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AI-guided design of a CXCR4-targeted core-shell nanocarrier for co-delivery of berberine/paclitaxel in cancer therapy

Yeonwoo Jang^{1†}, Amal Babu^{2†}, Sahil Chahal², Arathy Vasukutty², James J. Moon^{3,4,5}, In-Kyu Park^{2,6*} and Hansoo Park^{1*}

Abstract

An integrated three-step artificial-intelligence (AI) workflow was developed to accelerate the design of a CXCR4-targeted, dual-drug nanocarrier for colorectal cancer therapy. The workflow combined AI-based drug synergy prediction, peptide ligand discovery, and formulation optimization to create a core-shell nanocarrier consisting of a mesoporous silica core coated with a liposomal shell, co-delivering berberine (BBR) and paclitaxel (PTX). The nanocarrier exhibited efficient drug loading, sustained release, and selective uptake by CXCR4-positive cancer cells. In vitro, it synergistically inhibited cancer proliferation and migration, while in vivo it produced pronounced tumor regression and reversal of tumor-associated splenomegaly without systemic toxicity. These findings demonstrate that AI-guided synergy scouting and modular nanocarrier engineering can yield a receptor-targeted combination therapy with translational potential for next-generation cancer treatment.

Keywords AI-guided nanomedicine, CXCR4-targeted core-shell, Mesoporous silica nanoparticle, Liposome coating, Combination chemotherapy

Graphical abstract

AI-guided, CXCR4-targeted liposome-mesoporous silica nanocarriers co-load berberine and paclitaxel, sustaining dual release and markedly suppressing tumor growth without systemic toxicity.

Introduction

Combination chemotherapy, the simultaneous administration of two or more mechanistically distinct agents, has long been recognized for improving clinical response and delaying chemoresistance in solid tumors. In contrast to monotherapy, which often leads to tumor relapse

and drug-resistance mutations [1–3], rational drug pairing can provide complementary mechanisms, synergistic efficacy, and reduced resistance [4–7]. Clinical and preclinical studies consistently demonstrate that such regimens achieve stronger antitumor responses and improved survival, underscoring the need for systematic strategies to identify truly synergistic partners [8].

Nanoparticle-mediated drug delivery systems, such as inorganic nanoparticles, lipid nanoparticles, polymeric nanoparticles, and nano-scaffolds, are currently undergoing preclinical and clinical development for combination chemotherapy by delivering multiple drugs to various cancers, offering benefits over conventional anticancer therapy [2, 9–11]. These systems show passive targeting

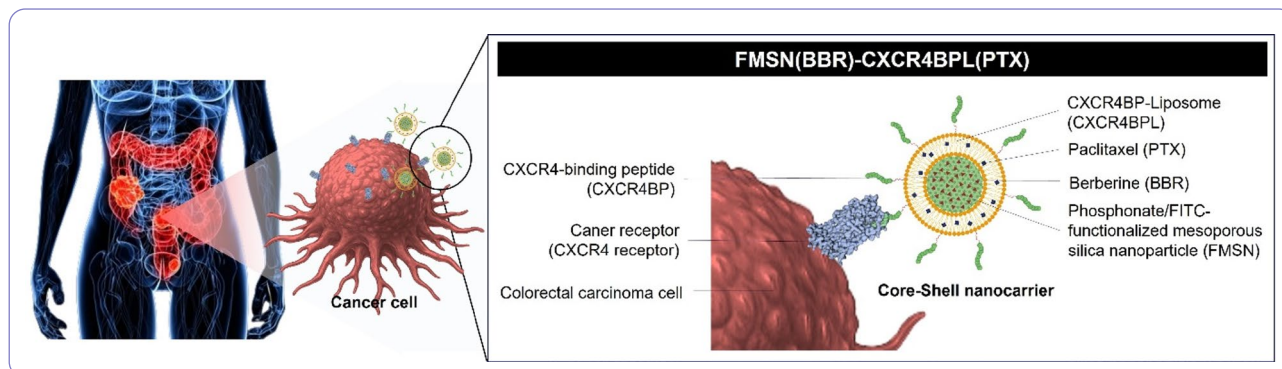
[†]Yeonwoo Jang and Amal Babu contributed equally to this work.

*Correspondence:
In-Kyu Park
pik96@jnu.ac.kr
Hansoo Park
heyshoo@cau.ac.kr

Full list of author information is available at the end of the article



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via enhanced permeability and retention (EPR) effects and active targeting by receptor-specific ligands [12–17]. Additionally, nanoparticles enable sustainable drug release, prevent agents from degradation, and minimize adverse effects, ultimately enhancing therapeutic efficacy [18–21]. Therefore, nanoparticle-mediated delivery of multiple therapeutic agents is an advanced approach due to the various physicochemical and pharmacological properties of nanoparticles for combination chemotherapy. Among various nanoparticle-mediated drug delivery systems, mesoporous silica nanoparticles (MSNs) are considered promising due to surface functionality, size tunability, high intrinsic stability, and efficient drug loading resulting from their unique mesoporous structure [22]. However, challenges such as rapid clearance [23–25], instability in biological media resulting in irreversible aggregation [26], and cytotoxicity at higher doses ($\approx 25 \mu\text{g/mL}$) [27, 28] limit their clinical translation.

To overcome these challenges, we turned to a combined strategy guided by AI-based design principles, which integrates surface modification of MSNs with liposomal coating to improve stability, dispersity, and targeting capacity. To address these critical issues, we employed two engineering strategies: (1) functionalization of MSNs through surface modification, and (2) coating functionalized MSNs (FMSNs) with liposomes. MSNs are surface-modified with various moieties (e.g., amino, phosphonate, and carboxylate groups) to prevent false-positive/false-negative signals and maintain the MSNs well-dispersed in aqueous solution with minimal to no aggregation. The positively or negatively charged MSNs improve solubility in biological solutions through electrostatic interactions, resulting in high permeability [29, 30]. Additionally, MSNs can be covalently bound to fluorescent dye molecules (e.g., fluorescein isothiocyanate [FITC] molecules) for bioimaging purposes [31]. The FMSNs can also be coated with liposomes that have opposite charges to enhance solubility and reduce cytotoxicity. Liposomes are a clinically proven delivery system as they exhibit non-immunogenicity, good biocompatibility, high delivery efficiency, and the ability to

encapsulate hydrophobic and hydrophilic drugs [32, 33]. Furthermore, ligand-conjugated liposomes possess active targeting abilities towards cancer [34, 35]. Therefore, liposome-coated FMSNs could be used as a promising multidrug delivery nanopatform for cancer treatment.

The selection of complementary drugs is crucial in combination chemotherapy, where distinct mechanisms maximize tumor eradication. Berberine (BBR), a natural alkaloid with broad antitumor activity against colorectal cancer, exerts its effects through mitochondrial accumulation, disruption of membrane potential, generation of reactive oxygen species, and induction of apoptosis [36, 37], while also modulating autophagy, enforcing AMP-activated protein kinase (AMPK)-driven metabolic reprogramming, and blocking epithelial-mesenchymal transition [38–42]. Despite these multifaceted actions and relatively low toxicity, BBR alone rarely achieves complete remission [43]. To identify a synergistic partner, we applied the AI-driven drug-synergy platform (MD-Syn), which screened FDA-approved oncology agents across multiple colorectal cancer models and consistently highlighted paclitaxel (PTX) as the top candidate [44]. PTX, a microtubule-stabilizing agent widely used in breast, ovarian, lung, and colorectal cancers [45–48], not only induces mitotic arrest but also perturbs mitochondrial function to amplify apoptosis [49]. Although its efficacy can be limited by multidrug resistance (MDR) [50, 51], the distinct yet complementary mechanisms of PTX and BBR provide a rationale for overcoming such resistance [52]. Together, the AI-predicted synergy and mechanistic complementarity establish a strong basis for the BBR/PTX combination explored in this study.

In this study, we integrated (i) AI-guided ligand discovery and drug-synergy selection, (ii) AI-rational drug allocation (PTX in the lipid shell, BBR in the FMSN core), and (iii) liposome-coated silica engineering to create FMSN(BBR)-CXCR4BPL(PTX), which is a C-X-C chemokine receptor type 4 (CXCR4)-targeted core-shell nanocarrier loaded with BBR and PTX as a multidrug delivery system (Fig. 1). CXCR4 is overexpressed in colorectal carcinoma and correlates with metastasis and

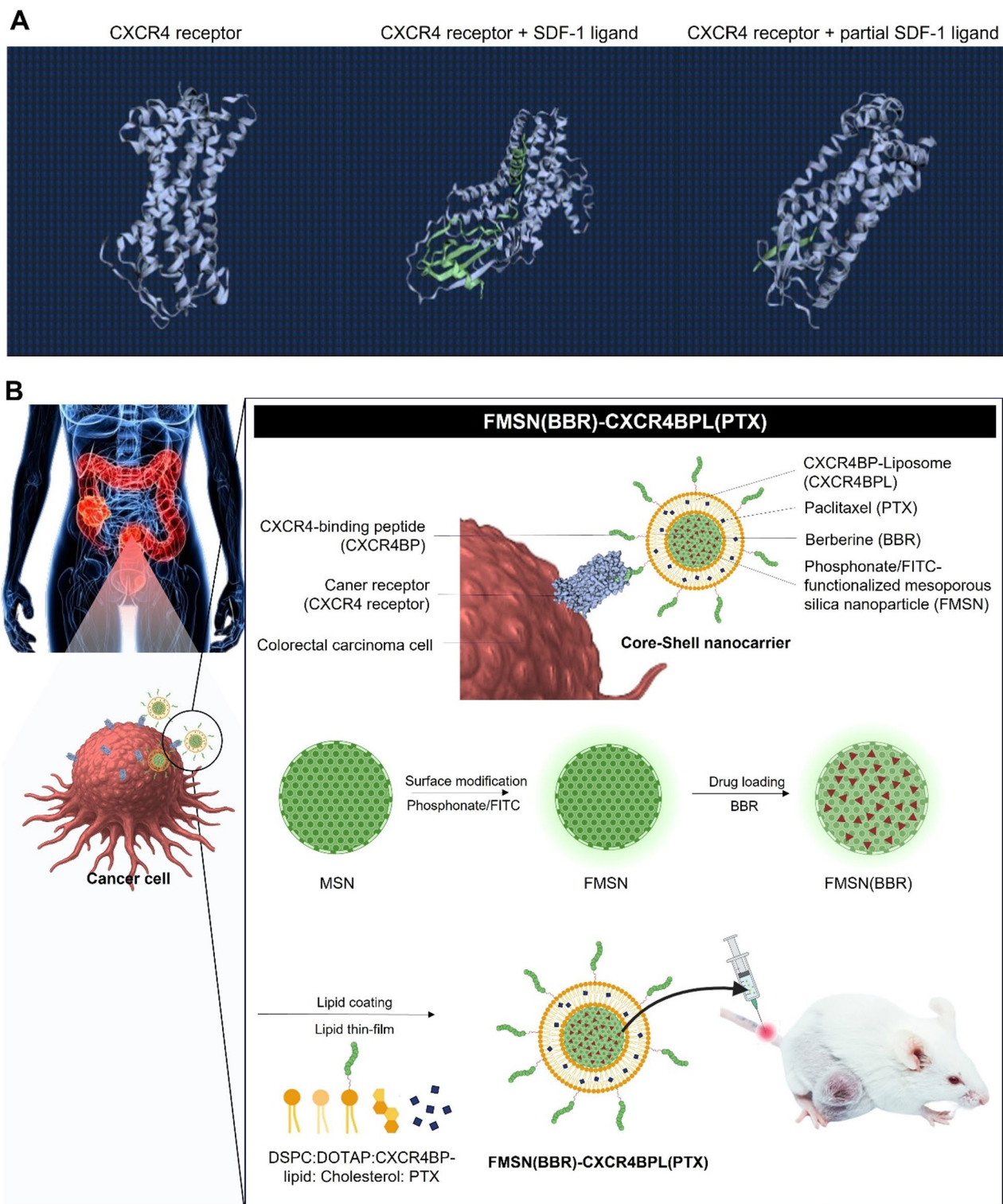


Fig. 1 AI-guided design and overall assembly of the CXCR4-targeted dual-drug core-shell nanocarrier, FMSN(BBR)-CXCR4BPL(PTX). **(A)** AI-assisted targeting motif selection. Flexible docking (CABS-dock) and AlphaFold-based interface scoring (NeuroSNAP-AI) identified a 9-mer fragment of SDF-1 (pSDF-1, green), hereafter referred to as the CXCR4-binding peptide (CXCR4BP), that binds the extracellular pocket of the CXCR4 receptor with high confidence (ipTM 0.96, low PAE/PDE). **(B)** Nanocarrier concept and fabrication. (1) Mesoporous silica nanoparticle (MSN) synthesis → (2) surface functionalization with phosphonate/FITC to yield FMSN → (3) BBR loading [FMSN(BBR)] → (4) thin-film hydration of DSPC : DOTAP : cholesterol : lipids containing CXCR4BP (CXCR4BP-lipid) and PTX → (5) electrostatic self-assembly, yielding the core-shell nanocarrier, FMSN(BBR)-CXCR4BPL(PTX). The CXCR4BP on the liposomal corona enables receptor-mediated uptake by colorectal carcinoma cells, while BBR resides in the silica core and PTX in the lipid shell for synergistic chemotherapy after intravenous administration

