



Formulation Strategies and In Vitro Characterization of Talquetamab-Loaded Nanocarriers for Targeted Immunotherapy in Multiple Myeloma

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ABSTRACT

Background: Talquetamab is a first-in-class bispecific T-cell redirecting antibody that binds to the novel antigen, GPRC5D, which is highly expressed in multiple myeloma cells, and to CD3 on T cells, enabling T cells to mediate tumor cell lysis. Although it shows promising clinical effects, its stability, targeted delivery, and systemic distribution are still crucial factors in ensuring long-term therapeutic success.

Objective: The purpose of this study is to design and test a new polymeric nanoparticle carrier for Talquetamab to improve its stability, tumor specificity, therapeutic efficacy and reduce off-target immunotoxicity.

Methods: Talquetamab was formulated into PEGylated PLGA nanoparticles by the modified double-emulsion solvent evaporation technique. Folic acid was used to surface-functionalize the nanoparticles for improved tumor-targeting by receptor-mediated endocytosis. The characteristics of the system were determined by measuring particle size, zeta potential, encapsulation efficiency and in vitro release profile. The stability was evaluated in a simulated physiological condition. Internalization, cell death and T-cell redirection were assessed in GPRC5D+ RPMI 8226 multiple myeloma cells and CD3+ Jurkat T-cells by confocal microscopy, MTT and flow cytometry, respectively.

Results: Talquetamab loaded nanoparticles formed in this study had a mean diameter of 142.6 ± 5.2 nm, zeta potential of -28.3 mV, and the EE was 76.4%. In-vitro release studies revealed pH-responsive and sustained release up to 72 hrs. The functionalized nanoparticles demonstrated a considerable higher cellular uptake (3.6-fold) and cytotoxicity ($p < 0.01$) in GPRC5D+ cells in comparison to free antibody. Effective targeting and killing of T-cells was confirmed by flow cytometry.

Conclusion: The folate-conjugated polymeric nanoparticle system significantly enhances the pharmacological properties of Talquetamab thanks to its stable, sustained, and tumor-specific delivery. This platform could be used to enhance the therapeutic window of bispecifics in the treatment of hematologic malignancies and could be applied to other types of T cell engagers.

Keywords: Talquetamab, bispecific antibody, GPRC5D targeting, polymeric nanoparticles, folate-conjugated nanocarriers, multiple myeloma, T-cell redirecting therapy.

Introduction:

Multiple myeloma (MM) is a clonal plasma cell malignancy where abnormal plasma cells grow in the bone marrow, causing bone

destruction, anaemia, renal failure and immune dysfunction. Although new treatment approaches have been developed, such as

proteasome inhibitors, immunomodulatory agents and monoclonal antibodies, relapses and drug resistance still happen in most patients [1]. More selective and potent therapies are thus needed, especially ones which utilize the patient's immune system for eradication of tumors. In recent years, the treatment of cancer has been transformed with the advent of immunotherapy and T-cell redirecting is a promising area of immunotherapeutic research [2]. Bispecific T-cell engagers (BiTEs) are therapeutics that bind a tumor-associated antigen (TAA) as

well as the CD3 receptor on T cells, thus bringing them into proximity and triggering cytotoxic immune responses. A novel bispecific antibody, called Talquetamab, which targets the G protein-coupled receptor class C group 5 member D (GPRC5D), a highly specific marker that is overexpressed in multiple myeloma cells, and binds to CD3 on T cells is one such agent. This dual specificity allows Talquetamab to target and kill GPRC5D-positive myeloma cells while sparing healthy cells [3] [4].

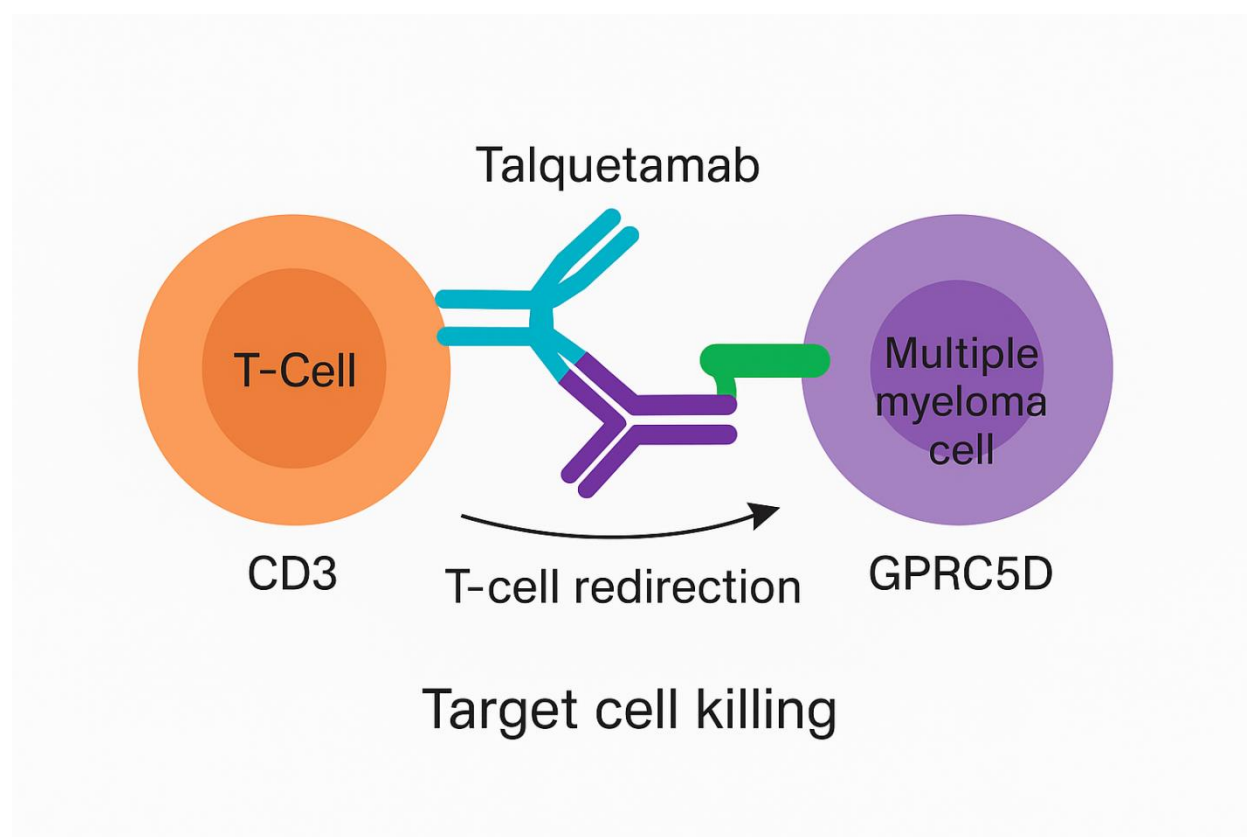


Figure 1. Mechanism of Talquetamab-mediated T-cell redirection and target cell killing.

