

Ph.D. Dissertation

Design of polymeric nanocarriers for tailoring functionality and enhanced therapeutic applications

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1. Introduction

The therapeutic efficacy of many pharmacologically active compounds is frequently hindered by unfavourable physicochemical properties and pharmacokinetic limitations. These challenges often originate from the drug's molecular structure, influencing solubility, absorption, metabolic stability, and overall bioavailability. For example, hydrophilic agents, including Mitomycin C (MMC), a widely used and versatile active substance in otorhinolaryngology, are rapidly cleared from systemic circulation, necessitating frequent dosing to achieve therapeutic efficacy. In contrast, hydrophobic molecules—including the calcium channel blocker Nimodipine (NIMO), the TRPV4 antagonist HC-067047 (HC), and the anti-inflammatory corticosteroid Methylprednisolone Acetate (MPA), prominent pharmacological agents for brain-ischemia-related complications and inflammatory diseases, respectively—exhibit poor aqueous solubility, which limits bioavailability and often necessitates formulation strategies with tolerability concerns.

Beyond these physicochemical and pharmacokinetic hurdles, a critical limitation of conventional therapeutics is their nonspecific release, which not only compromises therapeutic efficacy but also elevates the risk of adverse effects due to unintended interactions with healthy tissues. Ideally, drugs should be delivered at a precisely controlled rate to the intended site of action. However, achieving this level of control remains a formidable challenge using conventional delivery methods (e.g., capsules, tablets, ointments), which typically offer limited regulation over spatial localization and drug release kinetics.

To overcome these limitations, advanced drug delivery systems have been developed. Among these, polymer-based systems showed particular promise due to their structural versatility and tunable physicochemical characteristics. Advanced polymer engineering provides the essential tools to tailor the molecular architecture of nanocarriers, ensuring precise control over drug release kinetics or even site-specific therapeutic targeting. These macromolecular systems can encapsulate both hydrophilic and hydrophobic drugs, improve aqueous dispersibility and bioavailability of poorly water-soluble agents, and enable sustained drug release. Polymers can also be functionalized to facilitate triggered drug release in response to pathological cues, thereby reducing side effects.

Within this dissertation, we will investigate the possibilities of employing drug and applications customized polymers as drug delivery carriers, with a particular emphasis on the methods underlying their creation, from the early design phase to synthesis and characterization. Our research aims to develop innovative polymeric carrier systems for the above mentioned four pharmacological agents (MMC, NIMO, HC, and MPA) and evaluate their promise for enhancing therapeutic outcomes and promoting better patient adherence.

2. Literature background

2.1 Polymers

In recent years, polymers have garnered significant attention due to their exceptional resilience and versatile functionality across a broad spectrum of applications. Structurally, polymers are composed of long molecular chains formed by systematically arranged and chemically bonded repeating units. This molecular configuration enables a wide spectrum of mechanical and physical properties, ranging from rigidity and strength to elasticity and flexibility, depending on the polymer's composition and structure [1].

As modern society continues to demand materials that are efficient, adaptable, and economically viable, the reliance on polymeric materials has grown substantially. Their capacity for precise property modification has facilitated their integration into numerous sectors, spanning from consumer products to advanced biomedical technologies [2,3]. In everyday contexts, polymers are extensively employed in packaging, household containers, textiles, personal care products, electronic devices, and domestic tools [1,2,4]. In the medical sector, polymers are used in the production of syringes, catheters, blood bags, surgical gloves, and medical gauze [2,5]. Many polymers also play a pivotal role in the development of sophisticated biomedical innovations such as prosthetic limbs, dental implants, artificial organs, and intraocular lenses, where customized mechanical and biological properties are critical for safe and effective clinical outcomes [5,6]. Their durability, flexibility, and resistance to environmental stress make them suitable for such applications.

Beyond these domains, polymers contribute significantly to the construction industry, where those with corrosion resistance and structural integrity are ideal for insulation panels, waterproofing membranes, and sealants [7]. Moreover, the lightweight nature of certain polymers renders them advantageous in applications where mass reduction is crucial, such as aerospace structures and portable electronic devices [1,2]. Additionally, polymers with high mechanical robustness can withstand substantial stress, making them well-suited for automotive components, protective gear, and load-bearing structures [1,7,8]. Taken together, desirable properties such as mechanical strength, low density, long-term durability, environmental stability, ease of handling, processability, and cost-effectiveness have positioned polymers as cornerstone materials driving innovation across both everyday applications and advanced technological domains [1,2].

From an origin-based perspective, polymers are commonly segmented into three fundamental types: natural, semi-synthetic, and synthetic. Natural polymers refer to macromolecular substances that derived from biological sources such as plants, animals, and microorganisms [9]. These polymers play a vital role in sustaining life by providing structural

support, facilitating biological functions, and contributing to regulatory processes within living systems [9,10]. Among the major types of natural polymers are polysaccharides, proteins, and nucleic acids [11]. Polysaccharides represent the most abundant class of naturally occurring polymers on the earth. Composed of monosaccharide units that are interconnected by glycosidic linkages. These macromolecules function as structural frameworks in cell walls, serve as energy reservoirs, and contribute to cellular signalling and recognition across diverse biological systems [12,13]. Proteins also form a key group of natural polymers, composed of amino acid residues joined by peptide bonds [11]. They are essential to numerous biological processes, including enzymatic catalysis, structural support, molecular transport, metabolic regulation, immune defence, cellular communication, gene regulation, and many other specialized functions [14]. In addition to polysaccharides and proteins, Nucleic acids are nucleotide-based polymers that can encode and transmit genetic information, regulate gene expression, and facilitate protein synthesis [15]. All these polymers serve as the molecular foundation of living systems, and their diverse structural and functional roles are indispensable to the existence, regulation, and continuity of life.

