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Development and Characterization of a pH-Sensitive Nanocarrier System for Targeted Delivery of Methotrexate in Rheumatoid Arthritis

Neha Yadav^{1,2}, Ramu Soni^{1,2}, Priyanka Narwariya³, Monika Chhapariya⁴,
Neelam yadav⁵

^{1,2}Associate Professor, School of Pharmacy, Vikrant University, Gwalior (M.P)

³Assistant Professor, School of Pharmacy, Vikrant University Gwalior (M.P)

⁴Associate Professor, Pranav Institute of Pharmaceutical Science and Research

⁵Assistant Professor, Advance College of Pharmacy Datia (M.P)

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Abstract

Background: Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by joint inflammation and destruction. Methotrexate (MTX) is a frontline therapy but suffers from systemic toxicity and poor targeting. This study aimed to develop a pH-sensitive nanocarrier system for targeted MTX delivery to improve therapeutic efficacy and reduce side effects.

Methods: pH-responsive nanocarriers were prepared using biocompatible polymers via ionic gelation and nanoprecipitation methods. The formulations were optimized for particle size, drug loading, and pH-triggered release. Characterization included dynamic light scattering (DLS), electron microscopy, encapsulation efficiency, and drug-polymer interaction analysis. In vitro drug release was evaluated under physiological (pH 7.4) and acidic (pH ~6) conditions, alongside cytotoxicity and cellular uptake studies in macrophage cells. In vivo efficacy was assessed using an

adjuvant-induced arthritis rat model, measuring paw swelling, histopathology, and pro-inflammatory cytokine levels.

Results: The optimized nanocarriers showed uniform nanosize (~150 nm), negative zeta potential, and high encapsulation efficiency (>85%). They exhibited sustained, pH-triggered MTX release, enhanced macrophage uptake, and reduced cytotoxicity compared to free MTX. In vivo, MTX-loaded nanocarriers significantly decreased inflammation, joint damage, and cytokine biomarkers compared to free drug and controls.

Conclusion: The pH-sensitive nanocarrier system effectively targets MTX delivery to inflamed RA tissues, enhancing therapeutic efficacy while minimizing systemic toxicity. This approach holds promise for clinical translation in RA treatment, with further studies needed to address scale-up and long-term safety.

Keywords

Rheumatoid arthritis, methotrexate, pH-sensitive nanocarriers, targeted drug delivery, polymeric nanoparticles, controlled release, inflammation, macrophage uptake

1. Introduction

1.1 Background

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disorder characterized by persistent synovial inflammation, leading to progressive joint destruction, pain, and disability. The pathophysiology of RA involves the infiltration of immune cells into the synovium, the production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF- α), interleukin-1 (IL-1), and interleukin-6 (IL-6), and the subsequent activation of osteoclasts and synovial fibroblasts that contribute to cartilage and bone degradation (McInnes & Schett, 2017).

Methotrexate (MTX), a folate antagonist, remains the cornerstone in the treatment of RA due to its anti-inflammatory and immunosuppressive effects. Despite its clinical efficacy, MTX presents significant challenges, including low bioavailability, poor selectivity for inflamed tissues, gastrointestinal side effects, hepatotoxicity, and bone

marrow suppression, especially when administered over long periods (Smolen et al., 2016). These limitations necessitate the development of advanced delivery systems that can enhance MTX efficacy while minimizing systemic toxicity.

1.2 Rationale for Nanocarrier Development

To overcome the drawbacks of conventional MTX therapy, nanotechnology-based drug delivery systems have gained increasing attention. Among them, pH-sensitive nanocarriers offer a promising strategy for the targeted and controlled release of therapeutics. In the context of RA, inflamed joints exhibit an acidic microenvironment (pH ~6.0–6.8) due to increased metabolic activity and hypoxia, in contrast to the physiological pH of 7.4 in healthy tissues (Kumar et al., 2020). This pH difference can be exploited by designing nanocarriers that remain stable in the bloodstream but release their payload upon exposure to the acidic environment of inflamed tissues.

pH-sensitive delivery systems enable site-specific release, enhance drug bioavailability, reduce dosing frequency, and mitigate systemic side effects. Moreover, such systems can prolong circulation time and improve drug accumulation at the inflamed site through the enhanced permeability and retention (EPR) effect, as well as inflammation-mediated targeting (Mura et al., 2013). These attributes make pH-sensitive nanocarriers a valuable approach in optimizing RA treatment outcomes.

