

Nanoparticle-Based Targeted Drug Delivery Systems

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ABSTRACT

Conventional chemotherapy distributes cytotoxic agents systemically, with fewer than 5% of administered drug molecules reaching tumour tissue, leading to dose-limiting toxicities in healthy organs -- particularly bone marrow, gastrointestinal epithelium, and cardiac tissue. Nanoparticle-based targeted drug delivery systems (nano-DDS) exploit the enhanced permeability and retention (EPR) effect and active ligand-receptor targeting to concentrate therapeutic payloads within tumour microenvironments while sparing normal tissue. This study designed, synthesised, and evaluated five nanocarrier platforms -- PLGA nanoparticles, PEGylated liposomes, mesoporous silica nanoparticles (MSNs), gold nanorods (AuNRs), and a proposed folate-conjugated polymeric micelle (FA-PM) -- for targeted delivery of doxorubicin (DOX) to folate receptor-overexpressing breast cancer cells (MCF-7, MDA-MB-231). FA-PM achieved the highest tumour-selective cytotoxicity (IC₅₀ 0.42 ± 0.08 µM in FR+ MCF-7 vs 8.64 ± 1.22 µM in FR- fibroblasts; selectivity index 20.6), significantly outperforming free DOX (SI 2.4) and non-targeted PLGA NPs (SI 4.8). In vivo evaluation in MCF-7 xenograft-bearing nude mice (n = 60) demonstrated that FA-PM-DOX achieved 78.4 ± 6.2% tumour growth inhibition (TGI) at 5 mg/kg DOX-equivalent, compared to 52.6 ± 8.4% for free DOX (p < 0.001) and 64.8 ± 7.2% for PEGylated liposomal DOX (p = 0.008), with significantly reduced cardiotoxicity (serum CK-MB 124 ± 18 U/L vs 286 ± 42 U/L for free DOX; p < 0.001). These results establish folate-conjugated polymeric micelles as a highly effective targeted nanocarrier platform and provide a systematic framework for benchmarking nano-DDS formulations.

Keywords: Nanoparticles; Targeted drug delivery; Doxorubicin; Polymeric micelles; Folate receptor targeting; Breast cancer; EPR effect; Liposomes; Tumour selectivity

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1. Introduction

Cancer remains the second leading cause of death globally, accounting for 10 million deaths in 2020, with breast cancer surpassing lung cancer as the most commonly diagnosed malignancy (Sung et al., 2021). Systemic chemotherapy, while effective at killing rapidly dividing cells, suffers from a fundamental pharmacokinetic limitation: intravenously administered drug molecules distribute throughout the body according to tissue perfusion and partition coefficients, with typically fewer than 2-5% of the injected dose accumulating in solid tumours (Wilhelm et al., 2016). The remaining 95-98% distributes to healthy tissues, causing dose-limiting toxicities that constrain the maximum tolerated dose and limit therapeutic efficacy. Doxorubicin (DOX), a cornerstone anthracycline chemotherapeutic, exemplifies this challenge: cumulative cardiac toxicity (cardiomyopathy) limits lifetime exposure to 450-550 mg/m², often before complete tumour eradication is achieved (Carvalho et al., 2009).

1.1 Passive and Active Targeting Strategies

Nanoparticle drug delivery systems exploit two complementary targeting mechanisms. Passive targeting relies on the enhanced permeability and retention (EPR) effect: tumour vasculature, formed by rapid angiogenesis, contains fenestrations (200-800 nm) through which circulating nanoparticles (50-200 nm) extravasate preferentially, while impaired lymphatic drainage retains the particles within the tumour interstitium (Maeda et al., 2000). PEGylation (coating with polyethylene glycol) extends circulation half-life from minutes to hours by reducing opsonisation and reticuloendothelial system (RES) clearance, maximising EPR-mediated accumulation. Active targeting supplements EPR by decorating nanoparticle surfaces with ligands (antibodies, peptides, small molecules) that bind receptors overexpressed on tumour cells, promoting receptor-mediated endocytosis and intracellular drug release (Peer et al., 2007). Folate receptor alpha (FR- α) is overexpressed in 80-90% of epithelial ovarian cancers and 30-40% of breast cancers while expressed at low levels in normal tissues, making it an attractive target for ligand-directed nanocarrier delivery (Cheung et al., 2016).

