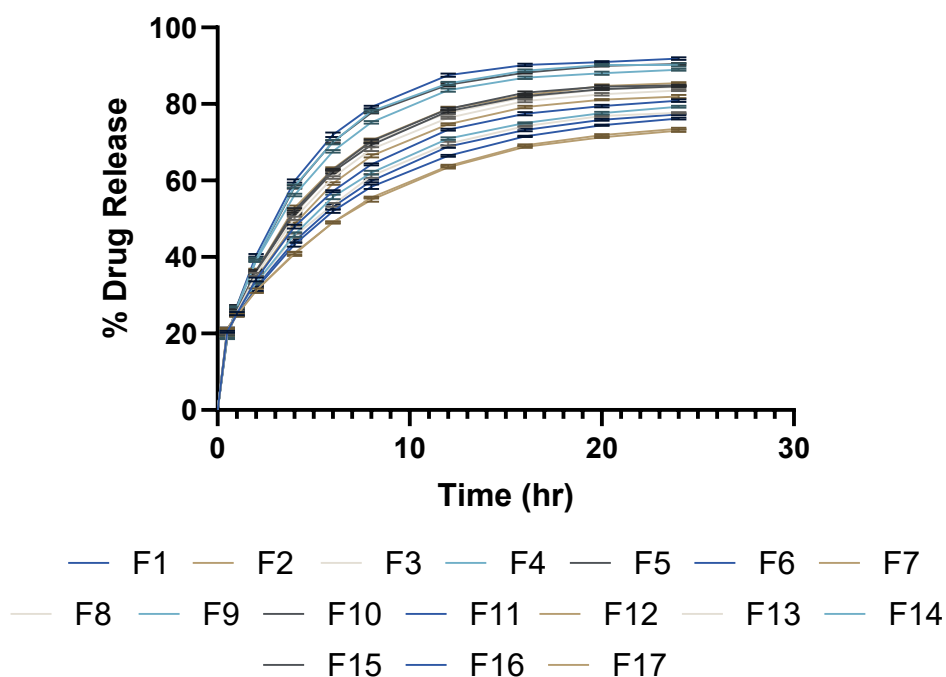


Figure 6: In vitro drug release profile of all batches of mesoporous silica nanoparticles (F1-F17)

SEM Analysis

The optimized formulation F16 is presented in Figure 7 in scanning electron microscopy image. The SEM image taken at 15,000 $\times$ magnification showed mostly spherical morphology and relatively smooth surface with morphology characteristic of sol-gel synthesized mesoporous silica nanoparticles. The particles shown a size distribution which was visually

supported by the DLS measured particle size, as shown in Table 5, 171.74 ± 4.42 nm. Surface roughness and slight aggregation tendency were observed and were believed to be due to the amino-functionalization using APTES and inherent van der Waals forces between particles during the preparation of the sample.

