

In Situ Needle-Free Injection of Multiretention Micelles for Melanoma Therapy with Multiomics Insights into Tumor Targeting and Immune Modulation

Kaichao Song,^{||} Yumei Hao,^{||} Peng Lei,^{||} Yige Yang, Yuxin Zhang, Suning Chen, Jiandong Jiang,*
Wensheng Zheng,* and Lulu Wang*



Cite This: <https://doi.org/10.1021/acsnano.6c01481>



Read Online

ACCESS |

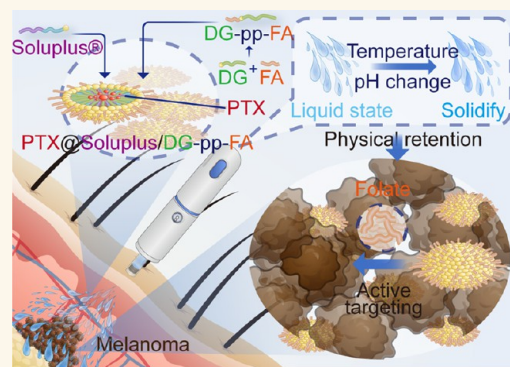
Metrics & More

Article Recommendations

Supporting Information

ABSTRACT: Melanoma is aggressive with limited treatment options. Paclitaxel (PTX) is effective but limited by poor solubility, systemic toxicity, and insufficient intratumoral retention. Here, we report an in situ needle-free platform that amplifies local PTX exposure via multiretention nanoscale mixed polymeric micelles composed of Soluplus and a dipotassium glycyrrhizinate-PEG-folic acid conjugate (DG-pp-FA). Needle-free intratumoral jet injection at the tumor site reduces needle-related barriers and discomfort. The micelles integrate thermoenabled local deposition and physical retention, pH-responsive stabilization and release in acidic tumor microenvironments, and folate-receptor targeting for enhanced uptake and selectivity. Central composite design optimization yielded stable micelles with a hydrodynamic size of 68 ± 3.8 nm, PDI of 0.08 ± 0.01 , and $98 \pm 0.11\%$ PTX encapsulation efficiency, showing sustained release. Compared with free PTX, NF#PTX@Soluplus/DG-pp-FA improved tumor retention, increased cellular uptake, and more strongly inhibited melanoma proliferation, migration, and invasion. Single-cell sequencing, spatial transcriptomics, and proteomics revealed dual actions of direct tumor suppression and immune microenvironment remodeling, including increased leukocyte infiltration, extracellular matrix (ECM) reprogramming to improve penetration, enhanced immune activation, and reduced fibrosis. *In vivo* studies confirmed improved tumor localization, prolonged retention, lower toxicity, and good biocompatibility.

KEYWORDS: melanoma, multiple omics, needle-free injection delivery system, tumor microenvironment, multiple retention effects



Melanoma is a highly lethal tumor, with its incidence rate continuing to rise globally.¹ Despite accounting for only 3% of skin cancers, melanoma is responsible for over two-thirds of skin cancer-related deaths.² Surgery remains the primary treatment option, particularly for early stage cases;³ however, its efficacy is limited in metastatic and advanced melanoma.^{3,4} Treatment strategies for metastatic and advanced melanoma mainly include chemotherapy, radiotherapy, targeted therapy, and immunotherapy.^{5,6} Paclitaxel (PTX), a chemotherapeutic agent, has been used for several decades to treat severe or metastatic melanoma.⁷

PTX is a well-established microtubule-targeting agent in cancer therapy,⁷ and it has been extensively studied in oncology.^{8–10} However, PTX has not yet realized its full therapeutic potential due to its low solubility and high cytotoxicity. PTX, although a first-line antineoplastic agent, is constrained by significant *in vivo* toxicities: microtubule stabilization can disrupt neuronal axonal transport and cause peripheral neuropathy;¹¹ it can induce myelosuppression by affecting rapidly dividing hematopoietic cells; and conventional Cremophor EL-based formulations may trigger severe hypersensitivity reactions.

In melanoma therapy, various nano drug delivery systems have been developed to overcome the limitations of conventional chemotherapy, such as poor solubility, high toxicity, and insufficient tumor targeting. Liposomes, with their high biocompatibility and drug-loading capacity, have been successfully used to deliver paclitaxel and immunomodulators, showing potential in delaying tumor growth and improving the immune microenvironment in melanoma mouse models.¹² However, their stability is affected by plasma protein adsorption and storage conditions, and they are prone to rapid clearance *in vivo*. Polymer-drug conjugates (PDCs), which exploit covalent bonds for controlled release, can effectively prolong drug half-life and reduce systemic toxicity, yet their complex synthesis and metabolic processes pose challenges for clinical translation.¹³

Received: January 24, 2026

Revised: February 24, 2026

Accepted: February 27, 2026

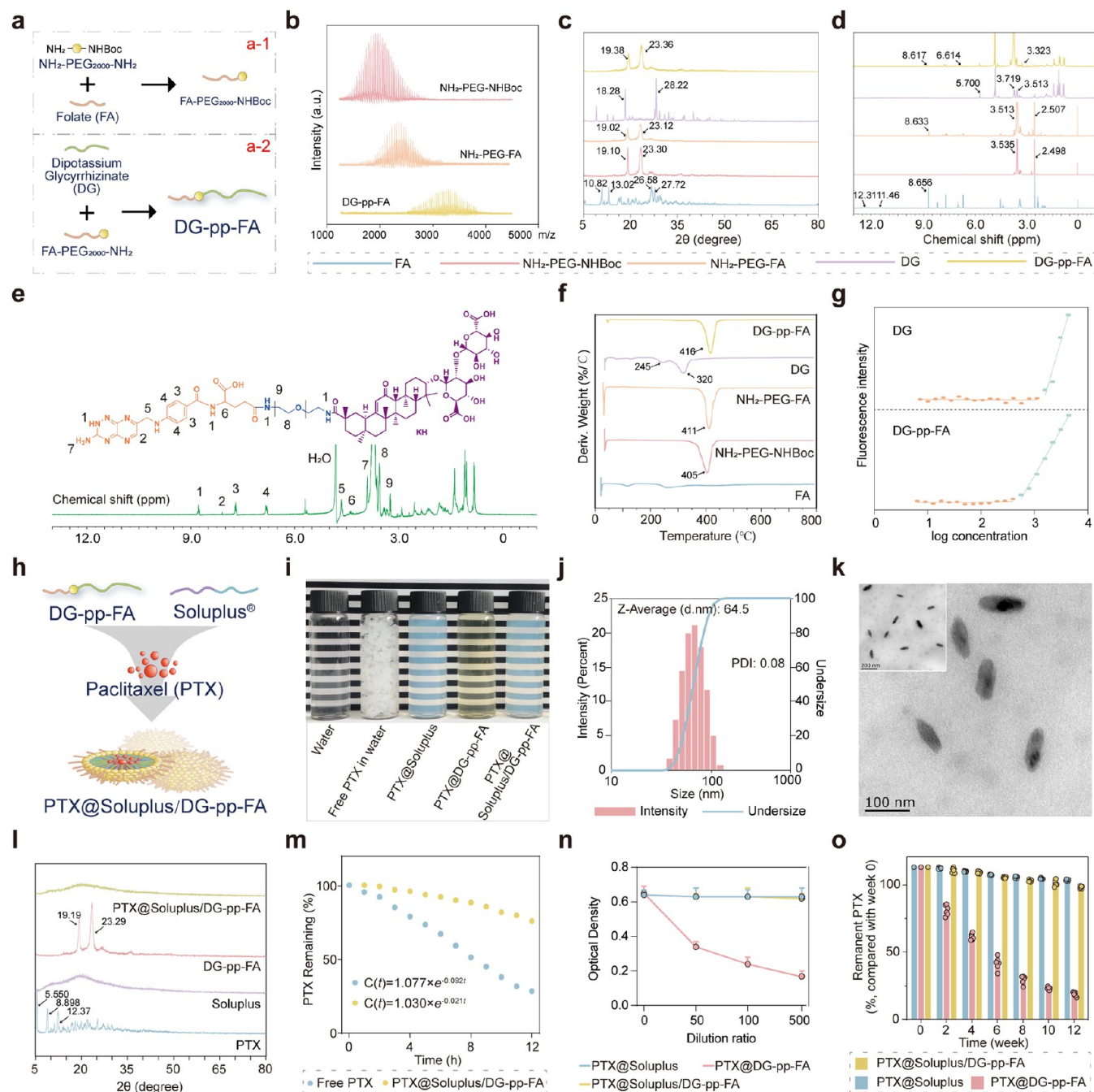


Figure 1. Synthesis of DG-pp-FA and preparation/characterization of PTX@Soluplus/DG-pp-FA micelles. (a) Synthetic diagram of DG-pp-FA; (b) MALDI-TOF spectra; (c) XRD patterns; (d) ¹H NMR spectra and (e) assignment of characteristic chemical shifts of NH₂-PEG-FA and DG-pp-FA; (f) DSC thermograms and (g) critical micelle concentration (CMC) of DG-pp-FA; (h) schematic illustration of PTX@Soluplus/DG-pp-FA mixed-micelle formation; (i) representative appearance of PTX@Soluplus/DG-pp-FA micelles; (j) hydrodynamic size distribution measured by dynamic light scattering (DLS); (k) TEM images of PTX@Soluplus/DG-pp-FA micelle morphology; (l) XRD patterns confirming amorphization of PTX after encapsulation; (m) chemical stability of PTX in micelles monitored by UV absorbance over time; (n) dilution stability after 50-, 100-, and 500-fold dilution; (o) storage stability over 12 weeks of PTX@Soluplus/DG-pp-FA.

Inorganic or organic–inorganic hybrid nanoparticles, including metal oxides and magnetic nanocarriers,¹⁴ have shown promise for photothermal therapy, multimodal imaging, and combination regimens in melanoma; however, concerns regarding long-term biosafety remain. In contrast, polymeric micelles as self-assembled nanocarriers are emerging as a promising platform for melanoma therapy due to their ability to encapsulate hydrophobic drugs, tunable particle size and surface functionality, passive or active tumor targeting, and excellent biocompatibility

with scalable manufacturing.^{15,16} Therefore, this study employs Soluplus and a functionalized polymer (DG-pp-FA) to construct a multiretentive polymeric micelle system to enhance paclitaxel accumulation, cellular uptake, and antitumor efficacy, aiming to offer a safer, more effective, and clinically accessible solution for refractory melanoma. Furthermore, conventional parenteral administration of PTX, including intravenous infusion, can compromise patient compliance due to needle-related anxiety

and discomfort, and it often requires trained personnel and clinic-based procedures.¹⁷

Soluplus, polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (PVCL–PVA-PEG) block copolymer developed by BASF, Germany, is widely used to solubilize poorly soluble drugs and improve bioavailability.^{18–20} In previous research, we successfully constructed micellar systems encapsulating myricetin,²¹ curcumin,²² and coumarin 6²³ using Soluplus, which demonstrated favorable physicochemical properties and stability, enhancing both *in vitro* and *in vivo* activity. These findings highlight Soluplus as a biocompatible micellar carrier. Glycyrrhizin, a natural extract derived from licorice,²⁴ and its salt form, dipotassium glycyrrhizinate (DG), have hydrophilic glycosyl side chains and hydrophobic triterpene structures, making them suitable for encapsulating poorly soluble drugs.^{25–27} In addition to various physiological activities, glycyrrhizinate salts are safe, exhibiting no significant reproductive or developmental toxicity in animal studies.²⁸ Although extensive research has focused on the biological activities of glycyrrhizin, its structural properties have been less thoroughly investigated.

Needle-free injection is a drug delivery method suitable for long-term administration and an alternative to traditional needle-based injections. Although the concept was first proposed in 1866, the first true needle-free injector was not developed until 1933.²⁹ Despite limited development, the needle-free injection market has expanded, with primary applications in insulin delivery,^{30–32} growth hormone administration,^{33,34} and vaccinations.^{35–37} This method offers distinct advantages, particularly in overcoming the limitations of conventional needle injections.

Advances in high-throughput profiling now enable integrated multiomics analyses that connect tumor-intrinsic programs with the immune microenvironment and extracellular-matrix barriers at single-cell and spatial resolution. In melanoma, such integration is particularly valuable for interpreting treatment responses under pronounced intratumoral heterogeneity and for linking local drug exposure to immune activation and stromal remodeling. Accordingly, we leveraged single-cell sequencing, spatial transcriptomics, and proteomics to elucidate how our local multiretention delivery strategy reshapes tumor biology and the tumor microenvironment.

In this study, we synthesized an amphiphilic carrier DG-pp-FA based on dipotassium glycyrrhizinate, where “pp” denotes a PEG linker covalently bridging DG and folic acid to enable folate-receptor-associated targeting. DG-pp-FA was combined with Soluplus to construct and optimize PTX-loaded mixed polymeric micelles, which were then administered using a needle-free jet injector to establish an *in situ* intratumoral delivery platform. This design integrates thermo-enabled local deposition and physical retention, acid-associated structural modulation in tumor-relevant microenvironments, and folate-receptor-assisted uptake to amplify intratumoral PTX exposure. We further applied multiomics analyses to clarify the mechanistic basis of antitumor efficacy and immune-microenvironment remodeling, providing a formulation-guided and minimally invasive strategy for localized melanoma therapy.

2. RESULTS

2.1. Synthesis and Characterization of DG-pp-FA

DG-pp-FA was synthesized via a stepwise heterobifunctional PEG strategy to ensure monofunctionalization and minimize

side reactions. Briefly, folic acid (FA) was first activated by EDC/NHS in anhydrous DMSO and subsequently coupled to mono-Boc-protected diamino-PEG (H₂N-PEG-NHBoc), yielding the monofunctionalized intermediate FA-PEG-NHBoc. The Boc protecting group was then removed using TFA in DCM to afford FA-PEG-NH₂ with a free terminal amine. Finally, dipotassium glycyrrhizinate (DG) was selectively grafted onto FA-PEG-NH₂ through a CDI-mediated amidation: DG was preactivated by CDI to generate the corresponding acyl-imidazolide intermediate, followed by nucleophilic substitution with the terminal amine of FA-PEG-NH₂ in the presence of DIPEA, producing DG-pp-FA. The overall synthetic route and key coupling steps are illustrated in Figures 1a and S1.

Following synthesis, MALDI-TOF analysis was performed to further confirm the successful stepwise construction of DG-pp-FA. As shown in Figure 1b, FA-PEG-NH₂ exhibited a dominant signal cluster centered at the expected mass range of PEG (Mn 1.8–2.2 kDa) plus the FA moiety, appearing as a series of regularly spaced peaks that reflect the intrinsic polydisperse nature of PEG. After CDI-mediated coupling with DG, the mass envelope of DG-pp-FA shifted to a higher *m/z* region relative to FA-PEG-NH₂, while maintaining the characteristic PEG peak spacing, indicating that the PEG backbone remained intact and that conjugation occurred primarily through end-group functionalization rather than nonspecific cross-linking. Importantly, the observed mass increase between FA-PEG-NH₂ and DG-pp-FA matched the theoretical contribution of a single DG unit, supporting the formation of the designed 1:1:1 architecture (FA-PEG-DG) rather than multi-DG substitution. In addition, the spectrum did not show pronounced high-mass shoulders or broad unresolved peaks that would be indicative of extensive intermolecular coupling or polymer–polymer cross-linking, consistent with the selective monofunctionalization achieved by the Boc-protection/deprotection strategy and the controlled CDI activation step.