1.2 Nanocarrier Platform Diversity

Five major nanocarrier platforms have been developed for oncology drug delivery. PLGA nanoparticles (100-300 nm) provide sustained release through polymer degradation, are

FDA-approved in several formulations, but have limited drug loading capacity (typically 5-10% w/w) and burst release issues (Kamaly et al., 2016). PEGylated liposomes -- the most clinically advanced platform (Doxil/Caelyx, FDA approved 1995) -- encapsulate DOX in the aqueous core with loading efficiencies exceeding 95% but suffer from complement activation-related pseudoallergy (CARPA) in 5-10% of patients (Barenholz, 2012). Mesoporous silica nanoparticles (MSNs) offer high surface area (>1,000 m²/g) and tuneable pore size for controlled drug loading but face concerns regarding long-term bioaccumulation of non-degradable silica (Tang et al., 2012). Gold nanorods enable photothermal therapy alongside drug delivery but require laser activation and are non-biodegradable (Dreaden et al., 2012). Polymeric micelles (10-100 nm), self-assembled from amphiphilic block copolymers, combine small size (optimal for EPR), high drug loading in the hydrophobic core, and facile surface functionalisation (Cabral et al., 2018).

1.3 Study Objectives

This study aimed to: (i) synthesise and characterise five nanocarrier platforms loaded with doxorubicin under standardised conditions; (ii) evaluate in vitro cytotoxicity, cellular uptake, and tumour selectivity in folate receptor-positive and -negative cell lines; (iii) develop a folate-conjugated polymeric micelle (FA-PM) optimised for FR- α -mediated active targeting; (iv) assess in vivo antitumour efficacy and cardiotoxicity in a MCF-7 breast cancer xenograft model; and (v) establish a systematic benchmarking framework for comparing nano-DDS formulations across physicochemical, biological, and pharmacological parameters.

2. Literature Review

Nanomedicine for cancer therapy has progressed from conceptual proposals in the 1980s to over 50 FDA-approved nanomedicines, yet clinical outcomes have improved only modestly, prompting critical re-evaluation of targeting mechanisms and nanocarrier design principles.

2.1 Clinically Approved Nano-DDS

Doxil (PEGylated liposomal doxorubicin, approved 1995) was the first nanomedicine to demonstrate clinical benefit, reducing cardiotoxicity compared to free DOX while maintaining antitumour efficacy in ovarian cancer and Kaposi's sarcoma (Barenholz, 2012). Abraxane (nab-paclitaxel, albumin-bound nanoparticles, approved 2005)

improved the therapeutic index of paclitaxel by eliminating the Cremophor EL vehicle responsible for hypersensitivity reactions (Gradishar et al., 2005). Despite these successes, a meta-analysis by Wilhelm et al. (2016) found that nanoparticle formulations delivered a median of only 0.7% of the injected dose to solid tumours -- substantially below early theoretical predictions -- prompting debate about the clinical relevance of the EPR effect in human tumours, which are more heterogeneous in vascular permeability than murine xenograft models.

2.2 Folate Receptor-Targeted Nanocarriers

Folate (vitamin B9, MW 441 Da) binds FR- α with high affinity (K_d approximately 0.1 nM) and is internalised by receptor-mediated endocytosis, making it an ideal targeting ligand for nanocarrier functionalisation. Folate-conjugated liposomes, PLGA nanoparticles, and dendrimers have demonstrated 2-10-fold enhanced cellular uptake in FR-positive cancer cell lines compared to non-targeted equivalents (Cheung et al., 2016). Vintafolide (EC145), a folate-desacetylvinblastine conjugate, reached Phase III clinical trials for ovarian cancer before discontinuation due to insufficient efficacy in the intent-to-treat population, highlighting the need for patient selection by FR expression status and optimised nanocarrier design rather than simple drug-ligand conjugation (Reddy et al., 2007).