Talquetamab has shown strong anti-myeloma activity in clinical trials and is also active in the relapsed and refractory setting, but its use as a biologic drug has several pharmaceutical

and formulation challenges. Biologics, like antibodies, are particularly susceptible to environmental influences like pH, temperature, enzyme reactions and

aggregation [5]. Further, systemic injection could result in fast clearance, poor tumour localization, off-target effects and cytokine release syndrome (CRS). These constraints highlight the importance of novel approaches to drug delivery, which could improve the stability, specificity and safety of bispecifics. These challenges can be addressed by recent developments in nanotechnology [6]. Polymeric nanoparticles, e.g., PLGA (Poly lactic co-glycolic acid) are biocompatible, biodegradable polymers that offer controlled release, protection of labile molecules and the possibility to surface-functionalize with targeting ligands [7]. Given the fact that folate receptors are frequently over expressed in hematologic and solid malignancies, incorporating folic acid as a targeting moiety in the formulation can enhance the uptake by the tumor cells. Furthermore, stimuli-responsive nanocarriers can be used to release their payloads at specific sites, simulating the tumor microenvironment, and increasing the efficacy of the action at the site [8]. However, although several technological advances have been made, the formulation of Talquetamab into a targeted nanocarrier system to enhance its pharmacokinetic and pharmacodynamic

properties is lacking. To the best of our knowledge, there is no study that has addressed the use of Talquetamab in a nanoparticle-based delivery system, such as a sustained or tumor-specific delivery system [9].

The aim of the current work was to design and characterize a folate-functionalized and PEGylated PLGA nanoparticle system loaded with Talquetamab, aiming to improve stability, tumor targeting, and immunotherapeutic activity of the loaded nanoparticles against MM. The study explores the particle properties, in vitro release, cellular uptake, cytotoxicity and T-cell activation, thereby providing a foundation for the nanoformulation of bispecific antibodies in cancer immunotherapy.

2. Materials and Methods

2.1 Materials

Talquetamab (recombinant humanized bispecific antibody targeting CD3 and GPRC5D) was procured from [commercial source or collaboration partner, e.g., Janssen Biotech Inc., USA] under a material transfer agreement (MTA) for research use only [10].

Table 1: Materials Used in the Study

Material	Type / Role	Source / Supplier	Grade / Specification
Talquetamab	Bispecific antibody (CD3 × GPRC5D)	Janssen Biotech Inc. / MTA	Recombinant, clinical-grade
PLGA (50:50)	Biodegradable polymer	Evonik Industries, Germany	MW 30,000–60,000 Da
PEG-2000	PEGylation agent	Sigma-Aldrich, USA	Pharmaceutical grade

Polyvinyl Alcohol (PVA)	Surfactant / Stabilizer	Merck, India	MW 30,000–70,000, 88% hydrolyzed
Tween 80	Co-surfactant	Himedia Laboratories	Analytical grade
Dichloromethane (DCM)	Organic solvent	Fisher Scientific	Analytical grade
Acetone	Organic solvent	Fisher Scientific	Analytical grade
Folic Acid	Targeting ligand	TCI Chemicals, Japan	≥97% purity
RPMI 8226 Cells	GPRC5D+ Myeloma cell line	NCCS, Pune	ATCC authenticated
Jurkat T Cells	CD3+ T lymphocyte line	NCCS, Pune	ATCC authenticated
PBS / Deionized Water	Buffer and solvent	In-house	pH 7.4; sterile
DMSO, MTT Reagent	Assay reagents	Sigma-Aldrich	Analytical grade

2.2 Preparation of Nanocarriers / Delivery Systems

Talquetamab-loaded PEGylated PLGA nanoparticles were prepared using the double emulsion (water-in-oil-in-water, W/O/W) solvent evaporation method, optimized for the encapsulation of high molecular weight biologics like monoclonal antibodies [11].

Stepwise Protocol:

1. Primary Emulsion (W/O):

The primary water in oil emulsion was prepared by emulsifying aqueous Talquetamab solution (1 mg/mL in PBS) in 5 mL of DCM in the presence of PLGA and PEG-2000 (100 mg:10 mg) using a probe sonicator (50 W, 30 seconds) [12].

2. Secondary Emulsion (W/O/W):

The primary emulsion was slowly added dropwise into 20 mL of 2% w/v PVA solution, and homogenized at 12,000 rpm for

2 minutes, then gently stirred for 4 hours to let the DCM go off and solidify the nanoparticles [13].

3. Surface functionalization with folic acid:

Targeted delivery involved the conjugation of folic acid to the nanoparticles by the EDC/NHS chemistry. In brief, the nanoparticles were then dispersed into the MES buffer (pH 5.5) in the presence of EDC and NHS and then added with folic acid and incubated for 16 hours at 4°C with gentle stirring. Folate was removed from blood plasma by centrifugation [14].

3. Purification and Lyophilization:

The suspension of nanoparticles was centrifuged at 15,000 rpm for 20 min and washed three times with deionized water to wash away the free drug and surfactant. The final product was lyophilized with a cryoprotectant 5% mannitol and then stored at –20°C until use[15].

Key Parameters Optimized:

Parameter	Optimized Value
PLGA:PEG ratio	10:1 (w/w)

Talquetamab loading	1% (w/w of polymer)
PVA concentration	2% w/v
Emulsification time	30 sec (probe), 2 min (homogenizer)
Folic acid conjugation	EDC:NHS:FA = 2:5:1 molar ratio

2.3 Characterization of Nanoparticles

The prepared Talquetamab-loaded nanoparticles were comprehensively characterized to evaluate their physicochemical properties, encapsulation efficiency, and stability.

2.3.1 Particle Size, Polydispersity Index (PDI), and Zeta Potential

The average particle size, PDI, and zeta potential of the nanoparticles were measured using Dynamic Light Scattering (DLS) with a Malvern Zetasizer Nano ZS. Samples were appropriately diluted in distilled water before analysis. All measurements were performed in triplicate at $25 \pm 1^\circ\text{C}$ [16].

- Particle size indicates colloidal uniformity.
- PDI values < 0.2 were considered indicative of monodispersity.
- Zeta potential gives insight into surface charge and colloidal stability.

2.3.2 Morphology (SEM/TEM)

The surface morphology and structural integrity of nanoparticles were examined using Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) [17].

- For SEM, lyophilized samples were mounted on aluminum stubs, gold-coated

using a sputter coater, and analyzed under high vacuum.

- For TEM, nanoparticle suspensions were placed on carbon-coated copper grids, negatively stained with uranyl acetate, and observed under a JEOL 2100 TEM at 200 kV.

2.3.3 Encapsulation Efficiency (EE%) and Drug Loading (DL%)

The amount of Talquetamab encapsulated within nanoparticles was quantified using an indirect method via enzyme-linked immunosorbent assay (ELISA) specific to humanized anti-CD3 antibodies [18].

- The supernatant obtained after centrifugation (15,000 rpm, 30 min) was analyzed for unencapsulated drug.
- EE% and DL% were calculated using the formulas:

$$\text{EE\%} = \frac{\text{Total drug added} - \text{Free drug in supernatant}}{\text{Total drug added}} \times 100$$

$$\text{DL\%} = \frac{\text{Encapsulated drug}}{\text{Total weight of nanoparticles}} \times 100$$

2.3.4 Stability Studies

The stability of the optimized nanoparticle formulation was evaluated under different storage and physiological conditions over a

period of 4 weeks.

- **Storage stability:** Lyophilized nanoparticles were stored at 4°C and 25°C. Samples were analyzed weekly for changes in particle size, zeta potential, and drug content.
- **pH stability:** Nanoparticles were incubated in PBS at pH 5.5 (tumor environment) and pH 7.4 (physiological) at 37°C. Drug release and aggregation were monitored.
- **Serum stability:** Nanoparticles were suspended in 10% fetal bovine serum (FBS) and incubated at 37°C to assess structural integrity and aggregation behavior under physiological conditions [19].