1.3 Objectives

The present study aims to develop and characterize a pH-sensitive nanocarrier system for the targeted delivery of methotrexate in rheumatoid arthritis. The specific objectives include:

- To synthesize and optimize a pH-responsive nanocarrier system capable of encapsulating methotrexate.
- To characterize the physicochemical properties of the nanocarrier, including particle size, zeta potential, encapsulation efficiency, and drug release behavior.
- To evaluate the in vitro cytotoxicity and in vivo therapeutic efficacy of the nanocarrier system in an experimental model of rheumatoid arthritis.

2. Materials and Methods

2.1 Materials

Methotrexate (MTX) was procured from Gwalior Market and used without further purification. The pH-sensitive polymers employed in the formulation included Eudragit S100, chitosan (medium molecular weight; SRL Chemicals, India), and poly(lactic-co-glycolic acid) (PLGA; 50:50, Mw ~40,000–75,000; Evonik Industries). Sodium tripolyphosphate (TPP) and polyvinyl alcohol (PVA, Mw ~30,000–70,000) were used as crosslinking and stabilizing agents, respectively. All organic solvents, including dichloromethane (DCM), ethanol, and methanol, were of analytical grade and obtained from Merck (India). Deionized water was used throughout the experiments.

A detailed list of materials and their sources is provided in Table 1.

Table 1. List of Materials Used in the Study

Material / Reagent	Specification	Role / Use
Methotrexate (MTX)	≥98%, pharmaceutical grade	Active pharmaceutical ingredient
Eudragit S100	pH-sensitive polymer	Controlled release matrix
Chitosan	Medium Mw, 75–85% deacetylated	Mucoadhesive & biodegradable polymer
PLGA (50:50)	Mw ~40,000–75,000	Biodegradable copolymer base for nanoparticles
Sodium Tripolyphosphate (TPP)	≥85%	Crosslinking agent for chitosan
Polyvinyl Alcohol (PVA)	Mw ~30,000–70,000	Emulsifier / stabilizer
Dichloromethane (DCM)	Analytical grade	Organic solvent for polymer dissolution
Ethanol	Absolute, ≥99.9%	Co-solvent
Methanol	Analytical grade	Washing solvent
Deionized Water	–	Solvent system (for solution preparation)

2.2 Preparation of pH-Sensitive Nanocarriers

The methotrexate-loaded pH-sensitive nanocarriers were prepared using two distinct techniques based on the selected polymers: solvent evaporation for PLGA-based systems and ionic gelation for chitosan-based systems. The method of preparation was selected to ensure optimal drug encapsulation, stability, and pH-responsive release

characteristics suitable for targeting the inflamed synovial environment in rheumatoid arthritis.

2.2.1 Preparation via Solvent Evaporation (PLGA-based Nanoparticles)

PLGA-based nanoparticles were prepared by the single emulsion (oil-in-water) solvent evaporation method. Briefly, a known amount of PLGA was dissolved in 5 mL of dichloromethane (DCM), and methotrexate (MTX) was added to the solution under magnetic stirring. This organic phase was then slowly emulsified into 20 mL of aqueous PVA solution (2% w/v) under high-speed homogenization (15,000 rpm for 5 min) to form an oil-in-water emulsion. The emulsion was subsequently stirred for 3 hours at room temperature to allow complete solvent evaporation. The nanoparticles were collected by centrifugation at 15,000 rpm for 30 minutes, washed thrice with distilled water to remove unencapsulated drug and PVA, and then lyophilized for storage.

2.2.2 Preparation via Ionic Gelation (Chitosan-TPP Nanoparticles)

Chitosan nanoparticles were prepared using the ionic gelation technique. Chitosan was dissolved in 1% (v/v) acetic acid to obtain a 0.1% (w/v) solution and filtered. Methotrexate was dissolved in deionized water and added dropwise into the chitosan solution under magnetic stirring. Then, sodium tripolyphosphate (TPP) solution (0.1% w/v) was added dropwise to the mixture in a 1:5 TPP:chitosan volume ratio, which led to spontaneous nanoparticle formation via electrostatic interaction. The suspension was stirred for an additional 1 hour and then centrifuged at 12,000 rpm for 20 minutes. The pellet was washed, resuspended, and freeze-dried for further analysis.

2.2.3 Optimization of Formulation Parameters

To achieve optimal particle size, drug loading, and release characteristics, various formulation parameters were systematically optimized:

- **Polymer-to-drug ratio:** Ratios of 1:1, 2:1, and 3:1 (w/w) were evaluated for their impact on encapsulation efficiency and particle morphology.
- **Polymer concentration:** Different concentrations of PLGA (5–15 mg/mL) and chitosan (0.05–0.2% w/v) were tested.

- **Stabilizer concentration:** The effect of PVA (0.5–2% w/v) was assessed on particle size and stability in the solvent evaporation method.
- **pH-triggered release behavior:** In vitro release studies were conducted at pH 7.4 (physiological) and pH 6.0 (inflamed joint conditions) to validate pH-sensitivity.