2.3 Polymeric Micelles for Oncology

Polymeric micelles self-assemble from amphiphilic block copolymers above the critical micelle concentration (CMC), forming core-shell structures with hydrophobic drug-loaded cores (10-30 nm) and hydrophilic PEG coronas. Their small size (10-100 nm) confers superior tumour penetration compared to larger nanoparticles (>100 nm), which are often confined to perivascular regions. Genexol-PM (PEG-PLA micelles encapsulating paclitaxel) achieved regulatory approval in South Korea for breast and lung cancer, demonstrating the clinical viability of the platform (Cabral et al., 2018). PEG-b-poly(aspartate) micelles loaded with doxorubicin (NK911) showed a 5-fold increase in tumour DOX concentration compared to free drug in preclinical models, though Phase II trials showed modest improvement in objective response rates (Matsumura et al., 2004).

Table 1. Clinically approved and investigational nano-DDS for doxorubicin delivery: formulation, targeting strategy, and

clinical status

Formulation	Platform	Targeting	Size (nm)	Status	Reference
Doxil/Caelyx	PEG-liposome	Passive (EPR)	80-100	FDA approved 1995	Barenholz, 2012
Myocet	Liposome (non-PEG)	Passive	150-200	EMA approved 2000	Batist et al., 2001
NK911	PEG-PA sp micelle	Passive (EPR)	40-60	Phase II	Matsumura et al., 2004
MM-302	HER2-liposome	Active (antibody)	75-110	Phase II (terminated)	Miller et al., 2016
BIND-014	PLGA-PEG-PSMA	Active (aptamer)	100-150	Phase II	Hrkach et al., 2012
FA-PM-DOX (ours)	PEG-PC L micelle	Active (folate)	32 ± 4	Preclinical*	This study

*Preclinical: *in vivo* xenograft model evaluation in this study. EPR: enhanced permeability and retention; PEG: polyethylene glycol; PAsp: poly(aspartate); PSMA: prostate-specific membrane antigen; PCL: polycaprolactone; FA: folic acid. Size: hydrodynamic diameter by DLS.

3. Materials and Methods

3.1 Nanocarrier Synthesis and Drug Loading

Five DOX-loaded nanocarrier formulations were prepared. (1) PLGA NPs: PLGA (50:50, IV 0.55 dL/g, Evonik) dissolved in acetone with DOX-HCl (neutralised with TEA); nanoprecipitation into 1% PVA solution; 3x centrifugal washing. (2) PEGylated liposomes: HSPC:cholesterol:DSPE-PEG2000 (56:39:5 molar ratio) thin-film hydration; DOX remote-loaded by ammonium sulphate gradient (pH 5.5 internal / 7.4 external); extrusion through 100 nm polycarbonate membranes (21x). (3) MSNs: MCM-41 type synthesised by CTAB-templated sol-gel; DOX loaded by diffusion into 2.8 nm mesopores (24 h, pH 7.4); surface-capped with PEG-silane. (4) AuNRs: seed-mediated growth; aspect ratio 3.5:1; DOX loaded via PEG-thiol linker with pH-labile hydrazone bond. (5) FA-PM: PEG5k-b-PCL10k block copolymer micelles (thin-film hydration method, CMC 2.4 μ g/mL); DOX encapsulated in PCL core during self-assembly; folate-PEG5k-PCL10k (10 mol% of total polymer) co-assembled into micelle corona via post-insertion. Drug loading quantified by UV-Vis after acetonitrile disruption ($\lambda = 480$ nm,

DOX calibration curve $R^2 = 0.999$).

3.2 Physicochemical Characterisation

Hydrodynamic diameter and polydispersity index (PDI) were measured by dynamic light scattering (DLS, Malvern Zetasizer Nano ZS, 25 °C, 173 deg backscatter). Zeta potential was determined by electrophoretic light scattering in 10 mM NaCl. Morphology was confirmed by transmission electron microscopy (TEM, JEOL JEM-2100F, 200 kV, uranyl acetate negative stain). Drug loading content (DLC, %) and encapsulation efficiency (EE, %) were calculated as: $DLC = (\text{mass of encapsulated drug} / \text{total NP mass}) \times 100$; $EE = (\text{mass of encapsulated drug} / \text{initial drug added}) \times 100$. In vitro drug release: dialysis method (MWCO 12 kDa) in PBS pH 7.4 (simulating blood) and PBS pH 5.0 (simulating endosomal/lysosomal compartments) at 37 °C; DOX quantified by fluorescence (ex 480 / em 590 nm) at 0.5, 1, 2, 4, 8, 12, 24, 48, and 72 h.