poor prognosis [53, 54]. To confer CXCR4 selectivity we implemented a dual-AI peptide screen: CABS-dock and NeuroSNAP-AI [55, 56]. We demonstrated that the platform (i) displayed high dual-drug encapsulation efficiency, (ii) released both agents in a sustained fashion, (iii) remained non-toxic up to 100 $\mu\text{g/mL}$, (iv) accumulated selectively in CXCR4-positive tumors, and (v) produced synergistic anti-proliferative and anti-migratory effects in vitro and significant tumor regression in vivo without systemic toxicity. Collectively, these findings showcase how AI-assisted selection of synergistic drug pairs and rational nanocarrier design can accelerate the development of next-generation, receptor-targeted combination nanotherapies. Here, we aimed to develop and validate an AI-guided, CXCR4-targeted core-shell nanocarrier co-delivering BBR and PTX, and to demonstrate

its therapeutic efficacy through integrated in vitro and in vivo studies.

Results and discussion

AI-guided selection of the CXCR4-binding peptide and fabrication of CXCR4BP-liposomes

An AI-driven pipeline was first used to identify an optimal ligand for CXCR4 (Fig. 1A). Flexible docking of SDF-1/CXCR4-derived sequences with CABS-dock ranked a 9-mer fragment of SDF-1 (Lys-Cys) highest [55, 56–58]. Subsequent AlphaFold-multimer analysis with NeuroSNAP-AI returned an inter-protein TM-score (ipTM) of 0.95 and uniformly low predicted-aligned-error (PAE) values ($< 10 \text{ \AA}$) across the interface, while the predicted-distance-error (PDE) map displayed a tight blue-purple distribution (Fig. 2B, C and D). These

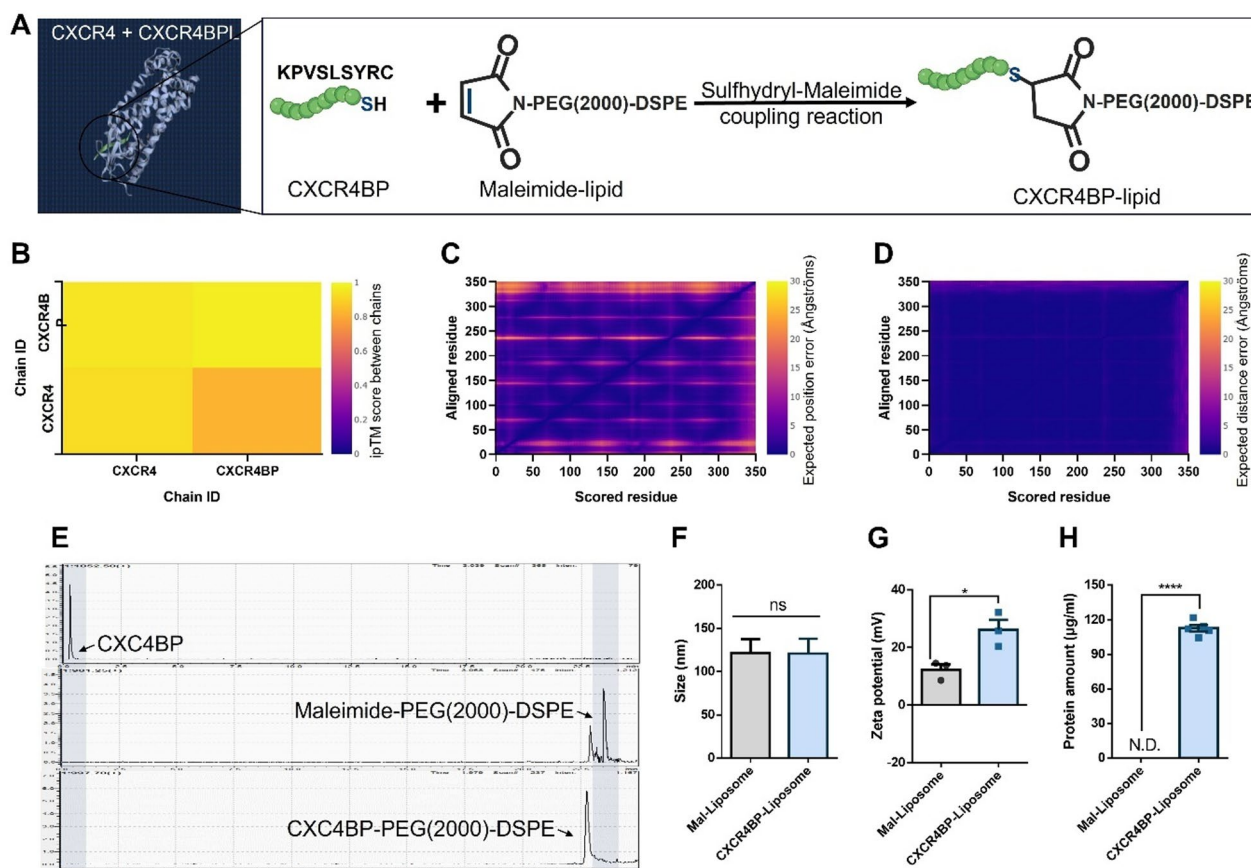


Fig. 2 AI-guided identification of the CXCR4-binding peptide (CXCR4BP), its chemical conjugation to lipid, and physicochemical validation of CXCR4BP-liposomes. **(A)** Design-to-lipid workflow. Left: CABS-dock/NeuroSNAP-AI docking of the 9-mer CXCR4BP (green) on the extracellular pocket of CXCR4 (grey). Right: sulfhydryl-maleimide coupling of the cysteine-terminated peptide (KPVLSYRC) with Maleimide-PEG(2000)-DSPE to give CXCR4BP-PEG(2000)-DSPE (CXCR4BP-lipid). **(B)** AlphaFold-multimer interface accuracy map (ipTM pairs); values approaching 1.0 (yellow) denote highly reliable chain-chain contacts. **(C)** Predicted aligned error (PAE) heat-map; low PAE ($< 10 \text{ \AA}$, purple/blue) indicates well-defined spatial relationships across the complex. **(D)** Predicted distance error (PDE) distribution further confirming high model precision. **(E)** LC/MS chromatograms of free CXCR4BP (top), Maleimide-PEG(2000)-DSPE (middle), and the purified conjugate (bottom), demonstrating successful peptide-lipid linkage (absence of individual starting-material peaks). **(F)** Hydrodynamic diameter of maleimide-liposomes and CXCR4BP-liposomes determined by NTA shows no significant size change after peptide insertion. **(G)** Zeta potential shifts upon introduction of the cationic peptide confirms surface display of CXCR4BP. **(H)** Protein content of maleimide-liposomes and CXCR4BP-liposomes quantified by BCA assay, validating peptide incorporation. Data represent mean $\pm$ SD; $n = 3$ (**F**, **G**), $n = 5$ (**H**); * $p < 0.05$, and **** $p < 0.0001$ vs. Control/Model. ns, not significant. N.D., non-detection

metrics indicate a high-confidence, well-defined binding pose; the peptide was therefore designated CXCR4-binding peptide (CXCR4BP). To anchor the ligand in a lipid membrane, CXCR4BP was conjugated to Maleimide-PEG(2000)-DSPE via a sulfhydryl-maleimide reaction (Fig. 2A, right). LC/MS confirmed disappearance of the free peptide and maleimide peaks, giving a single product at the expected m/z (Fig. 2E) with a coupling yield of $97.2 \pm 2\%$.

CXCR4BP-liposomes (CXCR4BPLs) were then prepared in a single thin-film hydration method. DSPC, DOTAP, cholesterol and the freshly synthesized CXCR4BP-lipid (67 : 8 : 20 : 5 mol %) were co-dissolved to form CXCR4BPL. For the controls, maleimide-PEG(2000)-DSPE was inserted into liposomes without CXCR4BP. Compared with peptide-free control liposomes (Mal-liposome), CXCR4BP-liposomes had a mean diameter (122 ± 4 nm) that was indistinguishable from maleimide control liposomes (119 ± 5 nm; Fig. 2F). In contrast, the ζ -potential shifted from 12.3 ± 3.2 mV to 26.1 ± 5.9 mV after peptide insertion, consistent with surface display of the mildly cationic ligand (Fig. 2G). A bicinchoninic-acid (BCA) assay quantified 112.6 ± 6.2 μ g peptide per mg lipid, confirming successful and reproducible incorporation (Fig. 2H).

Collectively, these results validated (i) the computationally selected CXCR4BP as a high-affinity ligand, (ii) its efficient chemical linkage to DSPE lipid, and (iii) the generation of size-conserved liposomes bearing a peptide-rich corona, laying the foundation for subsequent core-shell nanocarrier assembly.

Synthesis and physicochemical characterization of phosphonate/FITC-functionalized MSNs (FMSNs)

MSNs were produced by a base-catalyzed sol-gel reaction and subsequently co-functionalized with 3-trihydroxysilylpropylmethylphosphonate (THMP) and FITC-APTMS (see Methods). FITC offers a bioimaging function, while phosphonate confers high solubility in aqueous solution and enables cationic liposome coating.

The broad halo at $2\theta \approx 22^\circ$ in the X-ray diffractometer (XRD) profile confirmed the amorphous silica framework was retained after surface modification (Fig. 3A) [59, 60]. Field emission transmission electron microscopy (FE-TEM) revealed well-defined and radially aligned mesopores with an average particle diameter of ~ 150 nm (Fig. 3B, left). Scanning transmission electron microscopy with energy-dispersive spectroscopy (STEM-EDS) elemental maps displayed that phosphorus (magenta) was homogeneously distributed throughout the silica matrix (cyan) (Fig. 3B, centre and right), while the corresponding EDS spectrum exhibited clear Si and P peaks (Fig. 3C), further confirming surface modification. The exact amount of phosphorus was identified as 6.52 wt%

(Table 1). These results confirmed that the P element was successfully functionalized onto the MSNs. Fourier transform infrared spectroscopy (FTIR) comparison of pristine MSNs and functionalized FMSNs (Fig. 3D) provided further evidence of successful phosphonate functionalization. In addition to the common Si-O-Si bands at 1080 and 810 cm^{-1} [61], the FMSN trace displays a new P = O stretching band at $\sim 1230\text{ cm}^{-1}$ (inset), whereas the parent MSN lacks these features, indicative of successful THMP attachment on FMSNs. FITC labelling endowed the particles with bright green fluorescence, the intensity of which increased linearly with dispersion concentration and exhibited a maximum at 535 nm (Fig. 3E, inset photograph).

