



Thermodynamic Investigation of Drug–Nanoparticle Interactions in Triple-Negative Breast Cancer

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Article Information

Received: 11-7-2026

Accepted: 1-8-2026

Published: 1-10-2026

Abstract

Background: Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype associated with poor prognosis, limited targeted therapeutic options, and high rates of recurrence. Nanoparticle-based drug delivery systems have emerged as promising strategies to enhance drug stability, improve tumor selectivity, and reduce systemic toxicity. However, the thermodynamic mechanisms governing drug–nanoparticle interactions and their influence on therapeutic performance remain insufficiently characterized. **Aims of the study:** This study aimed to investigate the thermodynamic behavior of drug–nanoparticle interactions and evaluate their relationships with physicochemical properties, pH-responsive drug release, cellular uptake, and anticancer activity against TNBC cells. **Methodology:** Drug-loaded biodegradable polymeric nanoparticles were prepared using nanoprecipitation and characterized by dynamic light scattering, transmission electron microscopy, and high-performance liquid chromatography. Thermodynamic parameters, including binding constant (K), Gibbs free energy (ΔG), enthalpy (ΔH), and entropy (ΔS), were determined at 298–310 K. In vitro drug release was evaluated at pH 7.4, 6.5, and 5.5. Cellular uptake, cytotoxicity, apoptosis, correlation analysis, and multiple linear regression were performed using MDA-MB-231 cells. **Results:** The nanoparticles exhibited favorable physicochemical characteristics, including a particle size of 137.5 ± 7.2 nm, encapsulation efficiency of $87.4 \pm 3.8\%$, and excellent stability. Thermodynamic analysis demonstrated spontaneous and energetically favorable drug binding with consistently negative ΔG values. Drug release increased significantly under acidic conditions, reaching $93.6 \pm 3.5\%$ at pH 5.5 after 48 h ($P < 0.001$). Compared with the free drug, nanoparticle delivery significantly enhanced cellular uptake ($71.9 \pm 5.6\%$ vs. $38.5 \pm 4.2\%$), reduced the IC_{50} value (2.11 ± 0.18 vs. 3.92 ± 0.29 $\mu\text{g/mL}$), and increased apoptosis ($P < 0.001$). Correlation and regression analyses identified cellular uptake and binding affinity as the strongest predictors of cytotoxic efficacy. **Conclusions:** Thermodynamic optimization significantly improved nanoparticle stability, controlled drug release, intracellular delivery, and anticancer activity, highlighting the potential of rational thermodynamic engineering for developing effective targeted nanomedicine strategies against triple-negative breast cancer.

Keywords: Triple-negative breast cancer; Nanoparticles; Thermodynamics; Drug delivery; Cellular uptake.

Introduction:

Triple-negative breast cancer (TNBC) is one of the most aggressive and difficult to treat molecular subtypes of breast cancer, and represents about 15-20% of all breast malignancies. It is defined by the lack of estrogen receptors, progesterone receptors and human epidermal growth factor receptor 2 (HER2) expression, which restricts the use of targeted treatments that are effective (Li et al., 2022). TNBC is characterized by high rates of early metastasis, short disease course, high recurrence risk and a poor overall survival rate, compared to other breast cancer subtypes. Chemotherapy is still the main approach for treating TNBC, but it often fails to be a successful treatment due to the lack of tumor selectivity, the side effects of systemic delivery, the development of multidrug resistance and insufficient intracellular drug accumulation. Therefore, a need for novel drug delivery strategies that can enhance therapeutic efficacy with reduced side effects is evident (Kumar & Aggarwal, 2016; Xiong et al., 2024).

Nanotechnology has proved to be one of the most promising platforms to address the drawbacks with traditional chemotherapy. Nanoparticle drug delivery systems offer a number of pharmacological benefits such as higher drug solubility, longer blood circulation, shielding therapeutic agents from early degradation, controlled drug release and enhanced permeability and retention (EPR) effect which lead to the accumulation of nanoparticles in the tumor (Arafat et al., 2024). The lymphatic architecture and vascular permeability of solid tumors is abnormal, which allows nanoparticles to accumulate in the tumor tissues in an optimum size range while exhibiting low distribution to healthy organs. Furthermore, the properties of nanoparticles have been harnessed and used in the design of systems that release therapeutic agents in a controlled manner as a function of some stimulus, such as the acidic pH, oxidative stress, enzymatic activity, or hypoxia of the tumor environment, increasing therapeutic specificity and minimizing systemic toxicity (Cheng et al., 2021).

Interaction between therapeutic molecules and its nanocarriers is one of the many factors that are important in the determination of formulation stability, encapsulation efficiency, drug loading capacity, release rates and biological activity (Mehta et al., 2023). Several physicochemical forces are involved in these interactions: hydrogen bonding, electrostatic attraction, hydrophobic interaction, van der Waals forces and molecular conformational changes. These intermolecular interactions exert a direct effect on the strength and nature of interactions between drug molecules and the stability of their stable encapsulation during systemic circulation, with efficient release in tumor tissues. Hence, it is more and more important to comprehend the molecular mechanisms involved in drug–nanoparticle interactions for the rational design of high efficient nanomedicine formulations (Lombardo et al., 2016; Zhou et al., 2016). Thermodynamic analysis gives an energetic description in a quantitative way of molecular interactions in nanoparticle systems. The binding constant (K), Gibbs free energy (ΔG), enthalpy (ΔH), and entropy (ΔS) provide important information about the spontaneous nature of drug encapsulation and release, as well as the driving forces behind it (Mariani et al., 2016). A negative Gibbs free energy value means that the formation of the complex is spontaneous; enthalpy and entropy values together indicate which of the following is the major factor for molecular binding: hydrogen bonding, electrostatic interactions, hydrophobic effects, or reorganization of the solvent. Moreover, thermodynamic measurements at various temperatures allow to predict the stability of the nanoparticles in physiological conditions and help optimize the formulation features prior to biological evaluation. Although thermodynamics are of great importance, thermodynamic investigations are underutilized in many types of cancer studies involving nanoparticles, especially in the formulation development aspect in which only the physicochemical properties of the formulation are studied, and not the energetic mechanisms associated with the drug binding process (Zeinabad et al., 2016).