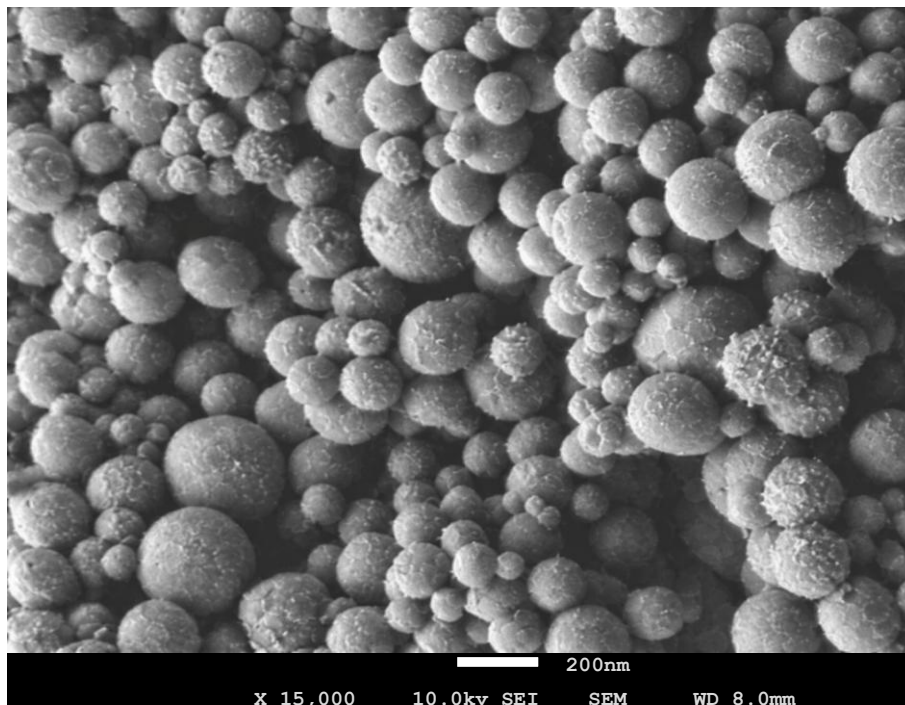


Figure 7: SEM of optimized batch F16

FTIR Analysis

Amino-functionalized mesoporous silica nanoparticles FTIR spectrum is shown in Figure 8. The spectrum showed characteristic N-H stretching bands at 3441.36, 3361.36, and 3304.36 cm^{-1} , indicating that grafting of APTES onto the silica surface was successful. C-H stretching vibrations of the aminopropyl chain were seen at 2935.36 cm^{-1} and 2868.36 cm^{-1} and N-H bending vibrations were confirmed at 1639.36 cm^{-1} and 1593.36 cm^{-1} . The presence of the SiO-Si stretching peak at 1091.36 cm^{-1} further indicated that the mesoporous silica framework had not been altered during amino-functionalization, thus establishing successful functionalization of the nanoparticles.

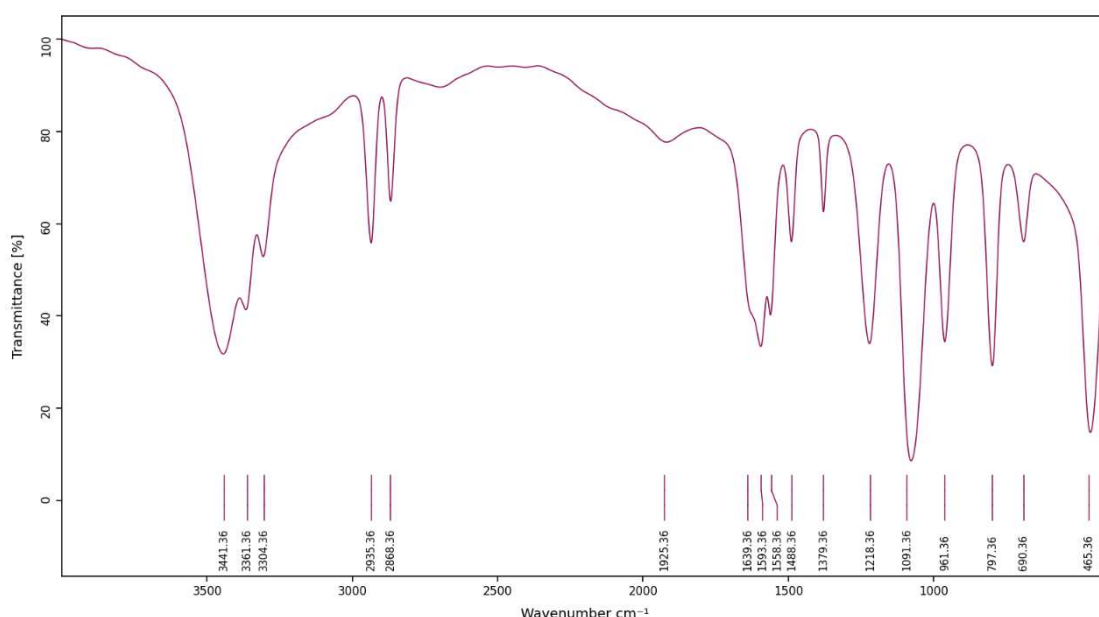


Figure 8: FTIR spectra of amino functionalized mesoporous silica nanoparticles

Results of characterization of mesoporous silica nanoparticles loaded capsule

Results of Average Weight Variation, Disintegration Time and Drug Content Uniformity

