Supplementary Information (SI) for Journal of Materials Chemistry B.
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Supplemental Material

S1: Peptide release kinetics

The *in-vitro* release studies of TMTP1 from solution form (PBS) and PPT-NA were carried out in simulated lung fluid (SLF) (Phosphate buffered saline, 0.02% tween 80, 0.05% sodium azide, pH 7.4) at 37 °C for the period of 6 days. The dialysis bag (molecular weight: cut off 14 KDa) was filled with 2 ml of formulation suspension (5 mg/ ml) or equivalent amount of TMTP1 peptide was suspended in 200 ml of release medium in a beaker over a magnetic stirrer (50 rpm) and the release temperature was maintained at 37 ± 2 °C. At predetermined time intervals, 1 ml of release medium was withdrawn followed by centrifugation (8000 rpm for 5min). The supernatant was filtered through 0.45μm filter and analysed with the RP-HPLC (Figure S1a). The experiments were performed in triplicates. The drug release profile was bi-phasic in case of encapsulated TMTP1, while peptide in solution form showed monophasic profile with faster release which liberate almost all the peptide within 6 h. Encapsulation of TMTP1 peptide in PEG-PLGA polymer resulted in a sustained release profile over 4 days, characterized by an initial burst release of approximately 32.78±8.56% of total encapsulated drug over the first day followed by sustained release over the remaining next three days (Figure S1b).

S2: Purity of PEG-PLGA copolymer

The purity of co-polymer was evaluated in terms of molecular weight of the synthesized PEG-PLGA copolymer by using gel permeation chromatography (Malvern VISCOTEK GPC-max system) coupled to the PL-gel columns. The data was recorded at 212 nm at temperature 25°C while polystyrene was used as standard for system calibration.

The chromatogram and molecular weight data resulting from the GPC analysis of the copolymer indicates a narrow molecular weight distribution (< 2) for the copolymer (Figure S2). The unimodal and symmetrical GPC curve, with a narrow molecular weight distribution, verifies the formation of the PEG-PLGA copolymer.

S3: Mucus penetration Studies:

Lung airway-Mucus layer is mainly composed of 97% water and 3% solids (mucins, non-mucin proteins, salts, lipids, and cellular debris). The mucins are foremost to interact with inhaled foreign materials via non-specific binding, including electrostatic and hydrophobic interactions. When administered, inhaled foreign particles have high probability to get trapped in airway by mucus via steric or adhesive forces and quickly removed by mucociliary clearance mechanism. The short transit or retention time in the airways may be insufficient for

nanoparticles to release a substantial portion of incorporated drugs, leading to lower bioavailability and ineffective efficacy. The diffusion of fluorescent particle in porcine-mucus gel was evaluated for 2 h by Confocal Scanning laser Microscopy. A major proportion of PLGA-NP could not disperse into the mucus-gel layer while, PLGA-PEG-NP demonstrated a better penetration with longer residence time (Figure S3b).

S4: Physical Stability Studies

The long-term physical stability evaluation of the PPT-NA formulations was performed at two different conditions (i) room temperature (25° ± 2°C and 60% Relative humidity) and (ii) 4°C temperature for up to 42 days (6 weeks) in tightly closed containers protected from light. After every 7 days, the formulation was evaluated for the morphology, particle size, zeta potential, and drug content (encapsulation efficiency). All the measurements were taken in triplicate and the results were presented as the mean ± standard deviation (Figure S4, Table S1).

The morphology and surface topography of PPT-NA nanoaggregates and their individual nanoparticles were observed under SEM at above time intervals, showed intact structure of particles at storage condition of 4°C temperature. In contrast, coalescence of nanoparticles was observed to a certain degree in PPT-NA powder samples after 5 weeks of storage at room temperature (25° ± 2°C) (Figure S4). A significant change in encapsulation efficiency of TMTP1 peptide was observed which reduced from 64.1±7.8% to 53.7±4.6% with the diminution in drug content by ~16.22% after 42 days of storage at room temperature, whereas a marginal change of ~4% was observed the samples stored at 4°C temperature. The particle size of the individual nanoparticles resulted marginal increase by ~11nm and ~3nm at 25°C and 4°C respectively, while zeta-potential of the particles did not show any specific pattern of change (Table S1).

