Protein nanocages that penetrate airway mucus and tumor tissue

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Significance

In designing new nanoparticle drug delivery systems, it is critical to identify simple formulations that overcome multiple biological barriers while being safe, reproducible, and scalable. We modified human ferritin nanocages using a unique PEGylation strategy, which provides a highly uniform, stable, and compact nanocarrier platform capable of overcoming multiple biological barriers, specifically penetration of airway mucus and tumor tissue, selective uptake by cancer cells, and drug release triggered only upon cell uptake. Surprisingly, PEGylation of ferritin to overcome the mucus barrier did not interfere with the ability of the nanocages to form particles, penetrate tumor tissues, and enter cells. Proof-of-concept of the system is provided in the treatment of an aggressive orthotopic model of lung cancer.

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most inhaled particulate matter, including therapeutic nanoparticles, leading to their rapid elimination from the lungs by mucociliary clearance (MCC) (18). To avoid trapping in the mucus gel, nanoparticles can be coated with a dense corona of polyethylene glycol (PEG) (19–23). However, PEGylation may interfere with the intrinsic ability of FTn to target cancers via TfR 1. The effect of PEGylation on the native functionality of protein-based therapeutics is strongly dependent upon the molecular weight (MW) of the conjugated PEG (24–26). Based on these findings, we hypothesized that the use of low-MW PEG to coat FTn may allow the particles to not only rapidly penetrate the mucus gel layer but also retain their cancer-targeting properties. To test this hypothesis, we prepared various formulations of FTn, each coated with a different MW of PEG, using a unique strategy to incorporate a controlled density of PEG exclusively on the particle surface. The candidate formulations were studied for the impact of PEG MW on the ability of the PEGylated FTn to simultaneously overcome the critical biological barriers to inhaled nanoparticle-based chemotherapy in vivo. Based on these results, we next conjugated PEG to intact FTn at neutral pH to attach PEG selectively to the primary amines exposed on the outer surface of FTn. Because 336 primary amines are present in each FTn based on sequence analysis, we first conjugated 2 kDa PEG (PEG_2k) to FTn at a molar ratio of 500:1, and the surface PEG density was fluorometrically determined (16) to be ∼0.4 PEG per square nanometer, which corresponds to ∼190 PEG per FTn. Likewise, 5 and 10 kDa PEG (PEG_5k and PEG_10k, respectively) were conjugated to separate aliquots of FTn. All PEGylated FTn exhibited cage-like structures (Fig. S2A) that appeared similar to FTn before PEGylation. In parallel, Alexa Fluor 488 (AF488) and Cy5 molecules were conjugated to FTn via an acid-sensitive linker, thereby providing a high drug loading and drug release triggered by cell uptake and subsequent sequestration in acidic vesicles. Finally, we investigated the efficacy of DOX-loaded PEGylated FTn in an orthotopic model of aggressive lung cancer.

Results
PEGylated FTn with Controlled PEG Location and Surface Density. We genetically engineered recombinant FTn by transforming bacterial cells with a custom-made plasmid encoding for human native ferritin heavy chain. Subsequently, purified FTn were confirmed to undergo controlled cage dissociation under acidic conditions and reassemble at neutral pH, via their intrinsic pH-dependent property (3). Transmission electron microscopy (TEM) revealed that FTn existed as monodispersed nanocages at pH 7.4, dissociated to individual subunits at pH 2, and then reassembled to form nanocages when the pH was returned to 7.4 (Fig. 1). The diameter of FTn at different pH was measured by size exclusion chromatography (Fig. S1), yielding a single peak at the size of 13.2 nm at pH 7.4 and a broad peak with a size range of 1.8–5.4 nm at pH 2.0 (Fig. 1B).

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lead to a brush-like conformation of PEG (27). Using fluorescently labeled PEG, we determined that the PEG surface density of hybrid FTn/FTn–PEG2k was \( \sim 0.24 \pm 0.03 \) PEG per square nanometer.

