Supplementary Information

In-situ Carbonization Metamorphoses Porous Silica Particles to Biodegradable Therapeutic Carriers of Lesser Consequence on TGF-β1 Mediated Fibrosis

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Experimental section

Materials

TEOS (Tetraethyl orthosilicate, SRL Pvt. Ltd., Product code: 93314), Cetyltrimethylammonium bromide (CTAB) (SRL Pvt. Ltd., Product code: 12779), *n*-tridecane (99%) (Acros Organics, Product code: 139511000), ammonium fluoride (98%) (SRL Pvt. Ltd., Product code:49813), ammonium hydroxide (28− 30%) (SRL Pvt. Ltd., Product code: 78719) were used for the synthesis of silica particles. Sucrose was purchased from Merck (Product code: 107687). Rhodamine 6G was purchased from Sigma-Aldrich, Product code: R4127 and curcumin was obtained from SRL Pvt. Ltd., Product code: 97461.

Table S1. Components and characteristics of various formulations developed for the study.

Components/ Characteristics	**Si-NP**	**Si-P**	**Si-P-CNP**	**Si-P-CNP-Cur**
Silica	Yes	Yes	Yes	Yes
Carbonization	No	No	Yes	Yes
Porosity	No	Yes	Yes	Yes
Curcumin	No	No	No	Yes
Induction possibility of lung fibrosis	Yes	Yes	No	No
Biodegradation	No	No	Yes	Yes

Synthesis of Silica particles

Silica based particles, Si-NP, were synthesized by Stöber method and with certain modifications as addition of carbon nanoparticles, as required. In a typical experiment a suspension containing 40 ml of ethanol, 10 ml methanol, and 10 ml of NH_4OH added in a round

bottom flask with continuous stirring. Then a mixture of 1 ml of ethanol and 1 ml of TEOS as source of siloxane was added in it. 2 ml (100 mg/ml) suspension of CNPs was added to the reaction mixture immediately after the addition of TEOS to prepare carbonized particles. The reaction continued for overnight at room temperature. Later these nanoparticles were kept for etching process and were able to produce Si-P and Si-P-CNP, which was further used to load curcumin as a model drug.

General protocol of etching process

The core-shell silica and silica carbon particles were generated by slightly modifying a method previously described[1, 2] . In brief, 1 g of synthesized silica particles with or without incorporated CNP were suspended in 100 mL of deionized water , and thereafter 1 g CTAB and 15.8 mL of *n*-tridecane was added with it. This mixture was sonicated for an hour. The final dispersed suspension was heated at 90 °C with continuous stirring. Afte that 16 ml aqueous ammonia solution and 25 mg of NH_4F was added and mixture was stirred for another 24 h. The produced particles were centrifuged and washed with water, ethanol, and diethyl ether. Finally, CTAB was removed by calcination to produce porous shell.

Hydrodynamic diameter & Zeta potential

Si-P, Si-P-CNP, Si-NP and Si-NP-CNP particles were suspended in water at concentration of 100 µg/ml and sonicated in an ultrasound bath for 10 minutes. CNPs were suspended in water at concentration 25 mg/ml. Hydrodynamic diameter and zeta potential were acquired at room temperature using a Zetasizer Nano ZS90 by Malvern.

Transmission electron microscopy (TEM)

(TEM) images were acquired on a FEI-Tecnai G2 12 Twin 120 KV TEM. The particles were suspended in ethanol and kept in an ultrasonic bath for homogenization for 10 minutes. The samples were drop casted on carbon coated copper grids for TEM analysis. The particle diameters were determined by using Image J software.

Scanning electron microscopy (SEM)

Particles were placed on an aluminium foil and dried under a vacuum desiccator for overnight. The sample containing aluminium foil was cut using scissors in an adjustable shape and fixed over a copper stub using carbon tape. All samples were gold coated and images were obtained under Zeiss scanning electron microscope.