S5. Determination of ROS levels in presence of ROS scavenger

Intracellular ROS is also measured using 2, 7, dichlorodihydrofluorescein diacetate (H2-DCFDA) with/without N-acetylcysteine (NAC as ROS scavenger, 10 mM). NAC was added to the cells at a concentration of 10 mM for 2 h before treatment and the cells were washed before adding the formulation.Briefly, A549 cells (1000 cells/well) were seeded in a 96-well cell culture plate and allowed to adhere for 24 h. Subsequently, cells were treated with free TMTP1 peptide (100 µg/ml) and two different concentrations of formulation PPT-NA (equivalent to 50 and 100 µg/ml) and incubated for 24 h. After the incubation time, the cells were washed and incubated with 10 µM of DCHF-DA dye for 30 min in the dark and the dichlorofluorescein (DCF) fluorescence intensity was recorded at 535 nm.[1]

In the presence of ROS scavenger, N-acetyl cysteine(NAC), ROS production is decreased with as compaired to NAC untreated cells. Reduction of ROS was also observed in case of positive control and pure TMTP1. There is a significant reduction in the ROS generation after pretreating the cells with NAC occurred in dose dependent manner (Figure-S5).

S6: In-house inhalation apparatus

The in-house inhalation apparatus is a simple device fabricated from a 15-ml centrifuge tube, silicon tubing and a pipette bulb as described elsewhere [2]. The procedure was well proven and verified for pulmonary delivery of microparticles to experimental rodents.

Fabrication of Inhalation apparatus**:** The components of the inhalable device for animal dosing will include: A tapered poly(propylene) centrifuge tube of capacity 15 ml that form the aerosol generation chamber; a Flexible tubing, to introduce a turbulent fluidizing air stream into the chamber; and a rubber pipette bulb that provided the source of turbulent air when pressed and released. Two holes was bored in the centrifuge tube. The first hole was made in the apex of the taper to admit the flexible tubing. The second hole was made in the wall of the tube, near the rim of the screw cap, to serve as the Delivery Port for nose-only inhalation (Figure S5).

Operation of the apparatus: pre-weighed microparticle powder (PPT-NA) was placed in the cap of the tube. A test animal was restrained with its nares inserted in the delivery port, without touching the powder bed. We have optimized the 30 actuations for dosing to mice using "in-house inhalation apparatus" with "One actuation per second". The pipette bulb was actuated (pressed and released) once every second over the desired period of exposure (30 sec), to fluidise the powder bed and create a 'dusty' atmosphere for the animal to breathe in**.** This provides comfortable "synchronization" of inhalation of particles with normal breathing of the mouse and avoid any damage of lungs.

S7: Estimation of retention of particles in lungs by Bronchio-alveolar lavage (BAL)

In order to investigate the residence time of formulations in the lung, the drug content of encapsulated drug in the broncho-alveolar lavage fluid (BALF) was analysed at different time points after single inhalation in mice according to the procedures reported previously. Briefly, twelve mice were randomly divided into four groups, and each group contained three mice dedicated to each time interval. All the animals received single inhalation of formulation (20 mg, PPT-NA) by in-house inhalation apparatus.

BAL procedure was conducted in individual animal groups at different time intervals (2, 6, 12, and 24 h). All the animals received single inhalation of formulation by in-house inhalation apparatus. BAL procedure was conducted in individual animal groups at different time intervals (Figure S7). Lungs were flushed with cold saline two times and BALF was pooled. BALF was vigorously vortexed for 10 min and then centrifuged at 1200g for 10 min at 4°C. The supernatant obtained was dried by nitrogen purging and then reconstituted with 0.5 ml of methanol. Drug/ Peptide content will be determined using RP-HPLC after processing the BALF using the method previously described (3% acetonitrile in acetate buffer, pH 7.0, at 1 ml/min).