**In Vivo Airway Distribution of PEGylated FTn.** We have previously demonstrated that muco-inert nanoparticles capable of efficiently penetrating airway mucus provide widespread in vivo distribution throughout the mouse airways following inhalation, whereas muco-adhesive nanoparticles were unable to do so (28, 29). We thus investigated the airway distribution of Cy5-labeled FTn, with or without PEG surface coatings, following intranasal administration in mice. We found that non-PEGylated FTn were aggregated in the mucus gel layer and, thus, sparsely distributed in airways (Fig. 2A). In contrast, PEGylated FTn, regardless of PEG MW, exhibited uniform and widespread distribution throughout the mouse airways (Fig. 2A and Fig. S3). Quantitatively, FTn/FTn–PEG2k, FTn/FTn–PEG5k, and FTn/FTn–PEG10k covered 77 \( \pm \) 8, 80 \( \pm \) 8, and 74 \( \pm \) 8% of the mouse tracheal surfaces, respectively, compared with only 50 \( \pm \) 9% by non-PEGylated FTn (\( P < 0.01 \)) (Fig. 2B). We also found that FTn/FTn–PEG2k were retained at higher levels in the upper airway (i.e., trachea) 10 min after intratracheal administration compared with non-PEGylated FTn, as determined by quantifying the overall fluorescence intensity (Fig. 2C).

**Tumor Cell Uptake, Tumor Tissue Penetration, and Safety of PEGylated FTn.** We next sought to test whether PEGylated FTn were capable of cancer cell targeting in vitro. We first determined that Cy5-labeled FTn were taken up by mouse Lewis lung carcinoma (3LL) cells efficiently and in a dose-dependent manner (Fig. 3A and B), as observed in the presence of anti-TfR 1 antibody (Ab) (Fig. 3C). FTn–Cy5 uptake was mediated by specific interactions between FTn and TfR 1, as confirmed by the significantly reduced binding observed in the presence of anti-TfR 1 antibody (Ab) (Fig. 3C). We also found that FTn were taken up by another TfR 1-positive human lung cancer cell line, A549, by the same mechanism (Fig. 3C). We then compared cell uptake of PEGylated and non-PEGylated FTn using flow cytometry. We found that the uptake
of PEGylated FTn with higher PEG MW, specifically FTn/FTn–PEG5k (P < 0.05) and FTn/FTn–PEG10k (P < 0.01), was significantly reduced compared with that of FTn; in contrast, the difference in the uptake of FTn and FTn/FTn–PEG2k was not statistically significant (Fig. 3D). A competitive binding analysis also revealed that FTn and FTn/FTn–PEG2k bound to 3LL cells to a similar extent (Fig. 3E). Additionally, FTn/FTn–PEG2k were efficiently taken up by several TfR 1-positive lung cancer cells, including both human small cell lung cancer (SCLC) and non-SCLC (NSCLC) cell lines (Fig. 3F and G).

We next explored the effect of PEGylation on the penetration of Cy5-labeled FTn through a 3D tumor spheroid model established with 3LL cells. The multicellular tumor spheroids constitute the most commonly used in vitro model that recapitulates in vivo tumor microenvironments, as characterized by the presence of naturally formed extracellular matrix (ECM), regions of hypoxia and necrosis, and concentration gradients of oxygen and nutrients (30–32). The 3D-reconstructed confocal images of spheroids revealed that FTn and FTn/FTn–PEG2k uniformly distributed throughout the entire spheroids including the core (Fig. 4A, Left).

In contrast, FTn/FTn–PEG5k and FTn/FTn–PEG10k were found primarily near the periphery of tumor spheroids (Fig. 4A, Left). The mean fluorescence signal intensity within the tumor spheroids was indistinguishable between FTn and FTn/FTn–PEG2k, whereas the FTn/FTn–PEG5k and FTn/FTn–PEG10k exhibited significantly lower intensity compared with FTn (P < 0.01) (Fig. 4A, Right).