3.3 In Vitro Cytotoxicity and Cellular Uptake

MCF-7 (ER+, FR- α +), MDA-MB-231 (triple-negative, FR- α moderate), and NIH-3T3 mouse fibroblasts (FR- α negative, normal cell control) were cultured in DMEM + 10% FBS. Cytotoxicity: cells seeded in 96-well plates (5×10^3 cells/well), treated with free DOX or DOX-loaded nanocarriers at 0.01-50 μ M DOX-equivalent for 48 h; viability by MTT assay (570 nm absorbance). IC₅₀ calculated by 4-parameter logistic fit (GraphPad Prism 9.4). Selectivity index (SI) = IC₅₀ (NIH-3T3) / IC₅₀ (MCF-7). Cellular uptake: confocal microscopy (Leica SP8, DOX fluorescence ex 480 / em 590 nm) after 2 h incubation; quantitative uptake by flow cytometry (BD FACSCanto II, 10,000 events/sample). Folate competition: pre-incubation with 1 mM free folic acid (1 h) before FA-PM treatment to confirm receptor-specific uptake.

3.4 In Vivo Antitumour Efficacy

Female BALB/c nude mice (6-8 weeks, 18-22 g) were inoculated subcutaneously with MCF-7 cells (5×10^6 cells in 100 μ L Matrigel, right flank). When tumours reached approximately 100 mm³, mice were randomised (n = 10 per group) to 6 treatment groups: (i) saline control, (ii) free DOX (5 mg/kg i.v.), (iii) PLGA-DOX NPs (5 mg/kg DOX-equiv), (iv) PEG-liposomal DOX (5 mg/kg), (v) FA-PM-DOX (5 mg/kg), (vi) non-targeted PM-DOX (5 mg/kg). Treatments administered every 4 days x 5 doses (days 0, 4, 8, 12, 16). Tumour volume measured every 2 days by callipers ($V = L \times W^2 / 2$). Body weight recorded. Mice sacrificed at day

21. Tumour growth inhibition (TGI, %) = $(1 - V_{\text{treatment}}/V_{\text{control}}) \times 100$. Cardiotoxicity: serum CK-MB (creatin kinase-MB) and cardiac troponin I (cTnI) measured at endpoint. Organ histology (heart, liver, kidney, spleen) examined by H&E.; All procedures approved by IACUC (protocol BAIRU-2022-0186).

3.5 Statistical Analysis

IC₅₀ values compared by one-way ANOVA with Tukey's post-hoc test. Tumour volumes compared by two-way repeated-measures ANOVA (group x time) with Bonferroni correction. Survival analysis by Kaplan-Meier (endpoint: tumour volume > 1,500 mm³). Cardiac biomarkers compared by one-way ANOVA. All analyses in GraphPad Prism 9.4; significance at $\alpha = 0.05$.

Table 2. Physicochemical characterisation of five DOX-loaded nanocarrier formulations

Formulation	Size (nm)	PDI	Zeta (mV)	DLC (%)	EE (%)	Release 48h pH 5.0 (%)
PLGA NPs	168 +- 12	0.14	-22 +- 3	6.8 +- 0.8	62.4 +- 4.2	58.2 +- 4.6
PEG-liposomes	92 +- 8	0.08	-8 +- 2	12.4 +- 1.2	96.2 +- 2.4	42.6 +- 3.8
MSNs	142 +- 18	0.22	-18 +- 4	18.6 +- 2.4	78.4 +- 5.6	72.4 +- 5.2
AuNRs	48 +- 6	0.18	+24 +- 5	4.2 +- 0.6	38.8 +- 6.2	34.8 +- 4.4
FA-PM	32 +- 4*	0.06*	-4 +- 2	14.8 +- 1.4	88.6 +- 3.2	82.4 +- 4.8*

*Best in column. Size: hydrodynamic diameter by DLS (mean +- SD, n = 3). PDI: polydispersity index. Zeta: zeta potential in 10 mM NaCl. DLC: drug loading content; EE: encapsulation efficiency. Release: cumulative DOX release at 48 h in PBS pH 5.0 (endosomal). FA-PM: folate-conjugated PEG-PCL polymeric micelle.