Figure 1c shows the XRD patterns of FA, H₂N-PEG-NHBoc, and FA-PEG-NH₂ intermediates. FA exhibited multiple sharp and intense diffraction peaks between 5° and 35°, reflecting its crystalline nature. The PEG precursor displayed typical diffraction peaks around 18° and 25°, characteristic of PEG-related crystallinity. In FA-PEG-NHBoc and FA-PEG-NH₂, the diffraction profiles were dominated by PEG-associated peaks, while the sharp crystalline peaks of FA were markedly attenuated, indicating successful conjugation and reduced FA crystallinity upon grafting. DG displayed several sharp, high-intensity peaks between 5° and 50°, confirming its crystalline nature. In DG-pp-FA, the diffraction profile remained primarily governed by the PEG-containing intermediate, consistent with the relatively lower mass fraction of DG in the conjugate and/or the reduced crystallinity of DG after covalent attachment, thereby supporting successful synthesis of DG-pp-FA.

FTIR spectroscopy was consistent with conjugation in Figure S2. Compared with pristine FA and H₂N-PEG-NHBoc, FA-PEG-NHBoc exhibited a characteristic absorption band at 1680 cm^{−1}, attributable to the amide I vibration, confirming formation of an amide linkage between FA and PEG. After deprotection, FA-PEG-NH₂ retained the amide-associated band, consistent with preservation of the FA-PEG amide bond while exposing the terminal amine for subsequent coupling. Likewise, DG-pp-FA displayed an evident amide-related absorption around 1680 cm^{−1} that was absent in DG, supporting the successful CDI-mediated coupling of DG onto FA-PEG-NH₂ to form DG-pp-FA.

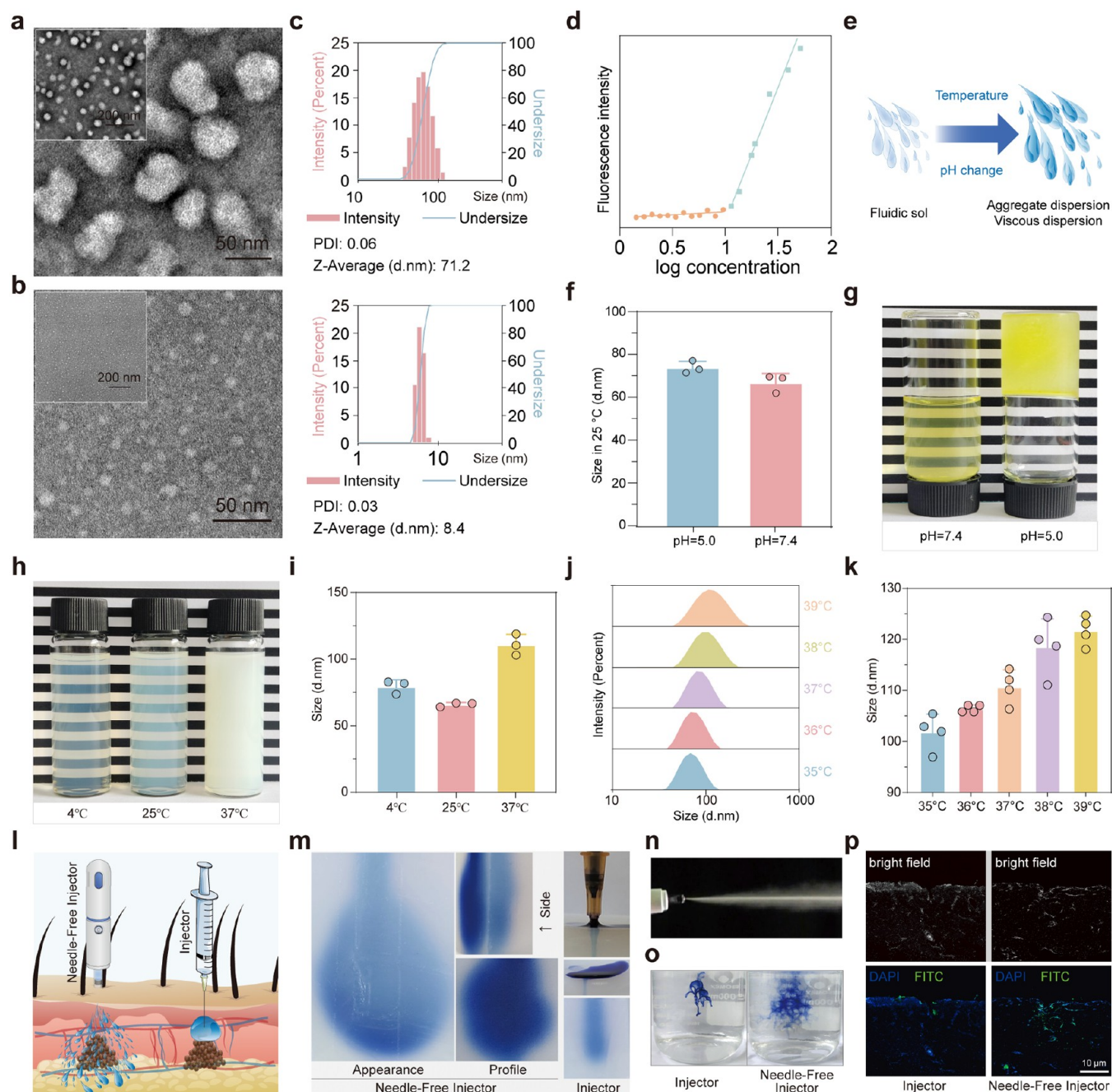


Figure 2. Tumor-microenvironment responsiveness and needle-free delivery performance of PTX@Soluplus/DG-pp-FA. (a,b) TEM images of single-component micelles PTX@Soluplus and PTX@DG-pp-FA; (c) particle-size distributions of PTX@Soluplus and PTX@DG-pp-FA by DLS; (d) CMC of the Soluplus/DG-pp-FA mixed carrier system; (e) schematic of pH/temperature-triggered phase and size modulation; (f) pH-dependent particle-size variation of PTX@Soluplus/DG-pp-FA; (g) pH-triggered sol-gel transition of PTX@DG-pp-FA; (h) representative appearance changes of PTX@Soluplus/DG-pp-FA at different temperatures; (i–k) temperature-dependent particle-size changes, including fine mapping within the 35–39 °C range; (l) schematic comparison of needle-free versus needle-based administration; (m) dispersion in agarose gel after needle-free versus needle injection; (n) jet dispersion in air; (o) dispersion pattern in aqueous medium; (p) *ex vivo* pig-skin distribution of Cou6-labeled micelles after needle-free versus needle injection.

The successful formation of FA-PEG intermediates and the subsequent DG grafting were further supported by ^1H NMR analysis (Figures 1d,e and S3). FA-PEG-NHBoc showed characteristic proton signals attributable to both FA and PEG segments, confirming FA grafting onto the PEG backbone. Importantly, after TFA-mediated deprotection, the Boc-associated *tert*-butyl signal disappeared in FA-PEG-NH₂, indicating efficient Boc removal and regeneration of the free

terminal amine. After DG coupling, DG-pp-FA exhibited additional resonances corresponding to DG moieties together with the retained FA and PEG signals, demonstrating successful conjugation of DG to FA-PEG-NH₂.

Differential scanning calorimetry (DSC) measurements were performed under a nitrogen flow (100 mL/min) with a heating rate of 10 °C/min from 25 to 800 °C. DSC thermograms of lyophilized FA, DG, FA-PEG-NH₂, and DG-pp-FA are

presented in Figures 1f and S4. the PEG precursor exhibited thermal transitions at 405 °C. After FA conjugation, FA-PEG-NH₂ showed shifted thermal features primarily in the 400–500 °C range, consistent with incorporation of FA into the PEG backbone and altered thermal behavior relative to the physical mixture. DG exhibited a characteristic thermal event at 245 and 320 °C, while FA-PEG-NH₂ displayed a transition around 416 °C, collectively supporting successful grafting of DG onto FA-PEG-NH₂ and the formation of DG-pp-FA.

2.2. Preparation and Characterization of PTX@Soluplus/DG-pp-FA

PTX@Soluplus/DG-pp-FA was prepared using the thin-film dispersion method, and the formulation was optimized through a central composite design (Figure 1h). The amounts of each component were adjusted with encapsulation efficiency, particle size, and polydispersity index as key indicators. The response surface is shown in Figures S5 and S6. The optimal preparation parameters for PTX@Soluplus/DG-pp-FA were determined as follows: the dosage of PTX was 10 mg/10 mL, the excipient ratio was 1:20, and the mass ratio of Soluplus to DG-pp-FA was 7:1. As shown in Figure 1i, the optimized formulation produced micelles with an encapsulation efficiency of 98% and a drug loading capacity of 5.5%.

The critical micelle concentrations (CMC) of Soluplus and DG-pp-FA were 8 µg/mL and 1 mg/mL, respectively. However, due to the 7:1 mass ratio of Soluplus to DG-pp-FA in the optimal formulation, the CMC of Soluplus/DG-pp-FA was measured at ~10 µg/mL, reflecting primarily the properties of Soluplus. This relatively low CMC facilitates the formation of a core-shell structure for PTX and improves dilution stability (Figures 1g, 2d, and S7c). All subsequent studies were conducted using the optimized PTX@Soluplus/DG-pp-FA formulation.

Figure 1i shows that the PTX@Soluplus/DG-pp-FA micelle solution prepared under these conditions was clear and transparent. The average particle size of the nanoparticles was 68 ± 3.8 nm (Figure 1j), with a polydispersity index of 0.08 ± 0.01 and a zeta potential of $-(0.1 \pm 0.01)$ mV (Figure S8). Transmission electron microscopy (TEM, Figure 1k) revealed that the PTX@Soluplus/DG-pp-FA nanoparticles were ellipsoidal or disc-shaped with a uniform size distribution. These findings indicate that the micelle sizes measured by dynamic light scattering were consistent with those observed via TEM. Correspondingly, we prepared the two single-component PTX-loaded micellar systems, PTX@Soluplus and PTX@DG-pp-FA, to establish formulation baselines. TEM images showed that PTX@Soluplus formed well-dispersed nanostructures with relatively uniform morphology (Figure 2a), whereas PTX@DG-pp-FA exhibited distinguishable micellar features with comparatively less uniform dispersion (Figure 2b). Consistently, DLS analysis confirmed clear differences in particle-size distributions between PTX@Soluplus (upper) and PTX@DG-pp-FA (bottom) (Figure 2c), supporting that the carrier chemistry markedly influences micellization behavior and colloidal homogeneity. In line with these observations, the mixed-carrier system PTX@Soluplus/DG-pp-FA displayed a low apparent CMC (Figure 2d), consistent with Soluplus-dominated micellization at the optimized 7:1 Soluplus:DG-pp-FA ratio.

To confirm the successful preparation of PTX@Soluplus/DG-pp-FA micelles and investigate the encapsulation of PTX within the micellar core, Fourier-transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD), and differential scanning

calorimetry (DSC) were employed. FTIR spectra (Figure S7a) contained characteristic bands attributable to PTX, Soluplus, and DG-pp-FA. In PTX@Soluplus/DG-pp-FA, several PTX-associated bands were markedly attenuated and overlapped by Soluplus and DG-pp-FA signals, consistent with PTX being molecularly encapsulated within the Soluplus/DG-pp-FA rather than present as free crystalline PTX. XRD patterns (Figures 1l and S7b) showed distinct diffraction peaks for free PTX at $2\theta = 5^\circ, 9^\circ, 12^\circ$, and 18° , whereas these peaks disappeared in PTX@Soluplus/DG-pp-FA, indicating an amorphous or molecularly dispersed state of PTX in the micelles. DSC thermograms (Figure S7d) further supported this observation, the sharp endothermic melting peak of free PTX at 220 °C was significantly reduced or absent in PTX@Soluplus/DG-pp-FA, confirming effective incorporation of PTX into the Soluplus/DG-pp-FA micellar matrix.

The chemical stability of the formulation was evaluated by monitoring the time-dependent UV absorbance at 227 nm. As shown in Figure 1m, the UV absorption of PTX in solution decreased over time due to degradation from prolonged exposure to UV light. However, this decline was significantly slower in PTX@Soluplus/DG-pp-FA micelles. The degradation curve of PTX was constructed by plotting the absorbance at the maximum absorption wavelength (227 nm) over time. Consistent with the UV spectra, PTX degradation in the PTX@Soluplus/DG-pp-FA micelle group was markedly slower than in the PTX solution group, suggesting that the core-shell structure of the micelles protected PTX, delaying its degradation and enhancing the drug's chemical stability.

As a natural product with an amphiphilic structure, DG-pp-FA can form micelles. However, its high CMC (1 mg/mL) makes its micelles unstable in solution. Dilution stability experiments further supported these results. After 50×, 100×, and 500× dilution, PTX@DG-pp-FA exhibited instability, with significant drug precipitation and reduced absorbance in the supernatant. Conversely, no significant changes in absorbance were observed in PTX@Soluplus and PTX@Soluplus/DG-pp-FA mixed micelles before and after dilution, indicating that the micelle structures remained stable in solution (Figure 1n).

A 12-week storage stability study assessed whether PTX@Soluplus/DG-pp-FA mixed micelles could improve the stability of PTX. As anticipated (Figure 1o), PTX in the PTX@DG-pp-FA formulation rapidly precipitated, with a significant decline in encapsulation efficiency. In contrast, PTX@Soluplus showed excellent stability, with over 90% of PTX retained in micelles after 3 months. The stability of PTX@Soluplus/DG-pp-FA mixed micelles was also improved, retaining nearly 90% of PTX after 3 months, suggesting that the mixed micelles effectively enhanced the stability of DG.

2.3. Tumor-Microenvironment Responsiveness of PTX@Soluplus/DG-pp-FA Micelles

PTX@Soluplus/DG-pp-FA micelles exhibited responsiveness to the tumor microenvironment, primarily due to the temperature sensitivity of Soluplus and the pH sensitivity of DG-pp-FA (Figure 2e).

The pH sensitivity of PTX@Soluplus/DG-pp-FA micelles was evaluated by observing particle size changes under varying pH conditions. Figure 2f shows that the micelle particle size increased from 65 ± 3.5 nm at pH 7.4 to 75 ± 4.4 nm at pH 5.0.