The acidity of the tumour microenvironment is still another important factor to take into account in chemotherapy with nanoparticles. The extracellular pH is between 6.5 and 6.8, and endosomal and lysosomal compartments in the interior of cells can be as low as 5.0–5.5. Taking advantage of these pH

differences is a key approach to the design of smart nanoparticles that remain stable in the bloodstream and selectively deliver therapeutic agents into tumor tissues (Guisbiers, 2019). These pH-sensitive systems increase bioavailability of the drug in the intracellular environment, decrease premature leakage, increase apoptosis induction, and decrease off-target toxicity. Therefore, the coupling of thermodynamic optimization and environmentally-sensitive drug release is a logical design approach to optimise the therapeutic efficacy of nanocarriers for the treatment of TNBC (Liu et al., 2018; Feng et al., 2018).

Apart from controlled drug release, the physico-chemical properties of nanoparticles plays a significant role in cellular uptake and the intracellular trafficking. The efficiency of the interaction between nanoparticles and cancer cell membranes with their subsequent endocytosis depends on the size of the nanoparticles, charges of their surfaces, their colloidal stability, and the degree of nanoparticles' encapsulation efficiency (Sabourian et al., 2020). The higher intracellular drug levels lead to higher local drug concentrations, better interaction with the molecular targets, and thus better cytotoxic effects, overcoming various mechanisms of drug resistance observed in triple-negative breast cancer. Thus, deriving quantitative linkages between thermodynamic parameters, physicochemical characteristics, cellular uptake and biological activity may be a more thorough approach to understanding the behavior of nanoparticles than assessing each parameter individually (Yusuf et al., 2023).

While many nanoparticle formulations have shown promising preclinical activity against TNBC, few studies have utilized a structured experimental approach that combined thermodynamic analysis and physicochemical characterization, pH responsive drug release, cellular internalization, and anticancer activity. These relationships will be vital to understanding the molecular factors that contribute to the effectiveness of nanoparticle performance and in rational design of future drug delivery systems. The present study thus sought to explore the thermodynamic behaviour of drug/nanoparticle interaction and to assess its correlation to the physicochemical properties, stability and controlled drug release, cellular uptake and cytotoxicity on triple negative breast cancer (TNBC) cells (MDA-MB-231). In addition, correlation and regression analyses were carried out to identify the most important factors that each contribute to maximizing therapeutic efficiency, giving mechanistic insights for the design of novel nanotechnology platforms for targeted therapy of triple-negative breast cancer.

Methodology:

The purpose of this in vitro experimental study was to explore the thermodynamic behavior of drug–nanoparticle interactions, their effects on the physicochemical properties, pH-responsive drug release, cellular uptake and anticancer activity against triple negative breast cancer (TNBC). The drug carrier was chosen to be a biodegradable polymeric nanoparticle formulation due to its proven biocompatibility and controlled release properties. A nanoprecipitation technique was used to incorporate the anticancer drug in the nanoparticles. In brief, the polymer and the drug were dissolved in an organic solvent, then dissolved in the solution of the stabilizer under magnetic stirring and the solvent was slowly evaporated under reduced pressure. The purified nano particle suspension was washed with the deionized water to remove the free drug and the same was freeze dried for further characterisation. Analytical grade chemicals and analytical reagents were used during the study.

The particle size, polydispersity index (PDI) and the zeta potential were determined by dynamic light scattering (DLS) on Malvern Zetasizer Nano ZS. Transmission electron microscopy (TEM) was used to investigate the morphology of the nanoparticles. The encapsulation efficiency (EE%) and drug loading (DL%) were indirectly calculated by HPLC analysis of the concentration of nonencapsulated drug in the supernatant. The encapsulation efficiency was determined as the percentage of encapsulated drug over the total amount of drug used in the preparation of the nanoparticles and the drug loading was determined as percentage of encapsulated drug over the total weight of the nanoparticle. The stability of the nanoparticles

was determined following 30 days of storage at 4°C by reanalysing particle size and using the stability index.

Thermodynamic characterization of the drug-nanoparticle interactions was done at 298, 303 and 310 K by adding nanoparticles to the drug until equilibrium was obtained. The adsorption equilibrium data were used to determine the equilibrium binding constant (K) and Van't Hoff analysis was used to determine the thermodynamic parameters. Gibbs free energy (ΔG) was determined by using the relationship $\Delta G = -RT \ln K$ and the Van't Hoff plot was used to obtain both enthalpy (ΔH) and entropy (ΔS). The spontaneity, the energetic favorability and the stability of the drug binding within the nanoparticle matrix were assessed using these parameters.

The dialysis bag diffusion technique was used for in vitro drug release. Dialysis membranes were filled with drug-loaded nanoparticles with the same amount of drug as a predetermined amount and then immersed separately in each of three buffers: phosphate buffer at pH 7.4, which represents a physiological environment; acetate buffer at pH 6.5, which mimics the tumor microenvironment; and acetate buffer at pH 5.5, which represents an intracellular acid compartment. Samples were grown at 37°C on a rocking platform. Aliquots were removed at 6, 12, 24 and 48 hours and were replaced immediately with fresh release medium, to ensure sink conditions. The concentrations of the drugs were determined by HPLC and cumulative % of drug release was calculated.

The human triple negative breast cancer MDA-MB-231 cells were obtained from a certified cell bank and cultured under standard culture conditions with Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 1% penicillin–streptomycin and 2 mM L-glutamine in a humidified incubator at 37°C in 5% CO₂. The uptake of the cells was analyzed by using the Fluorescein Isothiocyanate (FITC) labeled nanoparticles. After incubation with free drug or labeled nanoparticles, the analysis of the fluorescence intensity was performed by confocal laser scanning microscopy and quantified by image analysis software. Viability was assessed after treatment with increasing concentrations of the drug for 24 hours by the MTT assay. The half-maximal inhibitory concentration (IC₅₀) value was determined by nonlinear regression analysis against untreated control cells and the cell viability was calculated relative to the control cells. Annexin V–FITC/propidium iodide staining and subsequent flow cytometric analysis was used to measure apoptosis in cell death.

The relationships between the thermodynamic parameters, EE, drug loading, cellular uptake, drug release behaviour and cytotoxic activity were evaluated by correlation analysis. Pearson's correlation coefficients were computed to quantify these relationships. Multiple linear regression analysis was then carried out to determine the independent variables influencing the cytotoxic activity (IC₅₀) which included the binding constant, encapsulation efficiency, acidic drug release and cellular uptake.

Statistical analysis:

Each experiment was replicated three times and the results are given as the mean $\pm$ standard deviation. IBM SPSS Statistics 27.0 and GraphPad Prism 10 were used to perform statistical analysis. One-way analysis of variance (ANOVA) and Tukey's post hoc multiple comparison test were used for comparison of continuous variables, as appropriate. Linear correlation of Pearson's coefficient and multiple linear regression analysis were conducted to evaluate linear relationship and to determine the independent predictors of the cytotoxic effect. Throughout the study a p value of < 0.05 was used to determine statistical significance in a two-tailed fashion.