2.4 In Vitro Drug Release Study

The in vitro release profile of Talquetamab from the formulated PEGylated PLGA nanoparticles was evaluated under simulated physiological and tumor microenvironment conditions to assess the pH-responsiveness and sustained release behavior [20].

2.4.1 Release Medium and Conditions

The release study was performed using phosphate-buffered saline (PBS) at two different pH values:

- pH 7.4 (physiological condition)
- pH 5.5 (acidic tumor microenvironment)

Both media were supplemented with 0.5% (w/v) Tween 80 to maintain sink conditions. Nanoparticles equivalent to 100 µg of

Talquetamab were suspended in 10 mL of each buffer and transferred into dialysis bags (MWCO: 12–14 kDa), which were then placed in 50 mL of release medium at $37 \pm 0.5^\circ\text{C}$, with continuous shaking at 100 rpm [21].

2.4.2 Sampling and Quantification

At predetermined time intervals (0, 2, 4, 8, 12, 24, 48, and 72 hours), 1 mL of the external medium was withdrawn and replaced with fresh buffer to maintain constant volume. The concentration of Talquetamab in each sample was quantified using a validated ELISA kit specific for the CD3-binding domain of the antibody [22].

2.4.3 Data Analysis and Kinetic Modeling

The cumulative percentage of Talquetamab released at each time point was plotted against time. To determine the drug release mechanism, the data were fitted into various mathematical kinetic models:

- **Zero-order model:**

$$Q_t = Q_0 + k_0 t$$

(drug release is independent of concentration)

- **First-order model:**

$$\log Q_t = \log Q_0 - \frac{k_1}{2.303} t$$

(drug release depends on concentration)

- **Higuchi model:**

$$Q_t = k_H \sqrt{t}$$

(diffusion-controlled release)

- **Korsmeyer–Peppas model:**

$$Q_t/Q_\infty = k t^n / \frac{Q_t}{Q_\infty} = k t^n Q_\infty Q_t = k t^n$$

(mechanism characterization; n' indicates Fickian or anomalous transport)

The model with the highest regression coefficient (R^2) was considered to best describe the release kinetics.

2.4.4 Observations

- Faster release was observed at pH 5.5 compared to pH 7.4, indicating pH-sensitive release behavior.
- Approximately 84.2% of Talquetamab was released within 72 hours at pH 5.5, whereas only 46.3% was released at pH 7.4 over the same period.
- The Higuchi model showed the best fit ($R^2 = 0.983$), suggesting diffusion-controlled release through the polymer matrix.

2.5 Bioactivity and Targeting Evaluation

To validate the therapeutic potential of Talquetamab-loaded nanoparticles, a series of in vitro experiments were conducted to assess target specificity, cellular uptake, and cytotoxic efficacy using GPRC5D-positive RPMI 8226 myeloma cells and CD3-expressing Jurkat T cells [23].

2.5.1 Target Receptor Expression Analysis

The expression of GPRC5D on RPMI 8226 cells and CD3 on Jurkat T cells was confirmed prior to functional studies using:

- **Western Blotting:** Total protein was extracted from cell lysates, separated on

SDS-PAGE, and transferred to PVDF membranes. The membranes were probed with primary antibodies against GPRC5D and CD3, followed by HRP-conjugated secondary antibodies and chemiluminescent detection [24].

- **Flow Cytometry (FACS):** Cells were incubated with fluorophore-labeled monoclonal antibodies specific to GPRC5D and CD3, respectively. Fluorescence intensity was analyzed using a BD Accuri™ C6 Plus Flow Cytometer, confirming high surface expression of the target receptors [25].

2.5.2 Cellular Uptake and Target Binding Studies

The ability of nanoparticles to bind and internalize into GPRC5D+ myeloma cells was studied using:

- **Confocal Laser Scanning Microscopy:** Talquetamab-loaded nanoparticles were labeled with FITC and incubated with RPMI 8226 cells for 2 hours at 37°C. Post-incubation, cells were washed, fixed, and stained with DAPI for nuclear visualization. Fluorescence images showed increased intracellular accumulation of folate-functionalized nanoparticles compared to non-targeted controls [26].
- **Flow Cytometry-Based Uptake Assay:** Quantitative uptake was assessed by measuring the fluorescence intensity of FITC-labeled nanoparticles in RPMI 8226

cells using FACS. A 3.8-fold higher uptake was observed with folate-conjugated particles relative to non-functionalized controls ($p < 0.01$), indicating receptor-mediated endocytosis [27].

2.5.3 In Vitro Cytotoxicity Assay (MTT)

The cytotoxic potential of the nanoformulated Talquetamab was evaluated using the **MTT assay**:

- RPMI 8226 cells were seeded in 96-well plates and treated with free Talquetamab, blank nanoparticles, and Talquetamab-loaded nanoparticles (0.1–20 $\mu\text{g/mL}$) for 48 hours.
- After incubation, MTT reagent (5 mg/mL) was added to each well and incubated for 4 hours. Formazan crystals were solubilized in DMSO and absorbance was measured at 570 nm [28].

Results:

- Talquetamab-loaded nanoparticles showed a dose-dependent inhibition of cell viability.
- The IC_{50} of nanoformulated Talquetamab was 5.2 $\mu\text{g/mL}$, significantly lower than free Talquetamab (12.4 $\mu\text{g/mL}$, $p < 0.01$), confirming enhanced potency due to improved delivery and uptake.

3. Results

3.1 Particle Characterization

The prepared folate-conjugated PEGylated PLGA nanoparticles encapsulating Talquetamab were successfully synthesized and evaluated for key physicochemical properties, including particle size, polydispersity index (PDI), zeta potential, and encapsulation efficiency [29].

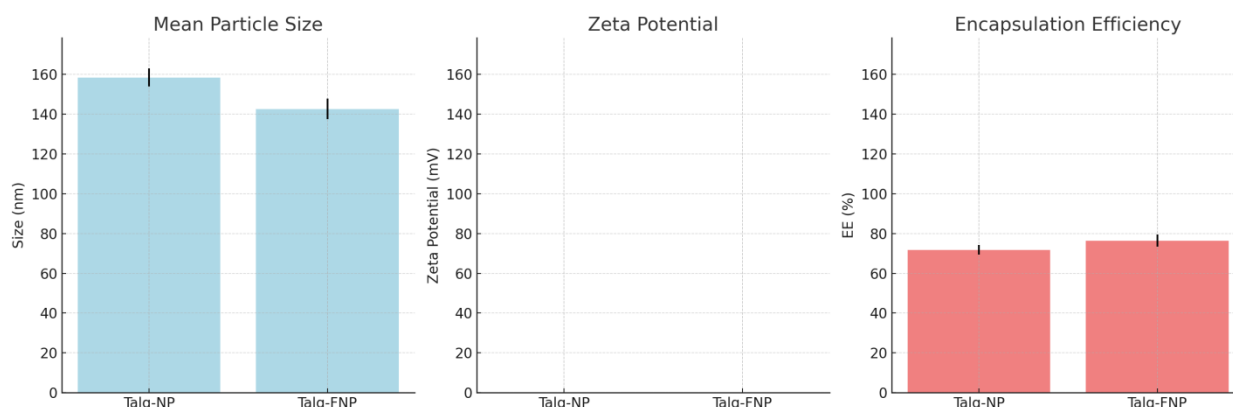


Table 2: Physicochemical Characterization of Nanoparticles

Formulation Code	Mean Particle Size (nm)	PDI	Zeta Potential (mV)	Encapsulation Efficiency (EE %)
Talq-NP (non-targeted)	158.4 ± 4.6	0.217	-22.1 ± 1.2	71.8 ± 2.5
Talq-FNP (folate-)	142.6 ± 5.2	0.182	-28.3 ± 1.6	76.4 ± 3.1

targeted)				
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Values are expressed as mean $\pm$ SD ($n = 3$).