Although the low proportion of DG-pp-FA in the formulation limited the extent of these changes, pH-induced phase transitions were still observed. To better understand the pH

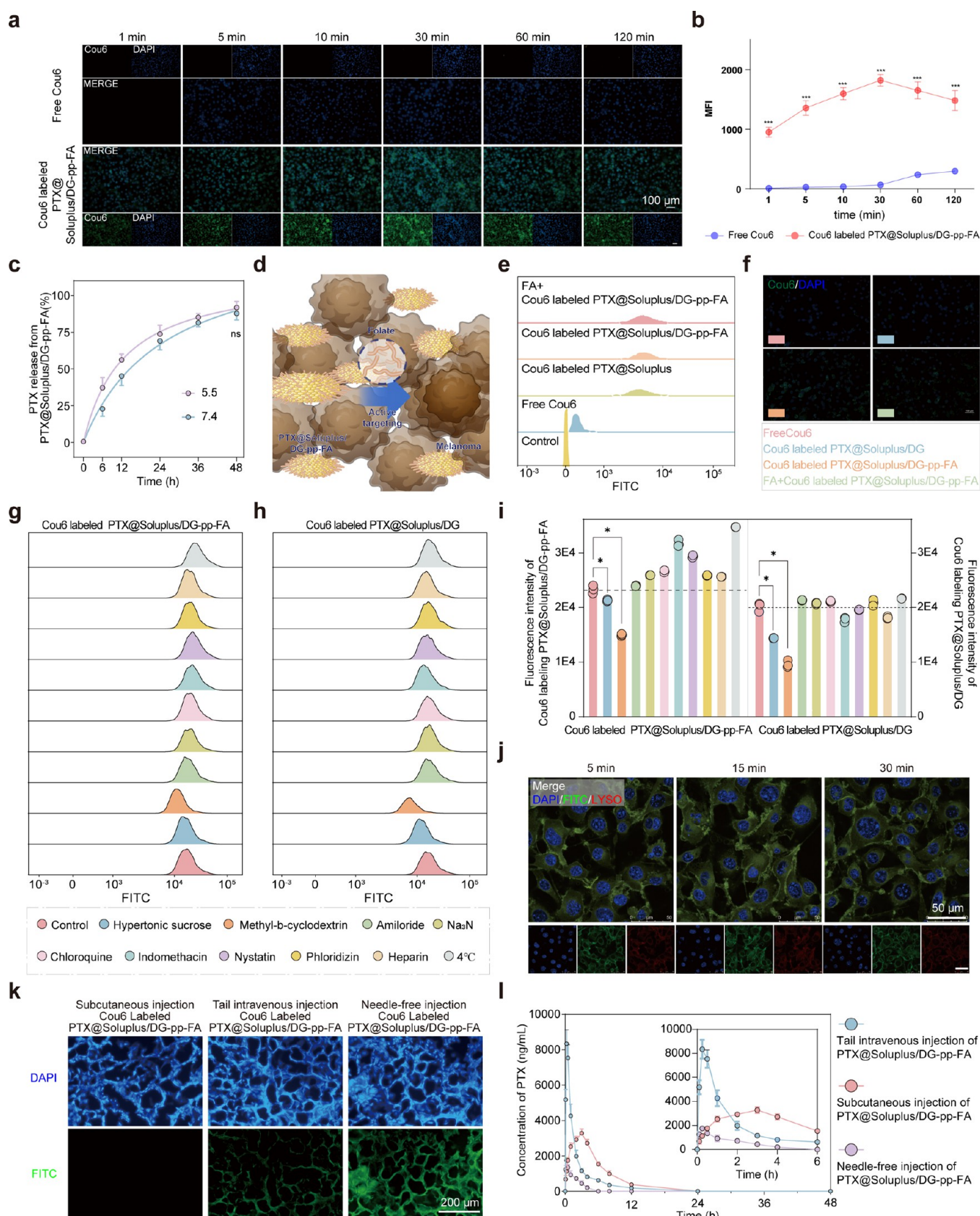


Figure 3. Cellular uptake, intracellular trafficking, tumor distribution, and pharmacokinetics of PTX@Soluplus/DG-pp-FA and NF#PTX@Soluplus/DG-pp-FA. (a) Representative fluorescence images of cellular uptake of Cou6-labeled micelles in B16–F10 cells; (b) time-dependent uptake kinetics; (c) *in vitro* PTX release profiles at pH 5.5 and pH 7.4; (d) folate-receptor competition assay design for validating FA-mediated targeting; (e, f) quantitative cellular uptake by flow cytometry under indicated conditions; (g, h) endocytosis-inhibitor studies with representative flow cytometry readouts; (i) summary of uptake inhibition across pathways; (j) lysosomal colocalization and escape analysis using LysoTracker; (k) tumor-section

Figure 3. continued

fluorescence distribution after intravenous, subcutaneous, and in situ needle-free administration; (l) serum pharmacokinetic profiles of PTX following intravenous, subcutaneous, and needle-free administration. Data are presented as mean $\pm$ SEM * P < 0.05.

response, we studied PTX@DG-pp-FA micelles alone, observing a transition from liquid to gel at lower pH values (Figure 2g). Although this transition was less pronounced in PTX@Soluplus/DG-pp-FA, the slight solidification due to DG-pp-FA was sufficient to physically retain the micelles at the injection site.

To investigate the pH sensitivity, PTX@Soluplus/DG-pp-FA micelles were stored at 4, 25, and 37 °C to simulate storage conditions and the tumor microenvironment, respectively. After 30 min, micelle morphology and particle size changes were observed under each condition (Figure 2h). At 4 and 25 °C, the micelles remained clear and transparent, whereas at 37 °C, the micelles turned white, and their transparency decreased.

Particle size studies following storage at different temperatures showed that the particle size of PTX@Soluplus/DG-pp-FA micelles increased by more than 50% between room and body temperature (Figure 2i). A detailed analysis of particle size variation within the 35 °C–39 °C range further confirmed the temperature sensitivity of the micelles (Figure 2j,k). This suggests that when PTX@Soluplus/DG-pp-FA micelles, stored at lower temperatures, are administered into the body via a needle-free injector, the temperature increase causes micelle size changes, allowing them to remain at the injection site. Combined with the active targeting effect of FA and the pH induced increase in particle size, PTX@Soluplus/DG-pp-FA micelles effectively accumulated in melanoma tissue.

2.4. Preparation and Characterization of NF#PTX@Soluplus/DG-pp-FA

The PTX@Soluplus/DG-pp-FA needle-free drug delivery system (NF#PTX@Soluplus/DG-pp-FA) was developed by combining PTX@Soluplus/DG-pp-FA mixed micelles with a commercially available needle-free injector (Figure 2l). The key features and functions of the injector are shown in Figure S11.

The ability of the NF#PTX@Soluplus/DG-pp-FA system to deliver PTX effectively to the tumor site is critically dependent on its injection performance, both *in vitro* and *in vivo*. Consequently, we evaluated the injection behavior of NF#PTX@Soluplus/DG-pp-FA under various conditions.

To first compare the NF#PTX@Soluplus/DG-pp-FA system with traditional needle-based injections, we examined its spray behavior in an air medium. A 200 μ L sample was drawn into the injector and sprayed into the air, following the manufacturer's guidelines, ensuring safety by directing the spray into an open space (Figure 2n). The results demonstrated that the NF#PTX@Soluplus/DG-pp-FA system generated sufficient spray force to complete the injection instantly, dispersing the drug into numerous small droplets widely spread in the air. Next, we evaluated the system's ability to penetrate skin-like media using agarose gel as a model (Figures 2m and S12a). The NF#PTX@Soluplus/DG-pp-FA system successfully delivered the drug into the interior of the gel, producing a diffuse drug distribution. This suggests the system can effectively deliver drugs into skin tissues, achieving sufficient diffusion within the melanoma region. In contrast, traditional needle-based injections of the same dose led to leakage along the needle path, thereby reducing the amount of drug delivered to the target, highlighting the superior delivery efficiency of the needle-

free system. The adjustable pressure feature of the NF#PTX@Soluplus/DG-pp-FA system also allows for precise control of the injection force to achieve the desired delivery location.

To simulate drug distribution postinjection, NF#PTX@Soluplus/DG-pp-FA was injected into a liquid medium, using trypan blue dye as a marker (Figure 2o). Trypan blue dispersed in a uniform cloud-like structure upon injection, whereas traditional needle-based injections created a concentrated liquid channel with a significantly smaller surface area covered.

We further tested the system using *ex vivo* pig skin to simulate *in vivo* conditions. PTX@Soluplus/DG-pp-FA micelles labeled with coumarin 6 (Cou6) were injected into pig skin using the needle-free injector. The injection site was then sectioned, and DAPI staining was used to label the cell nuclei. Fluorescent microscopy revealed fluorescence was concentrated near the skin surface after traditional needle injection, with substantial residual fluorescence. Conversely, needle-free injection resulted in widespread fluorescence distribution in a spherical pattern beneath the skin (Figure 2p), indicating that needle-free injection offers better drug coverage with the same dosage.

Validation experiments compared the set injection volume and drug content with actual outcomes. The results showed a proportional 1:1 relationship between the set and actual injection volumes and between the set volume and drug content, confirming the accuracy of drug delivery by the NF#PTX@Soluplus/DG-pp-FA system (Figure S12b).

2.5. In Vitro Release Study of NF#PTX@Soluplus/DG-pp-FA

The *in vitro* release study of PTX@Soluplus/DG-pp-FA is essential for determining whether PTX, the active ingredient, can be fully released under external conditions and the rate at which it is released. This indicates the drug release behavior of the PTX@Soluplus/DG-pp-FA needle-free delivery system *in vivo*. Figure 3c shows that at pH 5.5, PTX release from PTX@Soluplus/DG-pp-FA was rapid within the first 12 h, with 70% of the drug released. The release rate then slowed, with cumulative drug release exceeding 90% within 48 h. At pH 7.4, PTX release followed a similar pattern but was slightly lower, exceeding 90% overall. These results suggest that pH does not significantly affect the release profile of the PTX@Soluplus/DG-pp-FA system by altering its encapsulation structure. Instead, variations in pH and temperature may influence the physicochemical properties of PTX@Soluplus/DG-pp-FA, which could affect drug retention at the tumor site.

2.6. Cellular Uptake Evaluation of PTX@Soluplus/DG-pp-FA

To evaluate the uptake efficiency, the cellular uptake study assessed the fluorescence intensity of Cou6 and Cou6-labeled PTX@Soluplus/DG-pp-FA in B16–F10 cells. As shown in Figure 3a,b, the Cou6-labeled PTX@Soluplus/DG-pp-FA group exhibited significantly higher fluorescence intensity compared to the free Cou6 group, with rapid internalization by B16–F10 cells. Within a short period, cellular uptake reached a high level, stabilizing around 30 min. This indicates that Cou6-labeled PTX@Soluplus/DG-pp-FA is quickly absorbed by cells, potentially enabling faster pharmacological effects, likely due to the smaller particle size of PTX@Soluplus/DG-pp-FA, which enhances cellular absorption. Moreover, when FA was introduced as a competitive inhibitor (Figure 3e), a slight

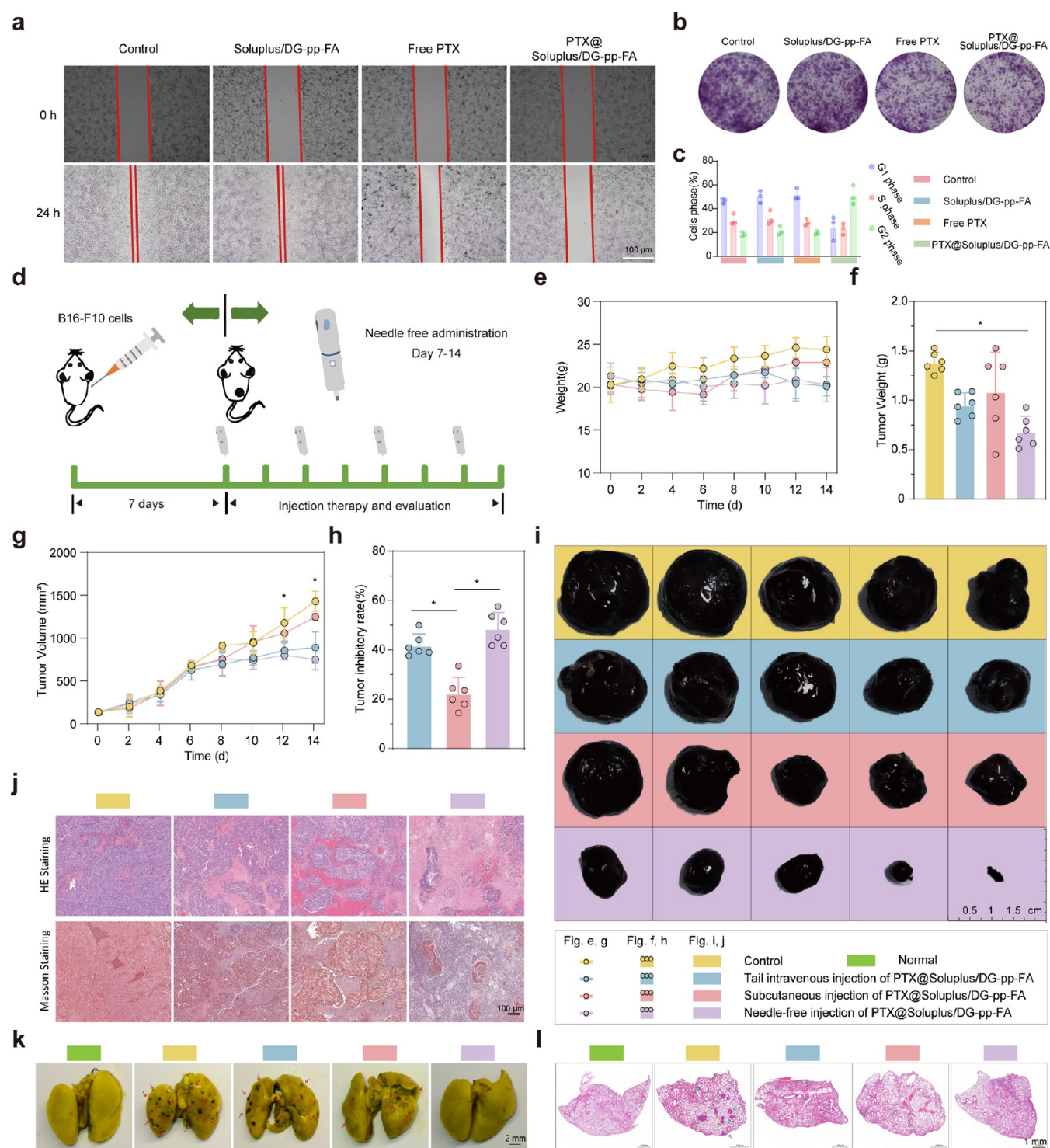


Figure 4. *In vitro* and *in vivo* antitumor efficacy of NF#PTX@Soluplus/DG-pp-FA, including lung-metastasis assessment. (a) Wound-healing assay for B16–F10 cell migration; (b) colony formation assay for long-term proliferative capacity; (c) cell-cycle distribution analyzed by flow cytometry; (d) *in vivo* treatment scheme in B16–F10 tumor-bearing mice; (e) body-weight changes during treatment; (f) tumor weight at end point; (g) tumor-growth curves; (h) tumor inhibition rate; (i) representative tumor images at end point; (j) H&E and Masson staining of tumor sections; (k) representative gross lung images in the pulmonary metastasis model; (l) representative H&E-stained lung sections showing metastatic lesions. Data are presented as mean $\pm$ SEM, * P < 0.05.

decrease in uptake was observed, suggesting that PTX@Soluplus/DG-pp-FA exhibits some degree of targeting via the folate receptor. These findings were further confirmed by flow cytometry (Figures 3e,f, and S13b).