Physisorption measurements

N_2-physisorption measurements were carried out on an Autosorb I (Quantachrome Instrument) at 77 K (am (N_2, 77 K). Prior to analysis, the samples thoroughly degassed at 250 °C for 4 h. The nitrogen adsorption branch of the isotherm was used to calculate the Brunauer-Emmett-Teller (BET) specific surface area for pure SiO_2 and hybrid materials in the relative pressure range of 0.07 to 0.15. The nitrogen desorption branch's pore size distributions (dV/dD) were determined using the Barrett- Joyner-Halenda (BJH) method[3].

FTIR

IR spectra were collected from PerkinElmer Spectrum Two spectrophotometer. A finely grained KBr was used for the background measurement.

X-ray diffraction

Aqueous suspensions of Si-P, Si-P-CNP, Si-NP and Si-NP-CNP were drop casted on a glass slide and allowed to completely evaporate the water at room temperature for 24 hrs. XRD investigation was performed with an X-ray diffractometer (XRD, PANalytical) using Cu-Kα radiation (λ = 1.54 Å).

Drug loading of curcumin

Curcumin and corresponding silica particles were mixed in ethanol at 1:1 ratio. The suspensions followed quick ultrasonication and were agitated for 4h. Subsequently, particles were centrifuged at 15,000 rpm for three min. The supernatants of the centrifuged samples were used to quantify the amount of loaded drug using the UV-Vis absorbance of curcumin at 425

nm. The equation given below has been used to determine the percentage of the drug that was loaded into the particles.

$$\text{Drug loading \%} = \frac{\text{Total amount of drug} - \text{Free drug}}{\text{Total amount of drug}} \times 100$$

Drug release studies and mode of loading

In vitro drug release studies of Si-P-CNP-Cur and Si-P-Cur were investigated at pH 7.4. The drug loaded Si-P-CNP-Cur and Si-P-Cur particles were suspended in 1 ml of phosphate-buffered saline (PBS) and transferred to a dialysis bag (molecular weight cut-off 2000 Da, Sigma-Aldrich, product code: D2272). The initial concentration of drug loaded particles was 100 µg/ml. The dialysis bag was placed inside a beaker containing PBS and incubated at 37 °C while stirring at 100 rpm. The released concentration of drugs from different particles were determined spectrophotometrically by measuring the absorbance at 425 nm at different time points of 0, 24, 48 and 72h.

Confocal microscopy

Nanoparticle samples were incubated on a shaker with rhodamine 6G solution in ethanol. After that, suspension of rhodamine loaded silica particles was centrifuged at 15000 RPM for three min. The pellet was resuspended in ethanol and drop casted over a glass slide. Confocal microscopic images were obtained under Zeiss LSM 700 confocal microscope.

NMR spectroscopy

Curcumin loaded Si-P and Si-P-CNP particles were suspended in D_2O and transferred into an NMR tube. ^{29}Si NMR spectra was obtained using a JEOL ECZ-500MHz NMR spectrometer.

Cell culture

A549 (lung epithelial carcinoma), Vero (kidney epithelial cell) and Raw 264.7 (Mouse macrophage) cells were cultured in a reconstituted media containing 89% Dulbecco's Modified Eagle's Medium (DMEM; Gibco; Thermo Fisher Scientific, Product code: 12800-017) 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Product code: 10270-106) and 1% antibiotic mixture of Penicillin-Streptomycin-Neomycin (PSN) (Gibco; Thermo Fisher Scientific, Product code: 15070-063) (V/V/V) inside an incubator maintained with 5% CO_2 and temperature of 37 °C. A 0.05% Trypsin EDTA (Gibco; Thermo Fisher Scientific, Product code: 15400-054) aliquot and phosphate buffered saline (PBS, pH 7.4) was used for passaging the cells[4].

Flow cytometric study

A549 and Vero cells (10×10^4 cells / well) were seeded in a 24 well plate and allowed to grow for 24h. Further, cells were treated with water suspended Si-P-CNP particles (5 mg/ml) loaded with curcumin. After 4hrs of treatment, cells were trypsinized and collected in a tube containing PBS supplemented with 0.2% FBS. Data acquisition was carried out in the green channel using flow cytometer (Cyflow space, Partec). Data was processed using FlowJo software.