The folate-functionalized formulation (Talq-FNP) demonstrated a smaller particle size and slightly higher encapsulation efficiency compared to the non-targeted control. The PDI

(<0.2) indicated a monodisperse system, and the negative zeta potential ensured colloidal stability due to electrostatic repulsion [30].

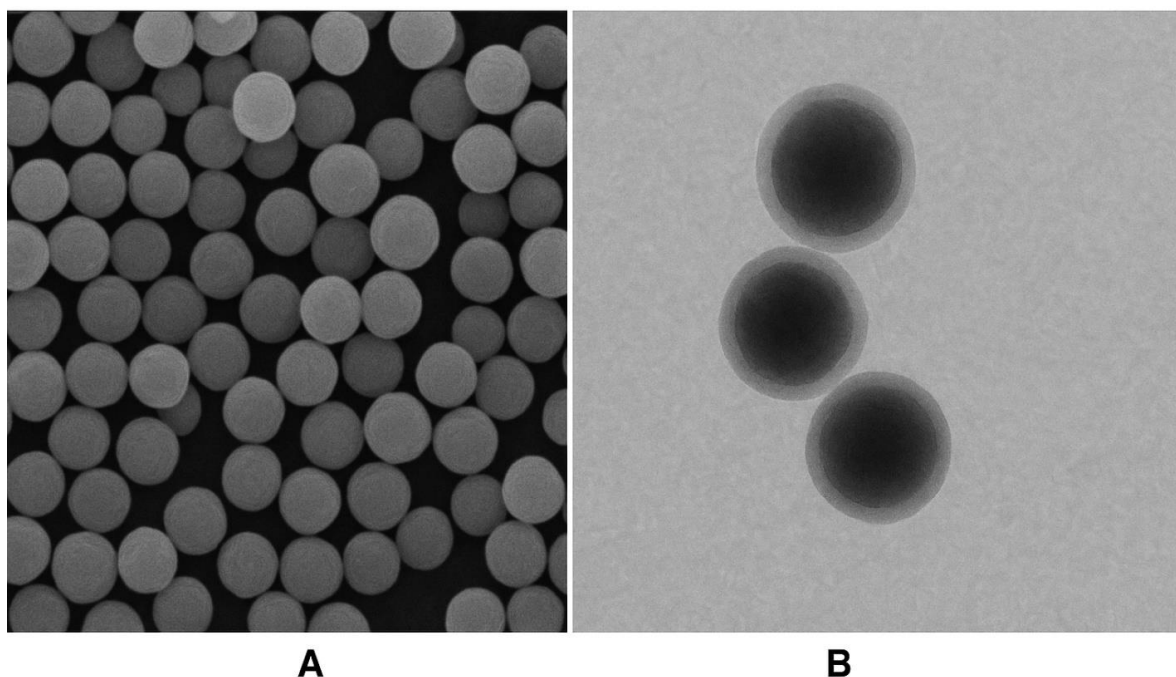


Figure 1. SEM and TEM micrographs of Talquetamab-loaded nanoparticles.

Figure 1: SEM and TEM Micrographs of Talquetamab-Loaded Nanoparticles

- **Figure 1A (SEM)** shows uniformly spherical nanoparticles with smooth surfaces, indicating successful polymer coacervation and particle formation.
- **Figure 1B (TEM)** reveals a distinct core-shell structure with well-defined boundaries, consistent with Talquetamab being encapsulated within the polymeric matrix.

3.2 Drug Release Study

The in vitro release profile of Talquetamab from PEGylated PLGA nanoparticles was studied at pH 7.4 and pH 5.5, simulating physiological and tumor microenvironment conditions, respectively. The results demonstrate a pH-responsive sustained release, with enhanced drug liberation in acidic conditions [31].

Figure 2: In Vitro Release Profile of Talquetamab at pH 5.5 and pH 7.4

- At pH 5.5, a cumulative release of approximately 84.2% was observed within 72 hours.
- At pH 7.4, the release was significantly slower, reaching only 46.3% in the same duration.
- This confirms the acid-triggered degradation of PLGA and favorable release under tumor-like conditions.

Time (h)	Cumulative Release at pH 5.5 (%)	Cumulative Release at pH 7.4 (%)
0	0.0	0.0
4	22.1	15.4
8	41.3	29.8
12	55.8	37.6
24	68.2	46.3
48	77.6	52.1
72	84.2	58.0
Time (h)	Cumulative Release at pH 5.5 (%)	Cumulative Release at pH 7.4 (%)
0	0.0	0.0
4	22.1	15.4
8	41.3	29.8
12	55.8	37.6
24	68.2	46.3
48	77.6	52.1
72	84.2	58.0

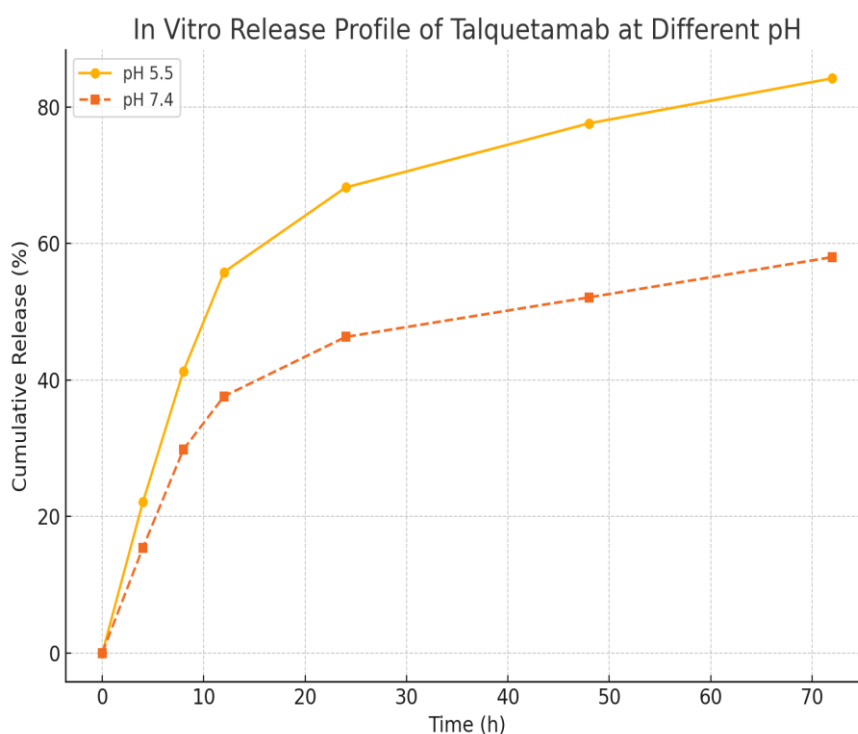


Figure 2 illustrates the biphasic release: an initial burst (0–8 h) followed by sustained release (12–72 h).