Average Weight Variation, Disintegration Time and Drug Content Uniformity results. The capsule evaluation parameters for formulations CF1- CF4 are given in Table 9. The average capsule weights were between 422 ± 3.24 mg (CF4) and 476 ± 2.84 mg (CF1) with maximum weight deviations within 1.79–2.30%, which are within the pharmacopoeial limits. The disintegration time increased with the progression of the blocks, from CF1 (12.84 ± 0.62 min) to CF4 (18.72 ± 0.92 min); this was inversely related to the decreasing MCC concentration. The drug content uniformity for all the formulations were within the range of 85–115% and the percentage results ranged from $96.48 \pm 1.08\%$ (CF4) to $99.14 \pm 0.76\%$ (CF3), with CF1 showing the best overall performance.

Table 9: Average Weight Variation, Disintegration Time, and Drug Content Uniformity of Pazopanib-Loaded Amino-Functionalized Mesoporous Silica Nanoparticles Capsule Formulations (CF1–CF4)

Sr. No.	Parameter	CF1	CF2	CF3	CF4
1	Average Capsule Weight (mg)	476 ± 2.84	458 ± 3.12	440 ± 2.96	422 ± 3.24
2	Maximum % Weight Deviation	1.79	2.04	2.02	2.30
3	Disintegration Time (min)	12.84 ± 0.62	14.26 ± 0.84	16.48 ± 0.74	18.72 ± 0.92
4	Drug Content Uniformity (%)	98.64 ± 0.84	97.82 ± 0.92	99.14 ± 0.76	96.48 ± 1.08

n = 20 for weight variation; n = 6 for disintegration time; n = 10 for drug content uniformity; values expressed as Mean $\pm$ SD

Results of Dissolution Study

The dissolution profiles of the capsule formulations CF1–CF4 in phosphate buffer pH 6.8 during 60 minutes are displayed in Figure 9. The drug release in all formulations was found to be progressive and dependent on concentration during the study. The cumulative drug release for CF1 ($72.46 \pm 0.40\%$) at 60 minutes was the highest, followed by CF2 ($67.82 \pm 0.54\%$), CF3 ($62.54 \pm 0.46\%$) and CF4 ($57.26 \pm 0.45\%$), indicating a decreasing dissolution trend as the amount of magnesium stearate increased. CF1 showed the best dissolution result which matched directly with the fastest disintegration time of 12.84 minutes as shown in Table 9, giving it an optimized capsule formulation.

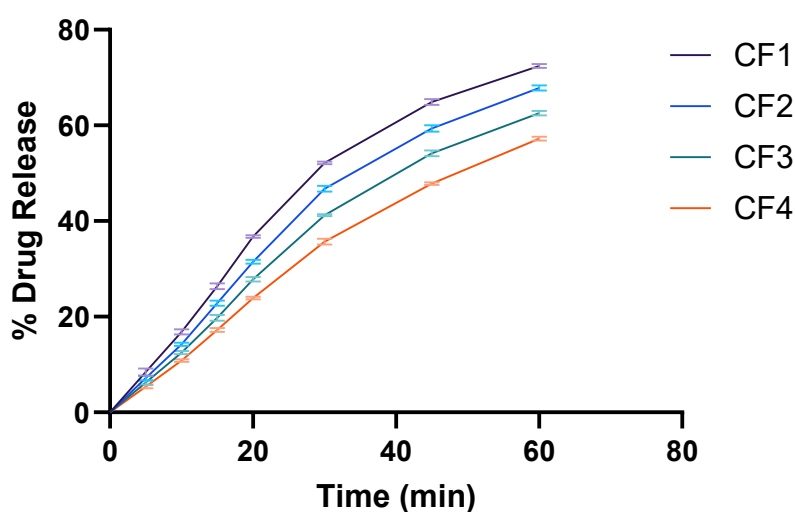


Figure 9: Drug dissolution profile of all batches of capsules (CF1-CF4)