S8: Inhaled PPT-NA Toxicity study: To evaluate the toxicological effect of PPT-NA pulmonary delivery in lung on healthy Balb/c mice. Cancer-free healthy mice were treated in the same way as mentioned in Section 2.13.2. The animals were divided into 4 groups and treated with either:

1) Control healthy mice (n=3)
2) Inhalation, 5 mg PPT-NA powder (low dose) (n=3)
3) Inhalation, 15 mg PPT-NA powder (medium dose) (n=3)
4) Inhalation, 25 mg PPT-NA powder (high dose) (n=3)

After 6 weeks of inhalation dosing (alternate days), the animals were sacrificed and lungs were excised off for morphological and histopathological evaluation for toxicological analysis. The lungs were perfused with buffered formalin, incubated for 24h and embedded in paraffin blocks. The tissues embedded parafilm blocks were sectioned using microtome at 5µm and then stained with Haematoxylin–Eosin (H&E) and observed under microscope for toxicological parameters including infiltration of cells[3]. Morphological evaluation of excised off lungs did not show any toxicological evidence (inflammatory redness, lesions, edema, surface nodules) after 42 days of inhalation dosing of PPT-NA. The H&E-stained tissue section of healthy Balb/c mice (Untreated) and the treatment groups were observed under optical microscope to observe any damage in alveolar architecture with any peribronchial-inflammation, epithelial damage, or oedema. After 6 weeks of treatment, the lung of all the treatment groups showed to have normal alveolar architecture with no signs of lung injury (oedema/ desquamation of epithelial cells of bronchioles/ peri-broncheal inflammation or any epithelial damage). No desquamation of epithelial cells of bronchioles were observed after dosing of three levels of dosing. Marginal lymphoid cell infiltration of the interstitial tissue or

in alveolar interstitium was recorded in higher (~15mg) dosed animals. This study concluded that the PPT-NA is compatible and do not show significant toxicity in lung tissue after inhalation delivery of the formulation at three different doses (Figure S8).

