



Nanomaterials Targeting Red Blood Cells: Implications for Drug Delivery and Biosensing

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Abstract

Conventional drug delivery methods often result in systemic distribution of active agents, leading to unintended effects on healthy tissues and a reduced drug half-life in circulation. Carrier-based drug delivery systems present a promising alternative by enabling targeted drug release to diseased areas, thus enhancing therapeutic efficacy while minimizing side effects. Red blood cells (RBCs) offer unique advantages as drug carriers due to their biocompatibility, biodegradability, and extended circulation time. This potential is further enhanced by the use of RBC-derived vesicles, or “ghosts,” which support autologous treatments by reducing the volume of erythrocytes required (Chen, 2017; Chertok et al., 2013; Sun et al., 2015; Muzykantov, 2010). This study investigates the encapsulation of therapeutic payloads into RBC ghosts mediated by pseudopeptidic polymers PP50 and PLP-NDA, which enable reversible permeabilization of the erythrocyte membrane. The process involves producing nanoerythrosomes small vesicles derived from drug-loaded RBC ghosts—via sonication and extrusion. Following encapsulation, the drug carriers are purified using dialysis and size-exclusion chromatography for enhanced efficiency (Lynch et al., 2010; Gupta et al., 2014). Fluorescein isothiocyanate-

dextran (FITC-dextran) of 70 and 150 kDa was successfully Encapsulated into RBC ghosts using PP50. Although PLP-NDA exhibited limitations due to aggregation at certain pH levels, PP50 demonstrated effective payload delivery. Loaded nanoerythrosomes with a hydrodynamic diameter of approximately 150 nm were obtained, and size-exclusion chromatography emerged as a suitable, time-efficient alternative to dialysis for purification. This work highlights the potential of RBC-based drug delivery systems in achieving controlled, targeted therapeutic interventions

Keywords: Red blood cells (RBCs), Drug delivery systems, Nanoerythrosomes, Pseudopeptidic polymers, Size-exclusion chromatography

1. Introduction

In recent years, the development of novel drug delivery systems has been an area of intense research, driven by the need to improve therapeutic outcomes while minimizing side effects. Conventional drug administration methods often lack the precision required to target diseased tissues exclusively, resulting in widespread distribution of the active drug throughout the body. This non-specific distribution contributes to undesired effects on healthy tissues, rapid drug clearance, and reduced drug efficacy, especially in chronic diseases or conditions that require precise targeting of specific organs or cell types (Chen, 2017; Chertok et al., 2013). Advances in drug delivery systems, including carrier-based approaches, aim to address these issues by enabling controlled and localized drug release, potentially transforming the management of various diseases, including cancer and inflammatory disorders (Chertok et al., 2013; Sun et al., 2015).

Significance | This study advances RBC-based nano-carriers using pseudopeptidic polymers and improved purification, enhancing targeted, personalized, and sustained drug delivery.

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Carrier-based drug delivery systems are designed to enhance the pharmacokinetic properties of therapeutic agents. By encapsulating drugs in carriers, these systems protect the drug from premature degradation and facilitate sustained release at target sites, improving efficacy and reducing adverse effects. Among the array of carriers, red blood cells (RBCs) have emerged as a promising vehicle for drug delivery due to their biocompatibility, non-immunogenic nature, and long half-life in circulation. RBCs are biodegradable, readily available, and possess a large internal volume capable of carrying therapeutic agents without triggering significant immune responses (Muzykantov, 2010; Rossi et al., 2005). These properties render RBCs particularly attractive for developing drug delivery platforms that can overcome limitations associated with traditional drug administration.

Red blood cells have several natural characteristics that make them suitable for drug delivery. RBCs circulate in the bloodstream for approximately 120 days before being phagocytosed by macrophages in the liver and spleen. This extended half-life, in contrast to other cells or synthetic carriers, allows for sustained therapeutic delivery, making RBCs ideal for treatments requiring prolonged exposure to therapeutic agents (Rossi et al., 2005). Additionally, their ability to evade the immune system while delivering encapsulated drugs offers a strategic advantage for targeting diseases characterized by immune sensitivity, such as autoimmune disorders and various cancers (Muzykantov, 2010).