Results of Stability study

The accelerated stability data of optimized formulation CF1 at 40 °C/75 %RH for three months is shown in Table 10, in accordance with the guidelines of ICH Q1A(R2). There was no change in physical appearance during the study period. There were only slight changes in all parameters: the encapsulation efficiency decreased from $89.12 \pm 0.60\%$ to $87.61 \pm 1.04\%$, the particle size increased slightly from 171.74 ± 4.42 nm to 176.43 ± 5.34 nm, and the zeta potential remained positive throughout the process: 20.81 ± 0.30 mV to 19.47 ± 0.54 mV. Cumulative drug release at 60 minutes was found to be slightly decreased from $72.46 \pm 0.40\%$ to $70.87 \pm 0.74\%$, both of which showed an excellent physicochemical stability of the optimized formulation in accelerated storage conditions.

Table 10: Stability Profile of Optimized Pazopanib-Loaded Amino-Functionalized Mesoporous Silica Nanoparticles Capsule Formulation CF1 at Accelerated Conditions (40°C/75% RH) as per ICH Q1A(R2) Guidelines

Sr.	Parameter	0 Month (T ₀)	1 Month	2 Months	3 Months
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No.					
1	Physical Appearance	Red opaque capsule; off-white fill; smooth; no deformation; no leakage	Unchanged	Unchanged	Unchanged
2	Average Capsule Weight (mg)	476 ± 2.84	475 ± 3.01	474 ± 3.14	473 ± 3.28
3	Maximum % Weight Deviation	1.79	1.84	1.90	1.95
4	Disintegration Time (min)	12.84 ± 0.62	13.18 ± 0.71	13.74 ± 0.84	14.32 ± 0.96
5	Drug Content Uniformity (%)	98.64 ± 0.84	98.22 ± 0.96	97.68 ± 1.08	97.08 ± 1.22
6	Encapsulation Efficiency (%)	89.12 ± 0.60	88.74 ± 0.72	88.18 ± 0.88	87.61 ± 1.04
7	Particle Size (nm)	171.74 ± 4.42	172.94 ± 4.68	174.52 ± 4.96	176.43 ± 5.34
8	Polydispersity Index	0.136 ± 0.003	0.140 ± 0.004	0.145 ± 0.005	0.149 ± 0.006
9	Zeta Potential (mV)	$+20.81 \pm 0.30$	$+20.38 \pm 0.38$	$+19.94 \pm 0.46$	$+19.47 \pm 0.54$
10	% Cumulative Drug Release at 60 min	72.46 ± 0.40	71.94 ± 0.54	71.38 ± 0.64	70.87 ± 0.74

All values are expressed as mean $\pm$ SD, n = 3; RH = Relative Humidity; Storage condition: $40^\circ\text{C} \pm 2^\circ\text{C}$ / $75\% \pm 5\%$ RH

Results of MTT assay

The in vitro cytotoxicity against 786-O renal cell carcinoma cells after 48 hours is provided in Table 11, Figure 10 and Figure 11. Cell viability reduction was shown to be concentration-dependent for all treatments. The cytotoxicity of the pazopanib loaded MSNPs was even better ($\text{IC}_{50}=18.37\pm0.72\mu\text{g/mL}$) than of pure pazopanib ($\text{IC}_{50}=28.64\pm0.84\mu\text{g/mL}$) and blank MSNPs demonstrated negligible toxicity ($\text{IC}_{50}>100\mu\text{g/mL}$), indicating the biocompatibility of the carrier. The data was supported by phase contrast microscopy in figure 11, which showed that cell density decreased considerably in the nanoformulation of pure drug (panel D) as compared to pure drug (panel B) and purified material (panel C), demonstrating the significantly enhanced anticancer efficacy of the nanoformulation as compared to pure drug. In Vitro Cytotoxicity (MTT Assay) of Blank Amino-Functionalized Mesoporous Silica Nanoparticles, Pazopanib-Loaded Amino-Functionalized Mesoporous Silica Nanoparticles and Pure Pazopanib against 786-O Human Renal Cell Carcinoma cell line after 48 hrs of treatment.