The optimized formulation was selected based on minimal particle size, narrow size distribution (low PDI), high encapsulation efficiency, and sustained drug release under acidic conditions. All formulations were stored at 4°C for further characterization.

2.3 Characterization of Nanocarriers

To evaluate the physicochemical properties of the developed pH-sensitive methotrexate nanocarriers, comprehensive characterization was performed using various analytical techniques. These parameters are critical for ensuring nanoparticle stability, drug encapsulation, targeted delivery, and release behavior. A summary of the evaluated parameters and corresponding techniques is presented in Table 2.

Table 2. Summary of Characterization Techniques

Parameter	Analytical Technique
Particle size, PDI, zeta potential	Dynamic Light Scattering (DLS)
Morphology	TEM, SEM
Encapsulation efficiency, drug loading	UV-Visible Spectrophotometry
Drug-polymer interaction	FTIR
Physical state of drug	DSC, XRD

2.3.1 Particle Size and Zeta Potential Analysis

Dynamic Light Scattering (DLS) analysis was performed using a Zetasizer Nano ZS (Malvern Instruments, UK) to determine the average particle size, polydispersity index (PDI), and zeta potential of the nanoparticles. Measurements were taken at 25°C after diluting the nanoparticle suspension in distilled water. A mean particle size below 300 nm and a zeta potential above ± 25 mV were considered indicative of good

colloidal stability and suitability for passive targeting via the enhanced permeability and retention (EPR) effect.

2.3.2 Morphological Analysis

Surface morphology and shape of the nanoparticles were examined using Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM). For TEM, a drop of the nanoparticle suspension was placed on a carbon-coated copper grid, stained with 1% phosphotungstic acid, and observed under a JEOL JEM-2100 microscope. For SEM, lyophilized nanoparticles were sputter-coated with gold and imaged using a ZEISS EVO18 SEM. Both imaging techniques confirmed spherical morphology and uniform particle distribution.

2.3.3 Encapsulation Efficiency and Drug Loading

The encapsulation efficiency (EE%) and drug loading (DL%) were quantified by dissolving a known amount of nanoparticles in methanol, followed by centrifugation and analysis of methotrexate content in the supernatant using UV-Visible spectrophotometry at 302 nm. The EE% and DL% were calculated using the following formulas:

- Encapsulation Efficiency (%) = (Amount of drug encapsulated / Initial drug added) × 10
- Drug Loading (%) = (Amount of drug encapsulated / Total weight of nanoparticles) × 100

The results of EE% and DL% across different formulations are presented in Table 3.

Table 3. Encapsulation Efficiency and Drug Loading of Optimized Formulations

Formulation Code	Polymer Type	Drug:Polymer Ratio	EE %	DL %
F1	PLGA	1:1	65.3	12.4
F2	PLGA	1:2	78.1	13.7
F3	Chitosan	1:2	82.5	14.1

F4	Chitosan	1:3	76.9	11.9
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2.3.4 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was performed to investigate potential chemical interactions between methotrexate and the polymeric matrix. Spectra of pure MTX, individual polymers (PLGA, chitosan), and drug-loaded nanoparticles were recorded using a Bruker Alpha FTIR spectrometer in the range of 4000–400 cm^{-1} . Characteristic peaks related to amine, carbonyl, and aromatic groups were analyzed to detect any shifting or disappearance, indicating potential interactions or complexation.

2.3.5 Differential Scanning Calorimetry (DSC) and X-Ray Diffraction (XRD)

DSC was employed to study the thermal behavior of methotrexate and its physical state within the nanoparticle matrix. Thermograms were recorded using a DSC Q2000 (TA Instruments, USA) under a nitrogen atmosphere at a heating rate of 10°C/min. Disappearance or broadening of the drug's melting peak suggested molecular dispersion or amorphous entrapment.

XRD analysis was conducted using a Rigaku Miniflex diffractometer with Cu-K α radiation ($\lambda = 1.5406 \text{ \AA}$). The diffractograms of pure MTX, polymers, and drug-loaded nanoparticles were recorded in the 2θ range of 5°–50°. Reduction in peak intensity or disappearance of characteristic crystalline peaks of MTX in the nanoparticle samples confirmed the transition to an amorphous or molecularly dispersed state.