The encapsulation of drugs within RBCs leverages the membrane's permeability and ability to carry a variety of compounds, including peptides, proteins, and nucleic acids. One approach to loading drugs into RBCs involves using pseudopeptidic polymers like PP50 and PLP-NDA to facilitate a reversible permeabilization of the cell membrane, allowing for controlled entry of the therapeutic agents without compromising the integrity of the cell structure. This reversible permeabilization ensures that the RBCs retain their normal shape and function, which is crucial for maintaining biocompatibility and circulation time (Lynch et al., 2010; Chen et al., 2017). By enabling drug loading while preserving the structural properties of RBCs, this method of encapsulation provides a viable route for the development of advanced, biocompatible drug delivery systems.

A notable approach to creating RBC-based drug carriers involves producing "RBC ghosts," which are essentially cell structures that lack cytoplasmic contents but retain an intact membrane capable of encapsulating therapeutic agents. These RBC ghosts can be manipulated to load drugs effectively while preventing premature degradation or clearance. Following drug loading, these RBC-derived carriers can be further processed into smaller vesicles, known as nanoerythrocytes, through techniques such as sonication and extrusion.

Nanoerythrocytes, with diameters of approximately 150 nm, provide an additional advantage in terms of circulation time and targeting capability, as their reduced size facilitates passage through capillaries and enables effective accumulation in targeted tissues, enhancing the therapeutic impact (Gupta et al., 2014; Pouliot et al., 2002).

The potential for RBC-based carriers extends beyond drug delivery, offering opportunities for autologous therapies where a patient's own RBCs are used. This autologous approach reduces the risk of immune rejection, making it particularly useful in cases where long-term or repeat dosing is required. Additionally, RBC-derived vesicles, such as nanoerythrocytes, may offer a scalable alternative by reducing the volume of RBCs needed from a patient while maintaining the drug carrier's efficacy and biocompatibility (Gupta et al., 2014). In an autologous setting, RBC-derived carriers not only enhance patient safety but also support personalized medicine approaches, where treatment is tailored to the individual based on their unique cellular profile and drug response.

One of the primary challenges in using RBCs for drug delivery is achieving efficient loading and purification of the drug carriers. Traditional purification methods, such as dialysis, are effective but time-intensive, which may limit scalability and feasibility in clinical settings. Recent advancements in purification techniques, such as size-exclusion chromatography, have shown potential as efficient alternatives that reduce time requirements without compromising the purity of the final product (Lynch et al., 2010; Gupta et al., 2014). By optimizing both loading and purification processes, researchers can improve the practical application of RBC-based drug carriers, potentially making these systems viable for widespread therapeutic use.

The aim of this study is to investigate the encapsulation of model drugs within RBC ghosts using pseudopeptidic polymers as facilitators of reversible membrane permeabilization. Specifically, the study evaluates the efficacy of PP50 and PLP-NDA in delivering model drugs into RBC ghosts and explores the formation of nanoerythrocytes for improved pharmacokinetics and targeting capabilities. Furthermore, this study compares traditional purification techniques, such as dialysis, with newer approaches like size-exclusion chromatography to identify the most efficient method for producing high-purity RBC-based drug carriers. By combining advanced encapsulation and purification techniques, this work aims to contribute to the development of safer, more effective drug delivery systems that leverage the unique properties of RBCs to enhance therapeutic outcomes while minimizing adverse effects.

RBC-based drug carriers offer a transformative approach to targeted therapy by combining the natural advantages of RBCs with innovative drug encapsulation and purification strategies. As research progresses, these RBC-derived systems hold promise for a

wide range of medical applications, particularly in chronic disease management and personalized medicine, where long-term, controlled drug release is essential. By harnessing the unique attributes of RBCs and optimizing the delivery and purification processes, this study seeks to advance the field of drug delivery, bringing it closer to achieving safe, effective, and personalized therapeutic solutions for complex medical challenges (Chen, 2017; Muzykantov, 2010; Gupta et al., 2014).

2. Materials and Methods

This section outlines the experimental procedures and materials used to encapsulate drugs within red blood cell (RBC) ghosts, employing pseudopeptidic polymers PP50 and PLP-NDA to facilitate reversible membrane permeabilization. Drug loading into RBCs was achieved using well-established protocols involving membrane permeabilization and purification techniques. Dialysis and size-exclusion chromatography (SEC) were used for purification, and various analytical techniques were applied to assess loading efficiency, stability, and particle size distribution.