Naturally occurring macromolecules can also be chemically modified to produce a second category of polymers known as semi-synthetic polymers. These polymers are engineered to improve specific properties or meet application requirements that natural polymers alone may not adequately fulfil [10,16]. Among the notable examples in this category is vulcanized rubber, which is renowned for its elasticity and heat resistance in automotive and industrial sectors [10,17]. Cellulose ethers and cellulose esters are also prominent members in this group [18]. The former are water-soluble and generally known for their stabilizing, thickening, binding and surface-active properties, which make them highly valuable in pharmaceutical formulations, food products, cosmetic preparations and construction materials [19,20]. The latter are commonly recognized for their water-insolubility and excellent film-forming ability, which support their widespread use in coatings and controlled-release pharmaceutical systems [19,21].

The third class of polymers, with respect to origin, is entirely synthesized through human-directed chemical processes and is referred to as synthetic polymers. The properties of these polymers are primarily governed by the type and spatial arrangement of their monomer units, the length of the polymeric chains, and the nature of the chemical bonding within the molecular structure [1,10,16]. Synthetic polymers play a pivotal role in nearly every aspect of modern life and industrial production. They serve as the foundational components of widely utilized materials such as synthetic fibres, plastics, adhesives, and high-performance coatings. Their versatility further extends to the manufacturing of packaging materials, electronic components,

automotive parts, and medical devices, making them indispensable across a wide range of sectors [22,23].

Synthetic polymers possess a distinctive set of properties that make them highly advantageous in certain applications compared to natural counterparts [24,25]. For instance, their molecular structure can be precisely engineered to achieve targeted levels of strength, flexibility, reactivity, and resistance to both physical and chemical environmental stressors [24,25]. This engineered resilience ensures consistent performance under demanding operational conditions. They also offer reliable uniformity and ease of processing, unlike natural polymers, which are subject to variability due to biological origin and environmental sensitivity—making them particularly suitable for applications requiring controlled composition and reproducible outcomes [24]. They can also be manufactured in large quantities with consistent quality and extended shelf life, enhancing their practicality for both commercial and industrial use. Moreover, they exhibit greater durability and generally more cost-effective than natural alternatives [25]. Despite all these functional advantages, they have some challenges. Their limited biodegradability can lead to long-term accumulation in natural ecosystems, raising environmental concerns [24,26]. Recycling is often complicated by chemical complexity and contamination during use [27]. Moreover, if not properly synthesized or managed, they may pose health risks due to the chemical agents involved in their production [3,10,16]. They can be more energy-intensive and costly compared to natural materials [26,28]. Nonetheless, their versatility, reliability, and broad applicability continue to make them indispensable in modern industry, with ongoing research focused on enhancing sustainability and safety.

2.2 Polymers in drug delivery

Polymers, regardless of their origin, have become foundational to the evolution of drug delivery technologies. The ability of certain polymers to be tailored for biological environments—combined with their structural diversity and potential biocompatibility—has enabled their use in transporting therapeutic agents with greater precision and control. The increasing reliance on polymer-based drug delivery platforms largely reflects the limitations that many therapeutic agents face when administered in their conventional forms.

Hydrophilic drugs, for example, are rapidly cleared through renal pathways, resulting in short circulation times, reduced therapeutic exposure, and the need for frequent dosing, which increases the risk of systemic toxicity [29,30]. In contrast, hydrophobic compounds inherently display poor aqueous solubility, leading to slow dissolution and restricted absorption in physiological fluids [31,32]. Chemical instability further complicates treatment, as numerous drugs are susceptible to degradation by oxidative conditions, pH variations, or enzymatic activity before reaching their intended site of action. Non-selective distribution add another

challenge by allowing drugs to accumulate in healthy tissues, reducing targeting accuracy and contributing to many adverse effects [31]. Collectively, these barriers not only compromise therapeutic reliability but also place considerable strain on patient adherence, often leading to delayed treatment initiation, inconsistent dosing schedules, or complete discontinuation—especially in chronic or long-term care settings. From this standpoint, the demand for advanced drug delivery systems has become increasingly evident to address these limitations and improve both therapeutic precision and patient adherence [31,33,34]. Advanced drug delivery systems are engineered technologies that employ carriers such as polymers, lipids, or inorganic materials to facilitate the transport of therapeutic agents within the body [26,31]. These systems offer substantial advantages across both clinical and industrial domains [33].

Clinically, they enhance the solubility of poorly absorbed compounds through tailored carrier design, thereby improving drug uptake and therapeutic efficacy. They also protect active agents from premature degradation by encapsulating them within stabilizing matrices, ensuring the drug remains intact until it reaches its target site [35]. Unlike conventional delivery methods, which often release the entire drug load rapidly and without kinetic or spatial control, controlled platforms enable precise modulation of the drug's release profile. This allows for site-specific delivery and helps maintain drug concentrations consistently within the therapeutic range—sufficiently high to be effective, yet low enough to avoid toxicity—over desired periods [33]. As a result, prolonged release can be achieved, dosing frequency can be reduced, treatment costs can be minimized, and patient adherence can be improved by reducing the likelihood of missed doses [36]. Furthermore, targeted delivery can also be achieved, concentrating the drug in diseased cells or tissues while limiting exposure to healthy regions [35].