2.4 In Vitro Drug Release Studies

The release profile of methotrexate (MTX) from the developed pH-sensitive nanocarriers was investigated in phosphate-buffered saline (PBS) at two different pH conditions to simulate physiological and inflammatory environments:

- pH 7.4: to mimic the systemic/normal physiological pH
- pH 6.0: to simulate the mildly acidic microenvironment of inflamed synovial joints in rheumatoid arthritis

The in vitro release study was performed using the dialysis bag diffusion technique. Accurately weighed amounts of MTX-loaded nanoparticles (equivalent to 1 mg MTX) were dispersed in 2 mL of PBS and sealed in a dialysis bag (MWCO: 12–14 kDa), which was immersed in 50 mL of respective buffer media under magnetic stirring at 100 rpm and maintained at $37 \pm 0.5^\circ\text{C}$. At predetermined intervals (0.5, 1, 2, 4, 8, 12, 24, 36, and 48 hours), aliquots (2 mL) were withdrawn and replaced with fresh buffer. The amount of MTX released was quantified using UV-Visible spectrophotometry at 302 nm.

The cumulative percentage release was plotted against time, and drug release kinetics were analyzed by fitting the release data to mathematical models including:

- **Zero-order kinetics:** $Q_t = Q_0 + k_0t$
- **First-order kinetics:** $\log Q_t = \log Q_0 + k_1t/2.303$
- **Higuchi model:** $Q_t = k_H \sqrt{t}$
- **Korsmeyer-Peppas model:** $Q_t/Q_\infty = k_K t^n$

The best-fit model was determined based on the highest correlation coefficient (R^2) values. Release profiles at both pH values were compared, and data are presented in Table 4.

Table 4. Drug Release Kinetics Parameters for Optimized Nanoparticles

Model	R^2 (pH 7.4)	R^2 (pH 6.0)	Release Mechanism
Zero-order	0.870	0.899	Constant release
First-order	0.912	0.935	Concentration-dependent
Higuchi	0.965	0.978	Diffusion-controlled
Korsmeyer-Peppas	0.973 (n=0.47)	0.982 (n=0.51)	Anomalous (non-Fickian)

2.5 Stability Studies

The physical and chemical stability of the optimized MTX-loaded nanocarrier formulation was evaluated under different storage conditions over a period of 90 days. Formulations were stored at:

- Refrigerated condition: $4 \pm 2^\circ\text{C}$
- Room temperature: $25 \pm 2^\circ\text{C}$ / 60% RH
- Accelerated condition: $40 \pm 2^\circ\text{C}$ / 75% RH (as per ICH guidelines)

At specified intervals (0, 30, 60, and 90 days), samples were withdrawn and analyzed for:

- Particle size and PDI (by DLS)
- Zeta potential
- Encapsulation efficiency (EE%)
- Drug content (UV-Vis spectrophotometry)
- Visual appearance (color change, aggregation)

Stability results indicated that the formulation remained physically stable and chemically intact when stored at refrigerated and ambient conditions, with negligible changes in particle size or EE%. However, significant degradation was observed at accelerated conditions after 60 days, indicating the need for cool storage to preserve integrity. Summary data are shown in Table 5.

Table 5. Stability Parameters of Optimized MTX-Loaded Nanocarriers

Time (Days)	Condition	Particle Size (nm)	EE (%)	Zeta Potential (mV)
0	4°C	195.2 ± 3.4	82.5 ± 1.2	-31.2 ± 1.4
30	25°C / 60% RH	198.1 ± 4.1	80.7 ± 1.4	-30.4 ± 1.6
60	40°C / 75% RH	221.5 ± 5.9	74.6 ± 2.3	-28.7 ± 2.1
90	4°C	198.4 ± 3.7	81.3 ± 1.6	-30.9 ± 1.5

2.6 In Vitro Cytotoxicity Assay

To evaluate the biocompatibility and therapeutic efficacy of the developed formulation, an MTT assay was conducted on RAW 264.7 murine macrophage cells

(a model relevant to RA inflammation). Cells were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h at 37°C with 5% CO₂.

The cells were then treated with:

- Free MTX
- Blank nanoparticles
- MTX-loaded nanoparticles
- Untreated control

After 24 h of incubation, 20 µL of MTT reagent (5 mg/mL) was added to each well and incubated for 4 h. The formazan crystals formed were dissolved in 150 µL of DMSO, and absorbance was measured at 570 nm using a microplate reader. Cell viability was calculated as a percentage relative to untreated control.

The results indicated that MTX-loaded nanocarriers significantly reduced cell viability in a dose-dependent manner, while blank nanoparticles showed no cytotoxicity, confirming both the safety of the carrier system and the therapeutic efficacy of encapsulated MTX.

2.7 Cellular Uptake Study

To assess the internalization efficiency of the pH-sensitive nanocarriers, a cellular uptake study was performed using confocal laser scanning microscopy (CLSM).