We adjusted the culture environment for B16–F10 cells to simulate tumor microenvironment conditions and assessed cellular uptake. The uptake trends under these conditions were similar to those in standard culture, though slightly higher overall. Among the parallel groups, PTX@Soluplus/DG-pp-FA

demonstrated the highest uptake and clear targeting behavior (Figure S13c).

The uptake mechanism was further investigated through single-factor control experiments using various inhibitors of cellular endocytosis to assess the uptake of Cou6-labeled PTX@Soluplus/DG-pp-FA in B16–F10 cells (Figure 3g–i). Results indicated multiple inhibitors reduced uptake, suggesting that PTX@Soluplus/DG-pp-FA is internalized through multiple pathways. Notably, methyl- β -cyclodextrin caused the greatest reduction in uptake, indicating that lipid raft- and caveolae-mediated endocytosis may be the primary uptake pathways for PTX@Soluplus/DG-pp-FA in B16–F10 cells. Additionally, results from sodium azide treatment and low-temperature incubation (4 °C) revealed that cellular uptake is not entirely energy-dependent.

To explore the potential for lysosomal escape, we stained lysosomes in B16–F10 cells following the uptake of Cou6-labeled PTX@Soluplus/DG-pp-FA. We performed a colocalization analysis of the green fluorescence from Cou6 and the red fluorescence from LysoTracker. Figure 3j shows that at 5 min chase, colocalization between the micelle signal and LysoTracker was low, consistent with early stage endocytic trafficking prior to extensive lysosomal delivery. Colocalization increased at 15 min, indicating prominent accumulation within the lysosomal compartment. By 30 min, colocalization decreased despite sustained intracellular fluorescence, supporting partial redistribution of the payload from lysosomes to the cytosol, consistent with lysosomal escape. Consistently, quantitative colocalization metrics showed that Pearson's correlation coefficient and Manders' overlap coefficients increased from 5 to 15 min and then decreased at 30 min (Figure S14), corroborating time-dependent lysosomal accumulation followed by partial lysosomal escape.

2.7. In Vivo Absorption Study of NF#PTX@Soluplus/DG-pp-FA

Cou6-labeled PTX@Soluplus/DG-pp-FA was administered through tail vein injection, subcutaneous injection, and in situ needle-free injection to evaluate drug distribution within the tumor. Tumor tissues were harvested, cryo-fixed, sectioned, and observed under a fluorescence microscope. Figure 3k shows that in situ needle-free injection group tumors exhibited significantly brighter green fluorescence than those from the tail vein and subcutaneous injection groups. This may be due to the in situ injection bypassing the body's clearance mechanisms, improving drug utilization. Additionally, the uniform distribution of green fluorescence throughout the tumor tissue suggests that needle-free injection provides better drug dispersion and broader coverage within the tumor.

The pharmacokinetics of PTX@Soluplus/DG-pp-FA were studied in tumor-bearing mice to assess the drug's *in vivo* behavior. As shown in Figure 3l, following the tail vein injection of PTX@Soluplus/DG-pp-FA, the blood concentration of PTX peaked at 30 min, followed by a gradual decline. In contrast, the subcutaneous injection group reached its peak concentration around 4 h, and the in situ needle-free injection group peaked at around 30 min. Overall, the area under the curve for the tail vein injection group was higher than that of the subcutaneous and needle-free injection groups. This difference could be due to a portion of the drug being directly absorbed into the tumor tissue in the in situ injection groups, especially in the needle-free injection group, where the drug exerted its effect at the tumor

site via active targeting and physical retention, reducing systemic absorption into the bloodstream.

2.8. Pharmacodynamic Evaluation of NF#PTX@Soluplus/DG-pp-FA In Vitro and In Vivo

The cytotoxicity results of the PTX@Soluplus/DG-pp-FA needle-free drug delivery system against B16–F10 cells (Figure S15b) demonstrated significant tumor cell inhibition within 1 to 4 h, outperforming the antitumor activity of free PTX *in vitro*. In the long term cytotoxicity study (24–72 h), the PTX@Soluplus/DG-pp-FA system exhibited notable cytotoxicity. However, the inhibition rate was slightly lower compared to the short-term results. This may be due to the lower drug concentration after dilution, as PTX's antitumor activity is concentration-dependent. Nevertheless, the PTX@Soluplus/DG-pp-FA system consistently demonstrated superior *in vitro* antitumor activity compared to free PTX.

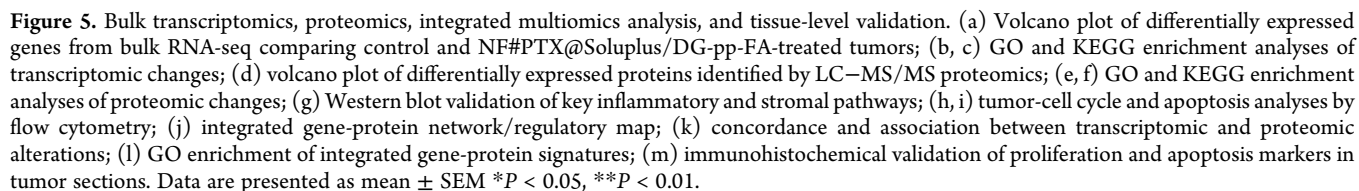
Inhibition of tumor cell migration is crucial for reducing tumor growth and metastasis. The results showed that PTX inhibited B16–F10 cell proliferation with an inhibition rate of 70% (Figures 4a and S15a). PTX@Soluplus/DG-pp-FA demonstrated an even more pronounced inhibitory effect on cell migration.

PTX significantly suppressed the clonogenic ability of B16–F10 cells shown in Figure 4b, with notable differences observed among the experimental groups. In the control group, cells formed numerous large and densely packed colonies, indicating a high proliferative capacity. Meanwhile, the Soluplus/DG-pp-FA group showed a similar colony formation pattern, suggesting that the carrier itself had no significant inhibitory effect on cell proliferation. In contrast, the Free PTX group exhibited a considerable reduction in both the number and size of colonies, demonstrating the proliferation inhibition effect of PTX on melanoma cells. Moreover, the most significant suppression of clonogenic growth was observed in the PTX@Soluplus/DG-pp-FA group, where only a few small colonies remained, indicating a strong inhibition of long-term proliferative potential.

PTX stabilizes microtubules and inhibits cell division, typically leading to cell cycle arrest at the G2/M phase. This was confirmed by flow cytometry. The results showed that, at a concentration of 40 nM PTX@Soluplus/DG-pp-FA, the proportion of cells in the G2/M phase was markedly elevated compared to the control group, while the proportion of cells in the G1 phase decreased (Figure 4c). These results suggest that PTX@Soluplus/DG-pp-FA disrupts mitosis in B16 cells, causing G2/M phase arrest.

The *in vivo* antitumor activity of the NF#PTX@Soluplus/DG-pp-FA needle-free delivery system was evaluated in tumor-bearing C57BL/6N mice by monitoring physiological characteristics and tumor changes during drug administration. A mouse model of melanoma was constructed, and treatment was initiated on Day 7 after the injection of B16–F10 cells (Figure 4d).

As shown in Figure 4e,f, the body weight of mice in the control group increased due to normal growth, while the negative control group experienced certain weight gain because of rapid tumor growth. In the other drug treatment groups, body weight fluctuated due to the combined effects of tumor burden, normal growth, and tumor progression, with greater variance observed within these groups. In the NF#PTX@Soluplus/DG-pp-FA needle-free group, the tumor-reducing effect alleviated the impact of tumor burden on body weight, resulting in minimal



Tumor volumes were measured throughout the treatment process. The control group (saline-treated) showed significant tumor growth, while all drug-treated groups exhibited varying degrees of tumor volume reduction. Notably, the NF#PTX@Soluplus/DG-pp-FA group displayed the most significant tumor volume reduction, with an average tumor size of 700 mm³ and an inhibition rate of 50% (Figure 4g). The tumor inhibition rate

(Figure 4h–i) further demonstrated that the NF#PTX@Soluplus/DG-pp-FA needle-free delivery system was the most effective in inhibiting tumor growth compared to other delivery methods. Postdissection, the tumor sizes closely aligned with the volumes measured during the study, with minor discrepancies likely due to measurement errors caused by tumor positioning. Tumor weights corroborated these findings, confirming the efficacy of the NF#PTX@Soluplus/DG-pp-FA in suppressing tumor growth (Figures 4i and S16b).

To assess whether NF#PTX@Soluplus/DG-pp-FA altered organ function in tumor-bearing mice, serum biochemical markers of liver and kidney function were measured. As shown in Figure S16c, AST and ALT levels were broadly comparable among all tumor-bearing groups, with no meaningful differences observed across treatments. Likewise, renal function indices showed no appreciable intergroup differences (Figure S16c).

Hematoxylin and eosin (H&E) staining revealed distinct histopathological differences among the treatment groups (Figure 4j). In the control group, tumor tissues displayed densely packed melanoma cells with irregular nuclei, abundant mitotic figures, and minimal necrosis, indicating aggressive tumor growth. The subcutaneous injection of PTX@Soluplus/DG-pp-FA group and tail intravenous injection of PTX@Soluplus/DG-pp-FA group exhibited increased tumor cell apoptosis and necrosis, with a reduction in overall cell density. The most pronounced histopathological changes were observed in the needle-free PTX@Soluplus/DG-pp-FA group, where extensive necrotic areas and reduced viable tumor cell regions were evident, suggesting enhanced antitumor efficacy. Masson's trichrome staining further highlighted differences in collagen deposition within the tumor microenvironment. The control groups showed abundant collagen fibers surrounding tumor cells, indicating a dense extracellular matrix that may facilitate tumor progression. In the subcutaneous injection of PTX@Soluplus/DG-pp-FA group and tail intravenous injection of PTX@Soluplus/DG-pp-FA group, collagen deposition was slightly reduced, while the needle-free PTX@Soluplus/DG-pp-FA group exhibited a significant decrease in collagen content, suggesting that this formulation not only inhibits tumor growth but also disrupts the tumor stroma, potentially reducing tumor invasion and metastasis (Figure 4j).

Gross lung imaging showed that NF#PTX@Soluplus/DG-pp-FA treatment markedly reduced pulmonary metastatic burden, with fewer and smaller pigmented metastatic nodules compared with the saline group and the nontargeted control (Figure 4k). Consistently, H&E staining confirmed a clear reduction in both the number and size of metastatic lesions in the NF#PTX@Soluplus/DG-pp-FA group, accompanied by a decreased metastatic area fraction (Figure 4l).

2.9. Multiomics Analysis Reveals NF#PTX@Soluplus/DG-pp-FA's Regulatory Mechanisms in Melanoma

To delineate the molecular basis of the therapeutic response to NF#PTX@Soluplus/DG-pp-FA, we implemented an integrated multiomics workflow using self-generated bulk transcriptomics and bulk proteomics from melanoma tumors collected in this study, followed by cross-modality integration at the gene-protein and pathway levels. Specifically, tumors from saline-treated control mice and NF#PTX@Soluplus/DG-pp-FA-treated mice were harvested at the treatment end point for downstream profiling. For transcriptomics, total RNA was extracted and subjected to bulk RNA sequencing (RNA-seq) to quantify global gene expression. For proteomics, tumor lysates were processed for LC-MS/MS-based quantitative proteomics to measure protein abundance changes.

In the bulk RNA-seq data set, principal component analysis (PCA) showed clear separation between control and NF#PTX@Soluplus/DG-pp-FA groups (Figure S17), indicating a robust treatment-associated transcriptional shift. Differential expression analysis identified 214 significantly dysregulated genes (191 upregulated and 23 downregulated) in treated

tumors relative to controls (Figure 5a). Functional annotation of differentially expressed genes using Gene Ontology (GO) enrichment revealed preferential activation of immune-associated processes, including leukocyte-mediated immunity, regulation of responses to biotic stimuli, cytokine-mediated signaling, and innate immune response activation (Figure 5b). Consistently, KEGG pathway analysis highlighted enrichment of immune-relevant pathways, such as phagosome, antigen processing and presentation, chemokine signaling, and B cell receptor signaling (Figure 5c), suggesting enhanced immune surveillance in the tumor microenvironment after treatment.

In the LC-MS/MS proteomics data set, quantitative profiling identified 690 differentially expressed proteins (508 upregulated and 182 downregulated) following NF#PTX@Soluplus/DG-pp-FA treatment (Figure 5d). GO enrichment of regulated proteins similarly emphasized immune activation and tissue remodeling programs, including regulation of peptidase activity, wound-healing-related processes, and leukocyte migration (Figure 5e). KEGG enrichment further supported these findings, with significant involvement of antigen processing and presentation, complement and coagulation cascades, lysosome, Fc gamma R-mediated phagocytosis, and PPAR signaling (Figure 5f), reinforcing an immunomodulatory and microenvironment-remodeling phenotype at the protein level (Figures S17–S19).

To validate key regulated axes suggested by the omics data sets, we performed targeted experimental verification on independent tumor samples, including Western blotting and flow cytometry. Western blot analysis confirmed modulation of tumor-associated inflammatory and stromal programs (Figure 5g), including reduced levels of TNF- α , IL-1 β , and IL-6 and attenuation of HMGB1/TLR4 signaling, alongside decreased collagen I abundance, consistent with reduced tumor-promoting inflammation and fibrosis. In parallel, flow cytometry of dissociated tumor cells quantified treatment-associated shifts in tumor cell state (Figure 5h,i), showing increased G2/M arrest and elevated Annexin V-positive populations, consistent with PTX-driven mitotic disruption and apoptosis induction.

For cross-modality integration, transcriptomic and proteomic profiles were jointly analyzed to evaluate concordant regulation at the gene-protein level and to resolve pathway programs supported by both data sets. Integrated analysis revealed strong concordance in treatment-associated regulation, particularly within immune activation modules (Figure 5j,k). Co-regulated features (Oas1g, Zfp385a, Plekhh1, and Trim) emerged as representative markers of the activated immune response and tumor suppression programs (Figures 5j and S21). GO analysis of the integrated gene-protein signatures highlighted upregulation of leukocyte migration, regulation of TNF superfamily cytokine production, lymphocyte proliferation, and mononuclear cell proliferation (Figure 5l). Pathway clustering further indicated that immune activation pathways were preferentially enriched in treated tumors, consistent with a treatment-driven shift toward an antitumor immune microenvironment.