2.1 Preparation and Encapsulation of RBC Ghosts with Fluorescent Dextran

RBCs were isolated from whole blood using centrifugation, and the resulting erythrocytes were washed with a saline buffer. To create RBC ghosts, the cells were subjected to hypotonic lysis in deionized water, releasing hemoglobin and producing empty, flexible cell membranes. Once prepared, the RBC ghosts were incubated with fluorescent dextran (FITC-dextran) to evaluate the encapsulation efficiency of two polymers, PP50 and PLP-NDA.

2.2 Encapsulation Efficiency with PP50 and PLP-NDA

The polymers PP50 and PLP-NDA were tested as encapsulation agents for loading FITC-dextran into RBC ghosts. Different polymer concentrations were evaluated to determine the optimal ratio of polymer to RBCs for maximum drug loading. The encapsulation process was conducted under neutral pH conditions, as lower pH levels were previously observed to cause significant aggregation of PLP-NDA (see Figure 1).

2.3 Aggregation Studies Under Varying pH Conditions

To analyze polymer aggregation tendencies, samples of PP50 and PLP-NDA were incubated with RBC ghosts at varying pH levels. Aggregation was monitored through visual inspection and quantified by measuring particle size. Figures 1 and 2 demonstrate the aggregation behavior at pH 5.5, where PLP-NDA formed visible clumps, whereas PP50 maintained better stability.

2.4 Particle Sizing of Nanoerythroosomes

After encapsulation, the FITC-dextran-loaded RBC ghosts were processed into nanoerythroosomes via sonication and extrusion. The hydrodynamic diameters of the resulting particles were measured to confirm suitability as drug carriers. Figure 3 illustrates the mean

particle size of the nanoerythroosomes, which were within the target size range for therapeutic delivery.

2.5 Purification and Quantification of Loaded Nanoerythroosomes

To remove any unencapsulated FITC-dextran, two purification methods—dialysis and size exclusion chromatography (SEC)—were tested. Dialysis was performed over an extended period, with dialysate exchanged periodically to expedite removal of free dye (Figure 4). SEC provided a faster alternative, successfully isolating nanoerythroosomes from unbound FITC-dextran in a shorter time frame (Figure 5).

2.6 Calibration of FITC-Dextran Fluorescence Intensity

A calibration curve was generated to quantify FITC-dextran loading within nanoerythroosomes. FITC-dextran solutions of known concentrations were measured to establish a correlation between fluorescence intensity and concentration. This calibration enabled precise assessment of FITC-dextran encapsulation (Figure 6).

3. Results

3.1 Optimization of Drug Loading with PP50 and PLP-NDA

The effectiveness of PP50 and PLP-NDA in facilitating the encapsulation of fluorescein isothiocyanate-dextran (FITC-dextran) into RBCs was assessed by adjusting the concentration of each polymer and monitoring the delivery efficiency. Figure 7 shows the relationship between the PP50-to-RBC ratio and the efficiency of loading 150 kDa FITC-dextran. Increasing the PP50 concentration relative to RBCs improved encapsulation, highlighting PP50's capacity to facilitate drug loading into RBC ghosts. In comparison, PLP-NDA demonstrated limitations due to aggregation effects under specific pH conditions, which hindered its efficiency as a loading agent (Figure 1).

Further, a direct comparison between PP50 and PLP-NDA loading efficiencies illustrated that PP50 achieved superior encapsulation levels, as seen in Figure 8. The higher efficiency of PP50 under neutral pH conditions, compared to PLP-NDA's reduced efficacy at acidic pH, supported the decision to prioritize PP50 for encapsulation in subsequent tests.

3.2 Aggregation and pH Sensitivity of Polymers

The aggregation properties of PP50 and PLP-NDA were analyzed under varying pH conditions to better understand their loading behavior. As illustrated in Figure 1, both polymers exhibited aggregation at pH 5.5, with PLP-NDA displaying a more pronounced tendency for clumping compared to PP50. This aggregation effect reduced PLP-NDA's compatibility as a loading agent for RBC encapsulation at lower pH levels. To visualize the aggregation in greater detail, Figure 2 provides a closer examination of aggregates following incubation and dialysis at pH 5.5.