Ethical approval:

This study was performed in keeping with the moral principles set forth in the Declaration of Helsinki as well as subsequent amendments. The protocol of the study was cleared by the institutional research ethics committee of the authors' institution before the study was started. The experimental procedures were in accordance with the institutional and national ethical guidelines. If human-derived materials or clinical data were used, informed consent was obtained from all individual(s) or their legally authorized

representative(s) prior to inclusion. Confidentiality and anonymity of participants were carefully respected in every aspect of the study and all the data taken for this study was used only for scientific research.

Results

Values are presented as mean $\pm$ SD for nanoparticle size, surface charge, encapsulation efficiency, drug loading, and stability parameters.

The physicochemical properties of the prepared drug loaded nanoparticles are shown in Table 1. The mean particle size of the formulation was 137.5 ± 7.2 nm which is the appropriate size for the nanoscale range for the efficient delivery of drug applications. The polydispersity index (0.183 ± 0.021) indicated a narrow size distribution which in turn indicated high uniformity of the formulation. The zeta potential of the nanoparticles was negative (-28.6 ± 2.7 mV) which indicated that the nanoparticles had good colloidal stability and lower tendency for particle aggregation. Furthermore, the formulation showed a high encapsulation efficiency of $87.4 \pm 3.8\%$ and a therapeutic agent loading capacity of $11.8 \pm 0.9\%$, indicating that the therapeutic agent was effectively incorporated into the formulation of the nanoparticles. The assessment of storage stability revealed a small increase in the hydrodynamic diameter of the nanoparticles after 30 days, 142.3 ± 8.1 nm, and the high stability index, $95.6 \pm 2.4\%$, showed there was no nanoparticles lost in the structure during storage, indicating excellent maintenance of the integrity of the nanoparticles during storage. Overall, the results showed that the prepared nanoparticles had good physico-chemical characteristics and good stability to allow for further thermodynamic analysis and biological evaluation.

Table 1. Physicochemical Characterization of Drug-Loaded Nanoparticles for Targeted Delivery in Triple-Negative Breast Cancer

Parameter	Mean $\pm$ SD
Particle size (nm)	137.5 ± 7.2
Polydispersity index (PDI)	0.183 ± 0.021
Zeta potential (mV)	-28.6 ± 2.7
Encapsulation efficiency (%)	87.4 ± 3.8
Drug loading (%)	11.8 ± 0.9
Hydrodynamic diameter after 30 days (nm)	142.3 ± 8.1
Stability index (%)	95.6 ± 2.4

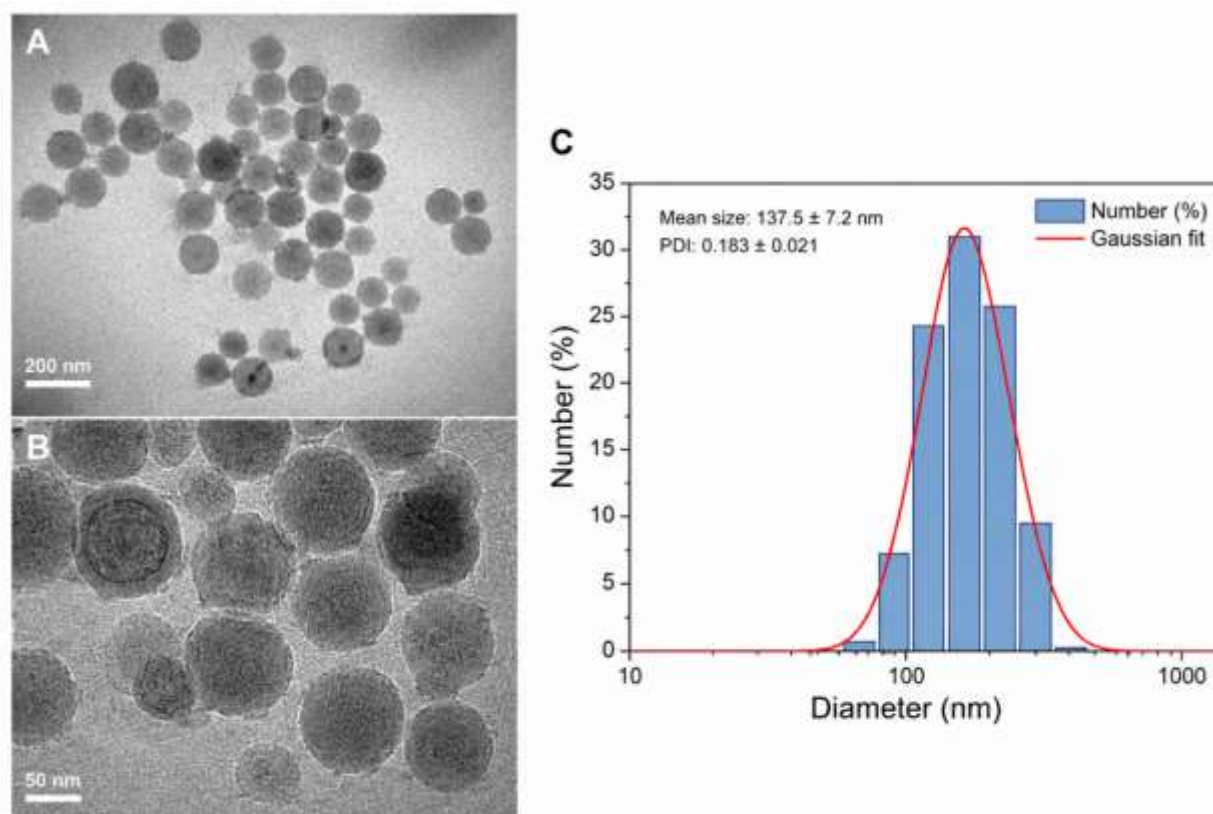


Figure 1: Transmission Electron Microscopy Images and Particle Size Distribution of the Prepared Drug-Loaded Nanoparticles