From a commercial standpoint, advanced drug delivery systems offer pharmaceutical companies a powerful tool to strengthen market position and enhance product value. By improving patient acceptance through simplified dosing and reduced adverse effects, they enable higher treatment uptake and stronger commercial returns. They also enable the revival of drug candidates previously discontinued due to toxicity or unfavourable pharmacokinetics, restoring their clinical potential. In addition, reformulating established therapies with these innovative delivery systems allows companies to extend market exclusivity and maintain competitiveness against generic alternatives [33].

Among the most effective drug delivery systems are those based on the use of polymers. Commercially, polymers have been successfully integrated into a wide array of pharmaceutical formulations, including transdermal patches, injectable suspensions, topical gels, oral solids (e.g. capsules and tablets), semi-solid preparations, and films. Within these systems, polymers do not merely serve as conventional excipients; they act as encapsulating carriers, conjugated

moieties, or surface-modifying coatings, providing protective, structural, or functional advantages that enhance drug stability, release behaviour, and overall therapeutic performance [37,38]. The effectiveness of these systems hinges on the careful selection of the polymeric material. This decision is governed by multiple factors, foremost among them the polymer's physicochemical attributes, which significantly impact the choice of preparation techniques, drug-polymer interactions, drug release kinetics, surface morphology, and electrostatic behaviour of the delivery system. These physicochemical characteristics include key factors such as molecular weight, structural composition, water solubility, crystallinity, biocompatibility, and biodegradability. Beyond polymer-specific considerations, the physicochemical profile of the therapeutic agent, the route of administration, and the intended therapeutic goal must also be considered during the selection process. Together, these elements determine the structural design and functional performance of the delivery system, thereby ensuring optimal therapeutic efficacy [39].

Once an appropriate polymer is selected, it can fulfil several critical roles within the biological system. One of the most important is regulating the drug release rate [40]. While drug-specific properties (e.g., molecular weight, molecular size, and solubility) can influence the release process, polymeric factors—such as coating thickness, molecular weight, and chemical composition—can significantly affect the release profile by modulating both the rate and the mechanism through which the active pharmaceutical ingredient is delivered [41–43]. Non-degradable polymers, for example can release active ingredients gradually over extended periods, as they do not degrade under physiological conditions and retain their structural integrity within the body. Consequently, these materials are widely utilized in the design of sustained-release systems intended for the long-term management of persistent health conditions. In contrast, degradable polymeric materials can gradually degrade within the body through enzymatic or hydrolytic processes. This property enables controlled release of the active ingredient over a defined period, making degradable polymers particularly suitable for targeted, time-limited therapeutic applications, such as chemotherapy regimens or vaccine delivery [44].

Beyond controlling release kinetics, polymer can also contribute to drug localization. By functionalizing polymer surfaces with specific moieties, targeted drug delivery can be achieved. These surface modifications enable drugs to accumulate at designated tissues or cells, enhancing therapeutic precision and minimizing off-target effects. This targeting strategy is particularly valuable in clinical applications that demand high specificity, such as oncology, gene therapy, and immunomodulation [39,44].

In addition to all these benefits, polymers can enhance both the efficacy and the safety of therapeutic agents. They are capable of incorporating both hydrophilic and hydrophobic compounds, shielding active pharmaceutical ingredients from systemic and immunological clearance [35,45]. This protective function extends circulation time and enhances bioavailability [35]. Additionally, polymers can alter the surface properties of drug molecules, improving solubility and promoting more efficient absorption within biological systems [3,45].

2.3 Structural categories of polymeric drug delivery systems

Building on the foundational roles and physicochemical properties of polymers in drug delivery, it becomes essential to explore the structural formats through which these materials are applied. Polymeric drug delivery systems encompass a broad spectrum of architectures—including colloidal carriers, polymer–drug conjugates, and injectable or implantable hydrogels—each distinguished by specific design characteristics and functional capabilities (**Figure 1**). In the following subsections, the first two key structural formats—polymeric colloidal carriers and polymer–drug conjugates—will be discussed with emphasis on their formulation techniques, therapeutic advantages, and representative examples from clinically approved drug products.

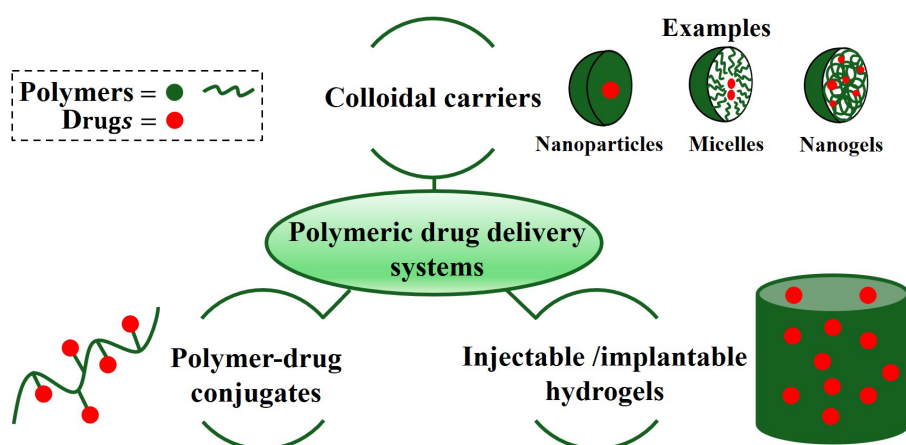


Figure 1. Types of polymeric drug delivery systems [3].