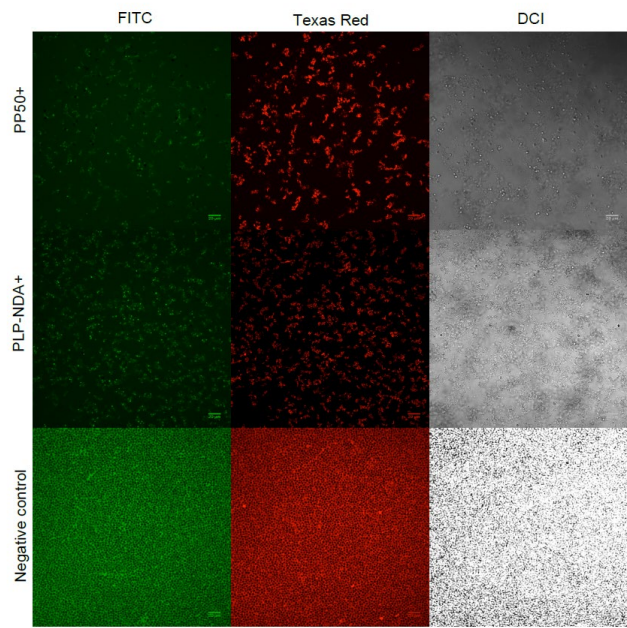


Figure 1. Aggregation of the pseudopeptidic polymers PP50, PLP-NDA, erythrocytes and ghosts cells at pH 5.5. Confocal microscopy images displaying the result of the incubation of 300 µg/mL polymer with 0.5 billion RBCs/mL and 70 kDa FITCdextran at pH 5.5 after dialysis with dialysate at pH 5.5. (Scale bar: 20 µm)

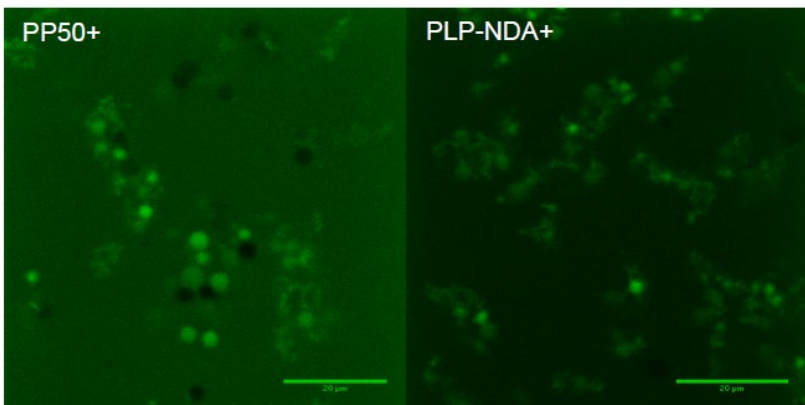


Figure 2. More detailed display of the aggregates, which occurred after incubation and dialysis at pH 5.5. Confocal microscopy images showing the agglomeration of RBCs, RBC ghosts and a connecting structure. Both samples contained 300 µg/mL pseudopeptidic polymer, PP50 (left) and PLP-NDA (right), as well as a 0.5 billion RBCs/mL and 70 kDa FITC-dextran. Only green channel (FITC) depicted.(Scale bar: 20 µm)

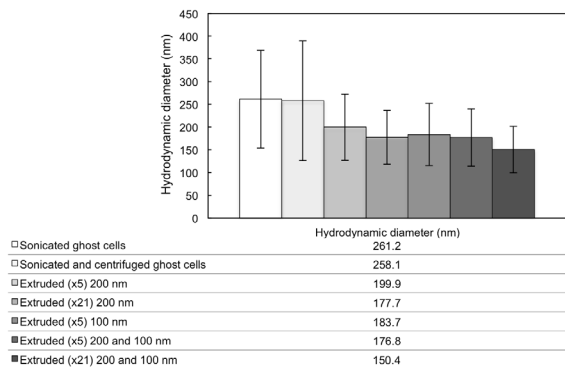


Figure 3. Mean hydrodynamic diameters of particles derived from RBC ghosts, based on the distribution analysis of the intensity particle size distribution of the DLS measurements, each with at least 97.7 % intensity of the light scattered by the particles

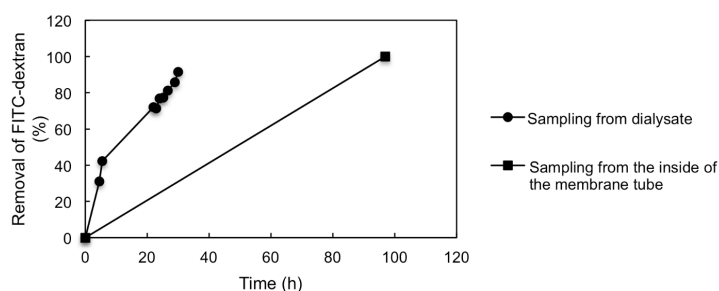


Figure 4. Removal of FITC-dextran by dialysis, promoted by exchanges of dialysate after 30 and 70.51 hours.