Finally, immunohistochemistry (IHC) was used as an orthogonal tissue-level validation of functional outcomes. NF#PTX@Soluplus/DG-pp-FA treatment reduced Ki67- and PCNA-positive tumor cells and increased apoptosis-related staining (Figure 5m), supporting the combined antiproliferative and pro-death effects inferred from multiomics and flow cytometry. Collectively, these multilayer data sets-bulk RNA-seq, LC-MS/MS proteomics, integrated gene-protein analysis, and targeted experimental validation-support that NF#PTX@

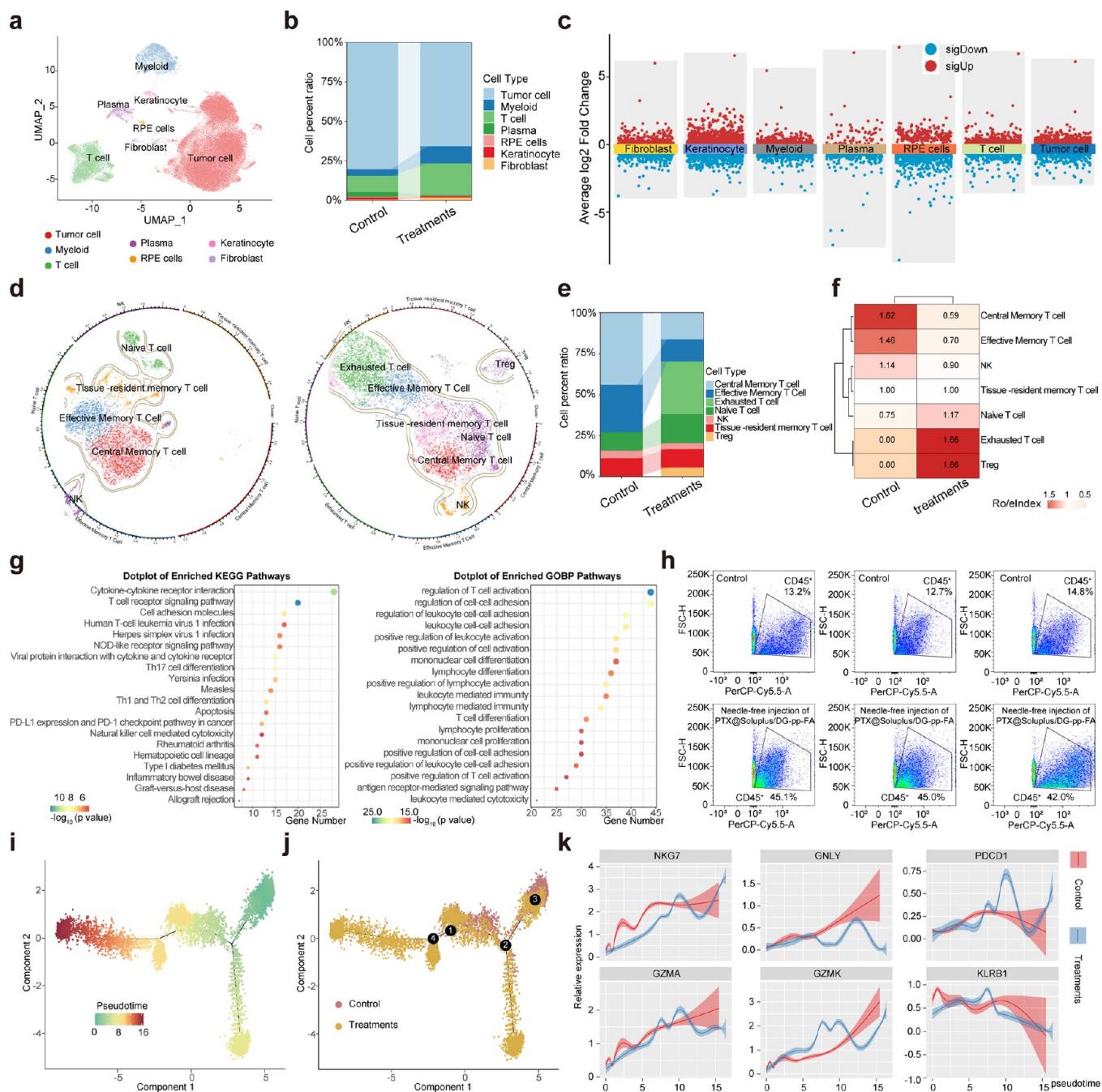


Figure 6. Public single-cell RNA-seq reanalysis defining melanoma cellular composition and immune-state programs. (a) UMAP visualization of major cell types in melanoma; (b) comparison of cell-type proportions across immune contexts; (c) overview of differential expression patterns across major compartments; (d) UMAP of immune-cell subpopulations; (e) proportion analysis of immune subsets; (f) heatmap and functional role index summarizing immune states; (g) pathway enrichment analysis of key T-cell subsets, including regulatory and exhausted T cells; (h) quantification of leukocyte infiltration across immune contexts; (i) pseudotime trajectory analysis of immune-cell state transitions; (j) comparative pseudotime analysis across contexts; (k) dynamic expression trends of representative immune genes along pseudotime.

Soluplus/DG-pp-FA exerts therapeutic activity through coordinated tumor growth inhibition, immune activation, and tumor microenvironment remodeling.

Based on reanalysis of publicly available single-cell RNA-seq data sets, we delineated the immune landscape and cellular heterogeneity of melanoma at single-cell resolution and used these data as an independent mechanistic reference to contextualize the immunoregulatory effects observed for NF#PTX@Soluplus/DG-pp-FA in this study. The UMAP visualization resolved the major cellular compartments within

the tumor microenvironment, including tumor cells, myeloid cells, T cells, fibroblasts, keratinocytes, plasma cells, and retinal pigment epithelium (RPE) cells, with clear separation across cell types (Figures 6a and S22–S23). Cell proportion analysis indicated that a tumor-cell-dominant state was associated with relatively limited immune infiltration, whereas an immune-activated state showed increased immune cell representation (Figures 6b and S24). This global pattern provides a single-cell framework supporting our working model that improved intratumoral drug coverage and retention-enabled by in situ

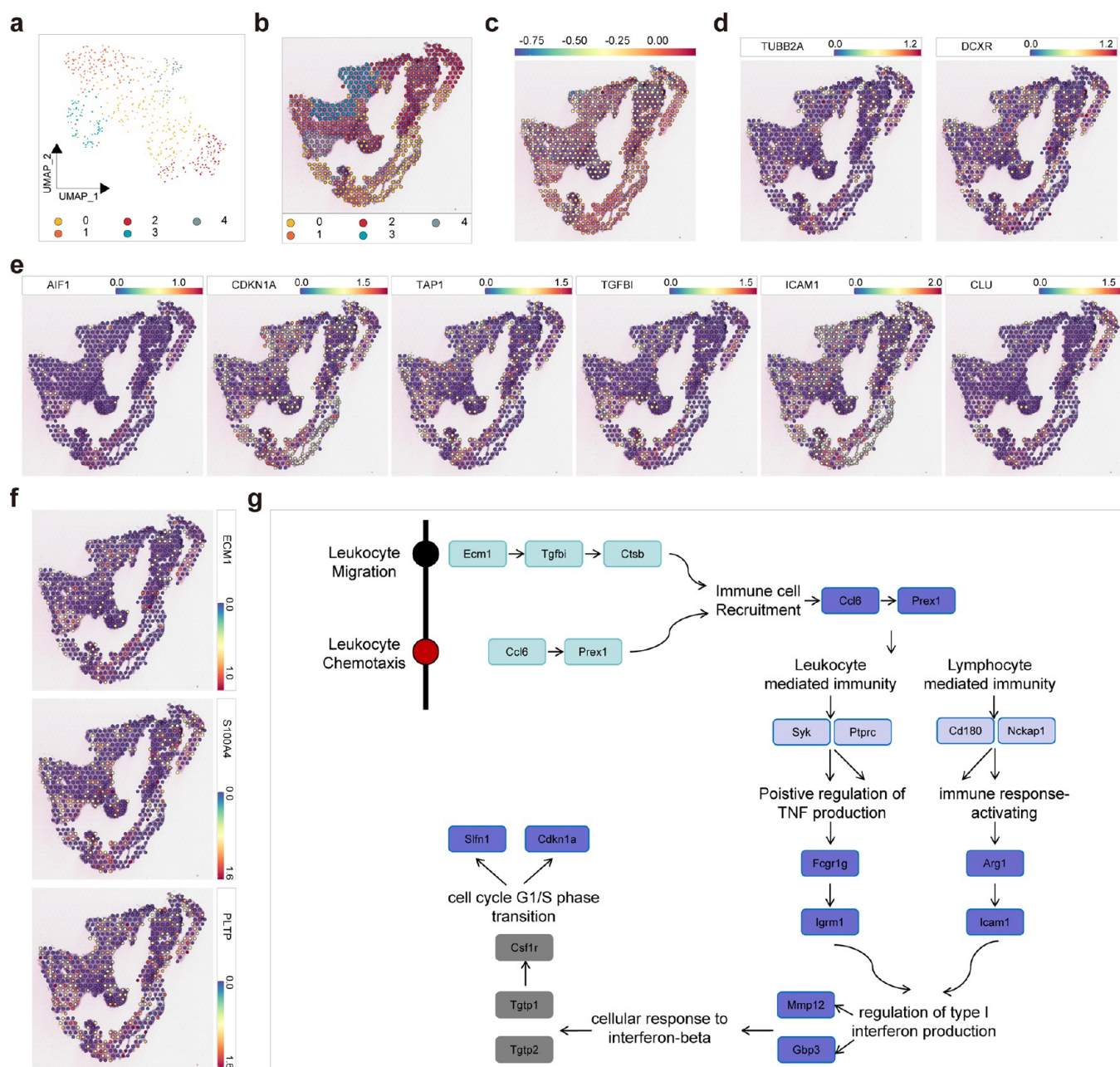


Figure 7. Public spatial transcriptomic reanalysis revealing spatial niches and immune–stroma interaction interfaces in melanoma. (a) UMAP clustering of spatial transcriptomic profiles; (b) clustering of spatial spots; (c) spatial mapping of spot clusters onto tissue sections; (d) spatial expression maps of TUBB2A and DCXR; (e) spatial expression of AIF1, CDKN1A, TAP1, TGFB1, ICAM1, and CLU; (f) spatial expression maps of ECM1, S100A4, and PLTP; (g) inferred regulatory network associated with leukocyte migration, chemotaxis, and immune-response modules.

needle-free delivery-can promote immune infiltration and reinforce immune surveillance.

Differential expression analysis further revealed that immune cell compartments displayed broad upregulation of immune activation-related genes, while tumor cells exhibited downregulation of proliferation- and survival-associated programs (Figure 6c). Heatmap clustering of canonical marker genes corroborated cell-type identities and highlighted systematic transcriptional differences across immune contexts (Figure S25). High-resolution annotation of the T-cell compartment identified multiple functional states, including naïve T cells, central memory T cells, effector memory T cells, tissue-resident memory T cells, regulatory T cells (Tregs), natural killer (NK) cells, and exhausted T cells (Figure 6d). Comparative

abundance and state analyses suggested substantial remodeling of T-cell composition across immune contexts, characterized by an increased fraction of naïve T cells with reduced central/effector memory T cells, accompanied by an elevated proportion of exhausted T cells (Figure 6e). This pattern is compatible with intensified antigen engagement and sustained immune activation, which may transiently drive activation-associated exhaustion phenotypes. Consistently, role index analysis indicated a higher proportion of functionally active immune cells in the immune-activated state (Figure 6f), providing a cell-state-level rationale for the immune pathway upregulation observed in our bulk multiomics results.

To probe the functional implications of these immune shifts, GO/KEGG enrichment analyses were performed across key T-

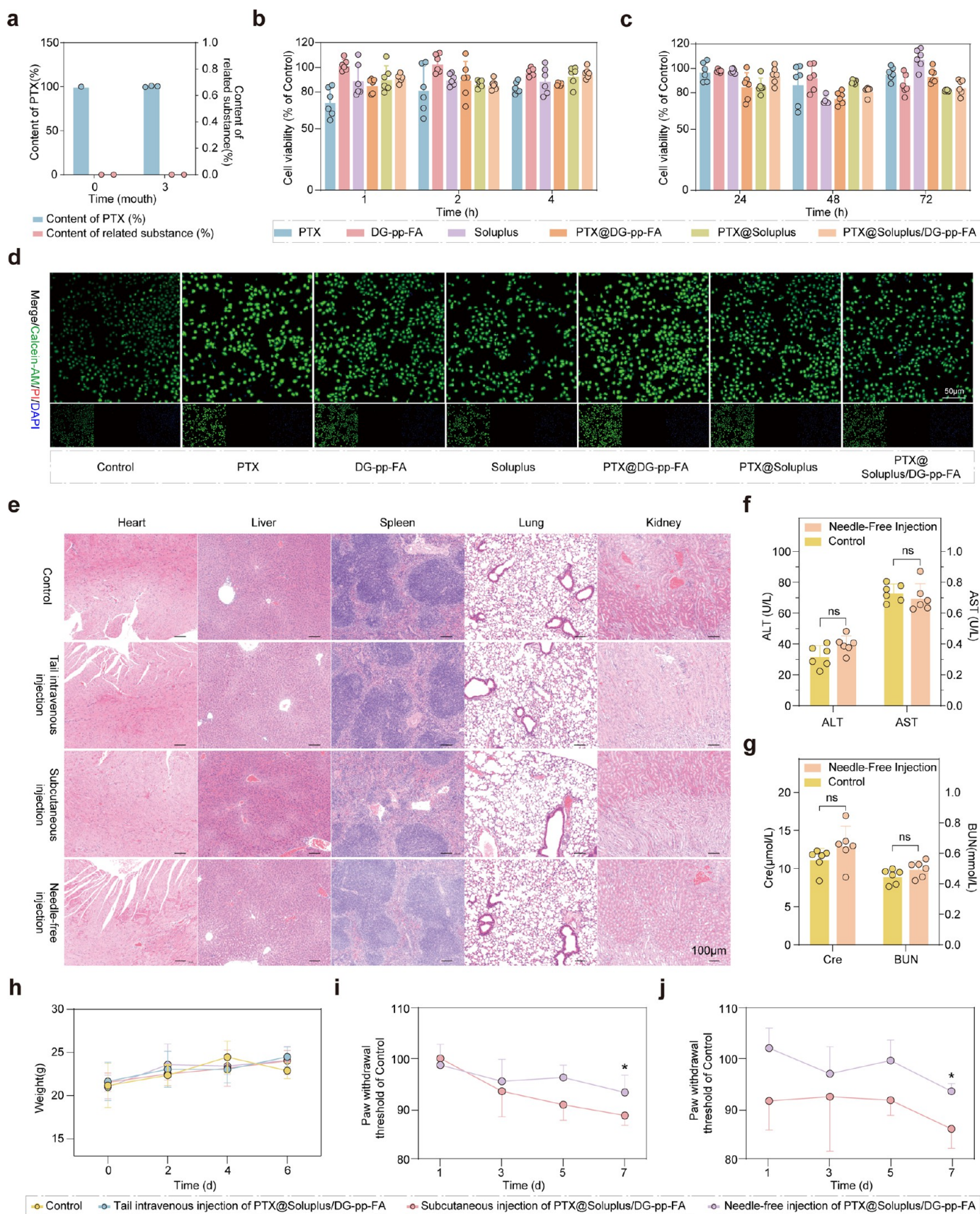


Figure 8. *In vitro* and *in vivo* safety, biocompatibility, and pain-related outcomes of NF#PTX@Soluplus/DG-pp-FA. (a) Packaging compatibility of PTX@Soluplus/DG-pp-FA in the needle-free cartridge assessed by HPLC; (b, c) cytotoxicity assessment in normal cell lines under indicated exposure conditions; (d) live/dead staining of normal cells; (e) H&E staining of major organs after treatment; (f, g) serum biochemistry for liver and kidney function; (h) body-weight monitoring during treatment; (i, j) pain-related behavioral assessments using Von Frey and hot-plate tests comparing needle-free versus needle-based delivery. Data are mean $\pm$ SEM; * P < 0.05.

cell populations, revealing significant enrichment of pathways related to T-cell receptor signaling, cytokine-cytokine receptor interactions, and immune response regulation (Figure 6g). Notably, pathway analysis within the exhausted T-cell subset suggested attenuation of immunosuppressive programs, including PD-1/PD-L1 signaling, implying partial relief of exhaustion-associated inhibitory signaling under immune-activated conditions. In parallel, GO enrichment in Tregs indicated significant modulation of immune regulatory processes, including antigen receptor-mediated signaling, leukocyte adhesion, and immune response activation (Figures 6g and S26). A prominent feature of the immune-activated context was a marked increase in leukocyte infiltration, rising from 13% to 43% of the tumor microenvironment affected by NF#PTX@Soluplus/DG-pp-FA (Figure 6h), directly reflecting a transition toward an immune-inflamed phenotype.