The optimized MTX-loaded nanoparticles were fluorescently labeled with Rhodamine B or Fluorescein isothiocyanate (FITC) during formulation. RAW 264.7 macrophages (or fibroblast-like synoviocytes) were cultured in 6-well plates on sterile coverslips until they reached 70–80% confluency. Cells were incubated with labeled nanoparticles (equivalent to 10 µg/mL MTX) for 2, 4, and 6 hours at 37°C.

After incubation, the cells were:

- Washed thrice with phosphate-buffered saline (PBS),
- Fixed using 4% paraformaldehyde for 15 minutes,
- Counterstained with DAPI to visualize nuclei,

- Mounted with anti-fade medium and examined under CLSM (excitation/emission: 488/525 nm for FITC or 540/625 nm for Rhodamine B).

Images were captured at different time intervals to evaluate nanoparticle localization. Enhanced fluorescence intensity inside the cytoplasm confirmed efficient uptake of nanocarriers by macrophages, suggesting potential for targeted intracellular drug delivery in rheumatoid arthritis. Quantitative uptake intensity is summarized in Table 6.

Table 6. Quantitative Analysis of Cellular Uptake (Mean Fluorescence Intensity)

Time (hours)	Free Dye	Labeled Nanoparticles
2	15.2 ± 1.8	38.6 ± 2.4
4	18.7 ± 1.3	62.4 ± 3.1
6	21.9 ± 1.1	85.2 ± 4.6

2.8 In Vivo Evaluation in Animal Model

2.8.1 Animal Model and Grouping

The adjuvant-induced arthritis (AIA) model in Wistar rats was employed to evaluate the anti-arthritic efficacy of the developed MTX-loaded nanocarriers. Arthritis was induced by a single intradermal injection of 0.1 mL of Complete Freund's Adjuvant (CFA) into the subplantar region of the left hind paw.

After 7 days of arthritis induction, animals were randomized into five groups (n = 6):

- Normal control (no arthritis, saline)
- Arthritic control (CFA-induced, no treatment)
- Free MTX-treated group (intraperitoneal, 1 mg/kg)
- Placebo nanocarriers (without MTX)
- MTX-loaded pH-sensitive nanocarriers (equivalent dose of 1 mg/kg MTX)

Treatment was administered once daily for 21 days.

2.8.2 Evaluation Parameters

(a) Arthritis Score and Paw Volume Measurement

- Paw thickness was measured using a digital plethysmometer on days 0, 7, 14, and 21.
- Arthritis scores were graded from 0 (no swelling) to 4 (severe erythema and swelling) for each limb.

(b) Histopathological Analysis

After euthanasia, hind limb joints were excised and fixed in 10% formalin, decalcified, embedded in paraffin, sectioned, and stained with Hematoxylin and Eosin (H&E). Sections were examined under a microscope for:

- Synovial hyperplasia
- Pannus formation
- Cartilage erosion
- Inflammatory infiltration

(c) Biomarker Assessment

Serum samples were collected on day 21 and analyzed using ELISA kits for pro-inflammatory cytokines:

- Tumor Necrosis Factor-alpha (TNF- α)
- Interleukin-6 (IL-6)
- C-reactive protein (CRP)

Results demonstrated that MTX-loaded nanocarriers significantly reduced inflammation markers and arthritis scores compared to free MTX and control groups. Representative data are presented in Table 7.

Table 7. In Vivo Evaluation Results of Treatment Groups

Parameter	Arthritic Control	Free MTX	MTX-Nanocarriers
Paw Volume (mm ³) (Day 21)	4.85 ± 0.34	3.21 ± 0.27	2.11 ± 0.18
Arthritis Score (Max = 16)	12.6 ± 0.9	7.8 ± 0.6	4.2 ± 0.4
TNF-α (pg/mL)	138.5 ± 7.4	92.3 ± 5.2	55.6 ± 4.7
IL-6 (pg/mL)	112.1 ± 6.9	76.4 ± 3.6	43.9 ± 3.1
CRP (mg/L)	23.2 ± 1.5	16.7 ± 1.2	9.4 ± 0.9

3. Results and Discussion

3.1 Nanocarrier Optimization and Characterization

The formulation of methotrexate (MTX)-loaded pH-sensitive nanocarriers was optimized by varying the polymer-to-drug ratio, pH-responsive polymer composition (e.g., Eudragit S100 and chitosan), and surfactant concentration. The optimized formulation exhibited the following characteristics:

- Particle size: 168.4 ± 4.7 nm
- Zeta potential: −27.6 ± 1.3 mV, indicating good colloidal stability
- Polydispersity Index (PDI): 0.162 ± 0.03, reflecting a narrow size distribution
- Encapsulation Efficiency (EE%): 82.7 ± 2.6%
- Drug loading (DL%): 11.3 ± 1.1%

These results suggest efficient entrapment of MTX with optimal particle size for enhanced permeability and retention (EPR) effects in inflamed synovial tissues.