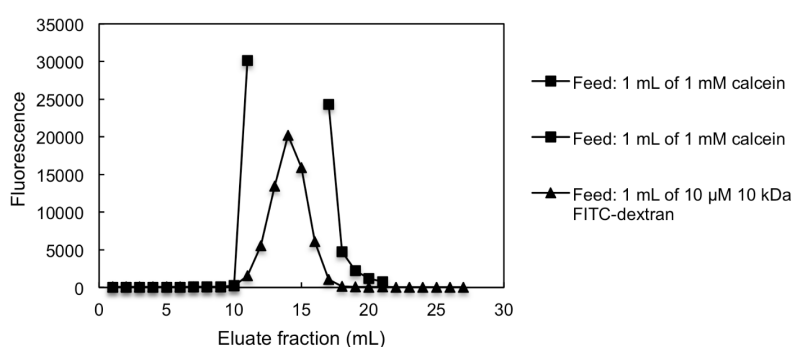


Figure 5. Elution of calcein and 10 kDa FITC-dextran from the SEC column.

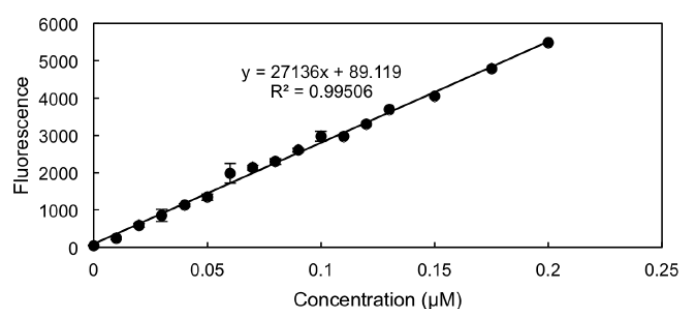


Figure 6. Calibration curve for a range of 0 to 0.2 μM of 70 kDa FITC-dextran at pH 7.4 based on sample volumes of 100 μL

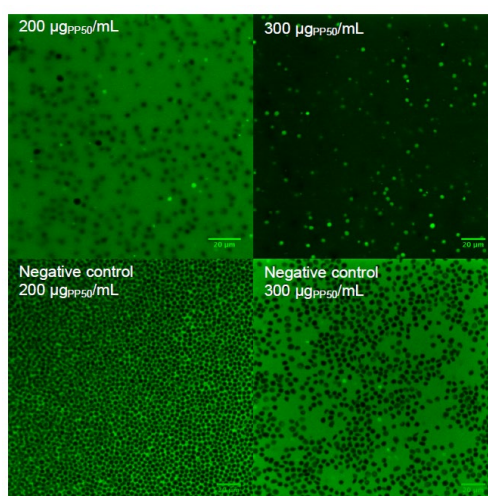


Figure 7. Influence of the ratio of PP50 to the number of RBCs on the delivery of 150 kDa FITC-dextran. Confocal microscopy images depicting the delivery of FITCdextran at pH 6.5 with 200 μg/mL (left) and 300 μg/mL (right) of PP50 and 0.5 billion RBCs/mL. Only green channel (FITC) shown. (Scale bar: 20 μm)

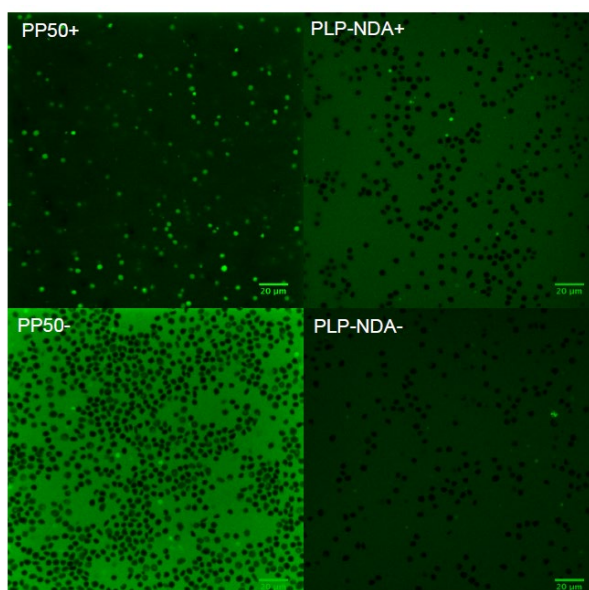


Figure 8. Comparison of PP50 and PLP-NDA for the delivery of 150 kDa FITC-dextran. Confocal microscopy images illustrating the delivery of FITC-dextran at pH 6.5 for 300 µg polymer/mL to 0.5 billion RBCs/mL, with PP50 (left) and PLP-NDA (right). Only green channel (FITC) shown. (Scale bar: 20 µm)