FIGURES

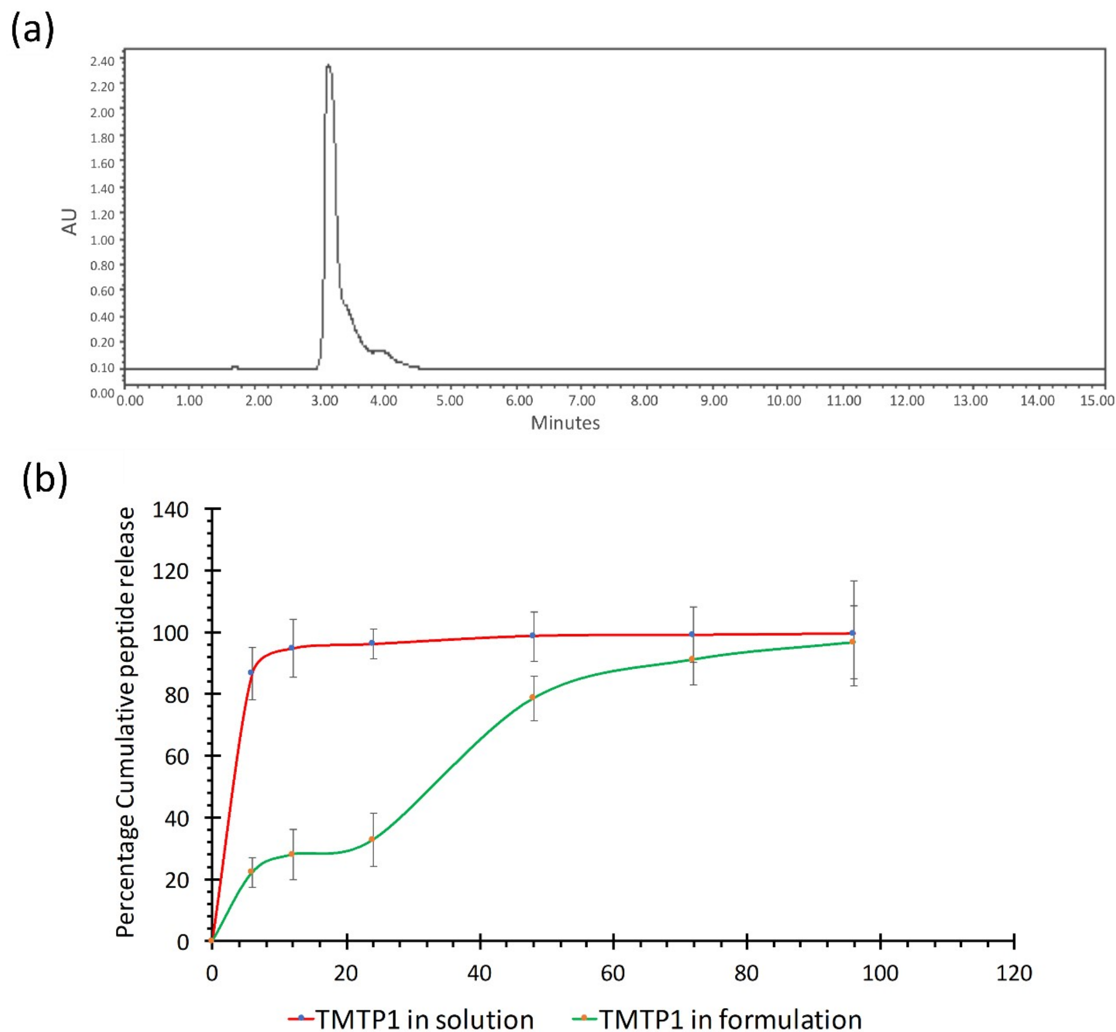


Figure S1: (a) **HPLC of peptide TMTP1:** The HPLC chromatogram show the purity of the TMTP1 peptide synthesized using a microwave peptide synthesizer at a wavelength of 214nm (b) **Cumulative TMTP1 peptide release:** Peptide release profile of TMTP1 from PPT-NA formulation.

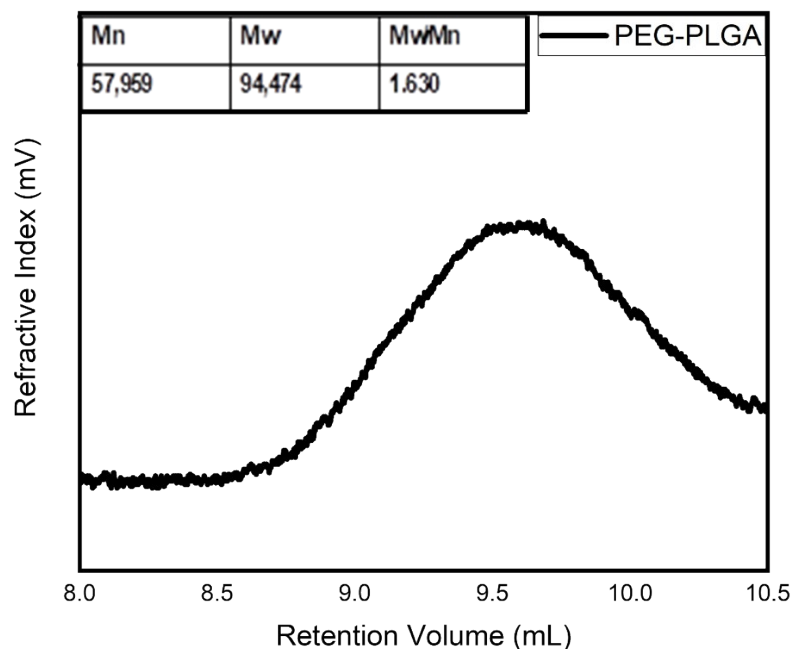


Figure S2: Gel Permeability Chromatography of PEG-PLGA: The single peak indicating the purity of the PEG-PLGA conjugate.