Pseudotime trajectory analysis further revealed coordinated shifts in immune cell differentiation and activation states across contexts (Figure 6i,j). Expression dynamics of key immune genes along the trajectory—including GZMA, GZMK, KLRB1, NKG7, GNLY, and PDCD1—supported a transition toward enhanced cytotoxic effector activation with reduced exhaustion-associated signatures (Figure 6k). Collectively, this reanalysis of independent public scRNA-seq data sets provides a coherent single-cell evidence chain-spanning cellular composition, immune subset remodeling, functional pathway enrichment, and differentiation trajectories—consistent with an “immune infiltration-effector activation-suppressed inhibitory signaling” axis. These insights offer a mechanistic reference to link our NF#PTX@Soluplus/DG-pp-FA strategy to tumor microenvironment remodeling and strengthened antitumor immunity, while acknowledging that the scRNA-seq cohorts were not treated with our formulation.

The spatial transcriptomic reanalysis provided a spatially resolved view of cellular heterogeneity and niche organization within melanoma tissues and was used here as an independent mechanistic reference to complement our study rationale. UMAP-based clustering of spatial transcriptomic profiles identified distinct transcriptional subpopulations, reflecting pronounced heterogeneity across tumor and stromal compartments (Figure 7a). Unsupervised clustering of spatial spots further revealed discrete spot communities with spatially segregated patterns, consistent with functional compartmentalization within the tumor microenvironment (Figure 7b). Mapping these spot clusters back onto the tissue section confirmed region-specific localization of distinct clusters and highlighted localized immune infiltration as well as tumor–stroma interaction interfaces (Figure 7c).

To interrogate spatially patterned molecular programs linked to tumor progression and immune modulation, we examined the tissue-level expression landscapes of TUBB2A and DCXR. Both genes displayed distinct regional enrichment, suggesting potential associations with tumor cell persistence and metabolic adaptation in specific intratumoral areas (Figure 7d). Spatial feature mapping of immune- and stress-response genes—including AIF1, CDKN1A, TAP1, TGFBI, ICAM1, and CLU—further underscored region-restricted transcriptional programs, consistent with localized immune activation and stress signaling hotspots within the tumor microenvironment (Figure 7e). In addition, the spatial distribution of ECM1, S100A4, and PLTP showed regionally enriched patterns, suggesting spatial coupling with extracellular matrix remodeling, tumor-associated stromal programs, and immune cell infiltration (Figure 7f).

We next inferred a spatially contextualized regulatory network centered on leukocyte migration, chemotaxis, and immune-response modules to nominate candidate molecular interactions associated with immune recruitment and activation in melanoma (Figure 7g). The inferred network implicated ECM1 and CTSB in leukocyte migration-related programs, while CCL6 and PREK1 were linked to immune cell recruitment. Additional nodes, including CD180 and NCXAP1, were connected to leukocyte chemotaxis and lymphocyte-mediated immunity. The network also suggested that CDKN1A-associated signaling may be positively associated with TNF-related inflammatory outputs within the tumor microenvironment. Moreover, key regulators connected to cellular responses to interferon- β —such as ICAM1, TGTPI, and TGTPI2—were identified, supporting a spatially partitioned immunomodulatory landscape.

2.10. *In Vitro* and *In Vivo* Safety and Biocompatibility Evaluation of NF#PTX@Soluplus/DG-pp-FA

The results of the compatibility study demonstrated that PTX@Soluplus/DG-pp-FA exhibited excellent stability within the neutral borosilicate glass cartridges (Figure 8a). The HPLC analysis showed no significant reduction in PTX content after the storage period, indicating that the packaging material did not induce drug degradation. Furthermore, no new impurity peaks were detected in the chromatographic profiles, confirming that no adverse interactions occurred between PTX@Soluplus/DG-pp-FA and the cartridge material. These findings suggest that the selected cartridge provides a protective environment for the needle-free injection formulation, effectively maintaining the stability of PTX@Soluplus/DG-pp-FA during storage. The study confirms that the borosilicate glass material is suitable for packaging the drug solution, ensuring both chemical stability and patient safety in the needle-free delivery system.

In the hemolysis assay, none of the tested formulations—PTX solution, DG-pp-FA, Soluplus, PTX@DG-pp-FA, PTX@Soluplus, or PTX@Soluplus/DG-pp-FA nanomicelles—exhibited significant hemolytic activity at either standard or diluted concentrations. The hemolysis rates for all groups were less than 5%, indicating excellent hemocompatibility compatibility for NF#PTX@Soluplus/DG-pp-FA. This suggests it is unlikely to damage blood cells when administered via needle-free injection (Figure S27).

The cytotoxicity assessment of PTX@Soluplus/DG-pp-FA on normal cells (Figures 8b,c, and S28) showed that free PTX, PTX@DG-pp-FA, PTX@Soluplus, and PTX@Soluplus/DG-pp-FA nanomicelles exhibited short-term safety (1–4 h). After a 10-fold dilution, these formulations maintained long-term safety (24–72 h) for normal cells. These findings indicate that NF#PTX@Soluplus/DG-pp-FA shows excellent *in vitro* biocompatibility without cytotoxicity, supporting its potential as a needle-free delivery system.

Live/dead staining results for NF#PTX@Soluplus/DG-pp-FA on C2C12, NIH/3T3, and HUVEC cells were consistent with the cytotoxicity results, showing no significant cytotoxic effects. This confirmed that NF#PTX@Soluplus/DG-pp-FA was not markedly toxic to the above cells (Figures 8d and S29).

Histopathological examination of major organs, including the heart, liver, spleen, lungs, and kidneys, was conducted after H&E staining of tissue sections. The results showed normal tissue morphology in the NF#PTX@Soluplus/DG-pp-FA group, with no significant pathological changes, indicating the absence of systemic toxicity (Figure 8e).

To evaluate the *in vivo* toxicity of NF#PTX@Soluplus/DG-pp-FA, liver and kidney function tests measured AST, ALT, creatinine (Cre), and BUN levels in blood samples collected after euthanizing the mice. Results showed no significant liver or kidney damage in the NF#PTX@Soluplus/DG-pp-FA needle-free group, confirming its safety as a treatment option for patients with melanoma (Figure 8f,g).

To further assess *in vivo* safety, body weight changes were monitored in mice following administration via tail vein, direct needle injection at the tumor site, and needle-free injection at the tumor site—mice injected with saline served as the control group. Results showed normal body weight growth across all groups, with no significant differences from controls, suggesting that NF#PTX@Soluplus/DG-pp-FA does not cause adverse physiological effects (Figure 8h).

PTX, a cytotoxic chemotherapy drug, is known to cause significant pain due to prolonged use, and repeated needle-based injections often result in subcutaneous hardening. Pain assessment using Von Frey filaments and hot plate tests in mice showed that the NF#PTX@Soluplus/DG-pp-FA needle-free system significantly reduced pain compared to traditional needle-based PTX injections. This reduction in pain may be attributed to the encapsulation of PTX within nanomicelles, which mitigates its systemic distribution and minimizes tissue irritation. Moreover, needle-free injection causes less pain and does not lead to subcutaneous hardening, making NF#PTX@Soluplus/DG-pp-FA a more comfortable and less burdensome delivery method for patients than conventional treatment (Figure 8i,j).

3. DISCUSSION

This study establishes an *in situ*, needle-free intratumoral delivery strategy that addresses key barriers limiting paclitaxel (PTX) efficacy in melanoma, namely poor aqueous solubility, rapid loss from the tumor after local administration, and dose-limiting systemic toxicity and discomfort associated with repeated injections. By integrating a mixed micellar carrier composed of Soluplus and a rationally designed dipotassium glycyrrhizinate-PEG-folate conjugate (DG-pp-FA) with a commercial jet injector, we demonstrate a “device-formulation” co-optimized platform (NF#PTX@Soluplus/DG-pp-FA) that improves local dispersion, prolongs tumor residence, enhances cellular uptake, and reshapes tumor biology through both direct cytotoxicity and tumor-microenvironment (TME) remodeling supported by multiomics readouts.

A central design element is the multiretention behavior achieved by combining rapid intratumoral dispersion driven by high-pressure jet injection and physicochemical transitions of the carrier under tumor-relevant conditions that favor local depot formation rather than rapid washout. The DG-pp-FA conjugate was constructed through stepwise monofunctionalization to yield a defined FA-PEG-DG architecture, enabling folate-receptor-associated interactions while preserving amphiphilicity from the DG moiety. Soluplus dominated micellization behavior at the optimized 7:1 Soluplus:DG-pp-FA ratio, producing stable mixed micelles with a low apparent CMC (10 $\mu\text{g/mL}$), narrow polydispersity, and high PTX encapsulation efficiency. Importantly, although dialysis-based release profiles were broadly similar at pH 7.4 and pH 5.5, the formulation still displayed clear pH- and temperature-associated changes in colloidal state. These observations suggest that pH/temperature primarily modulate local rheology, aggregation, and physical retention in tissue rather than directly accelerating PTX

diffusion across the dialysis membrane, thereby reconciling the apparent release-responsiveness discrepancy. In practical terms, the formulation is engineered to remain colloidally stable during preparation and handling but to become more locally retentive after deposition into the tumor.

Needle-free injection contributed a second, distinct layer of functional gain by improving coverage and reducing delivery losses typically seen with needle-based intratumoral dosing. In agarose and liquid dispersion models, as well as *ex vivo* porcine skin, jet injection created a broader, more homogeneous distribution pattern compared with needle injection, which tended to generate concentrated tracks and leakage along the needle path. Consistent with these physical observations, tumor-section fluorescence indicated that *in situ* needle-free delivery achieved stronger and more uniform intratumoral distribution than intravenous or subcutaneous routes at matched dose, supporting the premise that the spatial extent of exposure is a critical, underappreciated determinant of local chemotherapy performance in solid tumors. Pharmacokinetic behavior further supported this localization, the blood AUC of systemic exposure was higher after intravenous administration, whereas needle-free intratumoral delivery produced a delayed plasma peak with lower overall systemic burden, consistent with enhanced local deposition and reduced immediate spillover into circulation.

At the cellular level, the mixed micelles improved PTX internalization kinetics and supported time-dependent endolysosomal trafficking with partial lysosomal escape. Uptake inhibition profiles implicated lipid raft/caveolae-associated routes as major contributors, and folate competition produced a measurable reduction in uptake, indicating that folate-receptor interactions contribute but are not the sole determinant of cellular entry. This is consistent with a mixed mechanism in which nanoscale size, membrane microdomain engagement, and ligand-assisted interactions jointly promote cellular exposure.

Functionally, PTX@Soluplus/DG-pp-FA induced robust short-term cytotoxicity, G2/M arrest, apoptosis, and suppression of clonogenicity and migration in B16–F10 cells, aligning with canonical PTX activity while suggesting that enhanced intracellular delivery increases the effective pharmacodynamic impact at matched nominal dose.

In vivo, NF#PTX@Soluplus/DG-pp-FA produced superior primary tumor control relative to comparator administration routes and was accompanied by pronounced histological evidence of tumor injury. Reduced Ki67/PCNA staining and increased apoptosis markers supported the combined anti-proliferative and pro-death effects observed *in vitro*. Notably, Masson staining showed a marked reduction in collagen deposition in the needle-free micelle group, suggesting that the platform not only kills tumor cells but also remodels stromal architecture. This stromal effect is mechanistically important because dense collagen and fibrosis can act as physical barriers that impede both drug penetration and immune cell trafficking, thereby enforcing a resistant immune-excluded phenotype in many solid tumors. The observed reduction in pulmonary metastatic burden further supports that improving local control and microenvironmental constraints may translate into reduced dissemination or outgrowth at distant sites in this aggressive melanoma model.

Mechanistically, the most informative layer of this work is the multiomics evidence indicating that therapeutic benefit extends beyond direct PTX cytotoxicity to include coordinated immune and matrix remodeling programs. Self-generated bulk RNA-seq and LC–MS/MS proteomics consistently highlighted enrich-

ment of antigen processing/presentation, chemokine- and cytokine-associated signaling, phagosome/lysosome-related pathways, and leukocyte migration modules, indicating a shift toward an immune-engaged microenvironment. Targeted validation supported remodeling of inflammatory and stromal axes, including attenuation of HMGB1/TLR4-associated signaling and reduced collagen I abundance. While the decrease in canonical inflammatory cytokines (TNF- α , IL-1 β , IL-6) might appear counterintuitive to immune activation, it likely reflects suppression of tumor-promoting chronic inflammation and damage-associated signaling at the end point, while innate immune effector functions and antigen presentation programs become more prominent. In other words, the data support a transition from a tumor-supportive inflammatory state toward a more immune-productive milieu that is better aligned with antitumor surveillance rather than persistent, fibrosis-coupled inflammation.

Because we did not generate single-cell or spatial transcriptomic data from our treated cohorts, we intentionally used reanalysis of public scRNA-seq and spatial transcriptomic data sets as independent mechanistic references rather than as direct evidence of treatment-induced changes. In this context, the public single-cell data provide a high-resolution framework linking global immune infiltration to T-cell state composition, functional scoring, and differentiation trajectories, thereby helping interpret why bulk immune signatures may correlate with therapeutic benefit. The spatial reanalysis further reinforces the concept that immune activation and ECM/stress programs are spatially compartmentalized within melanoma tissues, and that local niches with differential ECM and adhesion signatures can plausibly gate leukocyte migration and drug penetration. Accordingly, these public multiomics layers strengthen biological plausibility for our bulk observations and histological remodeling readouts, while avoiding overattribution of causality to data sets that were not generated under our treatment conditions.