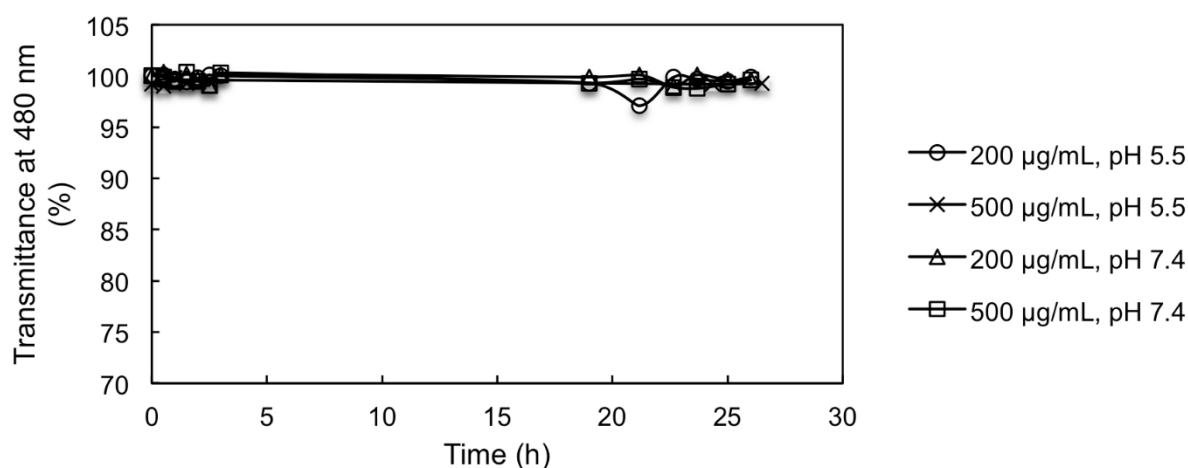


Figure 9. pH responsiveness of PLP-NDA in different concentrations at varying pH over Time

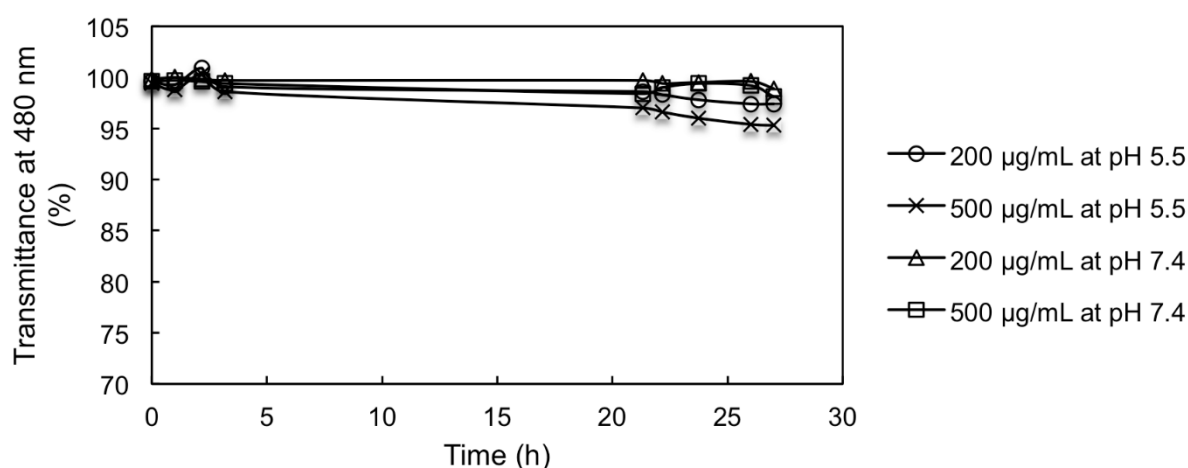


Figure 10. pH responsiveness of PP50 in different concentrations at varying pH over time

The polymers' pH responsiveness was further assessed over time and at different concentrations, as shown in Figures 9 and 10. These results indicate that PP50 maintained more stable solubility across a range of pH values, while PLP-NDA showed solubility issues at acidic pH, reinforcing the selection of PP50 as a preferred agent for encapsulation purposes.