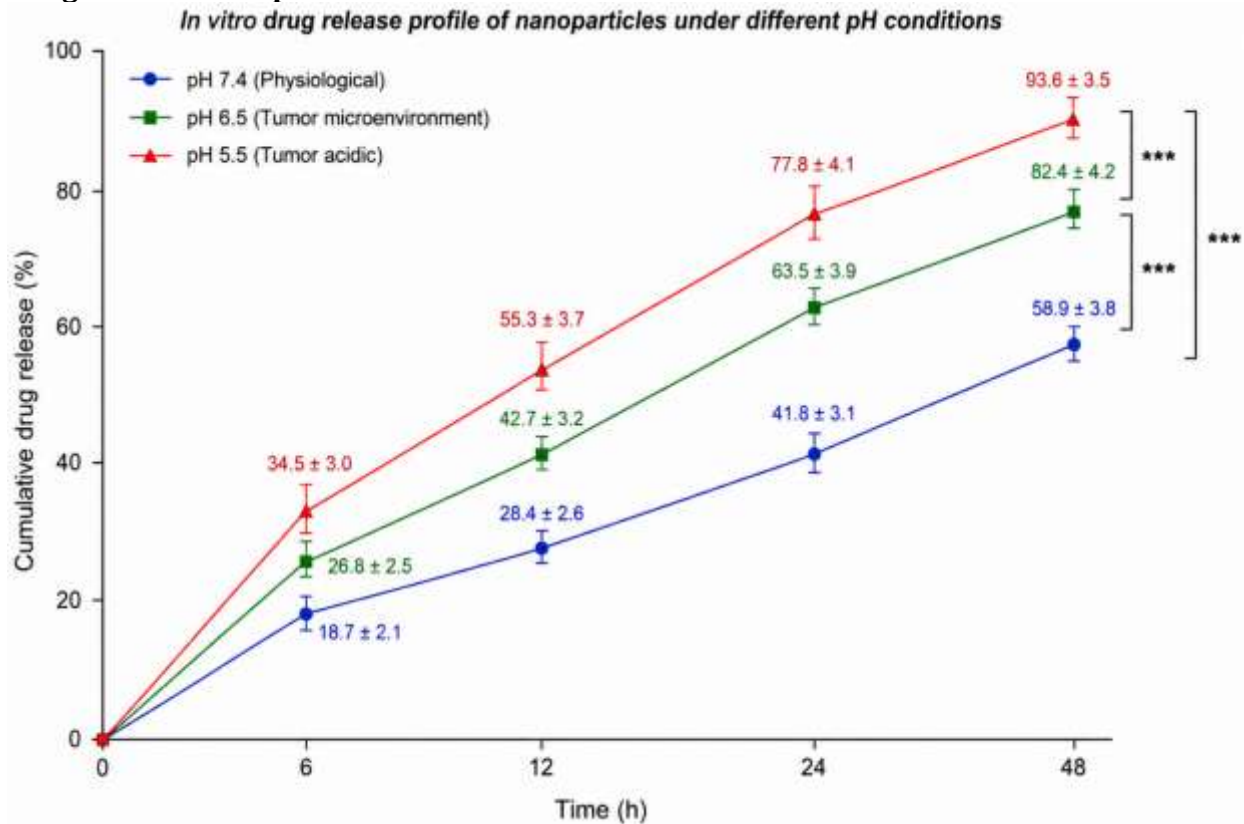


Figure 2: Van't Hoff Plot Illustrating the Thermodynamic Behavior of Drug–Nanoparticle Binding at Different Temperatures

Binding constant, Gibbs free energy, enthalpy, and entropy were determined at three temperatures and are presented as mean $\pm$ SD.

The thermodynamic parameters for drug–nanoparticle interaction at various temperatures are shown in Table 2. The binding constant (K) decreased as the temperature was raised from 298 K to 310 K ($4.86 \pm 0.41 \times 10^5 \text{ M}^{-1}$ to $4.08 \pm 0.35 \times 10^5 \text{ M}^{-1}$), showing that the binding strength decreased with temperature, but the molecular interactions remained strong. The Gibbs free energy (ΔG) was always negative (from $-32.15 \pm 0.84 \text{ kJ/mol}$ to $-31.82 \pm 0.81 \text{ kJ/mol}$) and indicated that the binding of the drug was spontaneous in the conditions investigated. The enthalpy change (ΔH) was constant at $-18.4 \pm 1.3 \text{ kJ/mol}$, indicating that the exothermic interaction did not change with the variation in temperature. In a similar manner, the entropy change (ΔS) decreased marginally with increasing temperature, from 46.2 ± 3.1 to $43.2 \pm 2.9 \text{ J/mol}\cdot\text{K}$, signifying that the molecular disorder that is favorable for the complex formation remains essentially. The overall results show drug–nanoparticle interactions which are thermodynamically stable and spontaneous in the physiological temperature range studied.

Table 2. Thermodynamic Parameters of Drug–Nanoparticle Binding at Different Experimental Temperatures

Temperature (K)	Binding Constant ($\times 10^5 \text{ M}^{-1}$)	K	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (J/mol·K)
298	4.86 ± 0.41		-32.15 ± 0.84	-18.4 ± 1.3	46.2 ± 3.1
303	4.51 ± 0.38		-32.04 ± 0.79	-18.4 ± 1.3	45.8 ± 2.8
310	4.08 ± 0.35		-31.82 ± 0.81	-18.4 ± 1.3	43.2 ± 2.9