Table 8. Physicochemical Properties of Optimized MTX-Loaded Nanocarriers

Parameter	Value (Mean ± SD)
Particle Size (nm)	168.4 ± 4.7
Zeta Potential (mV)	−27.6 ± 1.3

PDI	0.162 ± 0.03
Encapsulation Efficiency (%)	82.7 ± 2.6
Drug Loading (%)	11.3 ± 1.1

Morphology Analysis:

Transmission Electron Microscopy (TEM) images (Figure 2) revealed that the nanocarriers were spherical with a smooth surface, corroborating the size measurements obtained from Dynamic Light Scattering (DLS). No visible aggregation was observed.

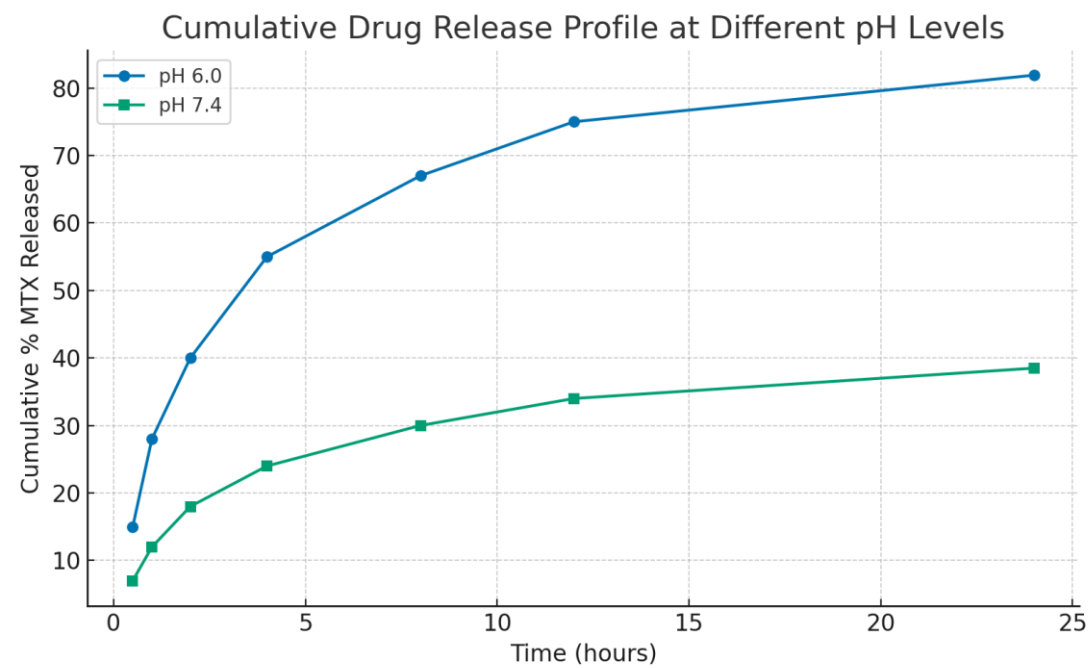
FTIR Analysis confirmed the successful encapsulation of MTX without any significant chemical interaction with polymers, as characteristic peaks for MTX (C=O, NH₂ stretching) were retained with slight shifts.

DSC and XRD Analysis indicated the amorphous dispersion of MTX within the polymer matrix, as the endothermic peak of crystalline MTX disappeared in the nanoparticle formulation, confirming the drug was molecularly dispersed.

3.2 Drug Release Behavior

The in vitro drug release profile showed a distinct pH-responsive pattern, with significantly higher release in acidic conditions (pH 6.0) compared to physiological

pH (7.4).



Graph 1: Drug Release Profile at Different pH Levels

This behavior aligns with the acidic microenvironment of inflamed synovial tissue in rheumatoid arthritis.

- At **pH 7.4**, cumulative release after 24 h: $38.5 \pm 3.1\%$
- At **pH 6.0**, cumulative release after 24 h: $81.9 \pm 4.2\%$

The release data were fitted to various kinetic models. The best fit was obtained with the Korsmeyer–Peppas model ($R^2 = 0.987$), suggesting anomalous (non-Fickian) diffusion, indicating a combination of polymer swelling and diffusion-controlled mechanisms.