Cell viability assay

Around ten thousand cells/well were seeded in a 96 well plate and grew for 24h. Thereafter, cells were treated with various samples of silica particles and curcumin loaded silica particles suspended in water. Curcumin dissolved in DMSO (Sigma-Aldrich, product code: D2650) was taken as a control. The concentrations of all samples were taken within 20% (v/v). After 44 h of incubation, a 20 µl volume of thiazolyl blue tetrazolium bromide (MTT) solution (Sigma-Aldrich, product code:M2128) (5mg/ml) was added to each well and incubated for another 4h. Then 100 µl of DMSO (Molychem, Mumbai, India, Product code: 14187) was applied to each well after removing all media. Absorbance values were obtained at 572nm by plate reader (En Spire 2300). Cells treated with DMSO were considered as a control for the cells treated with

curcumin solubilized in DMSO. Percent of cell viability was calculated using following formula[5].

$$\text{Cell viability (\%)} = \frac{Absorbance\ of\ treated - Absorbance\ of\ blank\ (DMSO)}{Absorbance\ of\ control - Absorbance\ of\ blank\ (DMSO)} \times 100$$

Interaction study with Raw 264.7

Around 10,000 Raw 264.7 cells were seeded over a 12 mm glass coverslip and allowed to grow for 24h. Cells were treated with Si-P-CNP-Cur (curcumin concentration 25.5 µM), washed with PBS and fixed using 4%(w/v) paraformaldehyde (Sigma Aldrich, Product code: 16005). Then cells were washed 3-4 times using PBS and 1,1,1,3,3,3-Hexamethyldisilazane (HMDS) (SRL Pvt. Ltd., Product code: 28437) was applied and removed immediately. Samples were kept inside a vacuum desiccator for complete drying. The coverslip was placed over a copper stub through carbon tape and gold coated to visualized under SEM.

Mucus adsorption assay

The simulated nasal mucus (SNM) was prepared using 0.5 % (w/v) porcine mucin (Sigma-Aldrich, Product code: M2378), 8.77 mg/ml NaCl (Sigma-Aldrich, Product code: S9888), 2.98 mg/ml KCl (HiMedia, Product code: GRM698) and 0.59 mg/ml $CaCl_2 \cdot 2H_2O$ (Sigma-Aldrich, Product code: C3881) in water [6, 7]. The SNM was properly mixed with rhodamine 6G (20 µM) and then incubated with 20 µg of Si-P-CNP particles in a total reaction volume of 200 µl for 4h at a constant rocking condition for mentioned cycles of treatment. Thereafter, the sample was centrifuged at 2000 RPM for 3 min and the absorbance of supernatant was collected at 530 nm wavelength. The percentage of loading was calculated by plotting a standard curve from the absorbance of known concentrations of rhodamine 6G.

Silicosis induction assay

An aliquot of 5 ng/ml TGF-β1 (Sino biologicals, China) was incubated per 20 µg/ml Si-P-CNP or Si-P particles in rocking condition at 4 °C for 2h. Then the suspension was centrifuged at 15,000 rpm for 3 min and the pellet was separated. Further, A549 cells were treated with TGF-β1 (5 ng/ml), Si-P-CNP particles incubated with TGF-β1 (20 µg/ml), Si-P particles incubated with TGF-β1 (20 µg/ml), Si-P-CNP (20 µg/ml) and Si-P (20 µg/ml) for 48h. Further cells were washed 3 times with PBS and detached using trypsin. Detached cells were fixed with 4% (w/v) paraformaldehyde for 5 min at 4 °C. Cells were washed using 1X PBS, 3 times, to remove paraformaldehyde and permeabilize with 90% (v/v) chilled methanol for 10 min. Methanol was removed by washing with 1X PBS, thrice. Cells were incubated with p-Smad 2/3 antibody (Cell signaling technology) (1:500 dilution in PBS containing 0.5% BSA) for 1h at room temperature. Excess antibody was removed through washing using antibody dilution buffer and incubated with alexa flour 594 conjugated with secondary goat anti-rabbit antibody (Invitrogen) (1:1000 dilution) for 30 min at room temperature. Excess amount of secondary antibody was removed by washing with 1X PBS (2 times) and resuspended in 1 ml volume of 1X PBS. Samples were investigated in the red channel of the flow cytometer (Cyflow space, Partec). Data was processed using FlowJo software [8].