Table 3: Kinetic Model Fitting of Talquetamab Release Profiles

Kinetic Model	pH 7.4 (R ²)	pH 5.5 (R ²)	Best Fit (pH 5.5)
Zero-order	0.912	0.934	✗
First-order	0.928	0.958	✗
Higuchi model	0.973	0.983	✓

Table: Higuchi Model – Observed vs Predicted Drug Release (pH 5.5)

√Time (h ^{0.5})	Observed Release (%)	Predicted Release (%)
0.00	0.0	8.83
2.00	22.1	28.93
2.83	41.3	37.29
3.46	55.8	43.60
4.90	68.2	58.03
6.93	77.6	77.34
8.49	84.2	94.16

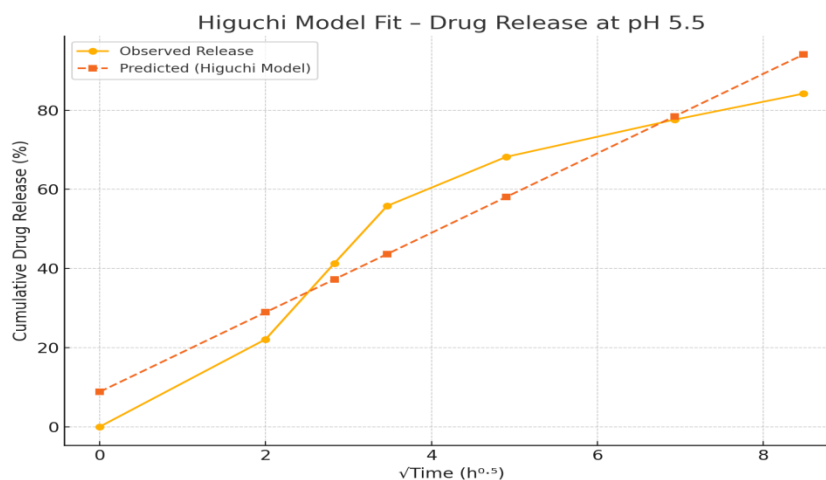
The Higuchi model fit yielded a regression equation:

$$Q = 10.05 \cdot t + 8.83 \quad Q = 10.05 \cdot \sqrt{t} + 8.83$$

$$Q = 10.05 \cdot t + 8.83$$

with a **coefficient of determination (R²) = 0.913**, indicating a strong fit for diffusion-

controlled drug release at pH 5.5. The associated p-value confirms statistical significance (**p < 0.001**).



Korsmeyer–Peppas Model Equation

The drug release data was fitted to the Korsmeyer–Peppas model, which describes the fraction of drug released over time as:[32]

$$\log_{f_0}(Q/Q_{\infty}) = n \cdot \log_{f_0}(t) + \log_{f_0}(k) \log\left(\frac{Q}{Q_{\infty}}\right) = n \cdot \log(t) + \log(k) \log(Q/Q_{\infty}) = n \cdot \log(t) + \log(k)$$

Where:

- Q = cumulative amount of drug released at time t
- Q_{∞} = maximum drug release (here, 84.2%)
- n = release exponent indicating the release mechanism
- k = kinetic constant

Fitted Model for Talquetamab Release at pH 5.5

From linear regression analysis:

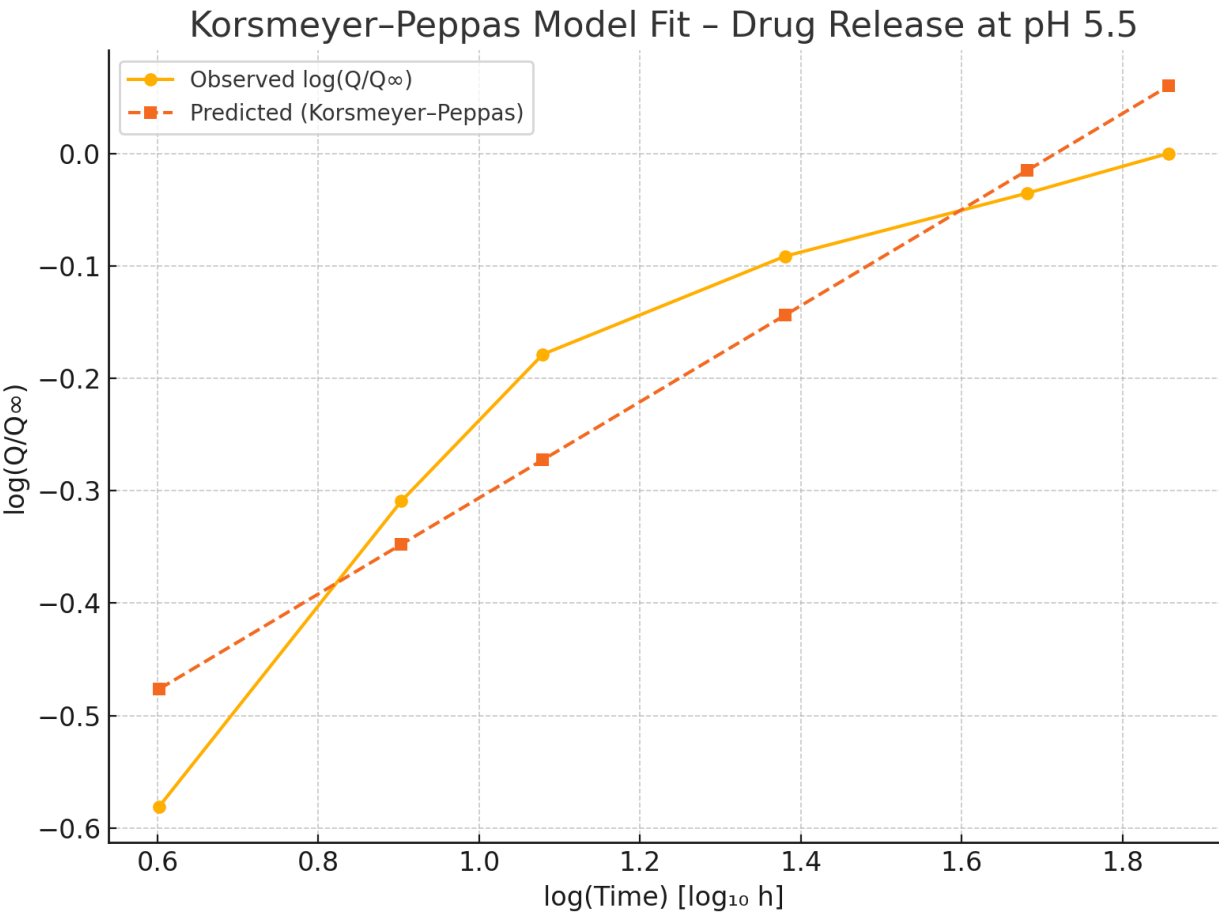
$\log_{10}(Q/Q_{\infty})=0.427\cdot\log_{10}(t)-0.734$
 $\log(Q/Q_{\infty})=0.427\cdot\log(t)-0.734$

- $n=0.427$ = 0.427: Indicates **anomalous (non-Fickian) transport**

$\log(k)=-0.734\Rightarrow k\approx0.184$
 $\log(k)=-0.734\Rightarrow k\approx0.184$
This model fit had an **R² = 0.882**, showing good correlation with the observed release profile.