Collectively, these results demonstrated that the dual functionalization strategy preserved the ordered mesoporous architecture while imparting aqueous dispersibility, traceable fluorescence and a phosphonate-rich surface for subsequent liposome coating and drug loading.

CXCR4BPL coating on FMSN and characterization of FMSN-CXCR4BPL

The self-assembly was initiated by hydrating a thin film of CXCR4BPL with FMSN using probe sonication to facilitate electrostatic interaction between the negatively charged FMSN and cationic CXCR4BPL [62]. BCA assay, FE-TEM with STEM-EDS mapping, field emission scanning electron microscopy (FE-SEM), and confocal laser scanning microscopy (CLSM) were used to confirm the liposome coating.

Following the liposome coating, FMSN-CXCR4BPLs exhibited a significantly higher protein content than FMSNs (Fig. 4A), attributed to the peptide portion of CXCR4BPLs. FE-TEM combined with STEM-EDS mapping visualized a clear core-shell architecture of FMSN-CXCR4BPL (Fig. 4B; Table 2). Silicon and phosphorus signals co-localize in the FMSN cores, whereas carbon is confined to the liposome shells within the same regions of the FMSN-CXCR4BPL particles. FE-SEM highlights the resulting surface transformation from a smooth silica texture to a blurred, uniformly coated surface indicative of the surrounding lipid layer (Fig. 4C). In addition, CLSM shows co-localization of FITC-FMSN (green) and DiI-labelled CXCR4BP (red) signals, confirming that each FMSN core is uniformly enveloped by the lipid shell (Fig. 4D).

We further characterized FMSN, FMSN-CXCR4BPL, and CXCR4BPL. Dynamic light scattering (DLS) analysis indicated that the hydrodynamic diameter of FMSN (~ 130 nm) was maintained after coating (Fig. 4E), confirming that liposome coating did not appreciably change particle size. As expected, the ζ -potential shifts from -7.3 ± 1.5 mV to $+8.0 \pm 2.4$ mV due to the positive charge

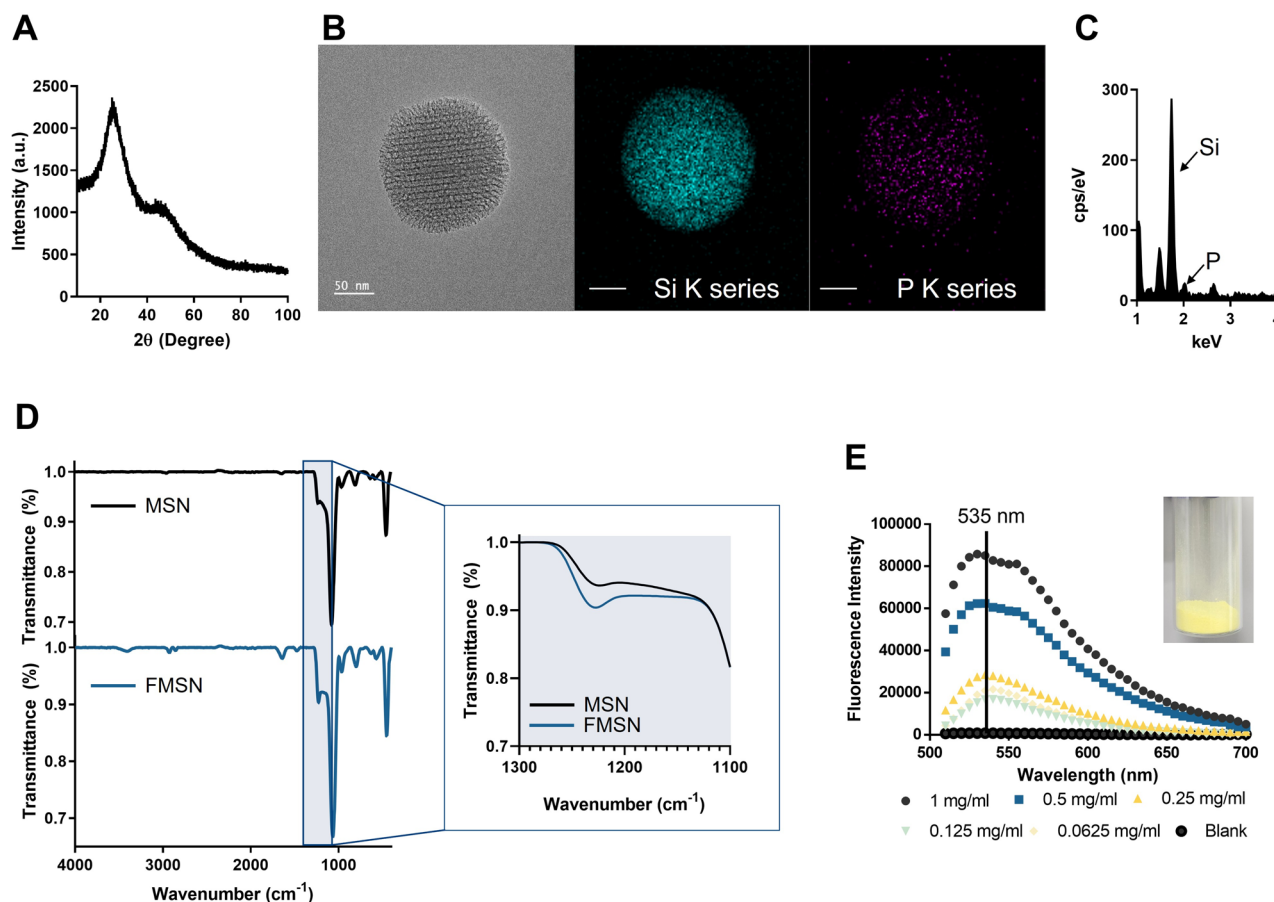


Fig. 3 Physicochemical characterization of phosphonate/FITC-functionalized mesoporous silica nanoparticles (FMSNs). **(A)** X-ray diffraction pattern showing the broad halo at $2\theta \approx 22^\circ$, characteristic of amorphous silica. **(B)** Left: FE-TEM image revealing radially aligned mesopores. Centre and right: STEM-EDS elemental maps of Si (cyan) and P (magenta) demonstrating homogeneous phosphonate distribution. **(C)** EDS spectrum confirming the presence of Si and P peaks. **(D)** FTIR spectra of unmodified MSNs (black) and phosphonate-grafted FMSNs (blue). Inset: enlargement of the $1300\text{--}1100\text{ cm}^{-1}$ region highlighting the new $\text{P}=\text{O}$ band ($\sim 1230\text{ cm}^{-1}$) validating successful THMP functionalization. **(E)** Fluorescence emission spectra of FMSN dispersions ($\lambda_{\text{ex}} = 480\text{ nm}$) at increasing concentrations (0.0625 to 1 mg/mL). All samples exhibit a peak at 535 nm, attributable to covalently bound FITC; inset photograph shows the green fluorescence of FMSN. **(B)** Scale bar = 50 nm

Table 1 STEM-EDS elemental composition of phosphonate/FITC-functionalized mesoporous silica nanoparticles (FMSNs). Quantitative analysis confirms successful phosphonate grafting, with phosphorus accounting for 6.52 wt % (5.95 atomic %)

Element	Line Type	k Factor	k Factor type	Absorption Correction	Wt%	Wt% Sigma	Atomic%
N	K series	1.807	Theoretical	1.00	0.00	0.00	0.00
Si	K series	1.000	Theoretical	1.00	93.48	1.83	94.05
P	K series	1.036	Theoretical	1.00	6.52	1.83	5.95
Total:					100.00		100.00

of peptides (Fig. 4F). The core-shell nanocarrier is colloidal stable in PBS for at least 7 days, with no significant change in PDI (Fig. 4G) or average size (Fig. 4H).

Synergy screening and BBR/PTX co-loading of FMSN-CXCR4BPL

BBR was chosen as the anchor drug because it triggers cell-cycle arrest, AMP-activated protein kinase (AMPK)-driven metabolic reprogramming, and inhibition of epithelial-mesenchymal transition in colorectal cancer cells, thereby suppressing tumor growth and metastasis

[43]. While BBR exhibits broad antitumor activity and relatively low toxicity, its monotherapeutic potential remains limited due to incomplete tumor eradication and risk of recurrence, necessitating a rationally selected co-therapeutic agent to enhance overall efficacy. To identify a truly cooperative drug, we employed the MD-Syn AI platform to screen BBR against 38 FDA-approved oncology agents across six human colorectal cancer cell lines (HCT116, HT29, LoVo, RKO, SW620, SW837) [44], which served as surrogates in lieu of a CT26 murine model. PTX emerged as the compound with a synergy