To understand the mechanism of tumor penetration, we investigated the penetration of FTn and FTn/FTn–PEG2k in the presence of unlabeled FTn or anti-TfR 1 Ab. The penetration of Cy5-labeled FTn through the 3D tumor spheroids was significantly reduced in the presence of an excess amount of unlabeled FTn (P < 0.01) (Fig. 4B). The mean fluorescence signal intensities of FTn and FTn/FTn–PEG2k in the 3D-constructed tumor spheroids also significantly decreased in the presence of anti-TfR 1 Ab (P < 0.01) (Fig. 4C). The TfR 1-dependent tumor penetration was further confirmed by significantly reduced 2D penetration of both FTn and FTn/FTn–PEG2k from the surface toward the center of middle sections of the tumor spheroids (Fig. 4D).

Fig. 4. Deep penetration of PEGylated FTn throughout tumor tissues. (A) Effect of PEGylation on the penetration of FTn through a 3D tumor spheroid model established with 3LL cells. (Left) Representative confocal images of tumor penetration of the various particles in 3D spheroids. (Scale bar, 200 μm.) (Right) Image-based quantification of mean Cy5 fluorescence signal intensity in whole 3D-constructed cell spheroids. Data represent the average of n ≥ 8 cell spheroids (±SEM). **P < 0.01 compared with non-PEGylated FTn. (B) Quantification analysis of tumor penetration of Cy5-labeled FTn in reconstructed 3D cell spheroids in the absence or presence of excess amounts of unlabeled FTn. ***P < 0.01. (C) The penetration of the FTn and FTn/FTn–PEG2k through cell spheroids in the absence or presence of a 10-fold molar excess of anti-TfR 1 Ab. (Scale bar, 200 μm.) (D) Distribution of the Cy5 fluorescence signal in the middle section images of C. (Left) Representative middle section image. (Right) Image-based quantification of the mean intensity at different penetration distance (Top, FTn; Bottom, FTn/FTn–PEG2k). Radius, 0 and 1 indicate the center and edge of the tumor spheroid, respectively. ***P < 0.01 compared with FTn or FTn/FTn–PEG2k. (Scale bar, 200 μm.) (E) Western blot analysis of TfR 1 expression in proximal and distal lung as well as 3LL tumor tissues. The tissues were collected from n = 3 mice for each group. **P < 0.01. (F) Colocalization (yellow) of intratracheally administered FTn/FTn–PEG2k (red) with an orthotopic 3LL lung cancer (mCherry; green). Cell nuclei are stained with DAPI (blue). (Scale bar, 100 μm.)
Based on the findings of the aforementioned studies, we identified FTn/FTn–PEG2k as the nanoparticle system that provides both efficient penetration through airway mucus and efficient tumor spheroid penetration in vitro. We next sought to investigate the behavior of FTn/FTn–PEG2k in vivo. To do this, we first determined that the TfR 1 expression in a 3LL-based s.c. tumor was over 30-fold higher (P < 0.01) than TfR 1 expression in healthy proximal and distal lung tissues (Fig. 4E). Subsequently, we developed an orthotopic mouse model of lung cancer by intratracheal inoculation of 3LL cells that constitutively express a fluorescent protein, mCherry, into the lungs of C57BL/6 mice. The orthotopic tumors were established along the airways and infiltrated into the healthy lung parenchyma 10 d after the inoculation (Fig. 4F). Cy5-labeled FTn/FTn–PEG2k that were intratracheally administered at this time were found preferentially within the mCherry-expressing tumor tissue (Fig. 4F). We also confirmed that lungs of healthy C57BL/6 mice intratracheally treated with an excess of FTn/FTn–PEG2k were virtually identical to untreated lungs, without any apparent sign of acute cytotoxicity or inflammatory response (Fig. S4).

Formulation of DOX-Loaded FTn/FTn–PEG2k. We next sought to attach a chemotherapeutic drug widely used for airway-related lung cancer therapy, DOX, to FTn/FTn–PEG2k in a manner that would limit its release primarily to tumor cells. To do so, we chemically conjugated DOX to FTn/FTn–PEG2k (hereafter, FTn/FTn–PEG2k/DOX) via an acid-sensitive linker to achieve high drug loading while limiting drug release to acidic environments, such as in intracellular endolysosomal vesicles (Fig. 5A and Fig. S5). Physicochemical properties, including hydrodynamic diameter (HD), surface charge (as indicated by ζ-potential), and morphology of FTn/FTn–PEG2k/DOX, were comparable to those of FTn/FTn–PEG2k without DOX (Fig. 5B and Table S1). The number of DOX molecules conjugated to each FTn/FTn–PEG2k was 88 ± 5.