Table 11: In Vitro Cytotoxicity (MTT Assay) of Pure Pazopanib, Blank Amino-Functionalized Mesoporous Silica Nanoparticles, and Pazopanib-Loaded Amino-

Functionalized Mesoporous Silica Nanoparticles against 786-O Human Renal Cell Carcinoma Cell Line after 48 Hours of Treatment

Sr. No.	Concentration ($\mu\text{g/mL}$)	Negative Control (Untreated)	Pure Pazopanib	Blank MSNPs	Pazopanib-Loaded MSNPs
1	0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
2	5	99.84 ± 0.42	78.34 ± 1.24	96.42 ± 1.18	68.47 ± 1.32
3	10	99.76 ± 0.38	65.28 ± 1.46	95.18 ± 1.34	54.83 ± 1.48
4	25	99.62 ± 0.51	48.72 ± 1.63	93.74 ± 1.52	37.26 ± 1.54
5	50	99.48 ± 0.44	32.54 ± 1.72	92.36 ± 1.61	22.48 ± 1.67
6	100	99.21 ± 0.57	18.46 ± 1.84	91.08 ± 1.78	11.34 ± 1.82
—	IC₅₀ ($\mu\text{g/mL}$)	—	28.64 ± 0.84	>100	18.37 ± 0.72

All values are expressed as mean $\pm$ SD, n = 3

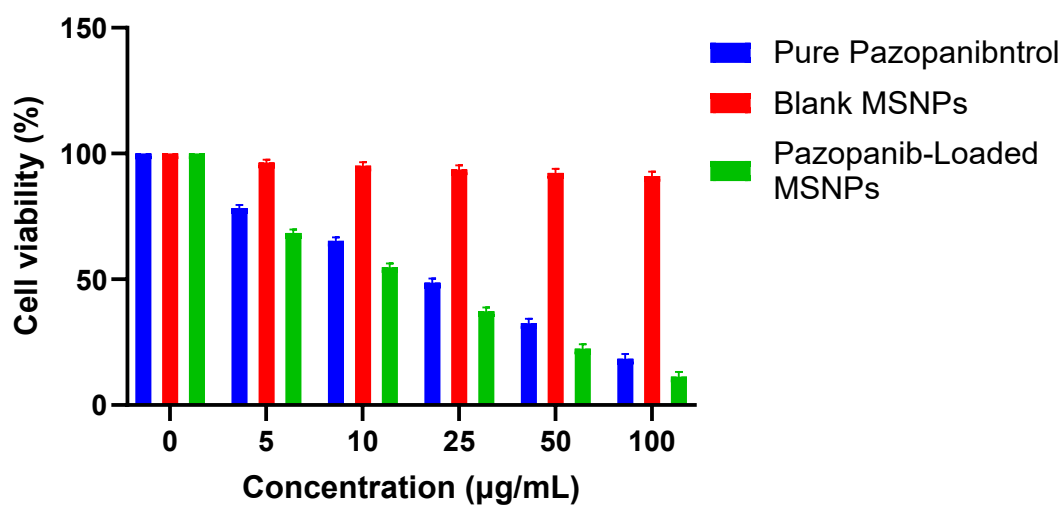


Figure 10: Concentration-dependent cell viability of 786-O human renal cell carcinoma cells treated with pure pazopanib, blank MSNPs, and pazopanib-loaded MSNPs for 48 hours as determined by MTT assay.