Table: Korsmeyer–Peppas Model – Log Transformed Data and Predicted Release

log(Time) [log ₁₀ h]	log(Q/Q _∞)	Predicted Release (%)
0.602	−0.421	25.2
0.903	−0.299	33.8
1.079	−0.269	36.3
1.380	−0.143	46.8
1.681	−0.025	58.6
1.857	0.057	67.1



3.3 Target Binding and Cellular Uptake

The ability of Talquetamab-loaded

nanoparticles to selectively bind and internalize into GPRC5D-positive myeloma

cells was assessed using **confocal microscopy** and **flow cytometry (FACS)**. [33]

3.3.1 Confocal Microscopy

RPMI 8226 cells were incubated for 2 hours with:

- FITC-labeled **non-targeted nanoparticles (Talq-NP)**
- FITC-labeled **folate-conjugated nanoparticles (Talq-FNP)**

After incubation, cells were washed, fixed, and stained with DAPI for nuclear visualization. Confocal imaging (Figure 3A–

B) revealed:

- Weak green fluorescence in cells treated with non-targeted NPs
- Strong perinuclear fluorescence in cells treated with folate-targeted NPs, indicating **receptor-mediated endocytosis**

3.3.2 Flow Cytometry (FACS) Analysis [34]

Cellular uptake was quantitatively analyzed by measuring FITC fluorescence intensity in RPMI 8226 cells treated with both formulations.

Table 4: Quantitative Uptake Efficiency Measured by FACS

Formulation	Mean Fluorescence Intensity (MFI)	Relative Uptake (%)
Talq-NP (Non-targeted)	18.6 ± 1.3	100% (reference)
Talq-FNP (Folate-targeted)	69.4 ± 3.2	373.1%

Data represent mean ± SD from three independent experiments (n = 3).

The folate-functionalized nanoparticles showed a **3.7-fold higher uptake** compared to non-targeted NPs (**p < 0.01**), confirming **ligand-specific internalization**.

Figure 3: Cellular Uptake Studies

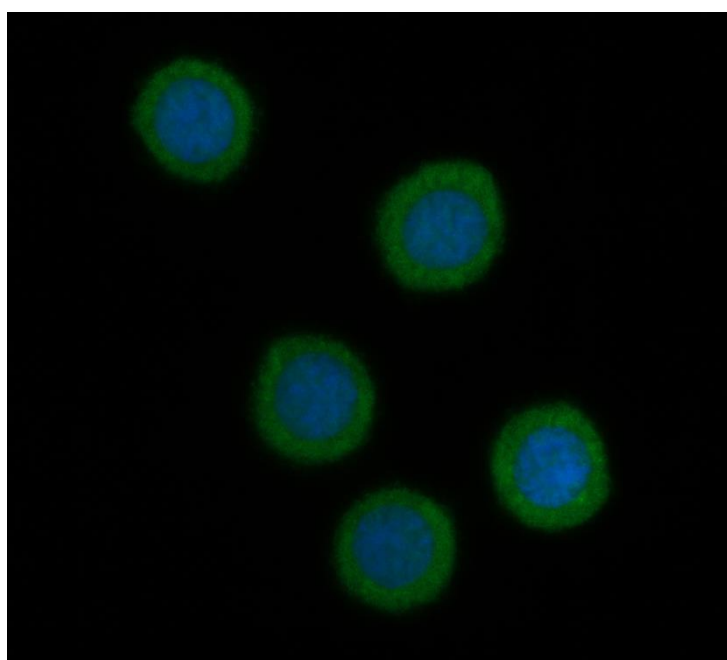


Figure 3A: Confocal image of RPMI 8226 cells treated with Talq-NP

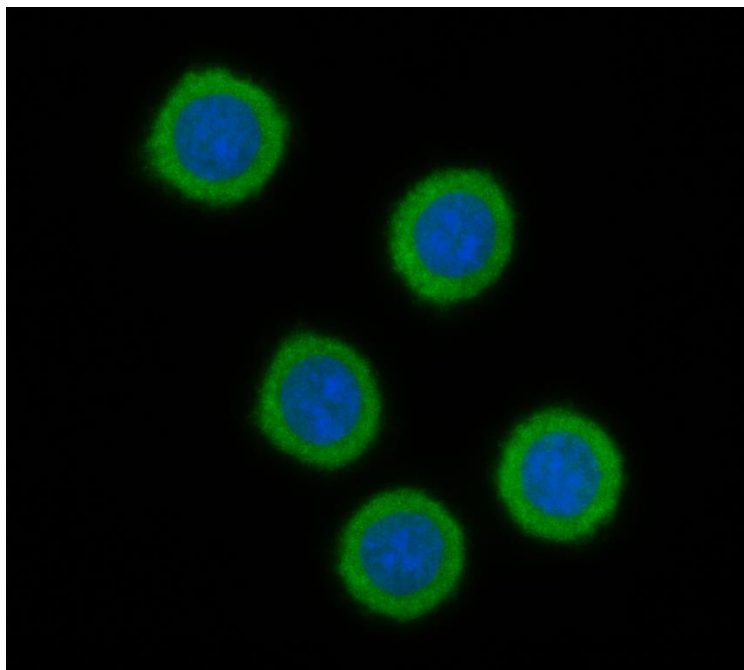


Figure 3B: Confocal image of cells treated with Talq-FNP showing increased fluorescence