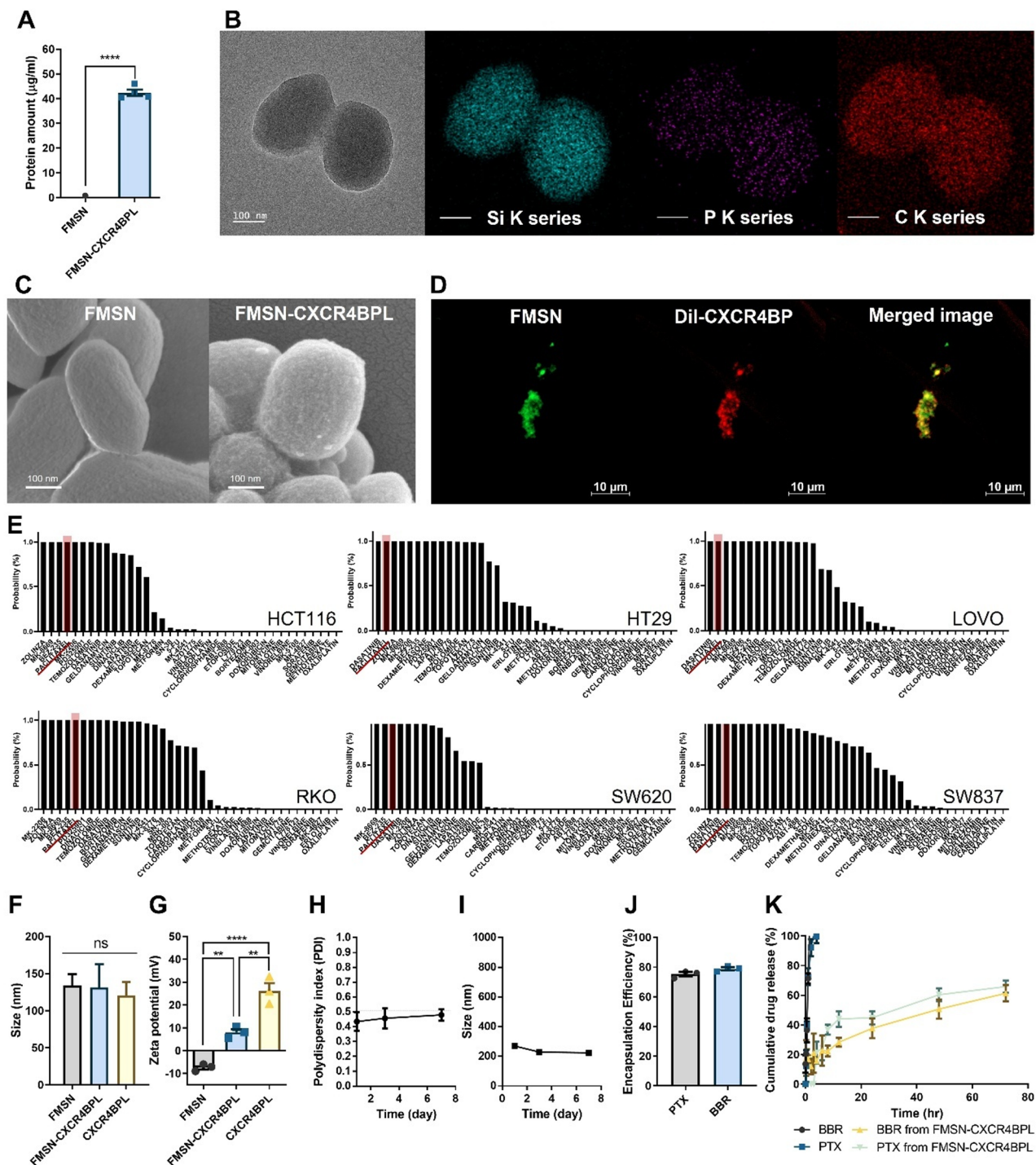


Fig. 4 Verification of liposome coating on FMSNs, and characterization of FMSN(BBR)-CXCR4BPL(PTX). **(A)** Protein quantification of FMSN and FMSN-CXCR4BPL using BCA assay. **(B)** FE-TEM and STEM-EDS mapping images of silica (Si), phosphorus (P), and carbon (C) of FMSN-CXCR4BPL. **(C)** FE-SEM images of FMSN (left) and FMSN-CXCR4BPL (right). **(D)** CLSM images showing co-localization of FMSN (green), DiI-labeled CXCR4BPL (DiI-CXCR4BP, red). **(E)** MD-Syn in silico synergy screen of BBR with 38 oncology drugs across six human colorectal cancer cell lines (HCT116, HT29, LoVo, RKO, SW620, SW837). PTX (highlighted in red) is the compound exhibiting a synergy probability > 0.9999 in every model (mean = 0.999999852). **(F)** Size of FMSN, FMSN-CXCR4BPL, and CXCR4BPL by NTA. **(G)** Zeta potential of FMSN, FMSN-CXCR4BPL, and CXCR4BPL by DLS. **(H)–(I)** Stability testing of FMSN-CXCR4BPL for 7 days. **(J)** BBR and PTX encapsulation efficiencies in FMSN-CXCR4BPL. **(K)** Cumulative drug release profile over time. Data are mean $\pm$ SD; $n=4$ **(A)**, $n=5$ **(E)**, $n=3$ **(F–J)**; ** $p < 0.01$, and **** $p < 0.0001$. vs. Control/Model. ns, not significant. **(B)** Scale bar = 100 nm. **(C)** Scale bar = 100 nm. **(D)** Scale bar = 10 μm

Table 2 STEM-EDS elemental composition of liposome-coated nanoparticles (FMSN-CXCR4BPL). The marked increase in carbon (~39 wt%) relative to bare FMSNs, together with the residual silicon and trace phosphorus from the core, confirms the presence of an organic lipid shell surrounding the silica core

Element	Line Type	k Factor	k Factor type	Absorption Correction	Wt%	Wt% Sigma	Atomic%
C	K series	3.115	Theoretical	1.00	39.49	0.26	51.45
O	K series	1.455	Theoretical	1.00	35.28	0.21	34.51
Si	K series	1.000	Theoretical	1.00	24.80	0.17	13.82
P	K series	1.036	Theoretical	1.00	0.43	0.04	0.22
Total:					100.00		100.00

probability > 0.9999 in every cell line (grand mean = 0.999999852; Fig. 4E), justifying its selection for combination therapy.

Prior to formulation, we employed the open-source FormulationAI drug encapsulation module [63]. The model predicted encapsulation efficiencies of 52.56% (BBR in liposome) and 68.65% (PTX in liposome). According to these AI-guided results, we decided to encapsulate PTX in CXCR4BPL part and load BBR in FMSN.

To further validate the MD-Syn prediction, in vitro viability data were analyzed using the Bliss independence model. Several combinations demonstrated clear synergy, including PTX 10 µg/mL + BBR 1 µg/mL ($\Delta = +30.1\%$), PTX 1 µg/mL + BBR 0.1 µg/mL ($\Delta = +14.3\%$), and PTX 0.1 µg/mL + BBR 1 µg/mL ($\Delta = +40.4\%$), whereas other dose pairs showed additive or mildly synergistic effects. These findings are consistent with the AI-guided prediction that PTX is a cooperative partner for BBR, strengthening the rationale for the selected dual-drug formulation.

After one-pot self-assembly, the resulting core-shell nanocarrier achieved BBR EE = $78.8 \pm 1.9\%$ and PTX EE = $75.2 \pm 2.4\%$ (Fig. 4J), suggesting an optimized loading strategy for this core-shell loading combination. From the feed composition (lipid: FMSN 10:1, w/w; PTX feed 5% w/w of lipid) and the measured encapsulation efficiencies, the idealized formulation ratio is $\approx 10:0.8:0.4$ (lipid: BBR: PTX, w/w). Importantly, the downstream in vitro and in vivo efficacy studies serve as experimental validation of the MD-Syn AI-predicted BBR/PTX synergy, linking the computational selection to biological outcomes in both cell-based assays and a CT26 tumor model. Because MD-Syn outputs synergy probabilities but not explicit ratios, the working BBR/PTX proportion was defined by the actual encapsulated content in the core-shell formulation. At a standard input of 1×10^8 particles, the measured entrapment efficiencies corresponded to ≈ 1 µg/mL BBR and 0.4 µg/mL PTX, yielding a reproducible ratio of $\approx 2.5:1$ (w/w). This formulation-driven outcome was applied consistently in downstream in vitro and in vivo experiments. Additionally, the release kinetics of BBR and PTX from FMSN(BBR)-CXCR4BPL(PTX) demonstrated sustained release over a 72-h period in PBS (pH

7.4, 37 °C), indicating prolonged drug availability that could enhance targeted cancer cell treatment while maintaining effective therapeutic levels (Fig. 4K). These results supported the rationale for combining BBR with PTX, as the core-shell architecture not only accommodated both drugs at high loading capacity but also ensures their sustained co-delivery, a crucial factor in achieving the predicted synergistic antitumor effects.

In vitro biocompatibility of FMSN-CXCR4BPL

The in vitro biocompatibility of bare FMSN and lipid-coated FMSN-CXCR4BPL was assessed using a live/dead cell assay and CCK-8 assay on CT26 colon-carcinoma cells and human adipose-derived MSCs (hASC) at various concentrations after 48 h incubation (Fig. 5).

As shown in Fig. 5A and B, CT26 and hASC exposed to bare FMSN at 100 µg/mL displayed an increase in dead cells (red fluorescence), whereas little or no red signal was detected at 1 µg/mL and 10 µg/mL. In contrast, cells treated with FMSN-CXCR4BPL retained an almost exclusively green fluorescence pattern even at 100 µg/mL, indicating minimal membrane damage. The lipid shell effectively masked the potential toxicity at high concentrations of the FMSN cores (Fig. 5A and B). These observations were corroborated by the CCK-8 assay results. For CT26, viability fell to $74.6 \pm 1.5\%$ with bare FMSN at 100 µg/mL but remained $93.9 \pm 9.5\%$ with FMSN-CXCR4BPL (Fig. 5C). hASC showed a similar trend (bare FMSN $77.1 \pm 3.8\%$; coated FMSN-CXCR4BPL $97.3 \pm 4.5\%$, Fig. 5D). No significant difference was observed between the two formulations at 1 µg/mL or 10 µg/mL. These results agree with previous reports that uncoated MSNs become dose-limiting above ≈ 25 µg/mL owing to exposed silanol groups [27, 28]. The phospholipid shell passivates reactive silanols, thereby suppressing membrane disruption and improving serum compatibility. Because the CXCR4BP is present at only 0.5 mol % of total lipid, the shell itself is essentially non-toxic while conferring receptor-mediated targeting capability.

Collectively, the lipid corona transformed a dose-limited inorganic carrier into a biocompatible platform that remained safe at 100 µg/mL, which is a concentration exceeding typical therapeutic doses used in subsequent in vivo studies.

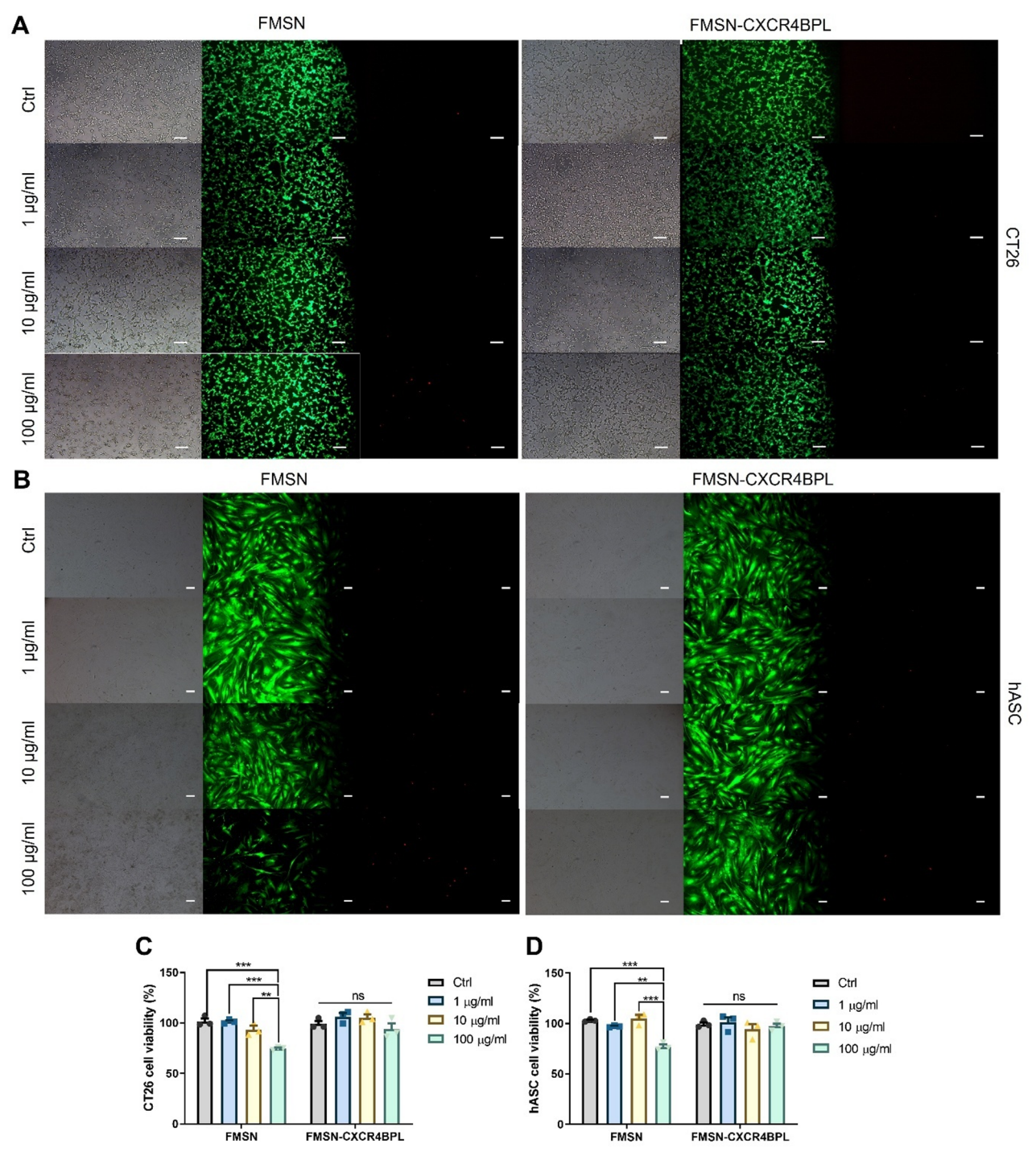


Fig. 5 In vitro biocompatibility of FMSN and FMSN-CXCR4BPL. **(A), (B)** Live/Dead staining fluorescence microscopy images of CT26 and hASC cells after 48 h of inoculation with FMSN and FMSN-CXCR4BPL (1, 10, and 100 $\mu\text{g/ml}$). Live cells were stained with calcein AM (green), and dead cells were stained with ethidium homodimer (EthD-1) (red). **(C), (D)** Cell viability assessed using the CCK-8 assay of CT26 and hASC cells after 48 h of inoculation with FMSN and FMSN-CXCR4BPL (1, 10, and 100 $\mu\text{g/ml}$). Data are mean $\pm$ SD; $n = 3$ **(C, D)**; ** $p < 0.01$, and *** $p < 0.001$ vs. Control/Model. ns, not significant. **(A)** Scale bar = 50 μm . **(B)** Scale bar = 20 μm

CXCR4-mediated cancer-targeting ability in vitro and in vivo

To evaluate whether CXCR4BPs enhance tumor selectivity, we compared bare FMSN with FMSN-CXCR4BPL in

CXCR4-overexpressing CT26 colon-carcinoma cells and in CT26-tumor-bearing mice (Fig. 6).