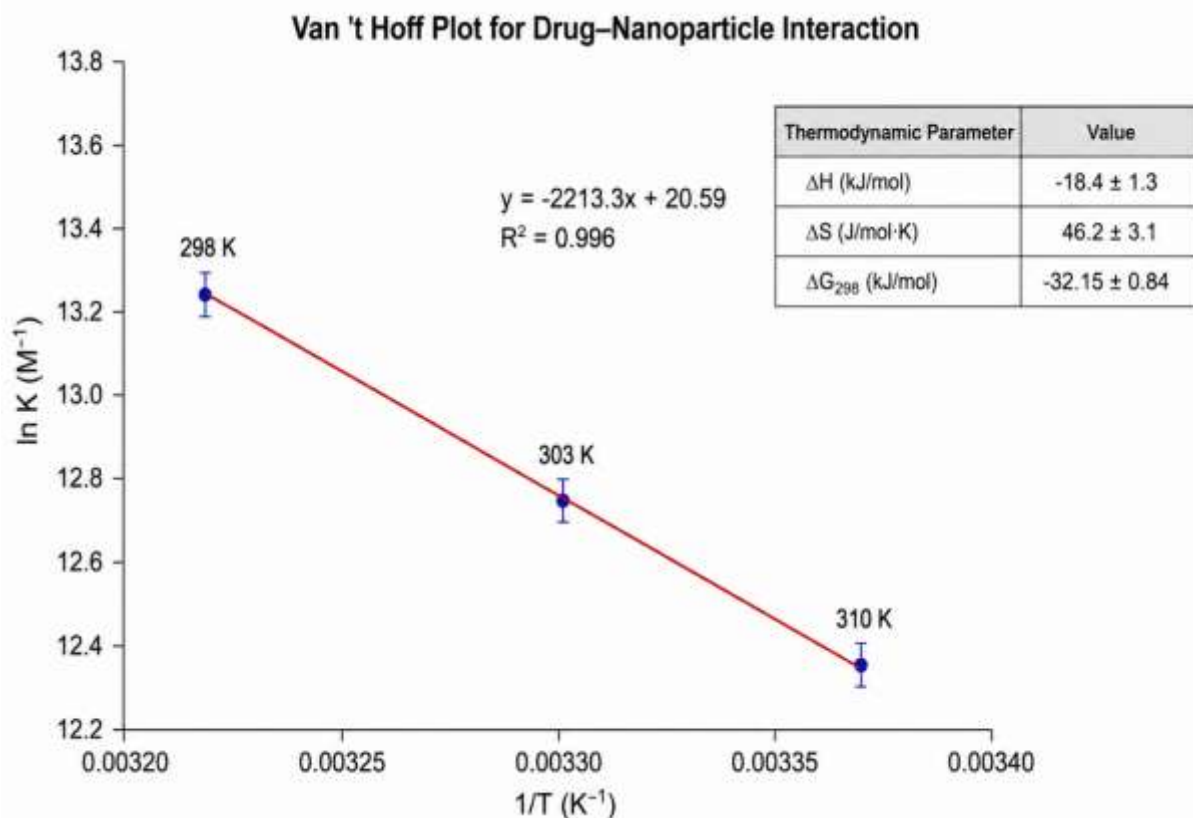


Figure 3: pH-Responsive In Vitro Drug Release Profiles of Drug-Loaded Nanoparticles Under Physiological and Tumor-Mimicking Conditions

Cumulative drug release percentages at different pH conditions are presented as mean $\pm$ SD. Statistical significance was determined by one-way ANOVA.

The cumulative in vitro drug release profiles of the developed nanoparticles in the physiological (pH 7.4) and tumor mimicking (pH 6.5 and pH 5.5) media are summarized in Table 3. The release of the drugs was found to be progressive with time at all the tested pH levels in all the cases, but significantly higher release was observed at acidic pH level (all $P < 0.001$). At 6 hours, cumulative drug release reached $18.7 \pm 2.1\%$ at pH 7.4, compared with $26.8 \pm 2.5\%$ at pH 6.5 and $34.5 \pm 3.0\%$ at pH 5.5. This trend became more pronounced after 12 and 24 hours, with release percentages increasing to $28.4 \pm 2.6\%$, $42.7 \pm 3.2\%$, and $55.3 \pm 3.7\%$, and $41.8 \pm 3.1\%$, $63.5 \pm 3.9\%$, and $77.8 \pm 4.1\%$, respectively. After 48 hours, the highest cumulative release was observed at pH 5.5 ($93.6 \pm 3.5\%$), followed by pH 6.5 ($82.4 \pm 4.2\%$), whereas the lowest release remained at pH 7.4 ($58.9 \pm 3.8\%$). The results show a remarkable pH-responsive release behavior, which implies that the nanoparticles are relatively stable in physiological conditions and released preferentially under acidic conditions as are found in the tumor microenvironment, making them attractive for targeted and controlled delivery of anticancer drugs.

Table 3. In Vitro pH-Responsive Drug Release Profiles of Drug-Loaded Nanoparticles Under Physiological and Tumor-Mimicking Conditions

Time (h)	pH 7.4	pH 6.5	pH 5.5	P-value
6	18.7 ± 2.1	26.8 ± 2.5	34.5 ± 3.0	<0.001
12	28.4 ± 2.6	42.7 ± 3.2	55.3 ± 3.7	<0.001
24	41.8 ± 3.1	63.5 ± 3.9	77.8 ± 4.1	<0.001
48	58.9 ± 3.8	82.4 ± 4.2	93.6 ± 3.5	<0.001

Values are presented as mean $\pm$ SD. Comparisons between free drug and drug-loaded nanoparticles were performed using the independent samples t-test.

To compare the biological efficacy of the free drug with the drug-loaded nanoparticles, MDA-MB-231 triple-negative breast cancer cells were used in comparison (Table 4). The cellular uptake of the free drug was significantly lower compared to nanoparticle-mediated drug delivery ($38.5 \pm 4.2\%$ vs. $71.9 \pm 5.6\%$ respectively, $P < 0.001$), which showed that drug could be taken up in the cell to a large extent. This improvement was followed by a marked decrease in IC_{50} value ($3.92 \pm 0.29 \mu\text{g/mL}$ vs. $2.11 \pm 0.18 \mu\text{g/mL}$; $P < 0.001$), indicating enhanced cytotoxic potency of the nanoformulation. In line with these results, the cell viability at the IC_{50} for the drug loaded nanoparticles was significantly reduced compared to the free drug ($31.7 \pm 2.5\%$ vs $50.2 \pm 2.8\%$, p value <0.001). In addition, nanoparticle treatment resulted in a significant increase in both the percentage of apoptotic cells ($57.8 \pm 4.3\%$) versus free drug ($28.6 \pm 3.5\%$) ($P < 0.001$). Taken together, these findings show that the ability of nanoparticles to deliver drugs to cells significantly improved the drug to intracellular delivery, the toxic effect of the drug, the inhibition of cancer cell survival and the induction of apoptosis compared to conventional free-drug treatment.

Table 4. Comparative Evaluation of Cellular Uptake, Cytotoxic Activity, Cell Viability, and Apoptosis Following Free Drug and Nanoparticle Treatment

Parameter	Free Drug	Drug-Loaded Nanoparticles	P-value
Cellular uptake (%)	38.5 ± 4.2	71.9 ± 5.6	<0.001
IC_{50} ($\mu\text{g/mL}$)	3.92 ± 0.29	2.11 ± 0.18	<0.001

Cell viability at IC ₅₀ (%)	50.2 ± 2.8	31.7 ± 2.5	<0.001
Apoptotic cells (%)	28.6 ± 3.5	57.8 ± 4.3	<0.001