2.3.1 Polymeric colloidal carriers

Polymers are widely recognized as versatile materials for constructing colloidal carriers at both nano- and microscale levels [44]. Among these, nanoscale systems have garnered particular attention due to their superior performance in therapeutic delivery. Common polymeric nanocarriers include nanogels, micelles, and nanoparticles (NPs) [46].

Thanks to their ability to accommodate a broad spectrum of drugs via encapsulation or surface adsorption, polymeric NPs hold high potential for versatile and effective drug delivery applications. Their efficient drug encapsulation, combined with their small size and high surface area, contributes to prolonged systemic retention of the drug, improved solubility, and enhanced

bioavailability [31,47]. Polymeric NPs are designed to release the active pharmaceutical ingredients in a rate-controlled manner, typically via mechanisms such as diffusion, polymer degradation, or stimuli-responsive release. These mechanisms help maintain therapeutic concentrations over extended periods [31].

Particle size plays a critical role in determining various biological and pharmacokinetic parameters, including biological fate, systemic stability, circulation time, cellular internalization, and drug release profiles [48,49]. For instance, spherical polymeric NPs smaller than 10 nm are typically subject to rapid renal clearance [50]. In contrast, NPs ranging from approximately 20 to 500 nm are less prone to rapid elimination through physical filtration processes, thereby exhibiting extended circulation times and improved potential for therapeutic delivery [51]. Numerous studies have further demonstrated that NP size significantly influences cellular internalization. Generally, as particle size increases, the surface-to-volume ratio decreases, leading to a reduced contact area with the cell membrane and consequently lower internalization efficiency [52]. For instance, in gastrointestinal drug delivery, transcellular uptake has been reported to show an inverse correlation with particle size, with 50 nm particles being taken up more efficiently than 500 nm particles [53]. NP size also affects drug release kinetics, whereby increasing particle size generally results in slower release rates due to extended diffusion pathways [49]. Additionally, the route of administration can be influenced by particle size, as different pathways present unique physiological constraints. For instance, in intravenous administration, particle dimensions should be significantly smaller than the diameter of the narrowest capillaries in the body (approximately 5–6 μm); otherwise, particles may obstruct capillary flow, leading to potential vascular blockage and an increased risk of circulatory complications [49]. Furthermore, studies have demonstrated that in oral administration, particles ≤ 300 nm are generally preferred, as they may enable efficient uptake by specialized intestinal cells such as M cells and enterocytes [54,55].

The shape of the polymeric NPs is also an important factor, as particle geometry directly influence interactions with biological barriers and cellular membranes. Sphere-shaped NPs, for example, can interact with cell surfaces regardless of orientation, whereas rod-shaped NPs often require specific alignment to achieve efficient uptake [56,57].

Given the impact of physical attributes on NP performance, selecting an appropriate synthesis method becomes crucial. Polymeric NPs can be synthesized using a variety of fabrication techniques, each characterized by distinct mechanisms of particle formation. Commonly employed methods include solvent evaporation, salting-out, nanoprecipitation, and supercritical fluid technology. These approaches typically utilize preformed polymers in the synthesis process, with each method defined by its own set of advantages and limitations.

In the solvent evaporation method, two principal strategies are typically employed: the single emulsion (O/W) and the double emulsion (W/O/W) techniques. The single emulsion is best suited for hydrophobic drugs, where both the drug and polymer are dissolved in an organic solvent and emulsified into a stabilizer-containing aqueous medium. As the solvent gradually diffuses into the water, the polymer begins to precipitate/ solidify out of the solution, forming drug-loaded particles that are subsequently recovered following solvent evaporation. In contrast, the double emulsion is more appropriate for hydrophilic drugs. Here, the drug is first dissolved in water and emulsified into a polymer-rich organic phase to form a W/O emulsion. This is then re-emulsified into an external stabilizer-containing aqueous medium to create a W/O/W system from which drug-encapsulated particles are obtained after solvent removal [58]. In both strategies, the characteristics of the produced NPs are significantly influenced by polymer and stabilizer concentrations, the physicochemical attributes of the selected solvent, and the homogenization techniques employed during emulsification [58]. Despite its widespread use, the solvent evaporation method demands considerable energy input, relies on toxic solvents (e.g., chloroform, and methylene chloride), and is prone to droplet coalescence, which may adversely affect particle morphology and size [45,59].

Among the more straightforward and safe techniques, the salting-out method offers an effective approach for producing polymeric NPs. This approach involves emulsifying a polymer–drug mixture, typically solubilized in a water-miscible organic solvents such as acetone, ethanol, or ethyl acetate into an aqueous medium enriched with salting-out agents (e.g., calcium or magnesium chloride) and, in some cases, stabilizing polymeric surfactants like polyvinyl alcohol (PVA) or poloxamers (e.g., Pluronic). The elevated ionic strength of the aqueous medium reduces the solubility of the polymers and the miscibility of the organic solvent, thereby enhancing emulsion stability. Subsequent dilution with water facilitates solvent diffusion into the aqueous phase, disrupting the solubility equilibrium and inducing NP formation via polymer precipitation [59]. The efficacy of this method hinges on precise regulation of ionic strength and emulsification parameters. It is regarded as environmentally favourable due to its mild operational conditions and avoidance of hazardous solvents. Nonetheless, it often requires extensive purification to eliminate residual salts, and its tendency to produce relatively large particles may limit its applicability in formulations demanding stringent size control [45,50].