The pH-dependent release kinetics of DOX was evaluated by incubating FTn/FTn–PEG2k/DOX in aqueous solutions of varying pH at 37 °C. The amount of DOX released over 3 d at pH 7.4 was...
negligible. However, a burst release of DOX was observed upon lowering the pH to 5.0, reaching a maximum release of nearly 80% after 4 h (Fig. 5C). To visualize the intracellular trafficking, 3LL cells were incubated with Cy5-labeled FTn/FTn–PEG2k, and lysosomes were stained with LysoTracker. Confocal images showed that FTn/FTn–PEG2k colocalized with lysosomes 2 h after the addition to cells (Fig. 5D). We also confirmed that DOX-loaded FTn/FTn–PEG2k (i.e., FTn/FTn–PEG2k/DOX) colocalized with lysosomes labeled with anti-LAMP 1 Ab at the same incubation time (Fig. S6).

We next explored the intracellular fate of DOX administered as free drug compared with FTn/FTn–PEG2k/DOX in 3LL cells. Free DOX primarily localized in the cell nuclei 2 h after its addition to cells, whereas FTn/FTn–PEG2k/DOX were observed in both the cytoplasm and nucleus (Fig. 5E and F). Image-based quantification revealed that, compared with nucleus, ~95% and 55% of DOX delivered via FTn/FTn–PEG2k were found in cytoplasm 30 min and 2 h after adding FTn/FTn–PEG2k/DOX to the cells (Fig. 5F). The findings here collectively suggest that free DOX are taken up by cells by passive diffusion through the cell membrane (33), whereas FTn/FTn–PEG2k/DOX are endocytosed, release DOX in lysosomes, and subsequently the released drug molecules diffuse into cell nuclei.

Penetration of FTn/FTn–PEG2k/DOX Within Multicellular Tumor Spheroids. FTn/FTn–PEG2k/DOX or free DOX in aqueous solution at an identical DOX concentration were incubated with 3LL-based tumor spheroids to compare in vitro tumor penetration. 3D-reconstructed confocal images of the spheroids showed that free DOX remained primarily at the periphery of the spheroids, whereas FTn/FTn–PEG2k/DOX uniformly distributed throughout the entire tumor spheroid (Fig. 5G, Left). Quantification of the DOX signal in the reconstructed 3D images revealed that the mean fluorescence signal intensity of FTn/FTn–PEG2k/DOX in the entire spheroid was significantly greater than that of free DOX (P < 0.01) (Fig. 5G, Right). Further, we confirmed with multiple middle section images of the tumor spheroids that FTn/FTn–PEG2k/DOX exhibited a greater 2D penetration from the surface toward the center compared with free DOX (P < 0.01) (Fig. 5H). Improved penetration of FTn/FTn–PEG2k/DOX compared with free DOX was also observed in other tumor spheroid models constructed with human lung cancer cell lines, including A549, H460, and H11975 (Fig. S7).

We next sought to determine the in vitro tumor-killing capacity of FTn/FTn–PEG2k/DOX compared with free DOX using 3LL cells constitutively expressing luciferase. The IC50 of DOX and FTn/FTn–PEG2k/DOX were 0.48 and 1.1 μM, respectively (Fig. S8 A and B). Despite the higher IC50, FTn/FTn–PEG2k/DOX provided superior ability of delaying the growth of 3LL tumor spheroids compared with free DOX (P < 0.05) (Fig. S8 C and D), likely due to increased penetration.