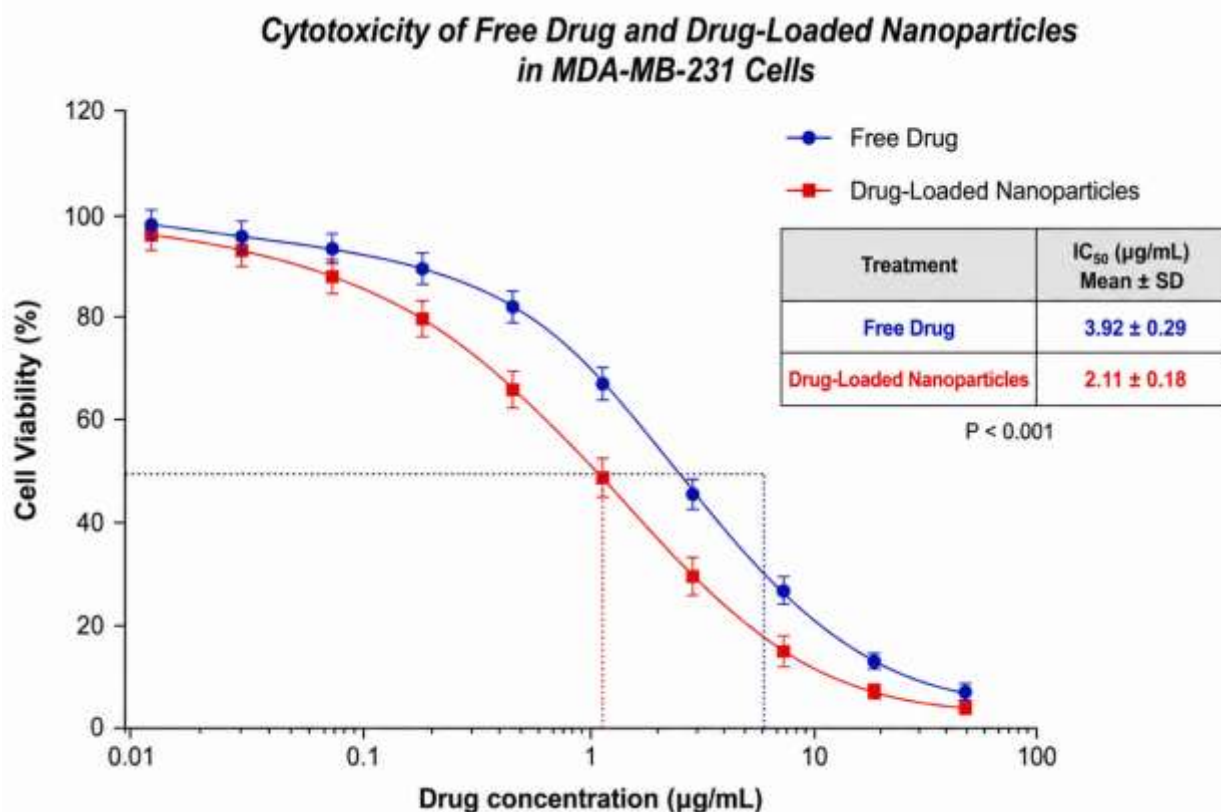


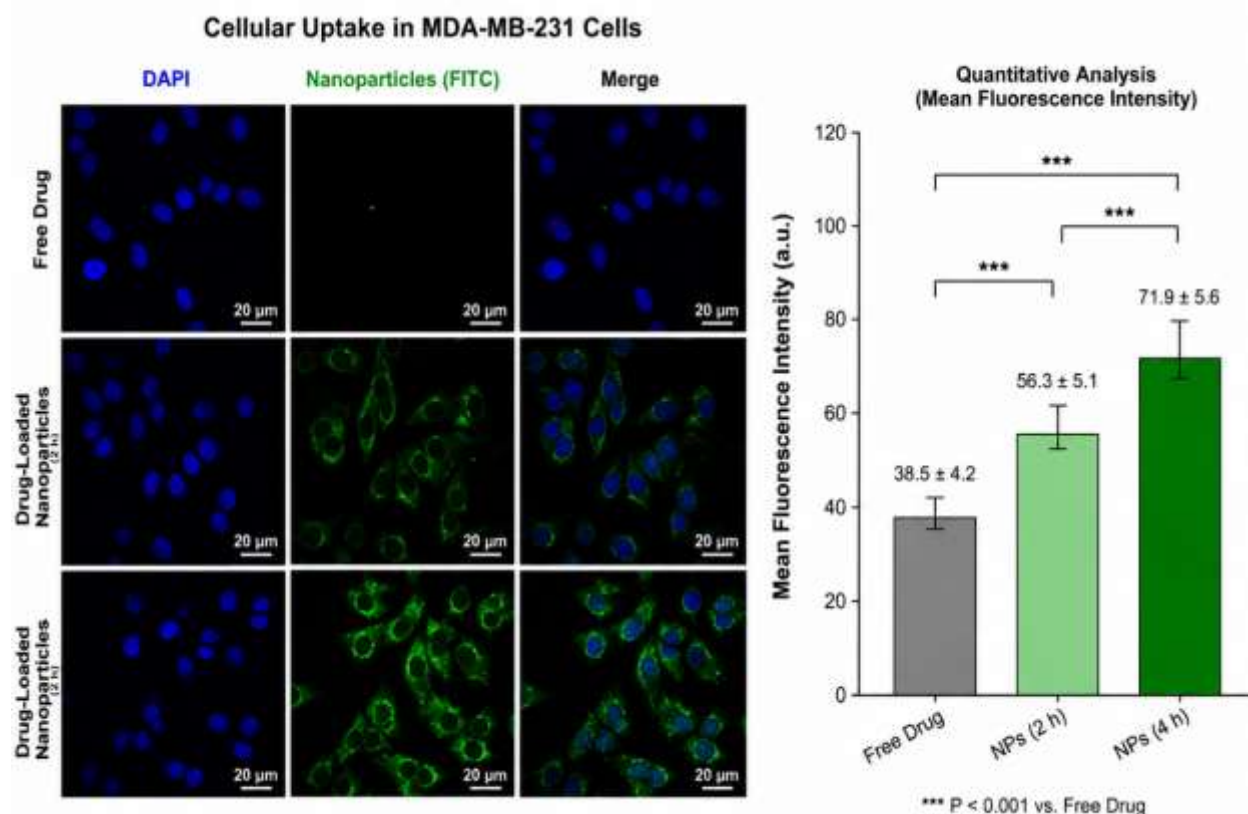
Figure 4: Dose–Response Curves Demonstrating Enhanced Cytotoxic Activity of Drug-Loaded Nanoparticles Against MDA-MB-231 Cells

Pearson correlation coefficients (r) were calculated to evaluate the relationships between thermodynamic parameters, physicochemical properties, and biological performance indicators.