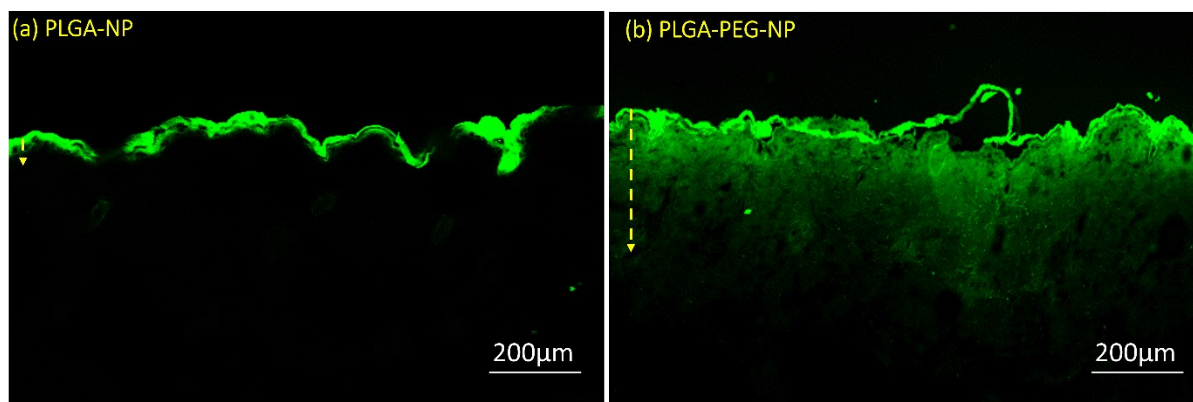
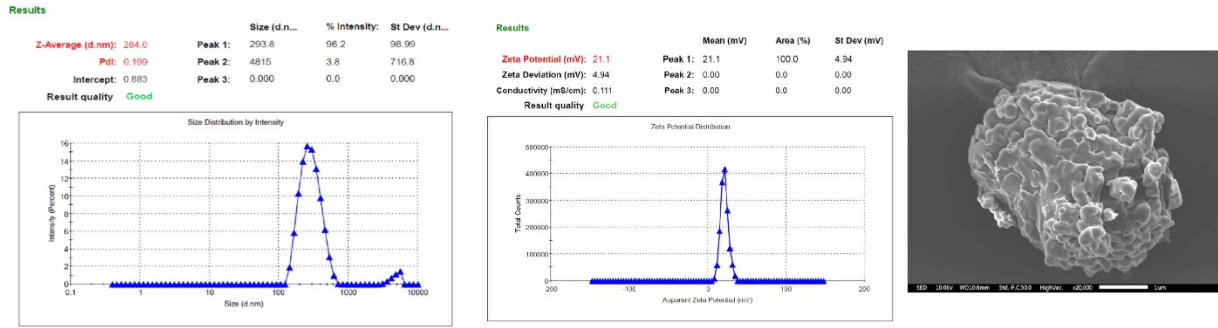


Figure S3: PEG conjugation enhances mucus penetration. Confocal Micrographs of for analysis of diffusion of each PLGA and PLGA-PEG nanoparticles (FITC-incorporated) in mucus. Enhanced PLGA-PEG-NP diffusion in mucus compared to uncoated counterpart.

(a) Storage condition: Room temperature (25° ± 2°C and 60% RH)



(b) Storage condition: 4° **temperature**

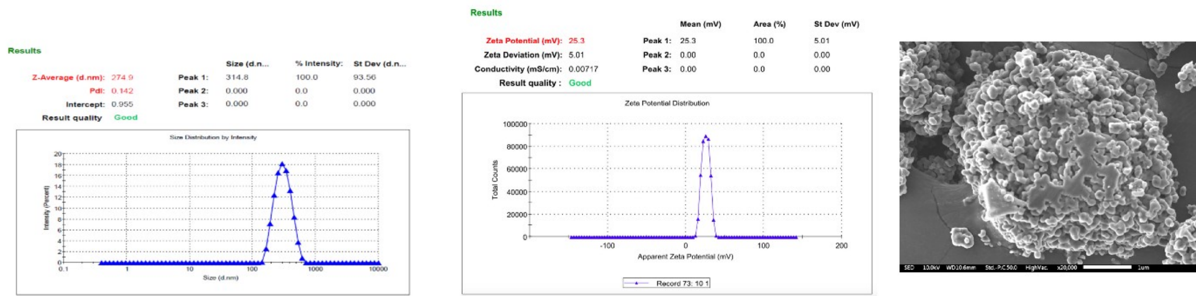


Figure S4. Change in physiochemical parameter of PPT-NA formulation after storage at (a) room temperature and (b) 4°C temperature for up to 42 days (6 weeks)