CLSM showed time-dependent internalization of both formulations, but FMSN-CXCR4BPL produced markedly

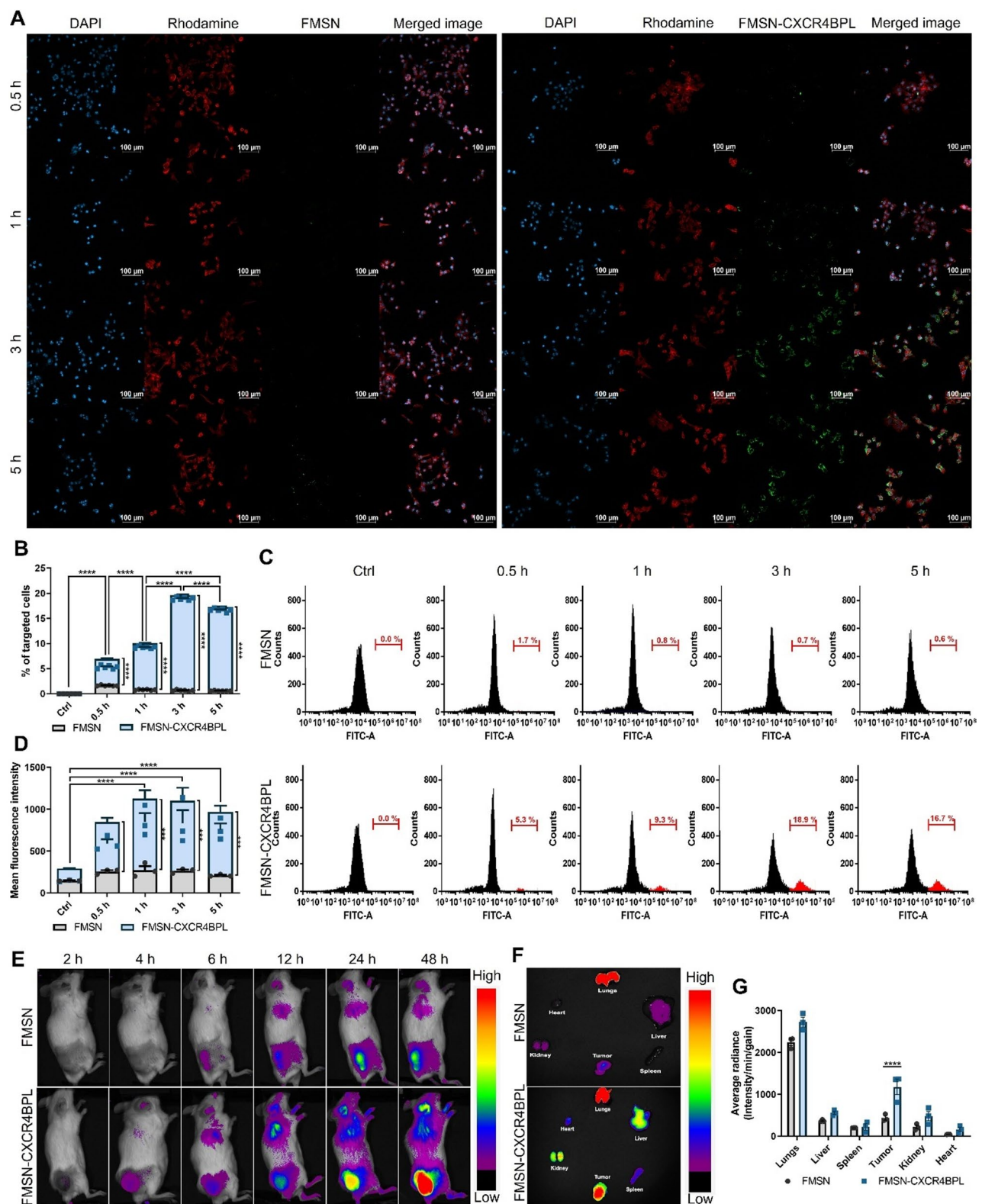


Fig. 6 (See legend on next page.)

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Fig. 6 CXCR4-mediated cancer-targeting ability of FMSN-CXCR4BPL in vitro and in vivo. **(A, B)** CLSM images of CT26 cells with FMSN and FMSN-CXCR4BPL (green) for 0.5, 1, 3, and 5 h. The nucleus was stained with DAPI (blue) and the cytoplasm with Rhodamine B (red). **(B)** Percentage of nanocarrier-positive cells quantified from **(A)** using ImageJ. **(C)** and **(D)** Flow cytometry analysis of the intracellular localization of FMSN and FMSN-CXCR4BPL after 0.5, 1, 3, and 5 h of incubation. **(E)** Whole-body IVIS images of CT26-tumor-bearing mice at 2, 4, 6, 8, 24, and 48 h post-intravenous administration of FMSN and FMSN-CXCR4BPL. **(F)** Ex vivo fluorescence images of excised tumors and major organs 48 h post-injection. **(G)** Quantitative radiant efficiency of excised tumor and major organs 48 h after injection. Data are presented as mean $\pm$ SD; $n=3$ **(D, G)**, $n=6$ **(B)**; *** $p<0.001$, and **** $p<0.0001$ vs. Control/Model. ns, not significant. **(A)** Scale bar = 100 μ m

stronger intracellular fluorescence from 3 h onward (green) than bare FMSN (Fig. 6A). ImageJ analysis confirmed that the percentage of nanoparticle-positive CT26 cells rose to $18.9 \pm 0.3\%$ for FMSN-CXCR4BPL versus $0.7 \pm 0.1\%$ for FMSN after 3 h (Fig. 6B). Flow cytometry analysis was conducted on CT26 cells to substantiate these findings with additional quantitative support. Flow cytometry histograms echoed this trend. FITC-positive populations appeared as early as 0.5 h and reached a three-fold higher mean fluorescence intensity for FMSN-CXCR4BPL at 3 h (Fig. 6C and D). These data demonstrate efficient CXCR4-mediated attachment and internalization of FMSN-CXCR4BPL in vitro.

To evaluate CXCR4-mediated tumor-targeting ability of FMSN-CXCR4BPL, IR780-loaded FMSNs and FMSN-CXCR4BPLs were injected i.v. (0.5 mg/kg IR780) into CT26-tumor-bearing BALB/c mice. Whole-body imaging revealed progressive tumor-specific fluorescence, peaking at 24–48 h. Signal in the FMSN-CXCR4BPL group was consistently higher than in the FMSN group, indicating improved targeting and/or retention (Fig. 6E). Ex vivo fluorescence imaging 48 h post-injection showed the strongest fluorescence in tumors relative to other organs (Fig. 6F). Quantification confirmed a three-fold higher radiant efficiency in tumors treated with FMSN-CXCR4BPL compared with bare FMSN ($p<0.0001$; Fig. 6G).

Together, the CLSM/flow cytometry in vitro data and the in vivo imaging results established that the CXCR4-binding lipid corona conferred robust and selective targeting to CXCR4-positive cancer cells, leading to enhanced tumor accumulation without off-target organ uptake.

In vitro anticancer efficacy of FMSN(BBR)-CXCR4BPL(PTX)

The targeted core-shell nanocarrier (FMSN(BBR)-CXCR4BPL(PTX)) was compared with a free BBR/PTX mixture and a non-targeted silica formulation, FMSN(BBR/PTX).

Because CXCR4 signaling is a key driver of tumor dissemination, we first assessed cell migration, a prerequisite for metastasis, alongside direct cytotoxicity [64]. In a scratch-wound assay (Fig. 7A and B), control monolayers almost completely closed > 97% of the gap within 24 h, and the free dual-drug mixture (BBR/PTX) reduced closure only modestly. Non-targeted FMSN(BBR/PTX) slowed migration further, yet a continuous front was

still visible. In contrast, FMSN(BBR)-CXCR4BPL(PTX) preserved $93.6 \pm 2.9\%$ of the initial gap after 24 h, significantly higher than all other groups (Fig. 7B).

Metabolic activity measured by CCK-8 assay followed the same drug-dose-dependent trend (Fig. 7C). After 72 h, cancer cell viability fell to $36.2 \pm 1.9\%$ with the targeted nanocarrier, whereas free drugs and non-targeted particles left $81.1 \pm 4.7\%$ and $68.9 \pm 20.1\%$ of viable cancer cells, respectively. Live/dead staining corroborated these data (Fig. 7D). Extensive EthD-1 (red) fluorescence was evident only in the FMSN(BBR)-CXCR4BPL(PTX) group at 72 h.

Thus, by combining CXCR4-specific delivery with complementary mitochondrial (BBR) and mitotic (PTX) mechanisms, the core-shell nanocarrier not only killed CT26 cancer cells more efficiently but also blocked the migratory behavior that underlies metastasis, highlighting its dual anti-cancer potential. Collectively, these migration and viability data were concordant with the AI-predicted synergy between BBR and PTX, thereby providing cell-level experimental confirmation that the AI-guided selection identified a truly cooperative drug pair when co-delivered by the CXCR4-targeted core-shell platform.

In vivo antitumor efficacy of FMSN(BBR)-CXCR4BPL(PTX)

Building on promising in vitro cytotoxicity and significant tumor accumulation, we evaluated the antitumor efficacy of FMSN(BBR/PTX) and FMSN(BBR)-CXCR4BPL(PTX) nanoparticles in a CT26 tumor-bearing murine model. The in vivo dosing was scaled to a BBR equivalent of 5 mg/kg, with the core-shell architecture assumed to release PTX proportionally at the established $\approx 2.5:1$ ratio, thereby maintaining consistency with in vitro conditions. When tumors reached approximately 100 mm³ in volume, mice were randomly assigned to six treatment groups: PBS (control), free BBR, free PTX, a free BBR/PTX combination, FMSN(BBR/PTX), and FMSN(BBR)-CXCR4BPL(PTX).