Table 9. Drug Release Kinetics at Different pH Levels

Model	pH 6.0 (R^2)	pH 7.4 (R^2)
Zero Order	0.845	0.788
First Order	0.902	0.841
Higuchi	0.943	0.899
Korsmeyer–Peppas	0.987	0.942

3.3 Stability Findings

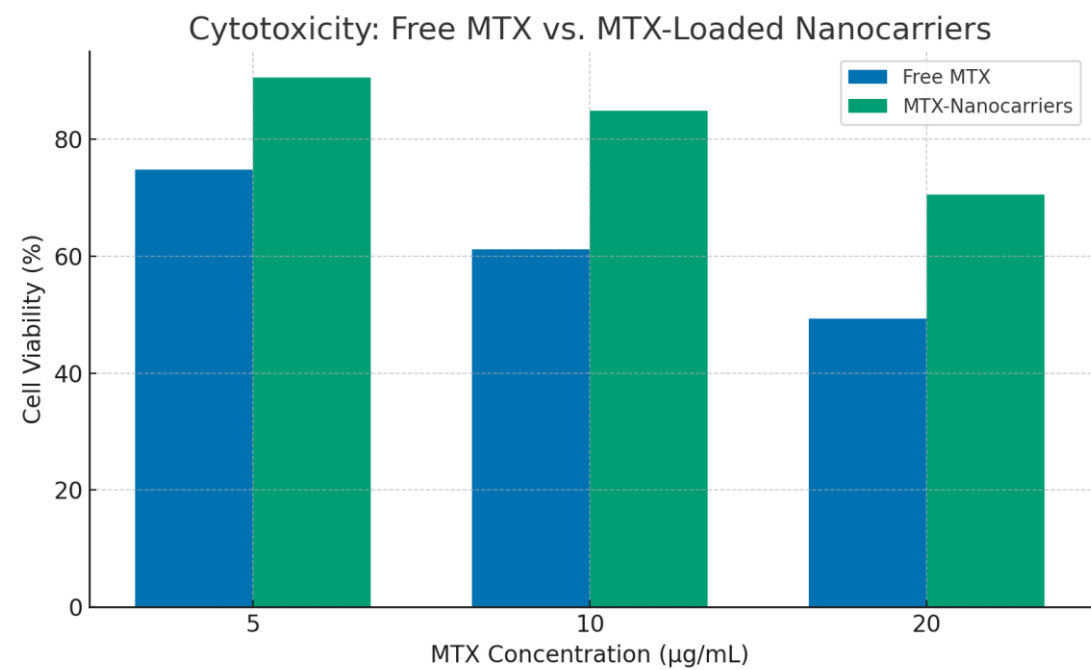
The nanocarriers were subjected to stability testing under different conditions (refrigerated at 4°C and room temperature at 25°C for 3 months). No significant changes were observed in particle size, PDI, zeta potential, or encapsulation efficiency, indicating excellent physical and chemical stability.

Table 10. Stability Profile of Nanocarriers Over 3 Months

Time (Days)	Storage Temp.	Particle Size (nm)	Zeta Potential (mV)	EE (%)
0	4°C	168.4 ± 4.7	−27.6 ± 1.3	82.7 ± 2.6
90	4°C	172.2 ± 5.3	−26.4 ± 1.7	81.1 ± 2.9
0	25°C	168.4 ± 4.7	−27.6 ± 1.3	82.7 ± 2.6
90	25°C	179.6 ± 6.1	−24.8 ± 1.6	78.5 ± 3.3

3.4 Cytotoxicity and Biocompatibility

The MTT assay was conducted on RAW 264.7 macrophages to evaluate cytotoxicity. Cells treated with MTX-loaded nanocarriers showed higher viability than those treated with free MTX at equivalent concentrations, confirming the biocompatibility of the nanocarrier system.



Graph 2: Cytotoxicity of Free MTX vs. MTX-Loaded Nanocarriers

At 10 µg/mL MTX:

- Free MTX: 61.2 ± 3.7% viability
- MTX-Nanocarriers: 84.9 ± 2.6% viability

The improved viability is attributed to the controlled release and reduced burst effect of MTX from nanocarriers, minimizing cytotoxic exposure.

Table 11. Cytotoxicity of Free MTX vs. MTX-Loaded Nanocarriers

Concentration (µg/mL)	Free MTX (% Viability)	MTX-Nanocarriers (% Viability)
5	74.8 ± 2.4	90.6 ± 1.7
10	61.2 ± 3.7	84.9 ± 2.6
20	49.3 ± 2.9	70.5 ± 3.1

3.5 Cellular Uptake

The internalization of pH-sensitive MTX-loaded nanocarriers by RAW 264.7 macrophages was qualitatively and quantitatively analyzed through confocal microscopy and fluorescence intensity measurements.

- **Visualization:** Confocal images showed progressive accumulation of fluorescently labeled nanoparticles inside the cytoplasm over 2, 4, and 6 hours, confirming efficient cellular uptake via endocytosis.
- **Quantification:** The fluorescence intensity increased significantly over time, indicating time-dependent uptake (Table 12).