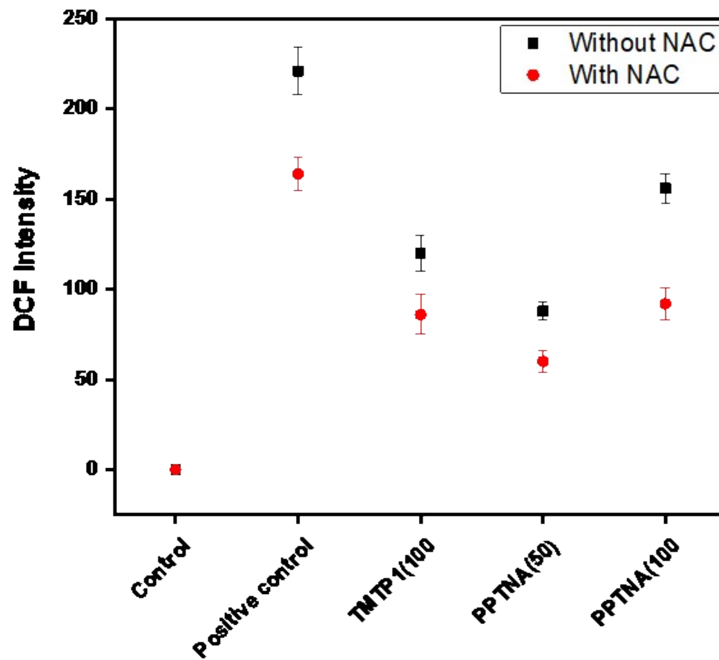


Figure S5. Estimation of ROS levels in presence of ROS scavenger.