A single i.v. administration of the CXCR4-targeted core-shell nanocarrier produced the most pronounced tumor control among the six treatment arms. Tumors in the PBS control group expanded steadily, reaching ≈ 1500 mm³ by day 21, whereas growth was only modestly delayed by free BBR or PTX alone. A combined injection of the two free drugs and the non-targeted FMSN(BBR/PTX) did slow tumor growth, yet a

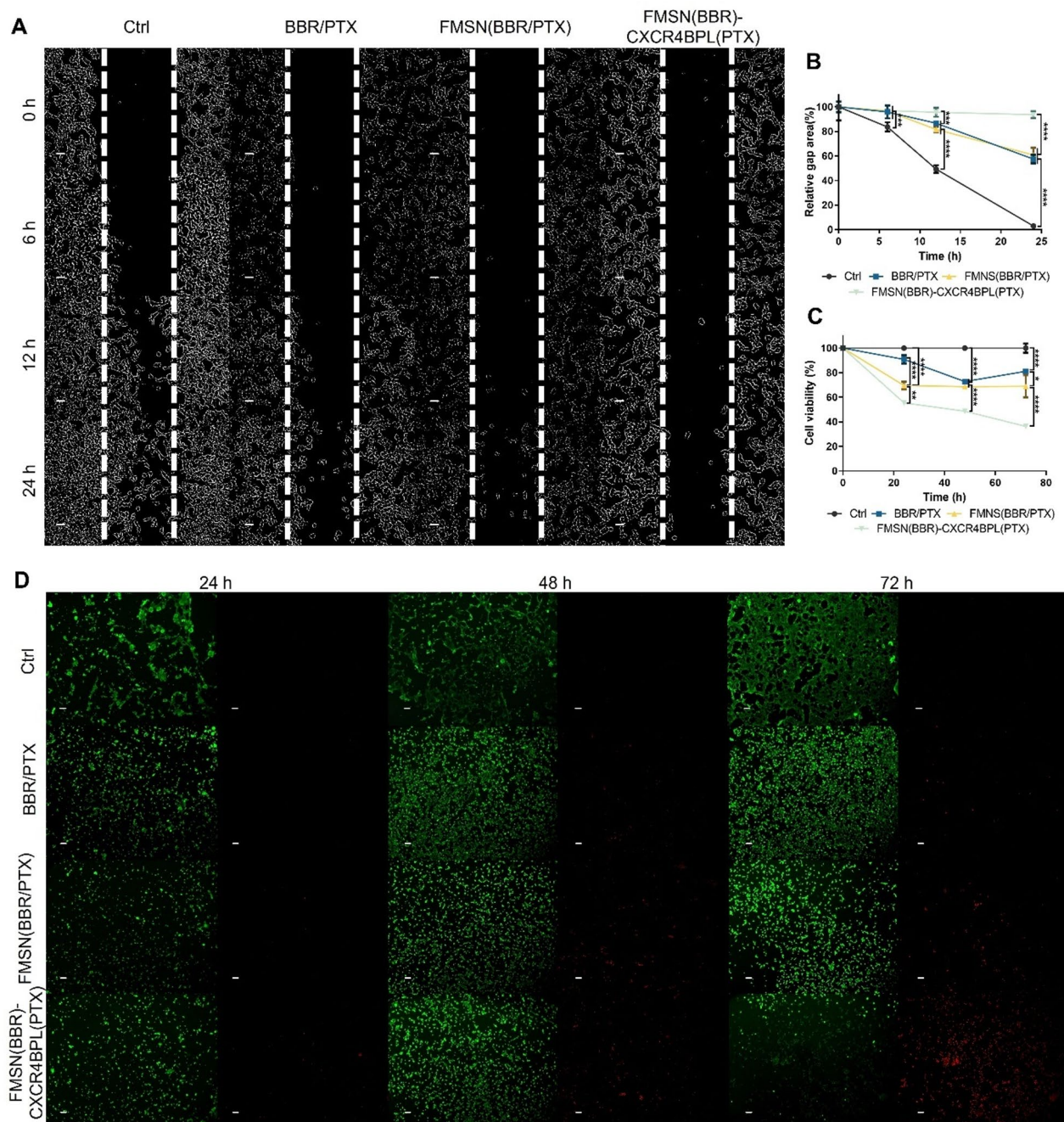


Fig. 7 In vitro cancer migration inhibition and cytotoxicity effects of FMSN(BBR)-CXCR4BPL(PTX). **(A)** Scratch-wound assay in CT26 cell monolayers treated with BBR (1 μ g/mL)/PTX (0.4 μ g/mL), FMSN including BBR (1 μ g/mL)/PTX (0.4 μ g/mL), and FMSN-CXCR4BPL including BBR (1 μ g/mL)/PTX (0.4 μ g/mL). Phase-contrast images were taken at 0, 6, 12, and 24 h. Dashed lines mark the initial wound edges. **(B)** Quantification of relative gap area versus time. FMSN(BBR)-CXCR4BPL(PTX) suppresses lateral migration most effectively. **(C)** CCK-8 viability assay of CT26 cells after 24, 48, and 72 h exposure to the same formulations. Targeted core-shell nanoparticles reduce viability significantly more than the free-drug mixture or non-targeted particles. **(D)** Live/dead staining (calcein-AM, green; EthD-1, red) at 24, 48, and 72 h corroborates the quantitative viability data. Extensive red fluorescence is observed only in the FMSN(BBR)-CXCR4BPL(PTX) group at 72 h. Data are presented as mean $\pm$ SD; $n=4$ (**B**, **C**); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ vs. Control/Model. ns, not significant. Statistical significance was assessed relative to the control group unless otherwise indicated. **(A)** Scale bar = 20 μ m. **(D)** Scale bar = 50 μ m

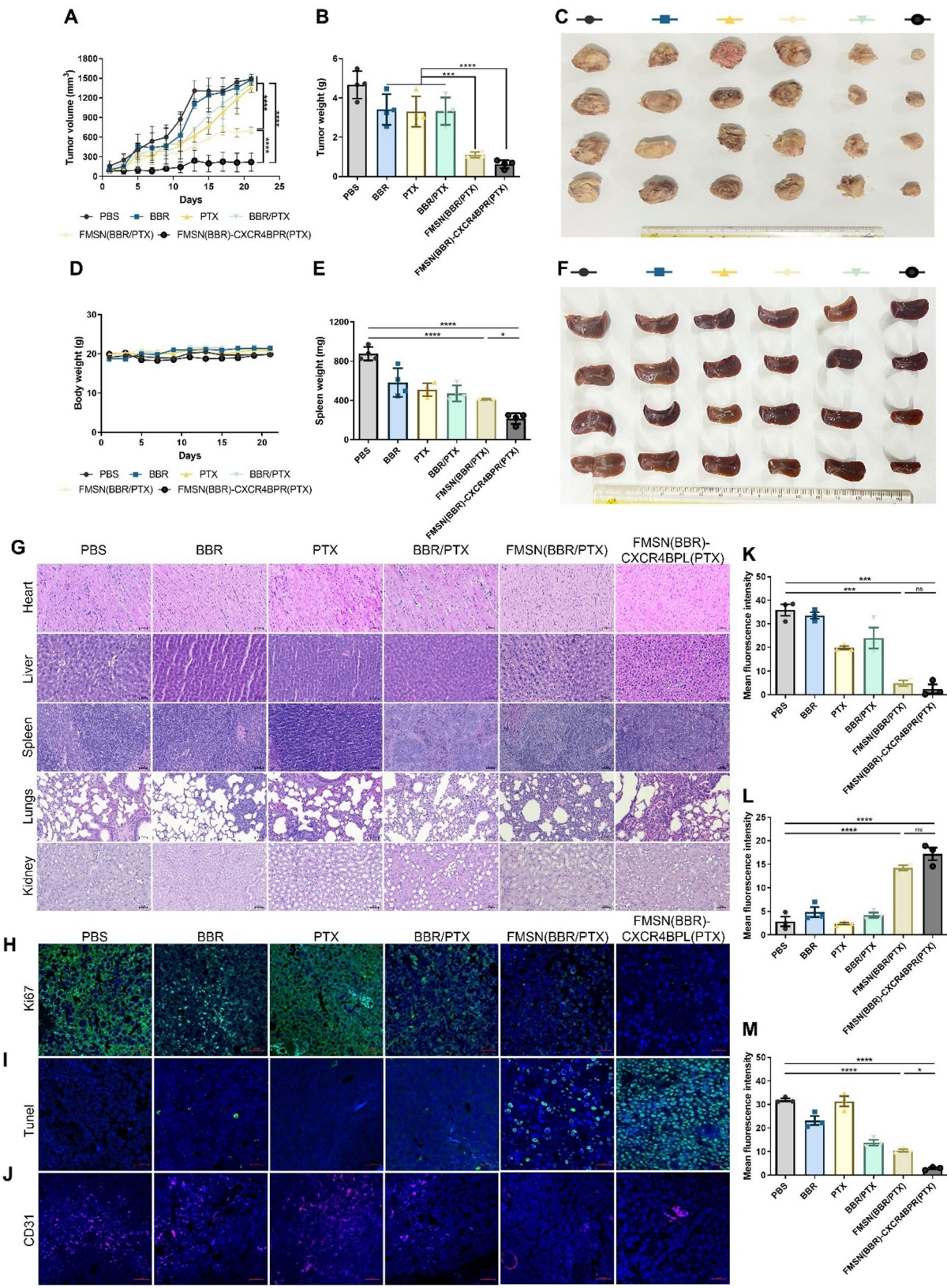


Fig. 8 (See legend on next page.)

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Fig. 8 In vivo antitumor activity of FMSN(BBR)-CXCR4BPL(PTX) in a CT26 subcutaneous mouse model. BALB/c mice bearing $\sim 100 \text{ mm}^3$ CT26 tumor received a single tail-vein dose of PBS, free BBR, free PTX, free BBR/PTX combination, non-targeted FMSN(BBR)/PTX or targeted FMSN(BBR)-CXCR4BPL(PTX) (100 μL ; BBR 5 mg/kg) on day 0. **(A)** Tumor growth curves over 21 days. **(B)** Endpoint tumor weights of mice. **(C)** Photographs of excised tumors. **(D)** Body weight evolution demonstrating systemic tolerance. **(E)** Spleen weights and **(F)** Representative spleen images showing reduced splenomegaly in the targeted group. **(G)** H&E staining of heart, liver, spleen, lung, and kidney. **(H)** Ki67 staining of tumor tissue sections to visualize tumor proliferation. **(I)** TUNEL staining to assess tumor apoptosis. **(J)** CD31 staining to analyze tumor angiogenesis. **(K–M)** Quantification of the mean fluorescence intensity of Ki67-positive cells, TUNEL-positive cells, and CD31 positive cells. Data presented are mean $\pm$ SD; $n = 3$ (**K, L, M**), $n = 4$ (**A, B, D, E**); * $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$ vs. Control/Model. ns, not significant. **(G)** Scale bar = 50 μm . **(H–J)** Scale bar = 20 μm

clear expansion persisted. In contrast, FMSN(BBR)-CXCR4BPL(PTX) virtually halted tumor enlargement, limiting the final volume to $14.6 \pm 4.8\%$ of the PBS group (Fig. 8A). Tumor growth data were corroborated by endpoint tumor weights. The targeted nanocarrier reduced mean tumor mass from $4.67 \pm 0.3 \text{ g}$ in PBS controls to $0.62 \pm 0.1 \text{ g}$, i.e. $\approx 13.3 \pm 2\%$ of the PBS control value (Fig. 8B). This weight reduction parallels the volumetric inhibition, and the excised tumors displayed the same hierarchy of mass (Fig. 8C). The results highlighted the superior in vivo potency of FMSN(BBR)-CXCR4BPL(PTX) over free drugs or the non-targeted formulation.

Systemic tolerance was excellent, since no significant body-weight changes were observed in any group over 21 days (Fig. 8D), and H&E examination revealed no histopathological damage to heart, liver, spleen, lung, or kidney (Fig. 8G). Spleen weight served as an additional systemic read-out. In this model, tumor-driven inflammation produces pronounced splenomegaly [65]. Notably, untreated mice exhibited marked splenomegaly ($876.2 \pm 68.9 \text{ mg}$), whereas the targeted formulation normalized spleen weight to $214.3 \pm 55.1 \text{ mg}$, roughly a four-fold reduction (Fig. 8E and F). The reversal of splenomegaly therefore indicates that the targeted therapy not only shrinks the primary tumor but also alleviates tumor-induced systemic immune dysregulation, further underscoring its favorable safety-efficacy profile.