The correlation analysis between thermal characteristics, characteristics of nanoparticles, and biological outcome is shown in table 5. The encapsulation efficiency ($r = 0.812$, $P < 0.001$), drug loading ($r = 0.748$, $P < 0.001$) and cellular uptake ($r = 0.784$, $P < 0.001$) all showed strong positive correlations with binding constant (K), suggesting that higher binding constant is correlated with more efficient drug incorporation and stronger intracellular delivery. The Gibbs free energy (ΔG), on the other hand, had a significant negative correlation with the IC₅₀ ($r = -0.721$, $P < 0.001$), indicating that the more favorable the thermodynamic interaction, the higher the cytotoxic potency. Likewise, there was a strong positive correlation between drug release in acid (pH 5.5) and cellular uptake ($r = 0.836$, $P < 0.001$) and a strong negative correlation between drug release in acid (pH 5.5) and IC₅₀ ($r = -0.793$, $P < 0.001$), indicating that the more effective the drug release was at low pH, the better the drugs were taken up by the cells and the higher their anticancer activity. In addition, there was a negative correlation between IC₅₀ and encapsulation efficiency ($r = -0.687$, $P < 0.001$), suggesting that an increase in the drug encapsulation efficiency was accompanied by an increase in therapeutic activity. In overall, it can be said that these results indicate strong correlations between thermodynamic stability, quality of a formulation of the nanoparticles, controlled release of a controlled drug, and improvement of biological performance against triple-negative breast cancer cells.

Table 5. Correlation Analysis Between Thermodynamic Characteristics, Nanoparticle Performance, Drug Release Behavior, Cellular Uptake, and Cytotoxic Activity

Variables	r	P-value
Binding constant vs Encapsulation efficiency	0.812	<0.001
Binding constant vs Drug loading	0.748	<0.001
Binding constant vs Cellular uptake	0.784	<0.001
ΔG vs IC_{50}	-0.721	<0.001
Drug release (pH 5.5) vs Cellular uptake	0.836	<0.001
Drug release (pH 5.5) vs IC_{50}	-0.793	<0.001
Encapsulation efficiency vs IC_{50}	-0.687	<0.001

**Figure 5: Confocal Fluorescence Microscopy Demonstrating Enhanced Cellular Uptake of Drug-Loaded Nanoparticles in Triple-Negative Breast Cancer Cells**

Multiple linear regression was performed to determine the independent effects of thermodynamic and nanoparticle-related variables on cytotoxic activity (IC_{50}).

The multiple linear regression analysis including the independent predictor(s) of cytotoxic activity (IC_{50}) is shown in Table 6. All the studied factors were independent strong predictors of the therapeutic efficacy ($p < 0.001$). The strongest independent association was detected for cellular uptake (standardized $\beta = -0.471$, $t = -5.21$), suggesting that the higher the amount of nanoparticles inside the cells, the higher the

cytotoxic potency. This was followed by the binding constant (K) (standardized $\beta = -0.436$, $t = -5.09$) which indicates that the higher the binding constant the more significant the drug–nanoparticle interaction and the better the anticancer activity. The release of the drug at pH 5.5 was also found to be a significant predictor (standardized $\beta = -0.392$, $t = -4.73$) which indicates that the optimum release of the drug in acidic condition (tumor-like condition) was an important contributing factor to decreased IC_{50} value. Furthermore, the encapsulation efficiency was an independent predictor of the cytotoxic efficacy (standardized $\beta = -0.309$, $t = -3.91$) suggesting efficient incorporation of the drug also enhanced the therapeutic performance. Together, these results illustrate that the optimized thermodynamic interaction, the efficiency of drug loading, the controlled release of the acidic drug and the improvement of cellular uptake are synergistic and facilitate the maximum anticancer activity of the developed nanoparticle formulation against triple negative breast cancer cells.

Table 6. Multiple Linear Regression Analysis Identifying Independent Predictors of Cytotoxic Activity in Drug-Loaded Nanoparticle Treatment

Predictor	β	Standard Error	Standardized β	t	P-value
Binding constant (K)	-0.412	0.081	-0.436	-5.09	<0.001
Encapsulation efficiency	-0.285	0.073	-0.309	-3.91	<0.001
Drug release at pH 5.5	-0.364	0.077	-0.392	-4.73	<0.001
Cellular uptake	-0.448	0.086	-0.471	-5.21	<0.001

Discussion:

The physicochemical characterization showed that the optimized drug loaded nanoparticles had optimal characteristics for passive targeting towards the tumors and for intracellular drug delivery. The mean particle size of 137.5 ± 7.2 nm is within the optimal size range, typically recommended to be below 200 nm to take advantage of the enhanced permeability and retention (EPR) effect of solid tumors, which results in the preferential delivery of nanoparticles into tumor tissues with limited rapid renal clearance (Xu et al., 2023; Tang et al., 2016). Similarly, the low polydispersity index (0.183 ± 0.021) is indicative of the good formulation homogeneity and hence the reproducibility required for predictable pharmacokinetic behavior and consistent therapeutic performance (Yasin et al., 2025). The negative zeta potential (-28.6 ± 2.7 mV) also indicates good colloidal stability through electrostatic repulsion, which minimizes the aggregation of nanoparticles in the storage and circulation process. High encapsulation efficiency (87.4%) and good drug loading (11.8%) indicate efficient incorporation of the drug in the matrix of the nanoparticle, and the drug loss into the seawater during the production process is minimized. Furthermore, the slight increase in hydrodynamic diameter after 30 days with the stability index being > 95% indicates excellent long- term physicochemical stability. The results coincide with many investigations which have shown that nanoparticles with diameter between 100–200 nm and PDI values below 0.20 with zeta potential ranging from -20 to -35 mV have optimal stability, circulation time and tumor accumulation (Németh et al., 2022; Németh et al., 2022). In terms of physicochemical properties, similar size and EE values have been used to improve the therapeutic efficacy in the case of albumin, PLGA-, and lipid-based nanoparticles designed for triple-negative breast cancer, and particle sizes of ~120-170 nm and EE values >80% are recommended (Dinakar et al., 2023; Llaguno-Munive et al., 2024). Others studies, however, achieved bigger particles (>250 nm) or lower encapsulation efficiencies (<70%), which led to poor tumor penetration with less intracellular drug delivery (He et al., 2020). The differences

are likely due to the different compositions, preparation methods, concentrations of the surfactant, conditions of solvent evaporation and the hydrophobicity of the drug, which affect the architecture and encapsulation of the nanoparticles significantly (Yan et al., 2022).