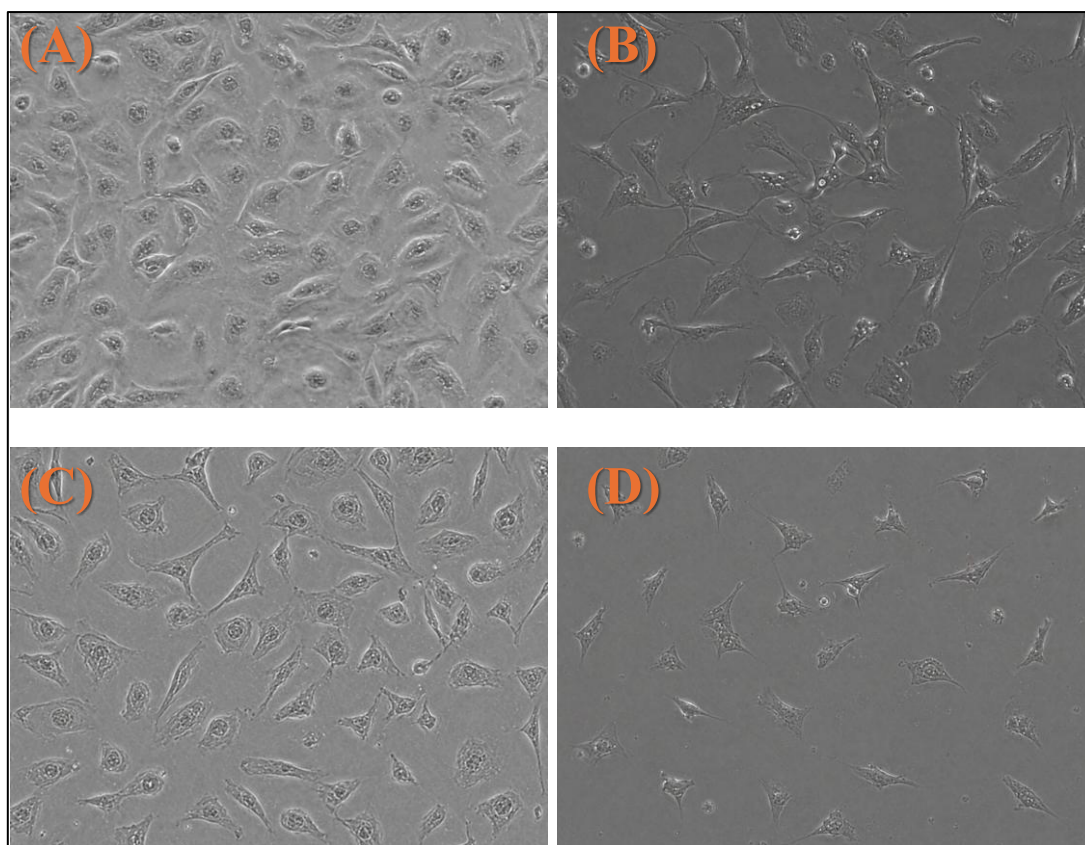


Figure 11: Phase contrast microscopic images of 786-O human renal cell carcinoma cells after 48 hours of treatment: (A) negative control (untreated cells showing confluent morphology), (B) pure pazopanib (moderate reduction in cell density with cytomorphological changes), (C) blank amino-functionalized mesoporous silica nanoparticles (near-normal cell density confirming carrier biocompatibility), and (D) pazopanib-loaded amino-functionalized mesoporous silica nanoparticles (markedly reduced cell density with significant morphological alterations, indicative of superior cytotoxic activity) at IC_{50} concentration.

DISCUSSION

In the present study, Box-Behnken design (BBD) was successfully utilized for optimization of formulation of pazopanib loaded amino-functionalized mesoporous silica nanoparticles (NH₂-MSNs) as targeted drug delivery system for cancer targeted therapy. From the solubility profiling (Table 4), pazopanib was verified to belong to BCS Class II which has practically negligible solubility in water (0.042 ± 0.003 mg/mL) and phosphate buffer pH 7.4 (0.038 ± 0.002 mg/mL), consistent with previous reports of approximately 0.043 mg/mL for pazopanib hydrochloride in phosphate buffer pH 7.4. The high hydrophobicity along with reported oral bioavailability of < 14–39% creates a major pharmacokinetic challenge, which required the use of an advanced nanocarrier-based delivery system in this study [38]. The DSC thermograms (Figure 2) showed the characteristic endothermic melting peak of pure pazopanib, which remained unchanged in the physical mixture and did not show any significant peak shifts, indicating no drug–excipient incompatibility before encapsulation. This was confirmed by FTIR spectroscopy (Figure 3), where the characteristic drug