Among the rapid and simple approaches that can offer a control over particle size are the nanoprecipitation and desolvation methods [59,60]. In the nanoprecipitation method, the polymer–drug mixture is typically solubilized in a water-miscible organic solvents. This organic phase is then added under gentle stirring to an aqueous phase, which acts as a non-solvent for

the polymer. Upon contact, the organic solvent rapidly diffuses into the aqueous phase due to its high miscibility, leading to a displacement of the solvent environment by the non-solvent. This abrupt change in solubility triggers polymer precipitation and leads to the formation of NPs [59]. This method is more suitable for hydrophobic drugs and synthetic polymers, offering simplicity, low energy input, and reproducibility [45,59].

In contrast, the desolvation method is primarily applied to natural polymers such as albumin and chitosan [60,61]. In this approach, the polymer is first dissolved in water (as good solvent) under controlled conditions, followed by the gradual addition of a desolvating agent like acetone or ethanol as poor solvent. This induces polymer precipitation, often requiring the addition of a chemical (e.g., glutaraldehyde) or physical (e.g., sodium tripolyphosphate, TPP) crosslinking agent to stabilize the NPs [60]. The drug can be incorporated either by mixing it with the aqueous polymer solution prior to desolvation or by adding it to the desolvating agent, depending on its physicochemical properties. Although this is a simple and cost-effective method, it requires precise control over process parameters to produce NPs with the desired properties [60,61].

Additionally, supercritical fluid (SCF) technology encompasses a group of advanced techniques that most predominantly employ supercritical carbon dioxide (CO₂) to facilitate the formation of NPs. Among these, the Rapid Expansion of Supercritical Solutions (RESS) and the Supercritical Anti-Solvent (SAS) methods are widely used. In the RESS process, the polymer and drug are co-dissolved in the SCF to prepare a homogeneous solution. This solution is then rapidly depressurized through a nozzle into a controlled lower-pressure chamber, resulting in solute supersaturation and subsequent particle formation [59]. Conversely, the SAS method involves injecting a polymer–drug solution—typically prepared in an organic solvent—into a controlled higher-pressure vessel containing the SCF. Due to the high miscibility of the SCF with the organic solvent, and its poor solvating capacity for the polymer and drug, the solvent is swiftly extracted, leading to the precipitation of composite particles [62]. These techniques are considered environmentally benign and are capable of producing NPs with uniform size distribution, high purity, and minimal solvent residues [50,62]. However, their implementation requires specialized equipment, high-pressure operation, and meticulous control of process parameters.

Although most polymeric NPs used in drug delivery are synthesized from preformed polymers, monomer units can also be polymerized directly into NPs using methods such as emulsion, interfacial, and controlled/living radical polymerization [45,50,59,63].

It is worth noting that several colloidal carriers—whether employing polymers as primary materials or as integral components in the construction of carrier systems—have achieved

clinical success, as evidenced by FDA-approved products such as Abraxane® (albumin-bound paclitaxel NPs for pancreatic and breast cancer) [64], Onpattro® (PEG-lipid NPs delivering siRNA for hereditary transthyretin-mediated amyloidosis) [65], and Doxil®/Caelyx™ (PEG-stabilized liposomal doxorubicin NPs for ovarian cancer, multiple myeloma, and Kaposi's sarcoma) [66].

2.3.2 Polymer–Drug conjugates

Polymer–drug conjugates involve the covalent linkage of therapeutic molecules to polymer chains, forming a unified chemical entity. They are also referred to as polymeric prodrugs. The foundational concept was first established in the mid-1970s through the work of Helmut Ringsdorf, who proposed that conjugating therapeutic agents to polymeric backbones could enhance their physicochemical stability, bioavailability, and solubility [67,68]. By attaching drugs to either the polymer backbone or its reactive pendant groups, a range of therapeutic enhancements can be achieved. These conjugates provide active ingredients with protection against premature degradation, reduced immunogenicity, extended circulation time and increased plasma half-life [45,69]. By remaining in circulation for extended periods, these conjugates can exploit the impaired lymphatic function and leaky vasculature typical of tumor tissues, resulting in preferential accumulation within the tumor microenvironment [67,70]. Polymer conjugation can also boost the aqueous solubility of poorly water-soluble active agents, increasing their bioavailability and therapeutic potential [69]. In some formulations, polymer–drug conjugates are further enhanced by the incorporation of functional components such as solubilizers, imaging agents, or targeting moieties. These additions can improve diagnostic precision, optimize pharmacokinetic behaviour, and promote selective accumulation at desired biological sites [69,71].

Due to the chemical linkage between the polymer and the drug, drug release can occur not only gradually but also in a stimulus-responsive manner, triggered by specific cues such as pH fluctuations or the presence of particular enzymes [69]. For instance, therapeutic agents conjugated via acid-labile linkers such as amide or hydrazone bonds, are more susceptible to be released under acidic conditions than under neutral pH. This design enables pH-responsive and targeted drug release, thereby enhancing therapeutic selectivity [72]. This approach is particularly effective in cancer therapy because cancer cells typically exhibit a lower extracellular pH (pHe) compared to normal cells. As is well established, the hypoxic microenvironment of tumours can reduce the pHe to values as low as 5.7–6.5 [73,74]. Furthermore, the acidic nature of intracellular compartments such as endosomes and lysosomes—where pH levels are approximately 5.5 or 5.8—can further facilitate site-specific drug release [75,76].