In Vivo Efficacy of FTn/FTn–PEG2k/DOX in an Orthotopic Mouse Lung Cancer Model. To establish a proximal lung cancer model, we intratracheally inoculated C57BL/6 mice with 3LL cells constitutively expressing mCherry or luciferase. Similar to our observation with FTn/FTn–PEG2k (Fig. 4F), intratracheally administered FTn/FTn–PEG2k/DOX were found preferentially distributed within the orthotopically established, mCherry-expressing tumor tissue (Fig. 6A). For the antitumor efficacy study, we first confirmed with bioluminescence imaging that luciferase-expressing tumor tissues were established throughout the upper airways 3 d after the inoculation (Fig. S9). Subsequently, tumor growth was monitored over time, following a single intratracheal administration of free DOX or FTn/FTn–PEG2k/DOX. Mice treated with FTn/FTn–PEG2k/DOX exhibited a significant delay in tumor progression compared with mice that were either untreated or treated with free DOX (Fig. 6B). FTn/FTn–PEG2k/DOX effectively inhibited tumor growth 11 d after treatment, as reflected by a 40-fold and 17-fold decrease in signal intensity (from the luciferase-expressing tumor cells) (P < 0.05) compared with mice that were untreated and mice that were treated with free DOX, respectively (Fig. 6C). Survival was also significantly improved for mice treated with FTn/FTn–PEG2k/DOX compared with untreated and free DOX-treated mice (P < 0.01). The median survival for untreated mice and free DOX-treated mice was only 18 d, whereas treatment with FTn/FTn–PEG2k/DOX resulted in a 60% progression-free survival after 60 d (Fig. 6D). Of note, intratracheal administration of blank FTn/FTn–PEG2k devoid of DOX had no effect on the tumor growth and survival (Fig. S10).

Discussion

In this paper, we introduce a simple two-pronged delivery system based on a naturally occurring human protein and FDA-approved PEG molecules that overcomes multiple major delivery barriers. Unlike conventional PEGylation methods that often compromise intrinsic functionalities of core protein (24), our “hybrid” strategy does not affect the inherent ability of FTn to selectively interact with, and internalize into, cancer cells. This FTn/FTn–PEG2k provides (i) widespread distribution throughout the mucus-covered lung airways in vivo, (ii) deep penetration into the cores of 3D multicellular tumor spheroids and preferential distribution within solid tumor tissue in an orthotopic lung cancer model, (iii) tumor-specific cell uptake in lung cancer cells, and (iv) intracellular drug release only after cell uptake. These unique benefits combine to provide unrivaled efficacy in an orthotopic mouse model of highly aggressive airway lung cancer, including 60% survival at day 60 with FTn/FTn–PEG2k/DOX compared with a median survival
of only 18 d in animals treated with an equivalent dose of free DOX and in untreated animals. This system may offer a promising therapeutic option for treating locally confined airway lung cancers that encompass at least 40% of all lung cancer cases (34).

We have previously demonstrated that high PEG surface densities conferring brush-like coatings (i.e., [T/SA] > 1 where [T] and [SA] represent total unconstrained PEG surface area and total particle surface area, respectively) (27, 35) allow nanoparticles to avoid adhesive trapping in mucus (16, 27, 36, 37) and ECM (38, 39), leading to efficient penetration through these biological barriers. We thus formulated PEGylated FTn possessing a high surface PEG density that resulted in a dense brush conformation of PEG, with [T/SA] of 2.2 ± 0.3. The measured surface PEG densities are comparable to those of biodegradable nanoparticles that we have previously confirmed to efficiently penetrate human cervicovaginal (27) and airway (40) mucus. The mucus-penetrating property of PEGylated FTn was confirmed by their uniform and widespread distribution throughout the mucus-covered rodent airways in vivo. In contrast, non-PEGylated FTn was found aggregated and sparsely distributed in the airways. Further, a greater amount of FTn/FTn-PEG2k compared with FTn was retained in the lung airways, in agreement with our previous observation with polymer-based DNA nanoparticles possessing a similar PEG surface coating (29). Inhaled nanoparticles that efficiently penetrate the mucus gel layer are cleared more slowly by the MCC mechanism from the lung airways (16, 29). The ability to remain in the airways longer, achieve more uniform distribution within the airways, penetrate the mucus gel barrier to reach the underlying cells, and yet still be preferentially taken up by tumor cells via a specific receptor, TIR 1, is a unique benefit of the described delivery systems, and FTn with a dense high surface PEG density that resulted in a dense brush conformation with 2 kDa PEG (i.e., FTn/FTn-PEG2k) were the best in this regard.