functional group peaks were also preserved in the physical mixture, which indicated the physicochemical compatibility. Concentration of TEOS (X_1) was the most statistically significant factor for the encapsulation efficiency ($F = 74.77$, $p < 0.0001$) and particle size ($F = 43.09$, $p = 0.0003$) (Table 7), which is in agreement with previous reports that the TEOS concentration is the basic parameter controlling the kinetics of the condensation of the silica framework and the subsequent pore architecture of the resulting materials [39]. The second most significant parameter was the concentration of CTAB (X_2), a surfactant template for the mesostructure, which led to the formation of smaller and more uniformly organized nanoparticles, as it is well known that CTAB assembles the MSN into smaller pores with more uniformly organized structures. Reaction temperature (X_3) was statistically significant but relatively small ($F = 10.12$, $p = 0.0155$ for Y_1 ; $F = 8.25$, $p = 0.0239$ for Y_2), affecting the condensation rate and the density of cross-links in silica network. The equations of the validated quadratic model ($R^2 = 0.8924$ for Y_1 ; $R^2 = 0.8485$ for Y_2) showed adequate predictability (Table 6) and the BBD statistical model was validated by the results of optimized formulation F16 which produced an EE of $89.12 \pm 0.60\%$ and particle size of 171.74 ± 4.42 nm (Table 5 and Table 8) with a minimum percentage relative error of 0.23% and 0.07% respectively [40]. The PDI value of 0.136 ± 0.3 (Table 5) indicated a narrow population, which had a positive zeta potential of $+20.81 \pm 0.30$ mV (Table 5) that confirmed successful amino-functionalization, as unfunctionalized MSNs usually have negative surface zeta potentials that result from surface deprotonation of the silanol groups [41]. The observation of characteristic N–H stretching (~ 3300 cm^{-1}) and N–H bending (~ 1550 cm^{-1}) bands, which can be assigned to grafted aminopropyl groups, by FTIR analysis (Figure 8) in this case confirms results that were obtained in similar pharmaceutical systems [42]. The SEM image of optimized batch F16 (Figure 7) showed a spherical shape of particles with uniform size exhibiting a smooth surface texture, which is similar to that observed in the well-ordered mesoporous silica nanoparticles produced by sol-gel technique [43]. The in vitro drug release profiles (Figure 6) showed a biphasic release for all formulations, with an initial burst release followed by a prolonged and sustained release over 24 hours, which is due to the porous MSN architecture, enabling drug to penetrate the surface layer and be released first, followed by the release of other drugs from the interior mesopores [44]. In agreement with recent literature reporting anomalous non-Fickian diffusion as the kinetic dominant mechanism in drug release from the functionalized mesoporous silica systems, the drug release kinetics of functionalized mesoporous silica systems were best described by the Korsmeyer–Peppas model, in which simultaneous diffusion and matrix erosion processes control the release kinetic [45]. The capsule formulation CF1, having the highest content of MCC (120 mg), showed the best disintegration (12.84 ± 0.62 min) and uniformity of drug content [8] ($98.64 \pm 0.84\%$) in comparison to the other formulations (CF1–CF4) while CF4 with highest concentration of MgSt (8 mg) showed the slowest disintegration time (18.72 ± 0.92 min), due to excessive hydrophobicity of the capsule fill blend.

The accelerated stability study at $40^\circ\text{C} \pm 2^\circ\text{C}$ and $75\% \pm 5\%$ RH for three months as per ICH Q1A(R2) guidelines showed excellent physicochemical stability of the optimized CF1 formulation (Table 10). The rigid silica matrix framework showed an encapsulation efficiency that decreased only slightly with time from $89.12 \pm 0.60\%$ to $87.61 \pm 1.04\%$, while the