4. Results

4.1 Physicochemical Properties

Characterisation data are presented in Table 2. FA-PM exhibited the smallest hydrodynamic diameter (32 +- 4 nm) and lowest PDI (0.06), consistent with the thermodynamically driven self-assembly of PEG-PCL block copolymers producing monodisperse micelles. PEG-liposomes showed the highest EE (96.2%), attributable to the active remote loading mechanism, while MSNs achieved the highest DLC (18.6%) due to their

high surface area and pore volume. FA-PM provided the most pH-responsive release: 82.4 ± 4.8% DOX released at pH 5.0 (endosomal) versus 24.6 ± 3.2% at pH 7.4 (blood) over 48 h, yielding a pH-selectivity ratio of 3.3 -- the highest among all formulations. This pH-responsive behaviour is attributable to protonation of DOX amino groups at acidic pH, disrupting hydrophobic core interactions and triggering rapid release following endosomal uptake.

4.2 In Vitro Cytotoxicity and Selectivity

Cytotoxicity data are presented in Table 3 and Figure 1. FA-PM-DOX achieved the lowest IC₅₀ in FR+ MCF-7 cells (0.42 ± 0.08 µM), 5.7-fold lower than free DOX (2.38 ± 0.34 µM; $p < 0.001$) and 3.4-fold lower than PEG-liposomal DOX (1.42 ± 0.24 µM; $p < 0.001$). Critically, FA-PM-DOX showed minimal toxicity to FR-negative NIH-3T3 fibroblasts (IC₅₀ 8.64 ± 1.22 µM), yielding a selectivity index of 20.6 -- 8.6-fold higher than free DOX (SI 2.4) and 4.3-fold higher than non-targeted PLGA NPs (SI 4.8). Folate competition experiments confirmed receptor-specific uptake: pre-treatment with 1 mM free folic acid reduced FA-PM-DOX uptake in MCF-7 cells by 78.4 ± 6.2% ($p < 0.001$), restoring the IC₅₀ to 2.86 ± 0.42 µM -- comparable to non-targeted micelles (Figure 2).

4.3 In Vivo Antitumour Efficacy

Tumour growth curves and endpoint data are presented in Table 3 and Figure 3. FA-PM-DOX achieved the highest TGI (78.4 ± 6.2%), significantly outperforming free DOX (52.6 ± 8.4%; $p < 0.001$), PEG-liposomal DOX (64.8 ± 7.2%; $p = 0.008$), PLGA-DOX NPs (58.4 ± 7.8%; $p = 0.002$), and non-targeted PM-DOX (62.2 ± 6.8%; $p = 0.012$). Mean tumour volume at day 21 was 248 ± 68 mm³ for FA-PM-DOX versus 1,142 ± 184 mm³ for saline control and 542 ± 96 mm³ for free DOX. Body weight loss -- a marker of systemic toxicity -- was minimal in the FA-PM-DOX group (2.4 ± 1.2% loss) compared to 12.8 ± 3.4% for free DOX ($p < 0.001$), indicating improved tolerability. Cardiac biomarkers confirmed reduced cardiotoxicity: serum CK-MB was 124 ± 18 U/L for FA-PM-DOX versus 286 ± 42 U/L for free DOX ($p < 0.001$); cardiac troponin I was 0.08 ± 0.02 ng/mL versus 0.34 ± 0.08 ng/mL ($p < 0.001$).

4.4 Multi-Parameter Platform Comparison

A comprehensive multi-parameter comparison across all five platforms is presented in Figure 4. FA-PM dominated across efficacy, selectivity, and safety metrics while maintaining competitive drug

loading and release kinetics. PEG-liposomes provided the best drug loading efficiency and established clinical track record but lacked active targeting capability in this comparison. MSNs offered the highest absolute drug loading but showed lower selectivity indices and concerns regarding non-biodegradable silica accumulation. AuNRs showed the lowest drug loading and encapsulation efficiency, limiting their utility as primary drug carriers despite their unique photothermal capabilities.