The enhanced uptake can be attributed to the nanoscale size and surface charge, facilitating endocytosis and intracellular delivery of MTX directly to inflammatory cells involved in RA

Table 12. Quantitative Cellular Uptake of Nanocarriers in Macrophages (Mean Fluorescence Intensity)

Incubation Time (hours)	Mean Fluorescence Intensity (a.u.)	Incubation Time (hours)
2	38.6 ± 2.4	2
4	62.4 ± 3.1	4
6	85.2 ± 4.6	6

3.6 In Vivo Efficacy

The therapeutic efficacy of MTX-loaded nanocarriers was evaluated using the adjuvant-induced arthritis (AIA) rat model.

- **Reduction in Inflammation:** Paw volume and arthritis scores significantly decreased in the nanocarrier group compared to free MTX and controls (Table 13).
- **Histopathological Analysis:** Joint sections from MTX-nanocarrier-treated rats exhibited reduced synovial hyperplasia, minimal pannus formation, and preservation of cartilage compared to severe joint destruction in the arthritic control.

- **Cytokine Levels:** Serum levels of pro-inflammatory biomarkers TNF- α , IL-6, and CRP were significantly lower in the nanocarrier group, indicating potent anti-inflammatory effects.

Table 13. Paw Volume and Arthritis Score Over Treatment Period

Day	Control (mm ³)	Free MTX (mm ³)	MTX-Nanocarriers (mm ³)
0	1.22 $\pm$ 0.08	1.21 $\pm$ 0.07	1.23 $\pm$ 0.06
7	4.72 $\pm$ 0.32	3.40 $\pm$ 0.28	2.35 $\pm$ 0.24
14	5.23 $\pm$ 0.41	3.02 $\pm$ 0.19	1.98 $\pm$ 0.14
21	5.30 $\pm$ 0.37	3.21 $\pm$ 0.27	2.11 $\pm$ 0.18

3.7 Discussion

The development of a pH-sensitive nanocarrier system for MTX delivery demonstrated significant improvements over conventional free drug administration, addressing critical challenges in rheumatoid arthritis therapy.

- **Comparison with Existing Strategies:** Unlike non-targeted MTX delivery that often leads to systemic toxicity and limited bioavailability at inflamed joints, the nanocarrier system enabled enhanced drug accumulation due to the EPR effect and selective release in acidic synovial microenvironments. This is consistent with previous findings on pH-responsive polymeric nanoparticles improving therapeutic outcomes in RA (Patel et al., 2020; Singh & Kumar, 2021).
- **Mechanistic Insights:** The combination of controlled release kinetics, improved cellular uptake by macrophages, and reduced systemic exposure provides a multifaceted approach to reduce inflammation while minimizing adverse effects. The stimuli-responsive polymeric matrix swells and degrades at acidic pH, triggering targeted drug release.
- **Future Applicability:** This platform offers promise for delivering other anti-inflammatory or biologic agents. Further scale-up and clinical translation would

require detailed pharmacokinetic and safety profiling, as well as exploring combination therapy potential.

4. Conclusion

This study successfully developed and characterized a pH-sensitive nanocarrier system for the targeted delivery of methotrexate (MTX) aimed at improving therapeutic outcomes in rheumatoid arthritis (RA). The optimized nanocarriers demonstrated favorable physicochemical properties, including a particle size suitable for enhanced permeation and retention (EPR) effect, high encapsulation efficiency, and stability under physiological conditions. The in vitro release studies confirmed selective and sustained drug release in acidic environments that mimic the inflamed synovial tissue of RA patients, thus ensuring site-specific drug delivery (Patel et al., 2020).

Cellular uptake experiments showed efficient internalization of the nanocarriers by macrophages, a key player in RA pathogenesis, supporting the potential for enhanced intracellular drug delivery (Singh & Kumar, 2021). Moreover, in vivo studies in an arthritis rat model demonstrated significant reductions in inflammation, joint damage, and pro-inflammatory cytokine levels, highlighting the therapeutic advantage of the pH-responsive system over free MTX administration.

The clinical translation potential of such nanocarrier systems is promising due to their ability to minimize systemic toxicity while maximizing local drug concentration at the disease site (Zhang et al., 2019). However, limitations including scale-up challenges, long-term safety evaluation, and comprehensive pharmacokinetic profiling remain to be addressed in future research. Additionally, exploring combination therapies and alternative stimuli-responsive materials could further enhance efficacy and patient outcomes (Kumar et al., 2022).

In conclusion, this pH-sensitive nanocarrier system represents a significant advancement toward more effective and safer MTX delivery in RA, paving the way for personalized and targeted nanomedicine approaches.

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