particle size increased marginally from 171.74 ± 4.42 nm to 176.43 ± 5.34 nm and the cumulative drug release at 60 minutes was largely unaffected (72.46% to 70.87%), which is consistent with their reported superior structural integrity and drug retention under accelerated stress conditions when compared to conventional amorphous dispersions [46]. The zeta potential of $+19.47 \pm 0.54$ mV at three months (Table 10) was also well above the recommended threshold of ± 15 mV for colloidal stability, indicating that there was no significant agglomeration or surface deamination during storage [47]. The *in vitro* MTT assay of the cytotoxicity activity of the 786-O human renal cell carcinoma cell line (Table 11, Figure 10) provided convincing evidence of the therapeutic superiority of the nanoformulation: pazopanib loaded with NH₂-MSNs showed a much lower IC₅₀ value of 18.37 ± 0.72 μ g/mL, an improved cytotoxic potency value of 1.56-fold compared to pure pazopanib (28.64 ± 0.84 μ g/mL) and blank NH₂-MSNs showed negligible cytotoxicity (IC₅₀ > 100 μ g/mL) confirming its intrinsic carrier biocompatibility. The superior electrostatic interaction between the positively charged amino-functionalized nanoparticles (+20.81 mV) and the negatively charged cell membrane of the cancer cells, which is a critical step for cellular internalization, was proposed to be one of the reasons for the enhanced cytotoxicity, while the passive tumor accumulation of amino-functionalized nanoparticles through the enhanced permeability and retention (EPR) effect mechanisms well documented for their ability of collectively reducing the IC₅₀ of nanoencapsulated drugs compared with their free drug counterparts, was proposed as the other reason [48]. The superior cytotoxicity observed is in line with those for other tyrosine kinase inhibitors nanoencapsulated either in PLGA or in nanogels, in which the nanoparticles administration is always associated with lower IC₅₀ in comparison to free drug, due to the increased intracellular accumulation [49]. These findings were further confirmed by the concentration-dependent cell viability curves (Figure 10) and phase contrast microscopy images (Figure 11) which showed that drug loaded MSN-treated cells (panel D) had significantly lower cell density and pronounced alterations in their morphology compared to pure pazopanib-treated cells (panel B) and blank MSN-treated cells (panel C), respectively. In the context of advanced renal cell carcinoma treatment, pazopanib suffers from principal [50]. pharmacokinetic drawbacks, such as poor solubility and variable bioavailability, and the present amino-functionalized MSN platform exemplifies a rational approach based on a combination of multiple components, which is capable of overcoming all these issues and can therefore be seen as a promising candidate for further *in vivo* evaluation and translational development [51].

CONCLUSION

The Box-Behnken design was successfully employed and optimized in developing and formulating amino-functionalized mesoporous silica nanoparticles loaded with pazopanib, which resulted in the formulation F16 with the highest EE of $89.12 \pm 0.60\%$, the lowest PDI of 0.136, and positive zeta potential of +20.81 mV, indicating successful functionalization with APTES. The optimized CF1 capsule formulation showed sustained drug release as well as good drug content uniformity ($98.64 \pm 0.84\%$) and good physicochemical stability under the ICH Q1A(R2) accelerated conditions for three months. *In vitro* MTT cytotoxicity assays against 786-O renal cell carcinoma cells showed that pazopanib has a significantly lower IC₅₀ value compared to pure pazopanib, suggesting that the biologic possesses excellent anticancer

activity with less frequent dosing, less systemic toxicity and better patient compliance. The exciting results obtained in this study are worthy of additional in vivo pharmacokinetic and antitumor efficacy studies in order to progress this nanoplatform toward clinical translation.

ABBREVIATIONS

ANOVA: Analysis of Variance; APTES: 3-Aminopropyltriethoxysilane; BBD: Box-Behnken Design; CTAB: Cetyltrimethylammonium Bromide; DSC: Differential Scanning Calorimetry; EPR: Enhanced Permeability and Retention; FTIR: Fourier-Transform Infrared Spectroscopy; ICH: International Council for Harmonisation; IC₅₀: Half Maximal Inhibitory Concentration; MCC: Microcrystalline Cellulose; MSN: Mesoporous Silica Nanoparticles; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; NH₂-MSN: Amino-Functionalized Mesoporous Silica Nanoparticles; PDI: Polydispersity Index; RCC: Renal Cell Carcinoma; SD: Standard Deviation; SEM: Scanning Electron Microscopy; TEOS: Tetraethyl Orthosilicate; TKI: Tyrosine Kinase Inhibitor; UV: Ultraviolet Spectroscopy; VEGFR: Vascular Endothelial Growth Factor Receptor.

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All authors are equal contributed

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