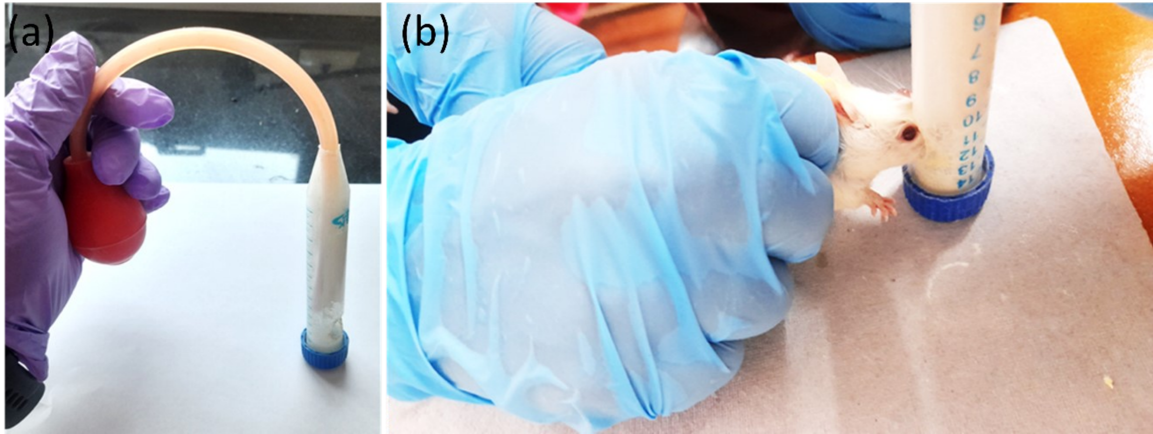


Figure S6: Inhouse inhalable apparatus (a) Design (b) Dosing to mice

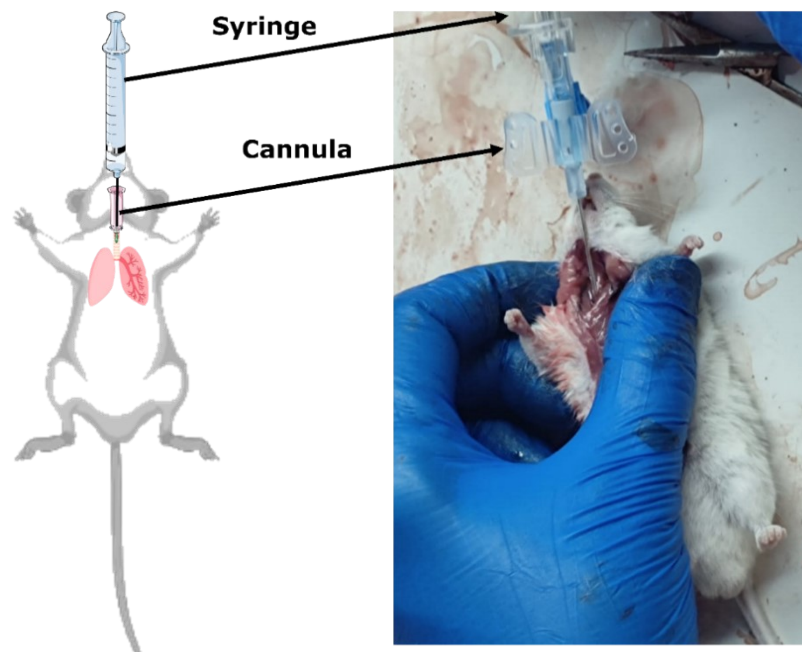


Figure S7. The broncho-alveolar lavage fluid collection after a single inhalation of the formulation from the trachea.

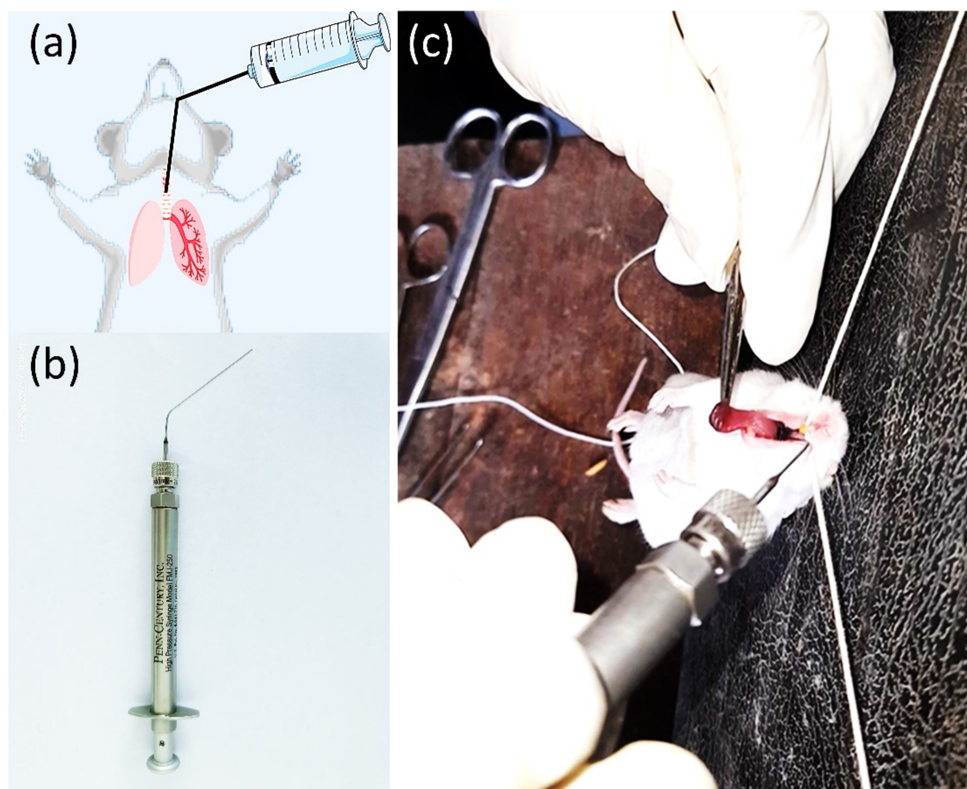


Figure S8. The intratracheal administration: (a) Skematic of intra-tracheal administration (b) Micro-sprayer IA-1C system (Penn-Century) for intra-tracheal delivery of the NNK (4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone) (c) Intra-tracheal administration of carcinogen in anesthetized mice.