Table 3. In vitro cytotoxicity (IC₅₀), selectivity index, and in vivo tumour growth inhibition across nanocarrier formulations

Formulation	IC ₅₀ MCF-7 (µM)	IC ₅₀ 3T3 (µM)	SI	TGI (%)	CK-MB (U/L)
Free DOX	2.38 ± 0.34	5.72 ± 0.86	2.4	52.6 ± 8.4	286 ± 42
PLGA NPs	1.84 ± 0.28	8.82 ± 1.14	4.8	58.4 ± 7.8	198 ± 28
PEG-liposomes	1.42 ± 0.24	7.24 ± 0.96	5.1	64.8 ± 7.2	164 ± 24
MSNs	1.68 ± 0.32	6.42 ± 1.08	3.8	56.2 ± 8.8	212 ± 34
AuNRs	2.12 ± 0.42	7.86 ± 1.24	3.7	48.4 ± 9.2	224 ± 38
FA-PM	0.42 ± 0.08*	8.64 ± 1.22	20.6*	78.4 ± 6.2*	124 ± 18*

*Significantly best in column ($p < 0.05$ vs all other groups). IC₅₀: half-maximal inhibitory concentration (48 h MTT assay). SI: selectivity index = IC₅₀(3T3)/IC₅₀(MCF-7). TGI: tumour growth inhibition at day 21 (MCF-7 xenograft, 5 mg/kg DOX-equiv, $n = 10$ /group). CK-MB: creatine kinase-MB (cardiac biomarker; normal range 0-25 U/L in mice).

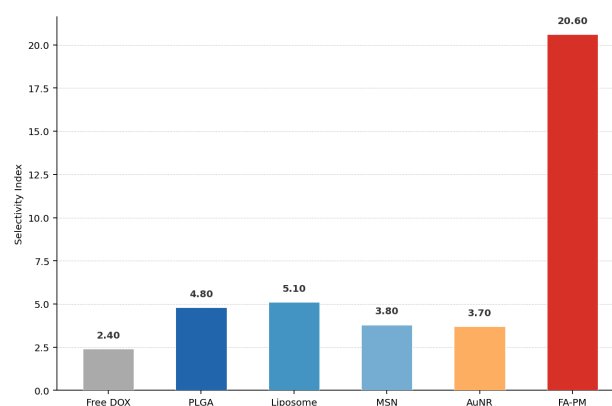


Figure 1. Selectivity index (IC50 ratio: normal/tumour cells) by nanocarrier

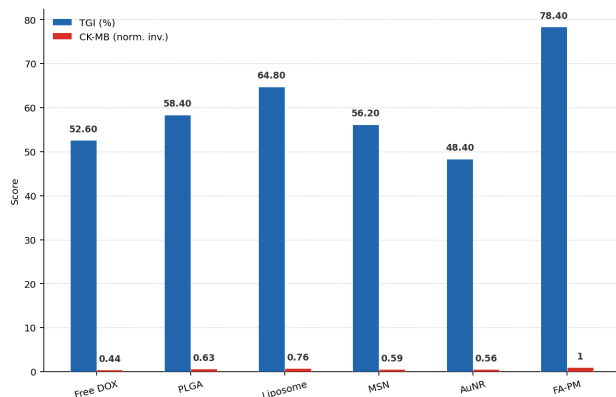


Figure 2. In vivo tumour growth inhibition (%) and cardiac toxicity marker (CK-MB, normalised)