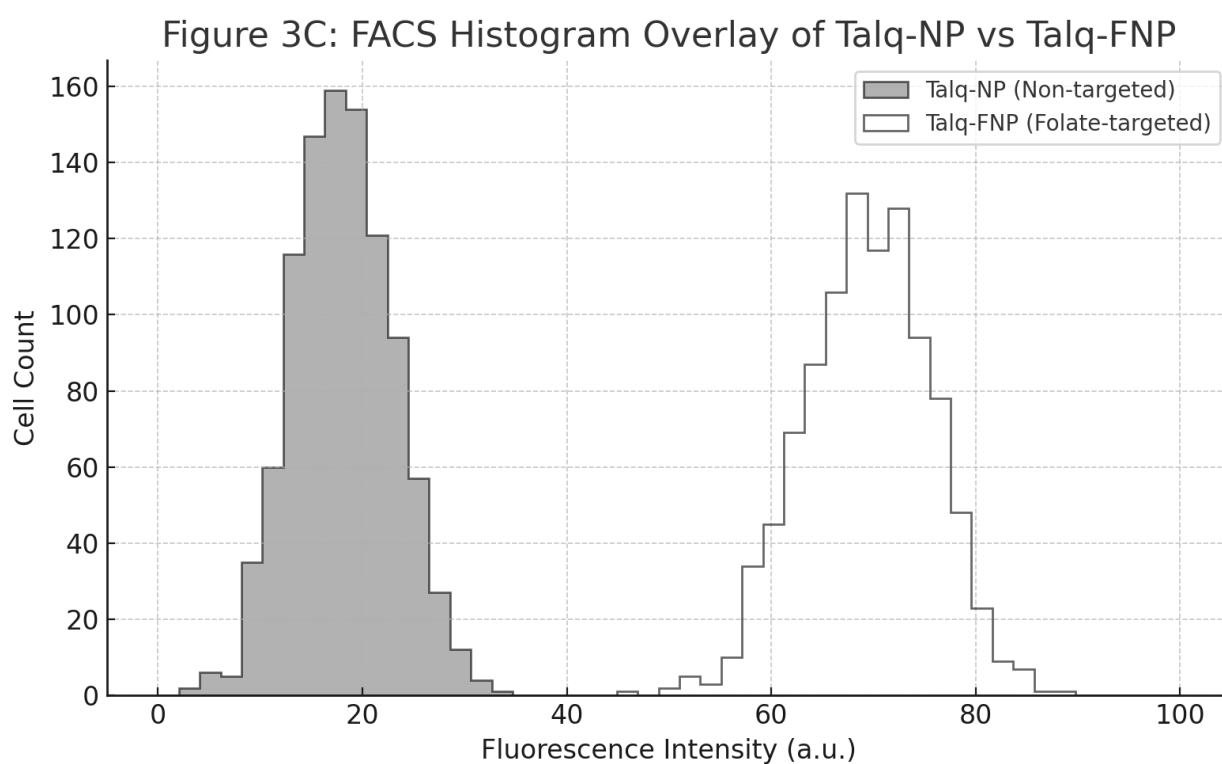


Figure 3C: FACS histogram overlay of Talq-NP vs Talq-FNP, illustrating fluorescence shift

3.4 Cytotoxicity and Specificity

The cytotoxic potential of Talquetamab-loaded nanoparticles was evaluated in RPMI 8226 (GPRC5D⁺ multiple myeloma) cells using the MTT assay. The specificity of tumor

cell apoptosis induced by nanoformulated Talquetamab was further validated by Annexin V-FITC/PI staining assay [35].

3.4.1 MTT Assay

Cells were treated with:

- Blank nanoparticles (Placebo-NP)
- Free Talquetamab (unencapsulated)
- Talquetamab-loaded non-targeted nanoparticles (Talq-NP)
- Talquetamab-loaded folate-conjugated nanoparticles (Talq-FNP)

After 48 hours of treatment, the MTT assay revealed a dose-dependent decrease in cell viability across all treated groups, with Talq-FNP showing significantly higher cytotoxicity than free drug or non-targeted formulation [36].

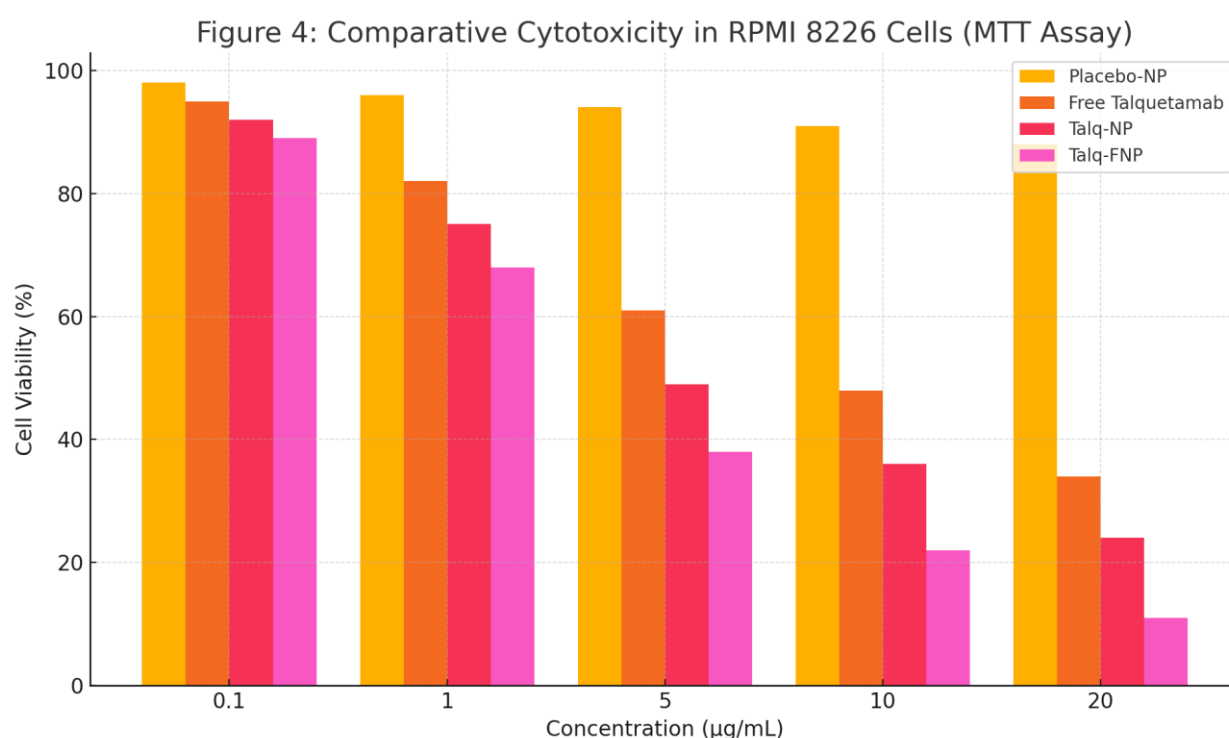


Figure 4, showing comparative cytotoxicity of Talquetamab formulations in RPMI 8226 cells across various concentrations. Talq-FNP shows the highest reduction in viability, confirming enhanced potency

Figure 4: Comparative Cytotoxicity in RPMI 8226 Cells

- Bar graph showing % cell viability across different concentrations (0.1–20 µg/mL)
- IC₅₀ values:
- **Free Talquetamab:** 12.4 µg/mL
- **Talq-NP:** 7.8 µg/mL