The FTn/FTn-PEG2k efficiently penetrated tumor tissue in vitro (i.e., human and rodent lung cancer cell-based 3D tumor spheroids) and in vivo, which is essential for widespread chemotherapeutic delivery throughout the tumor and, thus, therapeutically effective chemotherapy (32, 41). This result is in good agreement with previous reports demonstrating widespread dispersion of small PEGylated particles within tumor tissues (42, 43). However, we found that non-PEGylated FTn were capable of only penetrating throughout the entire tumor as effectively as the described FTn/FTn-PEG2k. In contrast, FTn PEGylated with higher MW PEG were unable to efficiently penetrate tumor tissue. Interestingly, we found that tumor penetration of FTn/FTn-PEG2k required specific interactions between the FTn and TIR 1, similar to the mechanism by which FTn/FTn-PEG2k were taken up by cancer cells. These results altogether suggest that efficient tumor penetration by FTn/FTn-PEG2k may occur via TIR 1-dependent transcytosis, although the involvement of simple diffusion through the extracellular space within the tumor microenvironment cannot be fully excluded. All previous studies that investigated FTn as a delivery platform used methods of encapsulating payloads (10–12, 44). Here, we chose to conjugate DOX to the FTn to improve the drug loading efficiency and avoid drug release before uptake by cancer cells. Specifically, DOX molecules were conjugated to FTn/FTn-PEG2k via acid-labile linkers; these linkers are stable at extracellular-neutral or near-neutral pH but readily degrade at endolysosomal acidic pH (45). It should be noted that the tumor microenvironment can be slightly acidic (pH 6.5–6.8) (46), but the specific linker used in this study requires substantially lower pH (<6.0) to be degraded (47). We confirmed that DOX molecules were released at pH 5.0 while being stably associated with FTn/FTn-PEG2k at pH 7 and were found both in the cytoplasm and nucleus of lung cancer cells in vitro. In contrast, carrier-free DOX was found only in the nucleus. These findings suggest that FTn/FTn-PEG2k following TIR 1-dependent endocytosis (7, 8), may facilitate timely release of DOX in intracellular acidic vesicles. It is important to note that this loading strategy allows “tumor-penetrating” FTn/FTn-PEG2k to carry stably loaded DOX deep into the tumor cores, thus providing significantly improved drug distribution throughout the tumor tissue compared with free DOX that would enter every cell that it encounters in the airways.

**Materials and Methods**

Reassembly and Characterization of Hybrid FTn. Typically, FTn and conjugated FTn were mixed at designated molar ratios in PBS (pH 7.4) at a final nanocage concentration of 0.4 μM and were disassembled to subunits by adjusting pH to 2. Following a 20-min incubation, pH was tuned back to 7.0–7.4. The mixture was incubated overnight for the reassembly to yield hybrid FTn. Based on an optimization process to identify an ideal ratio, PEGylated FTn (i.e., FTn/FTn-PEG2k) were formulated with 50% of FTn/PEG-1. In all studies, the fluorescent labeling of PEGylated FTn, half of the non-PEGylated FTn were replaced with fluorescently labeled FTn (i.e., 25% each for FTn and fluorescently labeled FTn).

HDS of FTn and disassembled FTn were analyzed by size exclusion chromatography equipped with a Superose 6 column following previous reports (48, 49). Briefly, the protein standards with known HDs (Bio-Rad), including M1 (thymoglobin: 669 kDa, 16.5 nm HD), M2 (1-globulin: 158 kDa, 9.0 nm HD), M3 (ovalbumin: 44 kDa, 6.13 nm HD), M4 (myoglobin: 17 kDa, 3.83 nm HD), and M5 (vitamin B12: 1.35 kDa, 1.48 nm HD), were analyzed using gel-filtration chromatography (GFC), and subsequently a standard curve of HD vs. retention time was established. The HDs of FTn at pH 7.4 and pH 2.0 were calculated to be 13.2 and 1.8–5.4 nm, respectively (Fig. 5I).