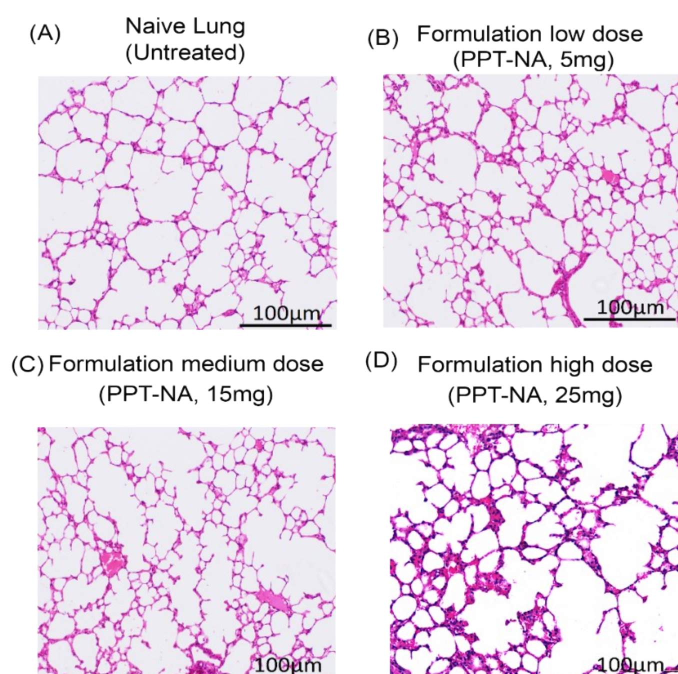


Figure S9. Lung Toxicity Study: The 6 weeks toxicity study was performed in healthy Balb/c mice for formulation with 5,15,25 mg of PPT-NA. The H&E staining tissue were observed under optical microscope at 100µm.

TABLES

Table S1: Stability studies of optimized PPT-NA formulation at different storage condition

		0 week	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
Room temperature (25° ± 2°C and 60% RH)	Particle Size	284.1±17.9	287.9±11.56	287.2±18.8	289.6±25.9	291.8±16.4	291.8 ±12.6	293.0±98.9
	Zeta Potential	17.8 ± 0.0	14.1 ± 0.1	15.3 ± 0.6	14.8 ± 0.0	14.9 ± 0.1	15.8 ± 0.0	21.1 ± 4.9
	Entrapment efficiency	64.1±7.8%	64.3±11.2%	62.8±14.7%	62.1±11.2 %	56.9±11.7 %	54.0±8.7%	53.7±4.6%
	Morphology	Intact nano-assemblies	Intact nano-assemblies	Intact nano-assemblies	Intact nano-assemblies	Intact nano-assemblies	Agglomeration of nanoparticles	Agglomeration of nanoparticles
4°C Temperature	Particle Size	284.1±17.9	284.8±18.5	285.9±11.7	287.6±12.8	287.8±6.9	287.6 ±11.8	287.9± 13.7
	Zeta Potential	17.8 ± 0.0	15.7 ± 0.6	14.6 ± 0.1	13.8 ± 0.7	16.8 ± 0.8	16.4 ± 1.6	25.3 ± 5.0
	Entrapment efficiency	64.1±7.8%	64.8±12.4%	62.7±10.1%	62.8±1.9%	61.8±4.8%	61.2±7.3%	61.5±8.3%
	Morphology	Intact nano-assemblies	Intact nano-assemblies	Intact nano-assemblies	Intact nano-assemblies	Intact nano-assemblies	Intact nano-assemblies	Intact nano-assemblies

Table S2: Retention of PPT-NA in the lung after pulmonary administration in mice at different time points based on broncho-alveolar lavage fluid (BALF) analysis.

Time interval	**Estimated peptide amount in lungs (μg)**	**Calculated PPT-NA amount in lungs (mg)**	**% Drug residue**
0	456.54±66.89	2.55±0.81	100
2	226.59±38.57	1.26±0.59	49.41
6	148.11±34.27	0.96±0.21	37.64
12	59.28±19.48	0.37±0.09	14.50
24	Not detected	Not detected	Not detected

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1. Chen, Y., et al., *Design and evaluation of inhalable nanocrystals embedded microparticles with enhanced redispersibility and bioavailability for breviscapine.* 2021. **377**: p. 128-138.
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3. Rauf, A., et al., *Lungs deposition and pharmacokinetic study of submicron budesonide particles in Wistar rats intended for immediate effect in asthma.* EXCLI J, 2017. **16**: p. 236-244.