Polymeric prodrugs are generally synthesized through one of three principal approaches. The first involves the attachment of therapeutic agents to preformed polymers via well-defined chemical linkers. The second strategy entails the prior linkage of drug molecules to monomer units, forming drug-conjugated monomers that serve as building blocks for polymerization. These monomers can be polymerized using techniques such as ring-opening polymerization (ROP), reversible addition–fragmentation chain transfer (RAFT) polymerization, or ring-opening metathesis polymerization (ROMP). The third approach employs drugs bearing two reactive functional groups directly as monomeric units, enabling the formation of so-called polymerizable drugs [77]. Among these strategies, the first is most frequently used for polymer–drug NP synthesis. However, it may lead to suboptimal drug loading depending on factors such as polymer architecture and steric hindrance during conjugation [77,78]. In such instances, the second approach can improve loading by uniformly incorporating drug moieties into monomers prior to polymerization. The third strategy may also enhance drug loading, but its applicability is limited to a narrow range of drug structures [77]. The linkers employed during the design of polymeric prodrugs should be carefully selected to achieve optimal therapeutic release rates and site-specific activation. Their nature—whether pH-sensitive, enzyme-sensitive, light-sensitive, or redox-sensitive—can influence the mechanism by which drug release is triggered [77,79]. However, it is important to note that, beyond linker chemistry, the physicochemical attributes of both the drug and the polymer also play a critical role in modulating the overall release behaviour in vivo [41,43,79].

One can conclude that optimal polymeric prodrugs should be designed with a strong emphasis on efficacy and safety to ensure precise drug release at the intended site while minimizing adverse effects on healthy tissues [77,80]. Accordingly, these systems must exhibit preferential responsiveness within pathological environments relative to non-target regions, thereby enhancing therapeutic precision and reducing systemic toxicity. Achieving this balance between systemic stability and site-specific responsiveness is central to effective design [79]. Nowadays, numerous polymeric prodrugs have demonstrated clinical success and reached commercial application, underscoring the translational potential of this strategy and its growing impact on modern therapeutics [80].

2.4 Biopolymer modification for enhanced drug delivery

So far, a range of biodegradable polymers has been identified, many of which display characteristics that are not ideally suited for drug formulation. Although their unique attributes can support the achievement of designated therapeutic objectives, they are not without limitations. Whether sourced from synthetic or natural origins, these polymers possess their own inherent strengths and weaknesses that influence their broader applicability in drug

delivery systems [39]. For instance, synthetic biopolymers are known for their outstanding tunable characteristics that facilitate formulation processing; however, their therapeutic performance is often constrained by limited biological interactions and insufficient responsiveness to cellular stimuli, unless specifically engineered for such functions [39,81,82]. On the other hand, unmodified natural biopolymers, despite their inherent biological compatibility, often exhibit limited physicochemical stability, inadequate drug-loading capacity, and uncontrolled, non-sustained release profiles [39,83]. So, based on that, a modification strategy should be employed to attain a specific set of functional attributes without transitioning between biopolymers. The multiple approaches that are frequently used to modify biopolymer properties are presented in **Figure 2**.

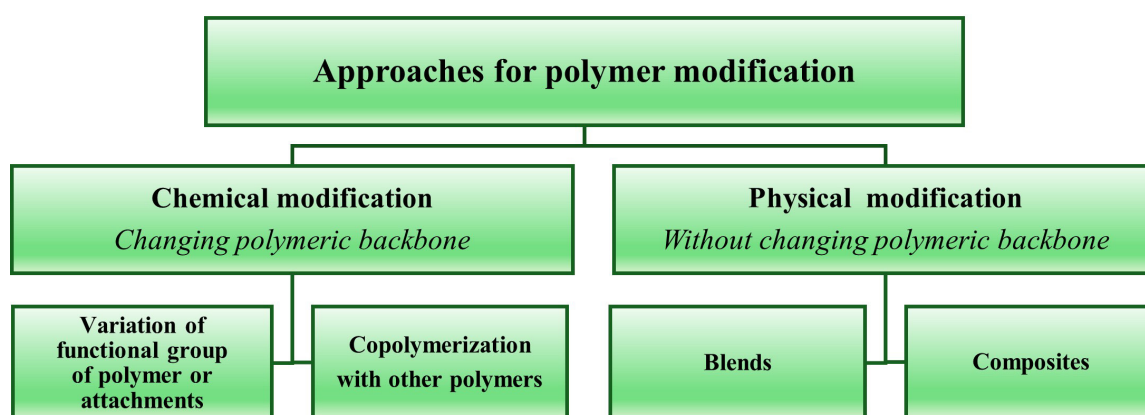


Figure 2. Various polymer-modification methods applied in tissue engineering and drug delivery [39].

While polymer modification is not a new concept, its application has gained increasing prominence in recent years to alter polymer attributes in ways that suppress instability, enhance drug loading, and fine-tune drug release profiles, thereby yielding polymers with improved biocompatibility and suitability for diverse formulation platforms [39,84]. Rational modifications can also impart advanced functionalities, including targeting capability, responsiveness to physiological stimuli, inhibition of P-glycoprotein activity, and enhanced pharmacokinetic performance for poorly water-soluble therapeutic agents [39]. To overcome the pharmaceutical limitations as well as the biological challenges (e.g., rapid systemic clearance, reduced bioavailability, limited specificity and selectivity, etc) that can diminish therapeutic efficacy, polymers should be strategically engineered to meet the demands of the intended therapeutic system [39]. In this context, we focus on modifications of polymers and their contributions to therapeutic efficacy, with specific emphasis on Polyvinyl Alcohol (PVA) and Chitosan (Chit).