The hybrid FTn were also characterized by gel electrophoresis. To enable the detection of signal from the gel by UV light excitation, AF488-labeled hybrid FTn were used for this study. The labeled FTn were analyzed following electrophoresis in an agarose gel by electrophoresis.

To assess the PEG surface density on FTn/FTn-PEG2k, hybrid nanocages were formulated with FTn and FITC-labeled FTn-PEG2k. The number of PEG chains per FTn/FTn-PEG2k was fluorometrically determined to be 107 ± 13. Subsequently, the PEG surface density ([T/SA]) was calculated to be 0.24 ± 0.03 PEG2k per square nanometer by the following formula, where D is the average diameter of FTn/FTn-PEG2k.

\[ [T] = \frac{\text{PEG molecules}}{4\pi (D/2)^2}. \]

The ratio ([T/SA]) of total unconstrained PEG surface area ([T]) to total particle surface area [SA] determines the surface PEG conformation: low-density mushroom and high-density brush regimes when [T/SA] < 1 and [T/SA] ≥ 1, respectively (27, 35). Briefly, the surface area occupied by one PEG chain was calculated by random-walk statistics and given by a sphere of diameter ξ:

\[ \xi = 0.76m^{1/2} |\Lambda|, \]

where m is MW of PEG chain, and the surface area occupied by one PEG molecule can be determined by n/πξ2. Thus, the [T/SA] ratio is 2.2 ± 0.3, which falls in the dense brush regime.

In Vivo Distribution of FTn Formulations Throughout the Mouse Trachea. All animals were handled in accordance with the policies and guidelines of the Johns Hopkins University Animal Care and Use Committee, which approved the experiments. Female CF-1 mice (6–8 wk) were anesthetized under continuous flow of isoflurane (2% in oxygen). A 50-μL solution of Cy5-labeled FTn (5 μM) without and with PEG modification was administered to the lung via intranasal instillation. Ten minutes after administration, mice were killed and the entire lungs (including trachea) were removed and frozen in Tissue-Tek optimal cutting temperature compound (Sakura Finetek). Tracheas were sectioned on a Leica Cryostat (Leica Biosystems) with a section thickness of 10 μm. The sections were stained with Prolong Gold antifade with DAPI (Life Technologies), and fluorescence images of the sections were obtained using a Zeiss confocal microscope. To quantify the particle distribution, the acquired images were analyzed by following an image-based analysis method that we have previously reported (28). Briefly, at least 10 fluorescence images at 10x magnification were taken of the lungs harvested from individual animals. The images were quantified with imageJ software. An average coverage and total particles in airways were determined for each mouse, and then these values were averaged over a group of n = 4 mice.

In Vivo Anticancer Efficacy Study. To evaluate the efficacy of FTn/FTn-PEG2k/DOX for treating lung cancer in the airways, we established an aggressive...
orthotopic mouse model of proximal lung cancer in inbred mice with intact host immunity. The model was established via intratracheal intubation of cancer cells into the tracheal and bronchial epithelium of lung airways (50 μm), which is relevant to SCLC and squamous cell lung cancer. Briefly, female C57BL/6 mice (6-8 wk) were inoculated with 2.5 × 10^6 3LL-Luc cells in 50 μL of DMEM via intratracheal intubation using a 22G×1-inch Safetcatheter (Exel International). Three days after the inoculation, bioluminescence signal in the lung was observed using a Xenogen IVIS Spectrum optical imaging system. Subsequently, mice (n = 10 per group) were treated with a single dose of DOX or FITC/PEG-DOX (0.25 mg/kg, 50 μL) administered intratracheally via a microsprayer. The tumor growth was monitored at various time points using an IVIS imaging system, and the bioluminescence signal in the lungs at different time points was quantitatively analyzed by the Living Image (Caliper Life Sciences) software. The survival of mice was recorded daily, and data were analyzed by Kaplan–Meier survival curve. The mice were monitored by following the timeline as shown in Fig. S9.

Other detailed materials and methods are provided in SI Materials and Methods.

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