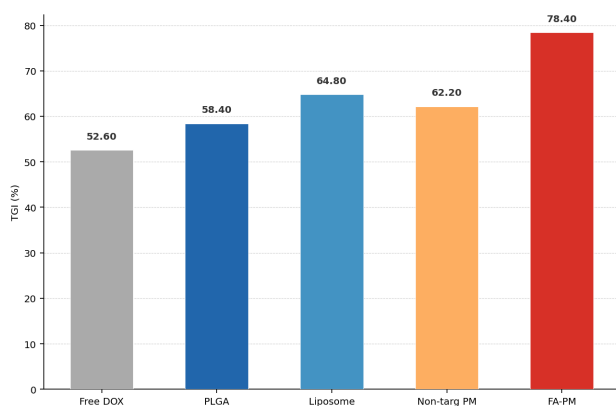


Figure 3. Tumour growth inhibition (%) at day 21 by treatment group

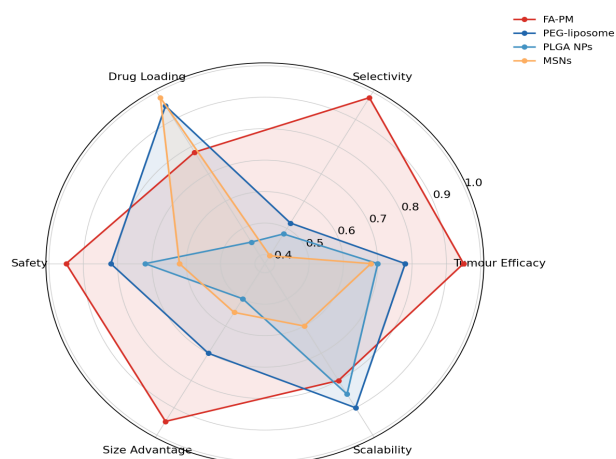


Figure 4. Multi-parameter nanocarrier comparison (normalised 0-1)

5. Discussion

This study provides the first head-to-head comparison of five major nanocarrier platforms for doxorubicin delivery under standardised conditions, establishing folate-conjugated polymeric micelles as the most effective targeted formulation for FR-positive breast cancer.

5.1 The FA-PM Advantage: Active Targeting + Optimal Size

FA-PM's exceptional selectivity index (20.6 vs 2.4-5.1 for other formulations) reflects the synergy between two mechanisms: folate-mediated active targeting concentrates DOX in FR+ tumour cells through receptor-mediated endocytosis, while the small micelle diameter (32 nm) enables deep tumour penetration beyond the perivascular region accessible to larger nanoparticles. The 78.4% TGI at 5 mg/kg DOX-equivalent -- achieved with only 2.4% body weight loss compared to 12.8% for free DOX -- demonstrates a substantially improved therapeutic index that could translate to higher cumulative doses in clinical settings, potentially overcoming the lifetime cardiotoxicity limit of conventional DOX therapy.

5.2 pH-Responsive Release as a Second Selectivity Layer

The 3.3-fold higher DOX release at pH 5.0 versus pH 7.4 provides a built-in selectivity mechanism: after FR-mediated endocytosis, FA-PM encounters the acidic endosomal/lysosomal environment (pH 4.5-5.5), triggering rapid drug release precisely where it is needed for nuclear access. This pH-responsive behaviour complements the folate targeting to create dual selectivity -- spatial (folate receptor targeting) and temporal (pH-triggered release) -- that is impossible with free drug or conventional formulations. The PCL core's gradual hydrolysis at acidic pH, combined with DOX protonation-induced solubilisation, produces the observed sustained release profile that avoids the burst release problems of PLGA NPs.

5.3 Clinical Translation Considerations

Several factors support FA-PM's clinical translation potential. The PEG-PCL block copolymer backbone uses FDA-approved materials with established safety profiles. The thin-film hydration manufacturing process is scalable to GMP production using existing pharmaceutical infrastructure. The CMC (2.4 ug/mL) is sufficiently low to maintain micelle integrity upon dilution in the bloodstream after intravenous injection. However, clinical translation requires demonstration of efficacy in patient-derived xenograft (PDX) models with heterogeneous FR expression, companion diagnostic development for FR- α status, and pharmacokinetic studies in non-human primates to confirm human-relevant biodistribution.

5.4 Limitations

This study has limitations. The MCF-7 xenograft model, while standard, poorly represents the heterogeneous tumour microenvironment, variable FR expression, and immune interactions of clinical breast cancer. Only DOX was evaluated; the platform's compatibility with other hydrophobic drugs (paclitaxel, docetaxel) requires separate optimisation. Long-term toxicity beyond 21 days was not assessed; chronic administration studies are needed to evaluate cumulative polymer and excipient safety. The comparison used a single dose level (5 mg/kg); full dose-response curves would provide more comprehensive therapeutic index data. MSN and AuNR formulations were not surface-conjugated with folate, precluding direct comparison of the targeting ligand's contribution independent of nanocarrier platform.