PVA is a synthetic water-soluble polymer that combines favourable physicochemical characteristics like mechanical strength, chemical stability, and functional modifiability. It is typically synthesized through the complete or partial hydrolysis of polyvinyl acetate, a process that replaces acetate groups with hydroxyl functionalities along the polymer backbone [24,85,86]. Due to its non-toxic nature, biological compatibility, weak protein-binding affinity, and biodegradability under physiological conditions, PVA has become a widely utilized material in drug delivery applications [85]. Its capacity to modulate physicochemical properties, enhance drugs' stability, and improve their bioavailability supports its utility as a versatile platform for encapsulation and controlled release applications [86–88]. PVA has been recognized by the FDA as generally safe and approved for use across multiple biomedical applications [85]. Nowadays, it is used in a variety of therapeutic delivery systems and further integrated into a broad spectrum of implantable and non-implantable medical devices, such as cartilage substitutes, neural guidance structures, anti-adhesion barriers, ophthalmic formulations, contact lenses, and catheter assemblies [85,89].

Multiple *in vivo* studies in animal models have yielded encouraging results regarding the biodistribution and bioelimination of PVA. Findings by Yamaoka et al. indicated that organ-level accumulation of PVA was too minimal to disrupt biological fate, thereby reinforcing its safety profile [90]. Additionally, Kaneo et al. suggested that following intravenous injection, PVA is cleared solely through routes independent of saturable transport processes [85,91]. Furthermore, Cerroni et al. observed no cytotoxic impacts in their *in vivo* investigation of the biodistribution and bioelimination of PVA-based microparticles in mice [92].

Chemical modification of PVA that introduces reactive carboxyl (–COOH) groups alongside its native hydroxyl (–OH) functionalities can expand its conjugation potential. In general, the presence of –COOH groups enable the formation of amide bonds with amino-containing bioactive agents, including peptides, proteins, and both natural and synthetic pharmaceutical compounds [93–95]. Dual functionalization can also allow for hydrogen bonding and metal–carboxylate coordination with biocompatible, nontoxic magnetic vectors, such as magnetic iron oxide nanoparticles (MNPs), which can be systemically administered, magnetically guided to target sites, and metabolically cleared via iron pathways—features that can support enhanced targeting strategies [96,97]. Additionally, the coexistence of –OH and –COOH moieties can facilitate electrostatic interactions with charged imaging agents such as Rhodamine B (RB) [98]. This organic fluorescent dye is widely utilized in imaging-guided therapy and cell labeling due to its favourable photophysical properties, including broad emission spectrum, strong fluorescence intensity, and relatively high quantum yield [99,100]. Its incorporation therefore enables efficient cell labeling and supports the evaluation of cellular internalization.

Chitosan (Chit) is a cationic, biodegradable polymer typically produced by chemical deacetylation of chitin, a structurally abundant biopolymer found in fungal and yeast cell walls, insect cuticles, and crustacean exoskeletons [101,102]. Compared to the yearly yield of synthetic polyethylene, which is around 0.1 billion tons [103], the biosphere contains more than 10 billion tons of chitin. Accordingly, Chit ranks among the most prominent renewable and green substances [101]. Beyond its inherent biodegradability, Chit exhibits nontoxicity and biocompatibility. These properties support its widespread use in regenerative medicine platforms, tissue-engineered constructs and therapeutic delivery systems [104]. Particulate Chit-based systems can encapsulate a broad spectrum of therapeutics, including anti-inflammatory drugs, antitumor compounds, antimicrobial agents, analgesics, and growth-promoting factors [102]. By accommodating both hydrophilic and hydrophobic therapeutics, Chit NPs can promote drug stability, minimize required doses and associated side effects, and support controlled release behaviour [105]. The ability of Chit to improve drug delivery performance is facilitated by its reactive amino groups, which enable surface functionalization and tailored physicochemical modifications [105,106].

Hydrophobic modification of Chit can expand its utility in drug delivery by influencing key dynamics such as cell membrane interaction, cellular uptake, drug encapsulation and drug release. For example, reacting Chit with hydrophobic moieties can reduce its aqueous solubility and alter its pH-dependent solubility behaviour, as Chit's solubility is governed not only by pH but also by factors such as extent of hydrophobic interactions [102,107]. Based on this, varying the hydrophobicity level of the applied molecule can allow the pH threshold for particle assembly or disassembly to be tuned, thereby enabling pH-responsive drug release. Such responsiveness can help mitigate premature drug leakage into healthy tissues and facilitate selective release in pathological regions such as brain ischemia, which are characterized by lower pH levels (typically pH 6.5–6.0) compared to normal tissue [105,108].

Moreover, the integration of biocompatible copolymers like Pluronic® F-127 (PF) into Chit-based systems can further enhance functional performance by supporting sustained drug release [109]. PF is a triblock copolymer composed of a central hydrophobic polypropylene oxide (PPO) block flanked by hydrophilic polyethylene oxide (PEO) chains. Consequently, due to its amphiphilic nature, it can self-assemble in aqueous environments to form small polymeric micelles. These micellar structures get more densely packed near physiological temperature, resulting in gelation. Owing to this distinctive thermo-responsive feature, PF-containing drug delivery systems are widely employed to achieve extended drug release profiles [109–111]. Thus, incorporating an appropriately hydrophobically modified Chit derivative together with

PF in a single delivery system may offer a promising strategy to combine pH-triggered targeting with sustained drug release in ischemic regions.