Safety and tolerability are essential for translating intratumoral chemotherapy strategies. NF#PTX@Soluplus/DG-pp-FA demonstrated favorable biocompatibility across multiple levels, including minimal hemolysis (<5%), reduced cytotoxicity on normal cells after clinically relevant dilution, normal organ histology, stable liver/kidney function markers, and no adverse body-weight trajectories. Importantly, behavioral pain assays indicated that the needle-free micellar system reduced both injection-associated discomfort and PTX-associated pain phenotypes relative to conventional needle-based PTX dosing, consistent with reduced local tissue trauma, avoidance of repeated needle injury and subcutaneous hardening, and mitigation of irritant exposure through micellar encapsulation. In parallel, cartridge compatibility studies supported formulation stability in neutral borosilicate glass with no detectable degradation or impurity formation, which is a practical prerequisite for a device-based delivery format.

Several limitations and next steps follow directly from these findings. First, the current work is based on murine melanoma models, and folate-receptor expression, stromal density, and immune contexture may differ across human melanoma subtypes and lesion sites; therefore, validation in additional models, including more clinically representative or orthotopic settings, will be important. Second, although public single-cell and spatial data sets offer valuable reference biology, definitive mechanistic attribution to NF#PTX@Soluplus/DG-pp-FA will require matched, treatment-specific single-cell/spatial profiling

and cell-type-resolved validation of immune recruitment, antigen presentation, and ECM remodeling pathways. Third, given the observed immune engagement signatures and T-cell state dynamics described in public references, rational combinations-particularly with immune checkpoint blockade-merit investigation to determine whether improved local drug coverage and stromal softening can convert immune-cold lesions into immune-inflamed, ICI-responsive states and yield durable systemic control. Finally, device parameters such as pressure, volume, injection geometry and repeat-dose scheduling should be optimized alongside formulation to maximize uniform intratumoral coverage while minimizing local tissue stress.

In summary, NF#PTX@Soluplus/DG-pp-FA integrates a clinically relevant needle-free injector with a stable, mixed micellar carrier engineered for local deposition and prolonged tumor retention. The platform enhances intratumoral distribution, improves pharmacodynamic efficacy, reduces fibrosis-associated barriers, and is supported by bulk multiomics evidence of immune and microenvironment remodeling, while maintaining a strong safety and tolerability profile. Together, these results position this *in situ* needle-free multiretention micelle strategy as a promising and translatable approach for localized melanoma therapy and as a potential enabling backbone for combination regimens aimed at sustained antitumor immunity.

4. CONCLUSION

In this study, we developed a paclitaxel-loaded hybrid micelle system, NF#PTX@Soluplus/DG-pp-FA, to amplify intratumoral exposure through coordinated multiretention mechanisms. By integrating Soluplus-driven thermo-enabled local deposition, DG-pp-FA-associated acid responsiveness, and folate-receptor-mediated uptake, the formulation achieves robust local residency while maintaining nanoscale colloidal stability. The optimized micelles exhibited a uniform hydrodynamic size of 68 ± 3.8 nm with a PDI of 0.08 ± 0.01 and a high paclitaxel encapsulation efficiency of $98 \pm 0.11\%$, supporting reproducible preparation and sustained drug release behavior. Needle-free intratumoral jet administration enabled *in situ* formation of a localized micellar depot, enhancing intratumoral distribution and cellular interaction while minimizing systemic exposure. Across *in vitro* and *in vivo* melanoma models, the platform improved antitumor efficacy relative to free paclitaxel, and multiomics analyses further indicated dual actions that combine direct tumor suppression with remodeling of the tumor immune microenvironment, including immune activation and extracellular matrix reprogramming that may facilitate drug penetration. Safety evaluations supported good biocompatibility with low systemic toxicity, and the needle-free approach reduces needle-related barriers and improves treatment acceptability. Collectively, these findings establish a formulation-guided and minimally invasive strategy for localized paclitaxel delivery and provide a translatable framework that may be extended to other poorly soluble anticancer agents and combination regimens.

5. METHODS

5.1. Materials, Cells, and Animals

Paclitaxel (PTX) and folic acid (FA) were purchased from Dalian Meilun Biotechnology (Dalian, China; $\geq 99\%$). Dipotassium glycyrrhizinate (DG) was obtained from Henan WanBang Industrial (Henan, China). Soluplus (PVCL-PVA-PEG) was provided by BASF (Ludwigshafen, Germany). Coumarin-6 (Cou6), DAPI, and other

analytical-grade reagents were purchased from commercial suppliers unless otherwise stated. Mouse melanoma cells (B16–F10) and murine myoblasts (C2C12) were obtained from a commercial vendor, and HUVECs were provided by the Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College. Cells were maintained under recommended culture conditions. Six-week-old male C57BL/6 mice were used for all *in vivo* studies. All experimental procedures were approved by the Ethics Committee of the Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College (Beijing, China; No. 00003620) and the Ethics Committee for Animal Experiments of the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College (Beijing, China; No. IMB-20241112D₁04).

5.2. Synthesis of FA-PEG-NHBoc

Folic acid (FA, 1.0 equiv) was suspended in anhydrous DMSO (10 mg/mL) under light protection. *N*-Hydroxysuccinimide (NHS, 1.5 equiv) and 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDC-HCl, 1.5 equiv) were added sequentially and the mixture was stirred for 2 h at room temperature to activate FA. Mono-Boc-protected diamino-PEG (H₂N-PEG-NHBoc, 1.0 equiv; PEG Mn = 1.8–2.2 kDa) dissolved in anhydrous DMSO was added dropwise, and the reaction proceeded for 18 h at room temperature under nitrogen and protected from light. The mixture was poured into 40 volumes of cold diethyl ether to precipitate the polymer conjugate. The precipitate was collected by centrifugation (6000 g, 10 min), washed with cold ether (3×), redissolved in deionized water, and purified by dialysis (MWCO 2 kDa) against deionized water for 72 h with frequent water changes. The dialysate was lyophilized to afford FA-PEG-NHBoc as a yellow solid.

5.3. Boc Deprotection to Obtain FA-PEG-NH₂

FA-PEG-NHBoc was dissolved in anhydrous dichloromethane (DCM, 5 mg/mL) and cooled to 0 °C under light protection. Trifluoroacetic acid (TFA) was added to reach DCM/TFA = 1:1 (v/v), and the mixture was stirred for 2 h while warming from 0 °C to room temperature. Volatiles were removed under reduced pressure at ≤30 °C. Residual TFA was removed by coevaporation with anhydrous toluene (3×). The residue was dissolved in deionized water and neutralized to pH 7.0–8.0 using dilute NaOH under gentle stirring. The solution was purified by dialysis (MWCO 2 kDa) against deionized water for 48 h and lyophilized to afford FA-PEG-NH₂.

5.4. Selective Grafting of DG onto FA-PEG-NH₂ to yield DG-pp-FA

Dipotassium glycyrrhizinate (DG, 1.0 equiv) was vacuum-dried (40 °C overnight) and dissolved in anhydrous DMF (5 mg/mL) under nitrogen. Carbonyldiimidazole (CDI, 1.20 equiv) was added portionwise at 0–4 °C, and the mixture was stirred for 30 min to generate the DG acyl-imidazole intermediate *in situ*. FA-PEG-NH₂ (1.00 equiv relative to DG) in anhydrous DMF/DMSO (1:1, v/v) was then added, followed by *N,N*-diisopropylethylamine (DIPEA, 4 equiv relative to the amine). The reaction proceeded for 12 h at room temperature under nitrogen and protected from light. The reaction was quenched with methanol (2% v/v) and concentrated under reduced pressure to remove most organic solvent. The residue was dissolved in deionized water, centrifuged to remove insoluble impurities, and filtered through a 0.22 μm membrane. The crude product was purified by dialysis (MWCO 3 kDa) against deionized water for 72 h with frequent water changes. The purified solution was lyophilized to afford DG-pp-FA as a pale yellow solid and stored at –20 °C protected from light.

5.5. Preparation and Optimization of PTX-loaded Micelles

PTX-loaded micelles (PTX@DG-pp-FA, PTX@Soluplus, and PTX@Soluplus/DG-pp-FA) were prepared using solvent evaporation-thin-film hydration. PTX and carriers were dissolved in ethanol, solvent was removed to form a thin film, and the film was hydrated with PBS followed by filtration through a 0.22 μm membrane. PTX content was quantified by HPLC and formulations were adjusted to a final PTX concentration of 1 mg/mL. A response surface design (central

composite design) was used to optimize the mixed micelles, using encapsulation efficiency as the primary criterion.

5.6. Physicochemical Characterization

Critical micelle concentration (CMC) was determined using DPH as a fluorescence probe. Micelle morphology was examined by TEM. Hydrodynamic size, PDI, and zeta potential were measured by DLS. PTX was quantified by HPLC (C18 column; detection 227 nm; methanol/water 60:40; 1.0 mL/min). Encapsulation efficiency and drug loading were calculated by comparing PTX levels before and after removal of unencapsulated drug and after micelle disruption. Storage stability (4 and 25 °C) and dilution stability were assessed by monitoring particle size/PDI/zeta potential and encapsulation efficiency over time. Temperature- and pH-responsiveness were evaluated by incubating micelles under relevant conditions and recording physicochemical changes.

5.7. Needle-Free Intratumoral Delivery System (NF#PTX@Soluplus/DG-pp-FA)

Sterile PTX@Soluplus/DG-pp-FA was loaded into sterile cartridges and administered using a commercial needle-free jet injector (QS-P, Beijing QS Medical Technology, China). The injector delivered 0.04–0.35 mL per shot (0.01 mL increments; discharge ≤ 0.3 s) using a disposable cartridge (nominal orifice 0.14 mm). For *in vivo* dosing, the injector was positioned perpendicular to the tumor surface and triggered in contact mode to achieve *in situ* intratumoral deposition. Delivered volume was verified gravimetrically.

5.8. *In Vitro* Release under Sink Conditions

PTX release was evaluated by dialysis (MWCO 3.5 kDa) in pH 7.4 and pH 5.5 media at 37 °C (100 rpm). PTX solubility was determined in each medium prior to release testing. Sink conditions were maintained by using a medium-to-formulation ratio ≥50:1 and replenishing fresh prewarmed medium after each sampling so that instantaneous PTX concentrations remained ≤10% of solubility. Samples were quantified by HPLC and cumulative release profiles were calculated.

5.9. Cellular Uptake and Intracellular Trafficking

Cou6-labeled micelles were prepared using the same procedure as PTX@Soluplus/DG-pp-FA, with Cou6 substituting for 10% (w/w) of PTX during formulation. B16–F10 cells were incubated with free Cou6 or Cou6-labeled micelles, and uptake was quantified by flow cytometry and visualized by fluorescence microscopy. Folate-receptor involvement was assessed by preincubating cells with free FA prior to treatment. Intracellular lysosomal colocalization was analyzed by confocal microscopy using LysoTracker staining, and colocalization was quantified using Pearson's and Manders' coefficients.

5.10. *In Vivo* Tumor Distribution and Pharmacokinetics

For intratumoral distribution, Cou6-labeled micelles were administered via intravenous injection, peritumoral subcutaneous injection, or *in situ* needle-free intratumoral injection at dose-matched levels. At predefined time points, tumors were excised, snap-frozen, cryosectioned (10 μm), counterstained with DAPI, and imaged to evaluate intratumoral dispersion and retention. For pharmacokinetics, blood was collected at scheduled time points after dosing via each route, serum was separated, and PTX concentrations were quantified by HPLC after appropriate sample processing.

5.11. *In Vivo* Antitumor Efficacy and Mechanistic Readouts

Subcutaneous melanoma tumors were established by injecting B16–F10 cells into the dorsum of C57BL/6 mice. When tumors reached comparable sizes, mice were randomized into treatment groups receiving PTX@Soluplus/DG-pp-FA via intravenous, peritumoral subcutaneous, or needle-free intratumoral injection, with saline and normal controls included as appropriate. Treatments were administered every 2 days for 1 week. Tumor volume and body weight were recorded serially; tumor volume was calculated as $V = L \times S^2/2$ (L , longest diameter; S , shortest diameter). At end point, serum biochemistry (AST/ALT, BUN/Cr) was analyzed, organs and tumors were collected for H&E and Masson staining, and tumor IHC (Ki67, PCNA, TUNEL) and Western blotting were performed to probe

inflammation, barrier/junction, fibrosis, and HMGB1/TLR4-associated signaling.

5.12. Multiomics Analysis

To elucidate treatment-associated molecular programs, bulk RNA-seq and LC-MS/MS proteomics were performed on melanoma tissues collected in this study. RNA-seq data were processed using standard QC, alignment, quantification, and differential-expression workflows followed by GO/KEGG enrichment. Proteomics data were acquired by LC-MS/MS and analyzed using established identification/quantification pipelines, with differential proteins subjected to functional enrichment. Transcriptome-proteome concordance and pathway interaction modules were assessed using correlation analysis and protein-protein interaction resources. Public melanoma scRNA-seq and spatial transcriptomics data sets (GEO access date: 2025-01-15) were reanalyzed using standardized workflows for cell-type annotation, pathway scoring, and cell-cell communication inference to support mechanistic interpretation.

5.13. Safety and Biocompatibility

In vitro biocompatibility was assessed in C2C12 and HUVEC cells using CCK-8 assays and live/dead staining. Hemocompatibility was evaluated by hemolysis testing. *In vivo* safety was assessed by body-weight monitoring, serum biochemistry, and histopathology (H&E) of major organs. Pain-related assessments were conducted at end point using mechanical and thermal threshold measurements to evaluate chemotherapy-associated peripheral neuropathy and injection-associated discomfort.

5.14. Statistical Analysis

All quantitative data are presented as mean $\pm$ SEM unless otherwise stated. Statistical analyses were performed using GraphPad Prism (version 8.0). Two-group comparisons used an unpaired two-tailed Student's *t* test. Comparisons among three or more groups used one-way ANOVA followed by Tukey's multiple-comparisons test. Time-course data (e.g., tumor volume and body weight) were analyzed using two-way repeated-measures ANOVA with appropriate multiple-comparisons correction. A *p* value < 0.05 was considered statistically significant. Sample sizes and specific tests are provided in the corresponding figure legends.

■ ASSOCIATED CONTENT

Data Availability Statement

The data sets used and/or analyzed during the current study are available from the corresponding author upon reasonable request. The transcriptomic raw data generated in this study have been deposited in the NCBI BioProject database under accession number PRJNA1413553, and the proteomics raw data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with data set identifier PXD073514. These data sets will be made publicly available upon publication of the article.

■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsnano.6c01481>.