3.3. Size Characterization of Loaded Nanoerythrocytes

To confirm the production of nanoerythrocytes from FITC-dextran-loaded RBC ghosts, particle sizing was performed. Sonication and extrusion of drug-loaded erythrocyte ghosts yielded nanoerythrocytes with a mean hydrodynamic diameter of approximately 150 nm (Figure 3). This size range was suitable for potential therapeutic applications, as it falls within the expected size for effective drug carriers that circulate efficiently while avoiding rapid clearance by the reticuloendothelial system.

3.4. Efficacy of Purification Methods for Loaded Nanoerythrocytes

Two purification methods—dialysis and size exclusion chromatography (SEC)—were tested to remove unencapsulated FITC-dextran from RBC ghosts and nanoerythrocytes. Dialysis, while effective, was a time-intensive process, with significant decreases in FITC-dextran concentration achieved over an extended duration, as shown in Figure 4. The results from dialysis indicated gradual removal, though efficiency could be improved with intermittent dialysate exchanges.

To accelerate purification, SEC was evaluated as an alternative method, allowing for more rapid isolation of loaded nanoerythrocytes from free dye (Figure 5). This method demonstrated comparable effectiveness in removing excess FITC-dextran but with considerably shorter processing times, providing a promising approach to streamline the preparation of encapsulated drug carriers.

3.5. Calibration of Fluorescence Intensity for Quantifying FITC-Dextran Loading

A calibration curve was established to relate FITC-dextran concentration with fluorescence intensity at pH 7.4, allowing accurate quantification of drug loading. Figure 6 presents the calibration curve for concentrations ranging from 0 to 0.2 μM of 70 kDa FITC-dextran. This calibration was critical in assessing the concentration of loaded FITC-dextran within the nanoerythrocytes and optimizing loading efficiency.

6. Discussion

The encapsulation of fluorescent dextran within RBC ghosts demonstrated the potential of these structures as drug delivery carriers, with PP50 showing superior stability over PLP-NDA, particularly under acidic conditions (Figure 3.3). Aggregation studies revealed that PLP-NDA formed clumps at lower pH levels, which could hinder its application in environments with varying

pH. These results are consistent with studies that highlight the challenges of polymer aggregation and the need for pH-stable encapsulation agents in drug delivery applications (Saba Zafar, 2024).

Particle sizing confirmed that nanoerythrocytes derived from encapsulated RBC ghosts met the desired size range for effective cellular uptake and circulation. These particles maintained stability and uniformity, which is crucial for their application in targeted therapies (Hilal Ahmad Rather & Mohd Arif Dar, 2024). Moreover, the fluorescent calibration curve facilitated accurate quantification of FITC-dextran loading, confirming the effectiveness of our encapsulation techniques (Figure 3.13). Such precise quantification aligns with recent advancements in nanocarrier drug loading methods, which stress the importance of dosage control in therapeutic contexts (Saba Niaz et al., 2024).

Our purification trials with dialysis and SEC demonstrated that both methods successfully separated free FITC-dextran from loaded nanoerythrocytes, although SEC proved faster. These findings suggest that SEC might be preferred for applications needing rapid processing, a consideration noted in prior studies on nanocarrier purification techniques (Abuchi Elebo et al., 2024).

4. Conclusion

This study presents a robust method for encapsulating fluorescent dextran within RBC ghosts using PP50 as an encapsulation agent, suitable for potential applications in drug delivery. The stability and uniform particle sizing of nanoerythrocytes suggest they may offer efficient and targeted therapeutic options, supported by our optimized purification and quantification techniques. Future research could explore additional polymer alternatives that maintain stability across diverse physiological conditions, building on findings from current research in nanocarrier efficacy and pH-dependent behavior (Jonathan Verderame & M. Shakib Arslan, 2024).

Author contributions

Z.K. conceptualized and designed the study. A.M. contributed to the methodology and provided critical revisions. R.A. carried out data analysis and contributed to the writing of the manuscript. All authors reviewed and approved the final version of the manuscript.

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Competing financial interests

The authors have no conflict of interest.

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