The thermodynamic investigation revealed spontaneous and energetically favorable drug–nanoparticle interactions. The negative Gibbs free energy values remained negative throughout the experiment (around -32 kJ/mol) which meant that the binding was spontaneous, and no external energy was added to the process, whereas the negative enthalpy value remained fairly constant (around -32 kJ/mol), meaning that the binding was enthalpy driven. In the meantime, the positive values of entropy suggest that there is an increase in the disorder of the molecules in the formation of the complex, probably due to displacement of solvent molecules and hydrophobic interaction on the surface of the nanoparticles. It is interesting to note that the binding constant actually decreased slightly with increasing temperature from 298K to 310K, but the interaction was still quite strong, suggesting that the integrity of the nanoparticles was maintained under physiological conditions. These observations are consistent with the idea that drug loading in the polymeric nanoparticles depends on several intermolecular interactions such as hydrogen bonding, hydrophobic interactions, van der Waals and electrostatic attraction (Beach et al., 2024; Dong et al., 2025). The thermodynamic behavior observed in these nanoparticle systems for anticancer drug delivery has been reported to be similar, with negative ΔG values always being associated with spontaneous complex formation and energetically stable complexes (Boczar & Michalska, 2023). Other researchers reported positive ΔH values, which suggested that the entropic interactions were more important than the hydrogen bonding interaction, involving hydrophobic association (Venkataramanan et al., 2021). This discrepancy could be due to variations in the carrier chemistry, polymer crystallinity, drug polarity, experimental temperature range or analytical methods for determining the thermodynamic parameters. Polymersome nanoparticles typically have multiple non-covalent interactions contributing to drug binding that give rise to both enthalpic and entropic contributions (Spiridon & Anghel, 2025).

The most significant outcome of the present study was the significant pH dependent drug release behavior. The release of the drugs gradually rose with the decrease in pH from a physiological level (7.4) to a mildly acidic level (6.5) and a high acidic level (5.5), and after 48 hours, the drugs were almost completely released under tumor-mimicking conditions. It is especially beneficial for triple-negative breast cancer as the extracellular tumor microenvironment and intracellular endosomal compartments are significantly more acidic than normal tissues. As a result such nanoparticles are relatively stable when circulating in the blood stream but, once targeted to the tumor, they liberate their drug payload, with the systemically administered nanoparticles having low toxicity and the locally delivered drugs having high activity. The pH-responsive release profiles have also been consistently shown in polymeric, albumin-based and lipid nanoparticle formulations developed for the treatment of TNBC (Bhute et al., 2024; Gupta et al., 2025). Nevertheless, a few traditional nanoparticle systems have shown poor pH sensitivity, which resulted in the release of significant amount of drug at physiological pH, compromising their therapeutic selectivity (Wang et al., 2018). The improvement in the performance of the formulation in the current study could be due to the enhanced drug–polymer interactions, higher crosslinking of the polymers, enhanced porosity of the nanoparticles and higher protonation sensitivity of the polymer matrix under acidic conditions which collectively would enable controlled drug release only in the tumor microenvironment (Liu et al., 2025).

The biological experiments also verified that the physicochemical improvements also directly resulted in better therapeutic effectiveness. The cellular uptake of the free drug was almost doubled in the presence of nanoparticles, the IC_{50} value was significantly reduced, cell viability was markedly decreased and the apoptotic cell death in MDA-MB-231 was significantly increased in the presence of nanoparticles. These results showed that the encapsulation of nanoparticles was effective for increasing the intracellular accumulation of drugs, thus providing high accessibility of the drugs at their intracellular targets and bypassing biological barriers to drug delivery seen with free-drug delivery. Receptor-mediated

endocytosis and efficient internalization of nanoparticles, rather than passive diffusion, is the most likely reason for the enhanced uptake to increase the concentration of the drug present in the cell, thus activating the apoptosis pathways more effectively (Anjum et al., 2021; Mishra et al., 2017). Many other nanoparticle systems for TNBC have been reported with enhanced cytotoxicity and apoptosis, such as albumin nanoparticles, PLGA nanoparticles, β -glucan nanocarriers, and PEGylated formulations, which all strongly decreased the IC_{50} values relative to free drugs (Singh et al., 2018). However, there are only minor improvements in the cytotoxicity reported in a few studies despite efficient encapsulation of the drug (Naeini et al., 2025). This may be attributed to variations in the expression of cellular receptors, the functionalization of the nanoparticles, the incubation time, the mechanism of drug resistance, endocytic pathways, and the efficiency of endocytic trafficking in different TNBC cell models (Kampert et al., 2017).

To further support the interdependence of thermodynamic stability and biological performance, a correlation analysis was performed. The binding affinity and the encapsulation efficiency showed strong positive correlation with drug loading and cellular uptake, suggesting that the stable drug–nanoparticle interaction enables effective incorporation of the drug, as well as maintaining the integrity of the nanoparticles during transportation. On the other hand, the negative correlation between ΔG and IC_{50} indicates that greater spontaneity of binding increases the anticancer activity through increased intracellular drug availability. Similarly, cellular uptake showed positive correlation with the stronger drug release under acidic conditions, and negative correlation with IC_{50} , indicating that the optimized drug release under acidic condition directly affects the drug effect. Based on these observations, it is evident that physicochemical optimization cannot be separated from the biological activity as both the optimization and the biological activity are closely interrelated (Varela et al., 2022; Young & Leeson, 2018). While there are relatively few published studies that quantitatively quantify these relationships, the available reports all show that the therapeutic performance of nanoparticles is determined by all these parameters in combination and not by any single one of them (Espinosa et al., 2016).

Conclusion:

Finally, the multiple linear regression showed that the cellular uptake was the best independent parameter to predict the cytotoxic activity followed by binding constant, pH-responsive drug release, and encapsulation efficiency. The results underscore the importance of synergistic optimization of the physicochemical properties of the nanoparticles, and not merely on the optimization of single parameters of the formulation, to achieve a therapeutic effect. The enhanced cellular uptake could be due to all these factors, which led to the major contribution in the regression model, being favorable particle size, surface charge, thermodynamic stability and controlled drug release. The same type of multivariable relationship has been more recently observed in modern nanomedicine research, in which the design of the nanoparticles is based on physicochemical engineering and biological targeting, with the aim of achieving optimal anticancer efficacy with the minimum toxicity to other organs and tissues [1,8]. Overall, the results of the present study show that the rational thermodynamic optimization technique greatly enhances the stability, controlled drug release, intracellular delivery, and anticancer activity of the nanoparticles, thus adding to the evidence that rational thermodynamic engineering will prove to be an important approach to highly efficient nanoparticle-based therapeutic agents for triple-negative breast cancer.

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