Biodegradation study in simulated body fluid (SBF):

SBF was prepared as described elsewhere[9, 10]. A 100 µg/ml sample of Si-P and Si-P-CNP particles were incubated with SBF at 37 °C in a continuous shaking condition. The hydrodynamic diameter of incubated samples was collected at different time period. Scanning electron microscopic images were also obtained at the initial and end time point of the experiment.

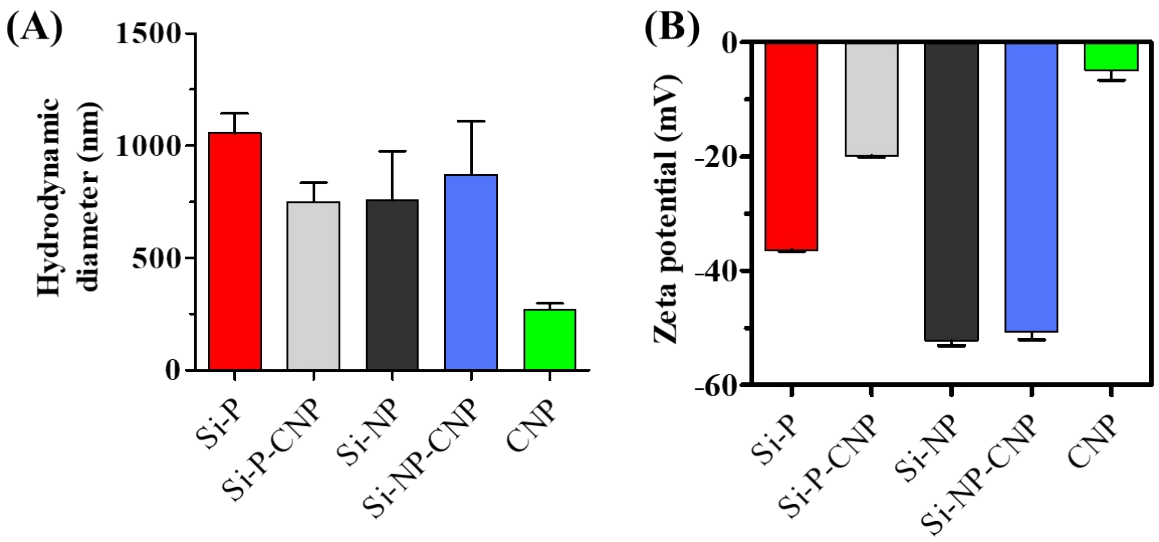

Fig. S1 (A) Hydrodynamic diameter and (B) zeta potential of Si-P, Si-P-CNP, Si-NP, Si-NP-CNP and CNP.

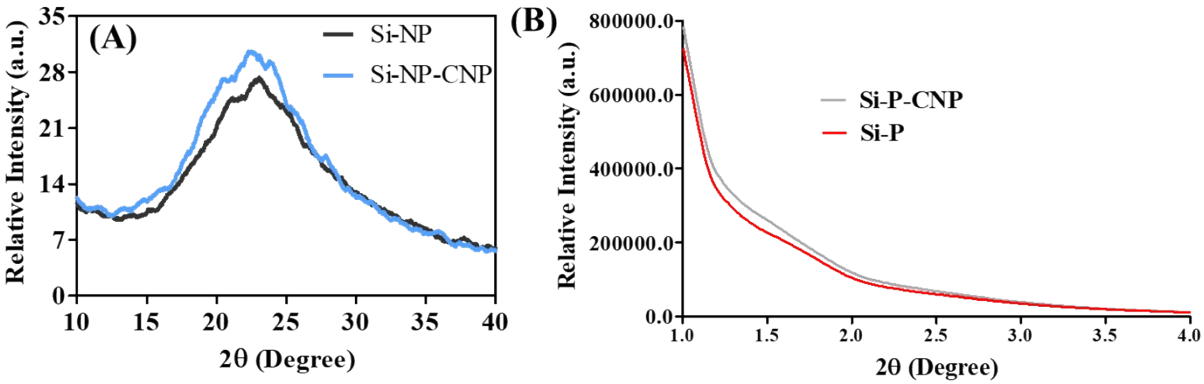

Fig. S2 (A) Wide angle XRD spectra of Si-NP and Si-NP-CNP**.** (B) Small angle XRD spectra of Si-P and Si-P-CNP.