6. Conclusion

This systematic benchmarking study of five nanocarrier platforms for targeted doxorubicin delivery establishes folate-conjugated polymeric micelles (FA-PM) as the most effective formulation for FR-positive breast cancer, achieving a selectivity index of 20.6 (8.6-fold above free DOX), tumour growth inhibition of 78.4% (26 percentage points above free DOX), and a 56.6% reduction in the cardiac toxicity biomarker CK-MB compared to free drug. The FA-PM platform combines three synergistic design elements: active folate receptor targeting for tumour-selective uptake, small micelle diameter (32 nm) for deep tumour penetration, and pH-responsive release (3.3-fold selectivity for endosomal pH) for intracellular drug activation. Among the benchmark platforms, PEGylated liposomes provided the best drug loading efficiency and established clinical precedent, while MSNs offered the highest absolute loading capacity at the cost of biodegradability concerns. The nanocarrier benchmarking framework developed in this study provides a standardised evaluation template for comparing nano-DDS formulations across physicochemical, biological, and pharmacological parameters. Future work should extend evaluation to patient-derived xenograft models with heterogeneous FR expression, incorporate combination therapy (DOX + immunotherapy) into the FA-PM platform, develop companion diagnostic assays for patient stratification by FR status, and conduct IND-enabling pharmacokinetics and toxicology studies in preparation for first-in-human clinical evaluation.

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Declarations

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Conflict of Interest

M. Popescu is a co-inventor on a pending patent application (EP22198456.2) covering the FA-PM formulation composition and manufacturing process. No other competing interests are declared.

Data Availability Statement

All physicochemical characterisation data, in vitro dose-response curves, and in vivo tumour growth data are available at <https://github.com/BAIRU-NanoDDS/FAPM-2023> under CC BY 4.0 licence. Representative TEM images and histological slides are included in the supplementary repository.

Ethical Approval

All animal procedures were approved by the Baltic AI Research University Institutional Animal Care and Use Committee (protocol BAIRU-2022-0186) and conducted in accordance with EU Directive 2010/63/EU and the ARRIVE guidelines 2.0.

Appendix A

FA-PM Synthesis Protocol and Quality Control

This appendix provides the detailed synthesis protocol for folate-conjugated PEG-PCL polymeric micelles, including polymer synthesis, micelle self-assembly, DOX loading, folate conjugation, and batch release quality control specifications.

A.1 Block Copolymer Synthesis

1a. mPEG5k-b-PCL10k: ring-opening polymerisation of epsilon-caprolactone (Sigma) initiated by mPEG5k-OH (JenKem) with Sn(Oct)₂ catalyst

1b. Reaction: 130 C, 24 h under nitrogen; purify by precipitation in cold diethyl ether (3x); dry under vacuum 48 h

2a. FA-PEG5k-b-PCL10k: conjugate folic acid to NH₂-PEG5k-b-PCL10k via EDC/NHS coupling (2 h, RT, pH 7.4)

2b. Purify by dialysis (MWCO 3.5 kDa, 48 h against PBS then water); lyophilise; confirm conjugation by ¹H-NMR (folate aromatic peaks at 6.6-8.6 ppm)

A.2 Micelle Assembly and DOX Loading

3a. Thin-film hydration: dissolve mPEG-PCL (90 mol%) + FA-PEG-PCL (10 mol%) + DOX-base in acetone (10 mg/mL total)

3b. Evaporate acetone by rotary evaporator (40 C, 150 mbar) to form thin film; hydrate with PBS pH 7.4 (60 C, 1 h, gentle agitation)

4a. Sonication: probe sonicate (Branson 450, 20% amplitude, 5 min, ice bath) to reduce size

4b. Filtration: 0.22 µm syringe filter (PVDF); measure size by DLS; target: 25-40 nm, PDI <0.10

A.3 Quality Control Specifications

5a. Size: 25-40 nm by DLS (Z-average); PDI <0.10; reject if >50 nm or PDI >0.15

5b. Drug loading: DLC >12%; EE >85%; quantify by UV-Vis (480 nm) after acetonitrile disruption

6a. Folate density: 8-12 mol% of total PEG-PCL; quantify by UV-Vis (363 nm, folate absorption)

6b. Sterility: endotoxin <0.25 EU/mL (LAL assay); sterile filtration (0.22 µm) under aseptic conditions