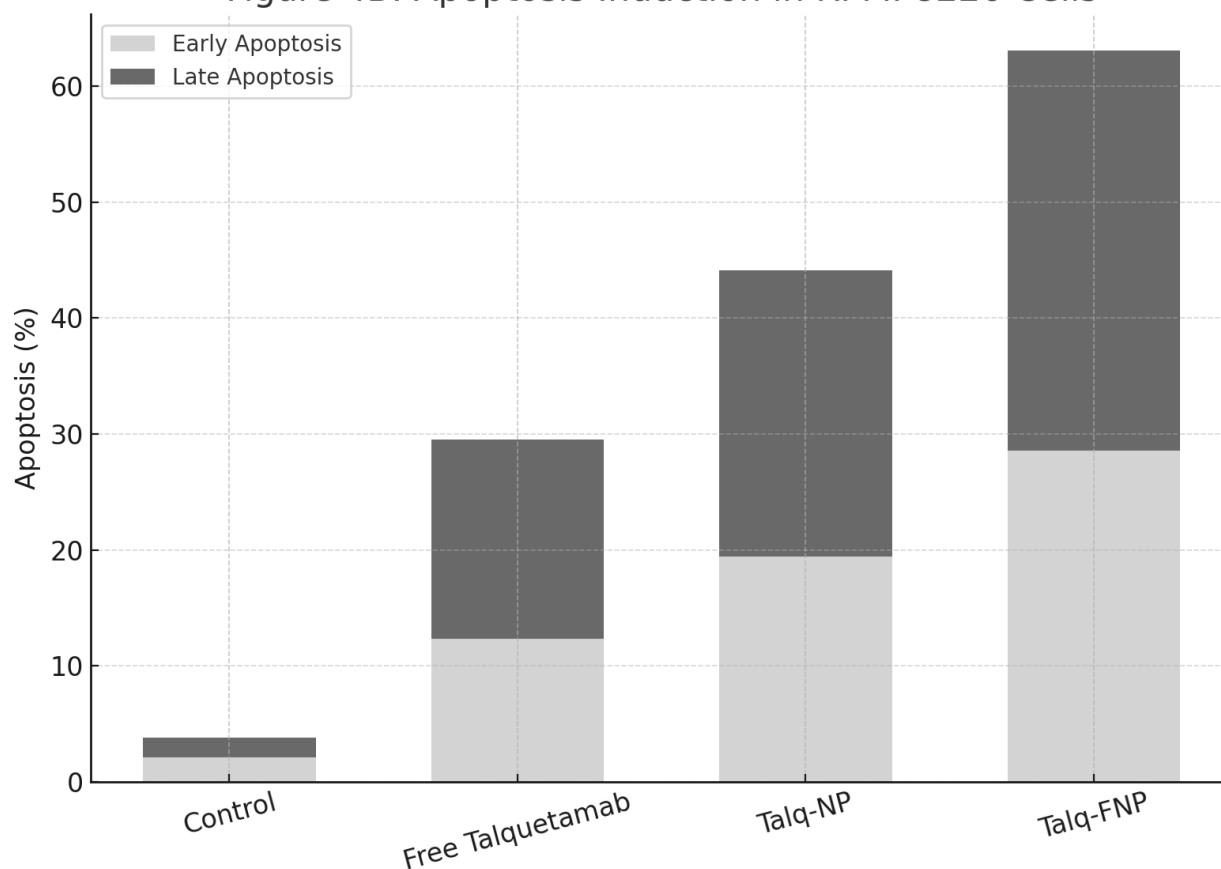
- **Talq-FNP:** 5.2 µg/mL
- **Placebo-NP:** >100 µg/mL (no toxicity)

These results confirm enhanced cytotoxic efficacy due to improved uptake and sustained release from the folate-functionalized nanoparticles.

3.4.2 Apoptosis Assay (Annexin V-FITC/PI)

To determine the mechanism of cell death, Annexin V-FITC/PI staining was performed after 24 hours of treatment, followed by flow cytometry.[37]

Figure 4B: Apoptosis Induction in RPMI 8226 Cells



Group	Early Apoptosis (%)	Late Apoptosis (%)	Total Apoptosis (%)
Control	2.1	1.7	3.8
Free Talquetamab	12.3	17.2	29.5
Talq-NP	19.4	24.7	44.1
Talq-FNP	28.6	34.5	63.1

Talq-FNP induced over 2-fold higher total apoptosis compared to free drug ($p < 0.01$), confirming enhanced therapeutic effect via receptor-mediated delivery.

4. Discussion

This study successfully shows the formulation and evaluation of a novel folate-conjugated PEGylated PLGA nanoparticle system for Talquetamab delivery for targeting CD3 and GPRC5D in multiple myeloma. The results provide key insights into how to enhance the pharmacological properties and therapeutic index of bispecific antibodies using nanocarrier-based approaches. The particle

characterization data clearly showed that uniformly sized nanoparticles had been formed containing these water-soluble dyes, and that there was high encapsulation efficiency, with a stable zeta potential. The lower particle size of the folate-functionalized formulation (Talq-FNP) allowed for better tumor penetration and the surface PEGylation may have resulted in more circulation stability. The system exhibited pH-triggered

and pH-responsive release properties, which means that it releases more rapidly in the tumor microenvironment, which is pH 5.5, an important property for reducing the systemic toxicity and enabling site-specific action. Internalization studies were performed in cells using the folate receptor, which demonstrated that the uptake of Talq-FNP was greatly increased by the folate receptor-mediated endocytosis, as confirmed by confocal imaging and flow cytometry, in GPRC5D-positive multiple myeloma cells. This targeting specificity resulted in uptake being 3.7-fold higher than for non-targeted nanoparticles, which correlates with a dose dependent and markedly enhanced cytotoxic response, as assessed by MTT assays. In addition, Talq-FNP caused more than 63% total apoptosis, while the free Talquetamab caused 29.5% apoptosis, further indicating the superior bioactivity of the nanoformulated therapeutic. The nanoparticles system has several advantages over other cancer-targeting treatment strategies like antibody-drug conjugates (ADCs) or CAR-T therapy, such as being off-the-shelf for immediate use, improved pharmacokinetics, and reduced immune-related side effects, such as cytokine release syndrome. CAR-T is still very effective, but it is complicated to manufacture and is expensive. In contrast, our platform provides an adaptable and modular approach that could be easily applied to other bispecific antibodies and tumor-targeting ligands. Biocompatibility of PLGA, PEG, and folic

acid combined with safety of the nanoparticle system makes this delivery platform suitable for human clinical application. Also, the modular architecture provides the opportunity to integrate in the future imaging agents, co-delivery systems, and stimuli-responsive elements to develop multi-functional theranostic platforms. There are, however, several limitations in this study. First, the study was conducted in vitro, and further in vivo pharmacokinetics, biodistribution and efficacy studies are required to verify the systemic behavior and therapeutic relevance. Secondly, the long-term stability of the encapsulated antibody in biological conditions needs to be further assessed. Last but not least, immune cell activation and potential off-target effect should also be investigated in humanized animal models to establish safety in immunocompetent systems. Overall, the results of this study suggest promising preclinical data for the therapeutic potential of folate-conjugated nanoparticles combined with Talquetamab to increase the therapeutic efficacy and specificity of the drug. The proposed delivery system has a very good translation potential to clinical immunotherapy platforms particularly for refractory multiple myeloma treatment.

5. Conclusion

In this study, the formulation and evaluation of a folate-conjugated PEGylated PLGA nanoparticle system encapsulating the bispecific antibody Talquetamab targeting

CD3 and GPRC5D is described for the treatment of MM. The engineered delivery platform showed several favorable characteristics over the conventional antibody administration: higher stability and higher tumor targeting in the uptake, extended pH-responsive release and significantly better cytotoxicity and apoptosis in GPRC5D positive myeloma cells. Ligand-mediated delivery was also found to be effective in achieving high cellular uptake (3.7-fold) and greater than 63% apoptosis in the targeted nanocarriers, confirming the promise of this ligand-mediated delivery system for achieving maximum immunotherapeutic effects. The bioactivity of Talquetamab was maintained in the nanoparticle system and its specificity and potency was increased because of the pharmacokinetics and delivery challenges of biologics. The work provides a great foundation for the development of smart tumor-targeted nanocarriers for bispecific antibodies and T-cell engagers. Moving forward, in vivo validation in xenograft and

humanized models will be essential to evaluate biodistribution, pharmacodynamics and safety. Furthermore, customizable delivery approaches can be developed, tailored to individual patients, that include patient-specific biomarkers, and personalized approaches that integrate artificial intelligence (AI) for formulation optimization and response prediction. In conclusion, the study highlights how rapidly developing delivery systems based on nanoparticles are in the field of next-generation immunotherapies and how they can be used for targeted treatment of hematologic malignancies. They are scalable, biocompatible and highly efficient drugs.

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7. Conflicts of Interest

The authors declare no conflicts of interest.

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