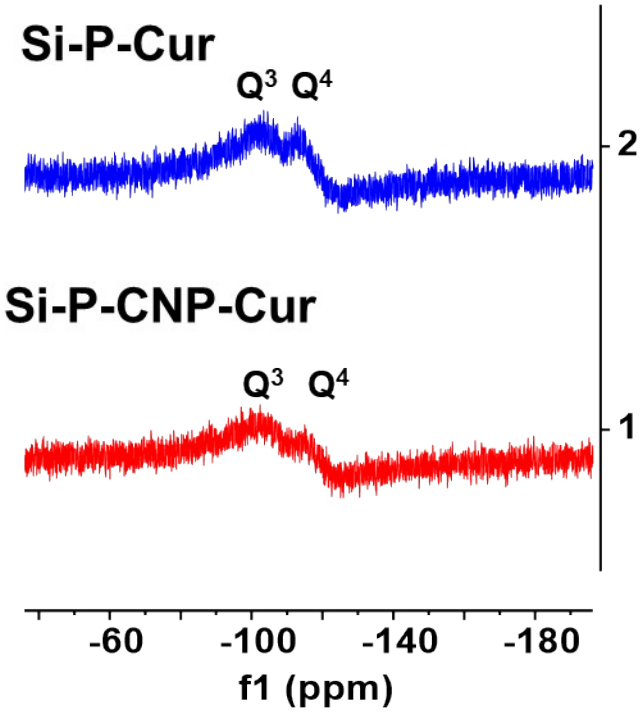

Fig. S3 ^{29}Si NMR spectra of Si-P-Cur and Si-P-CNP-Cur represents the existence of Q^3 ($Si(OSi)_3OH$)) and Q^4 ($Si(OSi)_4$) species.

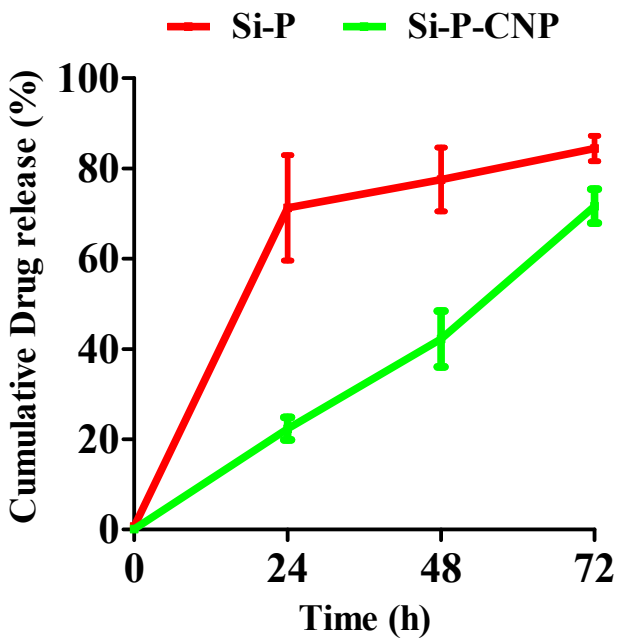

Fig. S4 Cumulative release profile of curcumin from Si-P and Si-P-CNP in different time point.