To further confirm the enhanced antitumor activity of FMSN(BBR)-CXCR4BPL(PTX), particularly in relation to cell proliferation, immunohistochemistry staining was performed. Ki-67 fluorescence fell to $7.0 \pm 5.2\%$ of control, indicating strong proliferation arrest (Fig. 8H and K). A TUNEL assay revealed a 6-fold rise in apoptotic nuclei relative to PBS (Fig. 8I and L), while CD31 labeling showed a one-tenth drop in micro-vessel density, confirming potent anti-angiogenic action (Fig. 8J and M).

These multi-parametric in vivo readouts, including tumor volume/weight suppression, reversal of splenomegaly, and favorable Ki-67/TUNEL/CD31 profiles, experimentally validated the AI-guided BBR/PTX selection at the organismal level, extending the in vitro findings to a relevant murine cancer model. Together, these results demonstrate that CXCR4-directed co-delivery of BBR and PTX achieves multi-modal tumor suppression, curbing proliferation, inducing apoptosis, and impairing

angiogenesis without systemic toxicity. This proof-of-concept study ($n = 4$ mice per group) showed statistically significant tumor inhibition, supported by IHC (Ki-67, CD31) and TUNEL analyses, confirming the efficacy of the treatment.

Conclusion

By chaining three orthogonal AI modules, we established a rapid, data-driven route to a receptor-targeted dual-drug nanocarrier for colorectal cancer. (i) MD-Syn screening of 38 FDA-approved oncology drugs across six human colorectal cancer cell lines identified PTX as a highly synergistic partner for BBR (> 0.9999 probability), nominating the BBR/PTX pair for formulation. (ii) A dual-AI peptide pipeline, combining CABS-dock with AlphaFold-multimer scoring in NeuroSNAP-AI, pinpointed a high-affinity 9-mer SDF-1 fragment (CXCR4BP; ipTM = 0.95) for selective CXCR4 targeting. (iii) FormulationAI predicted optimal drug placement, BBR in FMSN cores and PTX in CXCR4BPL shells, which was experimentally validated by high encapsulation efficiencies ($78.8 \pm 1.9\%$ for BBR, $75.2 \pm 2.4\%$ for PTX) and sustained 72-h release.

The $\sim 130 \text{ nm}$ core-shell nanocarrier markedly inhibited migration and proliferation of CXCR4-positive CT26 cells and, in vivo, achieved pronounced tumor regression, reversal of splenomegaly, and strong anti-proliferative (Ki-67 $\downarrow$), pro-apoptotic (TUNEL $\uparrow$), and anti-angiogenic (CD31 $\downarrow$) responses without systemic toxicity.

Collectively, these findings establish a proof-of-concept for AI-guided combination design, demonstrating that synergy scouting, ligand discovery, and formulation optimization can be integrated with modular nanocarrier engineering to produce potent, receptor-targeted therapeutics. Looking ahead, expanding validation to additional tumor models, dosing regimens, and resistance contexts will further define translational reliability. The modular workflow is adaptable to other receptor-ligand axes and drug pairs, offering a generalizable blueprint for accelerating next-generation anticancer nanomedicines.

Experimental section

Cell culture

Colon carcinoma cells (CT26, obtained from the Korean Cell Line Bank, Seoul, South Korea) and human adipose-derived stem cells (hASCs, isolated under approval of

the Chung-Ang University Hospital Institutional Review Board, IRB No. 2151-005-463, in accordance with the Declaration of Helsinki) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (*v/v*) fetal bovine serum (FBS) and 1% (*v/v*) penicillin/streptomycin. Cell lines were incubated at 37 °C in a 5% CO₂ humidified atmosphere.

Preparation of phosphonate/fluorescence-functionalized MSNs (FMSNs) and loading of BBR

The FMSNs were synthesized using a base-catalyzed sol-gel method at high temperatures, modifying previous protocols [66–68]. The FITC-labeled MSNs were prepared by reacting 1.4 mmol of FITC with 1.2 mmol of (3-aminopropyl)trimethoxy silane (APTMS) in ethanol under an argon atmosphere for 2 h. The FITC-APTMS product was then added to a co-condensation reaction of 5.0 mL of tetraethyl orthosilicate (TEOS), 1.0 g of hexadecyltrimethylammonium bromide (CTAB) in a mixture of 480 mL of distilled water, and 3.5 mL of 2 M sodium hydroxide. After 15 min of stirring at 80 °C, 1.3 mL of 3-trihydroxysilylpropylmethylphosphonate (THMP) was added dropwise to the mixture. The reaction mixture was vigorously stirred at 80 °C for 2 h. Once the reaction was complete, the solution was cooled to ambient temperature, and the FITC-labeled nanoparticles were filtered and washed thoroughly with methanol using a fritted funnel under vacuum for 24 h. To remove the CTAB surfactants, 1.0 g of the nanoparticles were dissolved in a mixture of 100 mL of methanol and 1.0 mL of 37% hydrochloric acid. After refluxing the solution for 24 h, the nanoparticles were filtered and washed thoroughly with methanol through a fritted funnel under vacuum for 24 h. FMSN characterization was performed using an X-ray diffractometer (XRD). The validation of fluorescence of FMSN was determined using a microplate reader (SynergyTM H1, BioTek Instruments Inc.) by exciting the samples at 480 nm and measuring the emission spectra between 500 and 700 nm. Phosphonate-surface modification was verified using a Fourier transform infrared spectroscopy (FTIR) and a field emission transmission electron microscopy (FE-TEM) coupled with energy-dispersive X-ray spectroscopy (EDS).

To load drug molecules into the pores of the particles, the FMSNs were soaked in a concentrated solution containing the drugs. Typically, 100 mg of FMSNs were gently stirred in a solution containing 100 mg of BBR in ethanol: distilled water (7:3 *v/v*) for 48 h. After stirring, the mixture was centrifuged at 23,000 g for 10 min several times, and the supernatant was removed. The FMSN(BBR)s were then lyophilized.

Preparation and characterization of peptide-conjugated lipids

Peptides that bind to CXCR4 and contain an additional cysteine at the N-terminus (custom-synthesized at >95% purity by PEPMIC Co., Ltd., Suzhou, China) were conjugated with Maleimide-PEG(2000)-DSPE through a sulfhydryl-maleimide coupling reaction [69]. In brief, Maleimide-PEG(2000)-DSPE was first diluted in chloroform and then rotary evaporated at 37 °C and 120 rpm to create a thin film, which was then hydrated with pure water at 37 °C. The peptides were dissolved in 0.1 M phosphate-buffered solution (PBS) and mixed with Maleimide-PEG(2000)-DSPE (peptides/Maleimide-PEG(2000)-DSPE molar ratio of 1.2:1) and left to react overnight under argon gas at room temperature. The resulting mixture was then placed in a dialysis bag with a molecular weight cutoff of 3.5 kDa for 48 h to remove the residual free peptides. After dialysis, the solution was dried through lyophilization. The peptide conjugation was confirmed by liquid chromatography-mass spectrometry (LC/MS) (Shimadzu, Japan) and the coupling efficiency was quantified using a bicinchoninic acid (BCA) assay, yielding 97.2 ± 2%.

Preparation of FMSN(BBR)-CXCR4BPL(PTX)

The conventional thin-film hydration method was applied to prepare PTX-loaded liposomes [70, 71] composed of 1,2-distearoyl-sn-glycero-3-phosphocholine, 1,2-dioleoyl-3-trimethylammonium-propane, cholesterol, and CXCR4BP-conjugated lipids (CXCR4BP-lipids) (67:8:20:5, molar ratio). Briefly, the lipids (lipid-to-FMSN mass ratio 10:1) and PTX (5%, *w/w*) were dissolved in chloroform, and the organic solvent was evaporated under reduced pressure at 70 °C using a hot drying oven for 2 h to form a thin film. The thin film was then hydrated with distilled water at 60 °C for 10 min. Following hydration, the lipid mixture was mixed with FMSN(BBR) solution and subjected to be stirred for 24 h. The mixture was then sonicated in an ice-water bath using probe sonication (on 2 s/off 2 s, 20 min, 26% amplitude). To remove any unencapsulated drugs, the FMSN(BBR)-CXCR4BPL(PTX) were centrifuged at 23,000 g for 10 min three times. Finally, the nanocarriers were dried through lyophilization. DiI-labeled CXCR4BPLs were prepared using the same methods, except that DiI was added before formation of the thin film.

Physicochemical characterization of FMSN-CXCR4BPL

The mean particle sizes and nanoparticle number concentration of FMSN, FMSN-CXCR4BPL, and CXCR4BPL were measured using nanoparticle tracking

analysis (NTA) with a NanoSight NS300 (Malvern Instruments Ltd., Worcestershire, UK, camera level = 12, detection threshold = 5). The polydispersity indexes (PDIs) and zeta potentials (surface charges) of FMSN, FMSN-CXCR4BPL, and CXCR4BPL were determined via dynamic light scattering (DLS) using a Zetasizer Nano ZS system (Malvern Instruments Ltd., Worcestershire, UK). The stability of the core-shell nanocarriers was assessed by monitoring changes in size and PDI over 7 days. Morphology and composition were confirmed through FE-TEM coupled with EDS and field emission scanning electron microscopy (FE-SEM). Additionally, CLSM was used to validate liposome coating and drug loading. Protein quantification of FMSN and FMSN-CXCR4BPL was performed using the bicinchoninic acid (BCA) assay.

BBR or PTX encapsulation efficiency and release profiles

The amount of loaded BBR or PTX was calculated using a microplate reader and high-performance liquid chromatography (HPLC, Agilent, CA, USA). To determine the amount of BBR or PTX in the core-shell nanocarrier, the FMSN-CXCR4BPL was processed with 1 N HCl using a bath sonicator (100 W). Encapsulation efficiency (EE) was then calculated using the following equation:

$$\text{Drug encapsulation (\%)} = \left(\frac{\text{Amount of drug encapsulated in FMSN - CXCR4BPL}}{\text{Total amount of drug}} \right) \times 100 \quad (1)$$

Using Eq. (1) and the measured encapsulation efficiencies (BBR $78.8 \pm 1.9\%$; PTX $75.2 \pm 2.4\%$), the resulting idealized composition corresponds to $\approx 10:0.8:0.4$ (lipid: BBR: PTX, w/w). At the working particle concentration applied in vitro (1×10^8 particles/mL), this equates to an effective BBR: PTX ratio of $\approx 2.5:1$ (w/w) in the assay medium. For in vivo studies, dosing was scaled to a BBR-equivalent 5 mg/kg, implying ≈ 2 mg/kg PTX delivery under the fixed core-shell formulation.

To measure the release profile of BBR or PTX, 400 μ L of FMSN(BBR)-CXCR4BPL(PTX) was placed in a dialysis bag and immersed in 14 mL of PBS (pH 7.4) with constant shaking (100 rpm) at 37 °C. At predefined time intervals (15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, 24 h, 48 h, and 72 h), 1 mL of the dialysis buffer was withdrawn for quantification and immediately replaced with an equal volume of fresh PBS, thereby maintaining sink conditions throughout the 72-h release period. The amount of released BBR or PTX was determined using a microplate reader or HPLC to quantify the concentration of each drug.