Detailed experimental procedures and additional methodological information; supplementary figures (Figures S1–S29); supplementary tables (Tables S1–S4); and abbreviations and supplementary notes (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Jiandong Jiang — State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking

Union Medical College, Beijing 100050, China;

Email: jiangjiandong@imb.cams.cn

Wensheng Zheng — Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China; orcid.org/0000-0002-1914-8924; Email: zhengwensheng@imm.ac.cn

Lulu Wang — State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China; Email: wanglulu@imb.cams.cn

Authors

Kaichao Song — State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China; orcid.org/0000-0003-3461-6033

Yumei Hao — Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China; orcid.org/0000-0002-6074-5202

Peng Lei — Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China

Yige Yang — Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China

Yuxin Zhang — Beijing QS Medical Technology Co., Ltd., Beijing 100176, China

Suning Chen — Beijing QS Medical Technology Co., Ltd., Beijing 100176, China

Complete contact information is available at:

<https://pubs.acs.org/doi/10.1021/acsnano.6c01481>

Author Contributions

[†]K.S., Y.H., and P.L. contributed equally to this work.

Funding

This work was supported by the CAMS Innovation Fund for Medical Sciences (Nos. 2024-I2M-ZH-012, 2025-I2M-KJ-018, and 2022-I2M-1-016), the China National Postdoctoral Program for Innovative Talents (BX20240044), and the Fundamental and Interdisciplinary Disciplines Breakthrough Plan of the Ministry of Education of China (JYB2025XDXM609).

Notes

The authors declare no competing financial interest.

■ REFERENCES

- (1) Hwang, M. P.; Fecek, R. J.; Qin, T.; Storkus, W. J.; Wang, Y. Single injection of IL-12 coacervate as an effective therapy against B16-F10 melanoma in mice. *J. Controlled Release* **2020**, *318*, 270–278.
- (2) D'Ambrosio, A. E.; Albonico, G.; Enide, I.; Morda, O.; Maisano, M.; Mondello, B. Pleural Melanoma Metastasis. *Ann. Thorac Surg* **2021**, *112* (2), e103–e105.
- (3) Lim, J. C. W.; Kwan, Y. P.; Tan, M. S.; Teo, M. H. Y.; Chiba, S.; Wahli, W.; Wang, X. The Role of PPARbeta/delta in Melanoma Metastasis. *Int. J. Mol. Sci.* **2018**, *19* (10), 2860.
- (4) Singh, M.; Vianden, C.; Cantwell, M. J.; Dai, Z.; Xiao, Z.; Sharma, M.; Khong, H.; Jaiswal, A. R.; Faak, F.; Hailemichael, Y.; Janssen, L. M. E.; Bharadwaj, U.; Curran, M. A.; Diab, A.; Bassett, R. L.; Tweardy, D. J.; Hwu, P.; Overwijk, W. W. Intratumoral CD40 activation and checkpoint blockade induces T cell-mediated eradication of melanoma in the brain. *Nat. Commun.* **2017**, *8* (1), 1447.

- (5) Soltantoyeh, T.; Akbari, B.; Karimi, A.; Mahmoodi Chalbatani, G.; Ghahri-Saremi, N.; Hadjati, J.; Hamblin, M. R.; Mirzaei, H. R. Chimeric Antigen Receptor (CAR) T Cell Therapy for Metastatic Melanoma: Challenges and Road Ahead. *Cells* **2021**, *10* (6), 1450.
- (6) Sullivan, R. J.; Atkins, M. B.; Kirkwood, J. M.; Agarwala, S. S.; Clark, J. I.; Ernstoff, M. S.; Fecher, L.; Gajewski, T. F.; Gastman, B.; Lawson, D. H.; Lutzky, J.; McDermott, D. F.; Margolin, K. A.; Mehnert, J. M.; Pavlick, A. C.; Richards, J. M.; Rubin, K. M.; Sharfman, W.; Silverstein, S.; Slingluff, C. L., Jr.; Sondak, V. K.; Tarhini, A. A.; Thompson, J. A.; Urba, W. J.; White, R. L.; Whitman, E. D.; Hodi, F. S.; Kaufman, H. L. An update on the Society for Immunotherapy of Cancer consensus statement on tumor immunotherapy for the treatment of cutaneous melanoma: version 2.0. *J. Immunother. Cancer* **2018**, *6* (1), 44.
- (7) Kappelman-Fenzl, M.; Kuphal, S.; Krupar, R.; Schadendorf, D.; Umansky, V.; Vardimon, L.; Hellerbrand, C.; Bosserhoff, A. K. Complex Formation with Monomeric α -Tubulin and Importin 13 Fosters c-Jun Protein Stability and Is Required for c-Jun's Nuclear Translocation and Activity. *Cancers (Basel)* **2019**, *11* (11), 1806.
- (8) Mu, J.; Zhong, H.; Zou, H.; Liu, T.; Yu, N.; Zhang, X.; Xu, Z.; Chen, Z.; Guo, S. Acid-sensitive PEGylated paclitaxel prodrug nanoparticles for cancer therapy: Effect of PEG length on antitumor efficacy. *J. Controlled Release* **2020**, *326*, 265–275.
- (9) Atrafi, F.; van Eerden, R. A. G.; van Hylckama Vlieg, M. A. M.; Oomen-de Hoop, E.; de Bruijn, P.; Lolkema, M. P.; Moelker, A.; Rijcken, C. J.; Hanssen, R.; Sparreboom, A.; Eskens, F.; Mathijssen, R. H. J.; Koolen, S. L. W. Intratumoral Comparison of Nanoparticle Entrapped Docetaxel (CPC634) with Conventional Docetaxel in Patients with Solid Tumors. *Clin. Cancer Res.* **2020**, *26* (14), 3537–3545.
- (10) Han, X.; Chen, J.; Jiang, M.; Zhang, N.; Na, K.; Luo, C.; Zhang, R.; Sun, M.; Lin, G.; Zhang, R.; Ma, Y.; Liu, D.; Wang, Y. Paclitaxel-Paclitaxel Prodrug Nanoassembly as a Versatile Nanoplatfor for Combinational Cancer Therapy. *ACS Appl. Mater. Interfaces* **2016**, *8* (49), 33506–33513.
- (11) Klein, I.; Lehmann, H. C. Pathomechanisms of Paclitaxel-Induced Peripheral Neuropathy. *Toxics* **2021**, *9* (10), 229.
- (12) Tran, M. A.; Watts, R. J.; Robertson, G. P. Use of liposomes as drug delivery vehicles for treatment of melanoma. *Pigment Cell Melanoma Res.* **2009**, *22* (4), 388–399.
- (13) Junyaprasert, V. B.; Thummarati, P. Innovative Design of Targeted Nanoparticles: Polymer-Drug Conjugates for Enhanced Cancer Therapy. *Pharmaceutics* **2023**, *15* (9), 2216.
- (14) Shevtsov, M.; Kaesler, S.; Posch, C.; Multhoff, G.; Biedermann, T. Magnetic nanoparticles in theranostics of malignant melanoma. *EJNMMI Res.* **2021**, *11* (1), 127.
- (15) Zhang, W.; Shi, Y.; Chen, Y.; Hao, J.; Sha, X.; Fang, X. The potential of Pluronic polymeric micelles encapsulated with paclitaxel for the treatment of melanoma using subcutaneous and pulmonary metastatic mice models. *Biomaterials* **2011**, *32* (25), 5934–5944.
- (16) Oerlemans, C.; Bult, W.; Bos, M.; Storm, G.; Nijssen, J. F.; Hennink, W. E. Polymeric micelles in anticancer therapy: targeting, imaging and triggered release. *Pharm. Res.* **2010**, *27* (12), 2569–2589.
- (17) Yang, Y.; Sun, H.; Sun, X.; Wang, Y.; Xu, F.; Xia, W.; Chen, L.; Li, M.; Yang, T.; Qiao, Y.; Geng, D. From mechanism to applications: Advanced microneedles for clinical medicine. *Bioact Mater.* **2025**, *51*, 1–45.
- (18) Shadambikar, G.; Kipping, T.; Di-Gallo, N.; Elia, A. G.; Knüttel, A. N.; Treffer, D.; Repka, M. A. Vacuum Compression Molding as a Screening Tool to Investigate Carrier Suitability for Hot-Melt Extrusion Formulations. *Pharmaceutics* **2020**, *12* (11), 1019.
- (19) Han, H.; Li, Y.; Peng, Z.; Long, K.; Zheng, C.; Wang, W.; Webster, T. J.; Ge, L. A Soluplus/Poloxamer 407-based self-nanoemulsifying drug delivery system for the weakly basic drug carvedilol to improve its bioavailability. *Nanomedicine* **2020**, *27*, No. 102199.
- (20) Pinto, J. M. O.; Rengifo, A. F. C.; Mendes, C.; Leao, A. F.; Parize, A. L.; Stulzer, H. K. Understanding the interaction between Soluplus(R) and biorelevant media components. *Colloids Surf. B Biointerfaces* **2020**, *187*, No. 110673.
- (21) Sun, F.; Zheng, Z.; Lan, J.; Li, X.; Li, M.; Song, K.; Wu, X. New micelle myricetin formulation for ocular delivery: improved stability, solubility, and ocular anti-inflammatory treatment. *Drug Deliv* **2019**, *26* (1), 575–585.
- (22) Li, M.; Xin, M.; Guo, C.; Lin, G.; Wu, X. New nanomicelle curcumin formulation for ocular delivery: improved stability, solubility, and ocular anti-inflammatory treatment. *Drug Dev. Ind. Pharm.* **2017**, *43* (11), 1846–1857.
- (23) Li, M.; Xin, M.; Song, K.; Sun, F.; Hou, Y.; Li, J.; Wu, X. Evaluation of polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer nanomicelle for trigeminal ganglion neurons delivering with intranasal administration. *Curr. Eye Res.* **2018**, *43* (3), 406–414.
- (24) Song, K.; Yan, M.; Li, M.; Geng, Y.; Wu, X. Preparation and in vitro-in vivo evaluation of novel ocular nanomicelle formulation of thymol based on glycyrrhizin. *Colloids Surf. B Biointerfaces* **2020**, *194*, No. 111157.
- (25) Wang, L.; Yang, R.; Yuan, B.; Liu, Y.; Liu, C. The antiviral and antimicrobial activities of licorice, a widely-used Chinese herb. *Acta Pharm. Sin B* **2015**, *5* (4), 310–315.
- (26) Hubert, P.; Roncarati, P.; Demoulin, S.; Pilard, C.; Ancion, M.; Reyniers, C.; Lerho, T.; Bruyere, D.; Lebeau, A.; Radermecker, C.; Meunier, M.; Nokin, M. J.; Hendrick, E.; Peulen, O.; Delvenne, P.; Herfs, M. Extracellular HMGB1 blockade inhibits tumor growth through profoundly remodeling immune microenvironment and enhances checkpoint inhibitor-based immunotherapy. *J. Immunother. Cancer* **2021**, *9* (3), No. e001966.
- (27) Cai, J.; Luo, S.; Lv, X.; Deng, Y.; Huang, H.; Zhao, B.; Zhang, Q.; Li, G. Formulation of injectable glycyrrhizic acid-hydroxycamptothecin micelles as new generation of DNA topoisomerase I inhibitor for enhanced antitumor activity. *Int. J. Pharm.* **2019**, *571*, No. 118693.
- (28) Cosmetic Ingredient Review Expert, P. Final report on the safety assessment of Glycyrrhetic Acid, Potassium Glycyrrhetinate, Disodium Succinoyl Glycyrrhetinate, Glyceryl Glycyrrhetinate, Glycyrrhetinyl Stearate, Stearyl Glycyrrhetinate, Glycyrrhizic Acid, Ammonium Glycyrrhizate, Dipotassium Glycyrrhizate, Disodium Glycyrrhizate, Trisodium Glycyrrhizate, Methyl Glycyrrhizate, and Potassium Glycyrrhizinate. *Int. J. Toxicol.* **2007**, *26*, 79–112.
- (29) Mitragotri, S. Current status and future prospects of needle-free liquid jet injectors. *Nat. Rev. Drug Discovery* **2006**, *5* (7), 543–548.
- (30) de Wit, H. M.; Engwerda, E. E.; Tack, C. J.; de Galan, B. E. Insulin administered by needle-free jet injection corrects marked hyperglycaemia faster in overweight or obese patients with diabetes. *Diabetes Obes. Metab.* **2015**, *17* (11), 1093–1099.
- (31) Engwerda, E. E.; Abbink, E. J.; Tack, C. J.; de Galan, B. E. Improved pharmacokinetic and pharmacodynamic profile of rapid-acting insulin using needle-free jet injection technology. *Diabetes Care* **2011**, *34* (8), 1804–1808.
- (32) Hu, J.; Shi, H.; Zhao, C.; Li, X.; Wang, Y.; Cheng, Q.; Goswami, R.; Zhen, Q.; Mei, M.; Song, Y.; Yang, S.; Li, Q. Lispro administered by the QS-M Needle-Free Jet Injector generates an earlier insulin exposure. *Expert Opin Drug Deliv* **2016**, *13* (9), 1203–1207.
- (33) Rohrer, T. R.; Ceplis-Kastner, S.; Jorch, N.; Muller, H. L.; Pfaffle, R.; Reinehr, T.; Richter-Unruh, A.; Weissenbacher, C.; Holterhus, P. M.; Ferring Arzneimittel GmbH, H. D. Needle-Free and Needle-Based Growth Hormone Therapy in Children: A Pooled Analysis of Three Long-Term Observational Studies. *Horm. Res. Paediatr.* **2018**, *90* (6), 393–406.
- (34) Bareille, P.; MacSwiney, M.; Albanese, A.; De Vile, C.; Stanhope, R. Growth hormone treatment without a needle using the Preci-Jet 50 transjector. *Arch Dis Child* **1997**, *76* (1), 65–67.
- (35) Suschak, J. J.; Bixler, S. L.; Badger, C. V.; Spik, K. W.; Kwilas, S. A.; Rossi, F. D.; Twenhafel, N.; Adams, M. L.; Shoemaker, C. J.; Spiegel, E.; Hooper, J. W. A DNA vaccine targeting VEE virus delivered by needle-free jet-injection protects macaques against aerosol challenge. *NPJ. Vaccines* **2022**, *7* (1), 46.

(36) Webb, S. Giving Needle-Free Flu Vaccines a Shot. *ACS Cent Sci.* **2017**, *3* (10), 1059–1060.

(37) Khobragade, A.; Bhate, S.; Ramaiah, V.; Deshpande, S.; Giri, K.; Phophle, H.; Supe, P.; Godara, I.; Revanna, R.; Nagarkar, R.; Sanmukhani, J.; Dey, A.; Rajanathan, T. M. C.; Kansagra, K.; Koradia, P. Efficacy, safety, and immunogenicity of the DNA SARS-CoV-2 vaccine (ZyCoV-D): the interim efficacy results of a phase 3, randomised, double-blind, placebo-controlled study in India. *Lancet* **2022**, *399* (10332), 1313–1321.



CAS INSIGHTS™

EXPLORE THE INNOVATIONS SHAPING TOMORROW

Discover the latest scientific research and trends with CAS Insights. Subscribe for email updates on new articles, reports, and webinars at the intersection of science and innovation.

Subscribe today

CAS
A division of the American Chemical Society