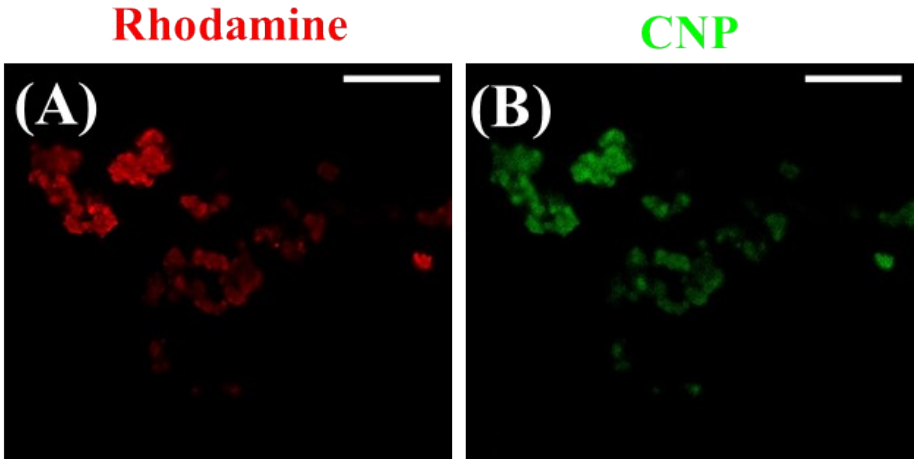

Fig. S5 Confocal microscopic images of rhodamine 6G loaded Si-P-CNP particles in (A) red emission channel for rhodamine and (B) green channel for CNP. Scale bar = 20μm.

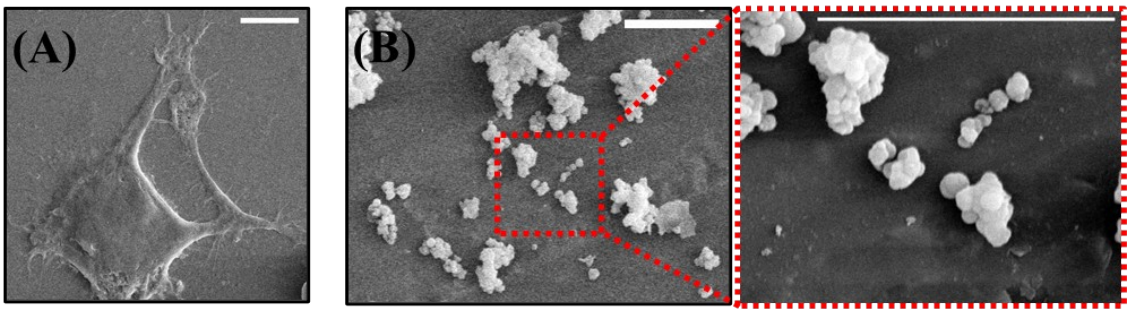

Fig. S6 SEM images of (A) Raw 264.7 cell and Si-P-CNP-Cur particles. Scale bar = 10μm.

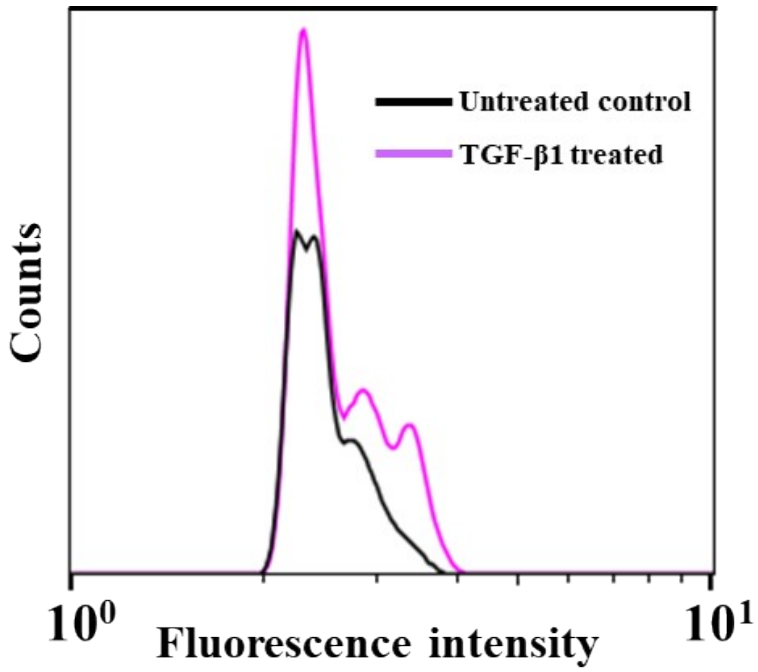

Fig. S7 Flow cytometry analysis of red subset from A549 treated with TGF-β1with respect to untreated control.

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