Biocompatibility of FMSN-CXCR4BPL

The LIVE/DEAD™ Viability/Cytotoxicity Kit (Invitrogen™, USA) was used to assess the cytotoxicity of FMSN and FMSN-CXCR4BPL via fluorescence microscopy. CT26 and hASC cells were seeded at a density of 3×10^4 cells/mL in 24-well plates. Regarding hASCs, the experiments were conducted using mid-passage cells (P5–P6). After 24 h, the cells were exposed to FMSN or FMSN-CXCR4BPL (1, 10, and 100 μ g/mL) in the cell-culture medium for 48 h. Following the 48-h incubation, the cells were treated with a live/dead solution (5 μ L of calcein AM for live cells and 20 μ L of ethidium homodimer (EthD-1) for dead cells in 10 mL of cell medium) for 30 min before observation under fluorescence microscopy. To evaluate the biocompatibility of FMSN and FMSN-CXCR4BPL, a cell counting kit-8 (CCK-8, DOJINDO, Japan) assay was performed. CT26 and hASC cells were seeded at a density of 1×10^4 cells/mL in 96-well plates and incubated for 24 h. The cells were then exposed to FMSN or FMSN-CXCR4BPL (1, 10, and 100 μ g/mL) for 48 h. After the 48-h incubation, the cell-culture medium was replaced with a 10% CCK-8 solution in each well and incubated at 37 °C for an additional 2 h. The absorbance of each

well was measured at 450 nm using a microplate reader. Cell viability was calculated based on the relative absorbance compared to the control absorbance. The use of hASCs was approved by the Institutional Review Board of Chung-Ang University Hospital (Approval No. 2151-005–463) and conducted in compliance with the principles outlined in the Declaration of Helsinki.

In vitro cellular uptake of FMSN-CXCR4BPL for validation of cancer-cell-targeting ability via confocal laser scanning microscopy (CLSM) and flow cytometry

The cancer cell targeting ability was determined via CLSM. CT26 cells were seeded (1×10^4 cells/chamber) on 4-well glass-chamber slides (SPL, Cell Culture Slide) in cell-culture medium. After 24 h, the cells were treated with fresh medium containing either 1×10^8 FMSN or 1×10^8 FMSN-CXCR4BPL for 0.5, 1, 3, and 5 h at 37 °C. Following incubation, the chambers were washed at least three times with PBS (pH 7.4). The cells were then incubated in PBS with rhodamine phalloidin for 2 h for cytoplasm staining and DAPI for 3 min for nuclear staining.

After washing several times with PBS, the chambers were mounted and examined using a Confocal Zeiss LSM 900 microscope, with ZEISS ZEN lite software used for setup. CLSM acquisition settings were: FITC ($\lambda_{\text{ex}}=488$ nm; emission 500–550 nm), DAPI ($\lambda_{\text{ex}}=405$ nm; emission 410–480 nm), and rhodamine–phalloidin ($\lambda_{\text{ex}}=561$ nm; emission 570–620 nm). For uptake experiments, fluorescence originated from FITC-labeled FMSN. CLSM images were analyzed in ImageJ with thresholds set from no-particle controls to quantify the percentage of FITC-positive cells and integrated density per cell.

Flow cytometry was used to determine the cancer cell targeting ability. CT26 cells (2×10^5 cells/mL) were cultured overnight at 37 °C under a 5% CO₂ atmosphere. The cellular uptake of FMSN and FMSN-CXCR4BPL was measured by incubating the cells with 1×10^8 nanoparticles for 0.5, 1, 3, and 5 h. Following incubation, the cells were washed twice with PBS and analyzed on a BD Accuri C6 Plus flow cytometer (BD Bioscience, USA) using 488 nm excitation with FITC collected in FL1 (533/30). Data were gated by FSC/SSC to exclude debris, and doublets were removed by FSC-A vs. FSC-H discrimination. The percentage of FITC-positive cells and mean fluorescence intensity (MFI) were quantified relative to unstained controls. Each experiment was performed in triplicate using independent replicates.

In vitro cancer migration and cytotoxicity test of FMSN(BBR)-CXCR4BPL(PTX) for validation of anti-cancer effect

Cell migration was assessed using a scratch assay. CT26 colon carcinoma cells were seeded at 8×10^6 cells/mL in a 60-mm dish and incubated at 37 °C for 24 h to form a confluent monolayer. Subsequently, scratches were made using a 10 μ L micropipette tip. The scratched cell monolayers were then treated with cell-culture medium including BBR (1 μ g/mL)/PTX (0.4 μ g/mL), FMSN containing BBR (1 μ g/mL)/PTX (0.4 μ g/mL), and FMSN-CXCR4BPL containing BBR (1 μ g/mL)/PTX (0.4 μ g/mL). After 0, 6, 12, and 24 h cell culture, images of the scratch areas were captured using phase-contrast microscopy and analyzed using Image J software. The extent of cell migration was determined using the following equation:

$$\text{Relative gap area} = \left(\frac{\text{gap area at the indicated time point}}{\text{gap area at 0 h}} \right) \quad (2)$$

Quantitative cytotoxicity was determined using a CCK-8 assay in 96-well plates. CT26 cells were seeded at a concentration of 7×10^3 cells/mL and incubated for 24 h. The cells were then treated with BBR (1 μ g/mL)/PTX (0.4 μ g/mL), FMSN containing BBR (1 μ g/mL)/PTX (0.4 μ g/mL), and FMSN-CXCR4BPL containing BBR (1 μ g/mL)/PTX (0.4 μ g/mL) for 24, 48, and 72 h. At each time point, 10%

CCK-8 solution was added to each well and incubated at 37 °C for an additional 2 h. The absorbance of each well was measured at 450 nm using a microplate reader. Cell viability was calculated as the relative absorbance compared to the control absorbance. Qualitative cytotoxicity of each group was assessed using the LIVE/DEAD™ Viability/Cytotoxicity Kit (Invitrogen™, USA). CT26 cells at a density of 3×10^4 cells/mL were seeded in 24-well plates. After 24 h of culture, the cells were treated with BBR (1 μ g/mL)/PTX (0.4 μ g/mL), FMSN containing BBR (1 μ g/mL)/PTX (0.4 μ g/mL), and FMSN-CXCR4BPL containing BBR (1 μ g/mL)/PTX (0.4 μ g/mL) for 24, 48, and 72 h. At each time point, the CT26 cells were incubated with the live/dead solution for 30 min before fluorescence microscopy.

In vivo evaluation of tumor targetability and biodistribution

Balb/c mice were subcutaneously (s.c.) injected with 1×10^6 CT26 cells into the right flank to induce tumor formation. Once the tumors reached an approximate volume of 100 mm³, the mice received intravenous (i.v.) injections of FMSN and FMSN-CXCR4BPL loaded with IR780 for imaging (IR780 dose: 0.5 mg/kg). The bio-distribution and tumor-targeting efficiency of FMSN-CXCR4BPL were monitored over time using optical imaging, with the assistance of the fluorescence-labeled organism bio-imaging system (FOBI; Neo-Science, Gyeonggi, Korea). Near-infrared fluorescence (NIRF) intensity measurements were used to evaluate the tumor accumulation of FMSN-CXCR4BPL. Regions of interest (ROIs) were defined for each organ using the Set ROI function in the Neo Image FOBI software, and fluorescence signals were quantified using the Measure tool. The resulting intensity was expressed as IU (Intensity/min/gain) and averaged across three animals per group. After 48 h post-injection, the mice were euthanized, and both tumors and major organs (liver, lungs, spleen, heart, and kidneys) were harvested. NIRF images of the excised tissues were then analyzed to determine the distribution and tumor accumulation of the treatment. All animal experiments were conducted in compliance with the Institutional Animal Care and Use Committee (IACUC) guidelines of Chonnam National University, South Korea (Approval No. CNU IACUC-H-2024-23).

In vivo antitumor activity

BALB/c mice were s.c. injected with 1×10^6 CT26 cells into the right flank to establish tumors. Once the tumors reached an approximate volume of 100 mm³, the mice were randomly assigned to one of six groups (four mice per group): PBS (control), free BBR, free PTX, a combination of BBR/PTX, FMSN(BBR/PTX), and FMSN(BBR)-CXCR4BPL(PTX). Treatments were administered

intravenously, with each mouse receiving 100 μ L of either PBS or the prepared nanoparticle formulations, corresponding to a BBR dose of 5 mg/kg, on day 0. Tumor size was measured every 2 d using Vernier calipers, and tumor volume was calculated using the equation:

$$\text{length} \times \text{width}^2 \times \frac{1}{2} \quad (3)$$

Body weight was recorded at each measurement point. On day 21 post-treatment, the mice were euthanized, and tumors, along with major organs (heart, liver, spleen, lungs, and kidneys), were harvested. Tumors and major organs were fixed in 10% neutral buffered formalin, then paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E). The H&E-stained sections were subsequently examined under an inverted light microscope.

Immunohistochemical analysis

Following deparaffinization, tissue slides underwent antigen retrieval through heat-mediated incubation in citrate buffer (pH 6.0) at 60 °C for 15 min. After retrieval, sections were blocked with 5% bovine serum albumin (BSA) to prevent non-specific binding. The slides were then incubated overnight at 4 °C with primary antibodies specific for Ki67 (Rabbit polyclonal anti-Ki67, Abcam, cat# ab15580; dilution 1:250) and CD31 (PECAM-1, D8V9E XP[®] Rabbit mAb, Cell Signaling Technology, cat# 77699; dilution 1:200), diluted in a blocking buffer containing 1% BSA in PBST (phosphate-buffered saline with Tween 20). Following primary antibody incubation, the sections were washed three times with PBST and subsequently incubated for 1 h at room temperature with secondary antibodies in PBST. Fluorescence images were acquired using a CLSM.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Tumor tissue sections were prepared for TUNEL assay to assess apoptotic cell death. The sections were first deparaffinized to remove embedding materials. Following deparaffinization, staining was conducted using the Dead End[™] Fluorometric TUNEL System (Promega) following the manufacturer's instructions. After staining, fluorescence images were acquired and analyzed with a CLSM to evaluate the extent of apoptosis in the tumor tissues.

Statistical analysis

Experimental data are presented as mean $\pm$ SD. Data distribution was not formally tested for normality due to the small sample sizes, and parametric tests were applied following common practice in exploratory nanomedicine studies. Unpaired Student's t-test was used for

comparisons between two groups, one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for comparisons involving more than three groups, and two-way ANOVA followed by Tukey's post-hoc test for analyses with two independent variables. Statistical significance was determined with the following criteria: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. All analyses were performed using GraphPad Prism 8.0.2 (GraphPad Software).

Acknowledgements

This research was supported by the National Research Foundation of Korea (NRF) funded by Ministry of Science and ICT (No. RS-2024-00449435 and No. RS-2025-00513306). This research was also supported by Korean Fund for Regenerative Medicine funded by Ministry of Science and ICT, and Ministry of Health and Welfare (RS-2025-02072977, Republic of Korea).

Author contributions

Y.J. conceived and designed the project, performed all AI-driven tasks, synthesized all materials, performed physicochemical characterization, executed and analyzed all in-vitro assays, curated the data, prepared the figures and tables, and wrote the manuscript. A.B. led the in-vivo studies, with technical assistance from S.C. and A.V. J.J.M. provided feedback throughout the study. I.K.P. and H.P. supervised the research, secured funding, and contributed to project administration. All authors approved the final version for publication.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Competing interests

The authors declare no competing interests.

Ethics approval

All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee Chonnam National University (CNU IACUC-H-2024-23), South Korea.

Author details

¹School of Integrative Engineering, Chung-Ang University, Seoul, Republic of Korea

²Department of Biomedical Sciences and BioMedical Sciences Graduate Program (BMSGP), Chonnam National University Medical School, Hwasun 58128, Republic of Korea

³Department of Pharmaceutical Sciences, University of Michigan, Ann Arbor, MI, USA

⁴BioInterfaces Institute, University of Michigan, Ann Arbor, MI, USA

⁵Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI, USA

⁶DRcure Inc., Hwasun 58128, Republic of Korea

Received: 6 August 2025 / Accepted: 7 November 2025

Published online: 22 November 2025

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