2.5 Thiolated aliphatic polyesters: characteristics and relevance in drug delivery

While modifying biopolymers—such as PVA and Chit—provides an effective strategy to overcome specific delivery challenges like poor solubility and lack of pH-responsiveness, other administration routes necessitate entirely different functional properties, such as mucoadhesivity. To meet these distinct requirements, researchers often explore inherently functionalized synthetic polymers alongside the previously outlined biopolymers. Among these, thiolated aliphatic polyesters are increasingly employed in developing therapeutic delivery systems, antibacterial hydrogel films, elastomeric biomedical materials and tissue engineering scaffolds [112,113]. Their well-established biodegradability, biocompatibility, and mucoadhesive features have positioned them among the prominent representatives of synthetic biodegradable polymers. The integration of the mucoadhesive properties of the thiolated polymers [114,115] with the inherent biocompatibility/biodegradability of aliphatic polyesters [113,116] makes them highly suitable for constructing mucoadhesive polymeric drug delivery systems.

Among thiolated polyesters, poly(ethylene thiomalate) (PET) has emerged as a promising candidate due to its demonstrated potential in biomedical applications, especially in therapeutic delivery systems and controlled-release formulations [112]. PET is typically synthesized by polycondensation of thiomalic acid (TA) with ethylene glycol (EG), using mineral acid catalysts (e.g., hydrochloric acid, HCl) or metal-based catalysts (e.g., scandium triflate, $\text{Sc}(\text{Otf})_3$) [112,113]. However, the use of these catalysts raises notable concerns. For instance, mineral acids are known for their corrosiveness, toxicity, poor separability from the reaction medium, and associated environmental and handling challenges [117]. At the same time, the potential risks associated with metal catalyst residues to both the environment and human health cannot be ignored, since most metal-based complexes have been shown to be biologically hazardous [118,119]. In response to these concerns, researchers have focused on synthesizing both polyesters and thiolated polyesters without the use of any catalysts [118,120]. In these direct melt polycondensation reactions, relatively long polyester chains can be produced solely from monomers at a carefully controlled temperature that prevent thermal degradation of both monomers and polymers, thereby enabling continued chain extension [120,121]. Although this approach has been applied to synthesize various polyesters, catalyst-free preparation of PET and its nanoparticle formulation remain unreported.

2.6 Drug release mechanisms

In polymer-based drug delivery systems, the term ‘drug release’ generally describes how therapeutic molecules move from their initial location within the polymeric network to the external surface of the polymeric framework, and how they are subsequently transferred into the external medium [122]. Therapeutic compounds can be released out of polymeric matrices by diffusing through water-filled pores. In this mechanism, spatial variation in the chemical potential of therapeutic molecules can direct their net movement across the matrix. Osmotic pressure-driven convection is also another mechanism that can drive the transport of drug molecules by inducing bulk solvent flow. Beyond diffusion and convection, therapeutic release may also proceed through matrix erosion, a process that generates pores and structural degradation and typically becomes evident following an early phase dominated by diffusion [122]. In addition to these classical release processes, therapeutic release may also be governed by stimulus-responsive mechanisms. All of these pathways are illustrated in **Figure 3** and are discussed in the following sections.

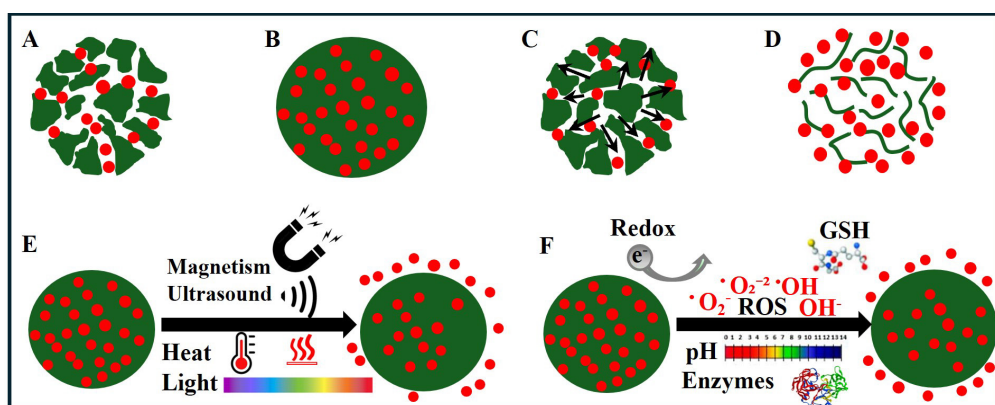


Figure 3. Therapeutic release mechanisms from polymeric systems: (A) diffusion through water-filled pores, (B) diffusion through the polymeric matrices, (C) osmotic pumping, (D) erosion, (E) stimulus-responsive release triggered by external cues, and (F) stimulus-responsive release triggered by internal cues [122].

In the mechanism of diffusion through water-filled pores (**Figure 3A**), the driving force behind the random motion of therapeutic compounds is the spatial variation in chemical potential, for which the concentration gradient serves as a practical approximation. Within degradable polymer matrices, the therapeutic release rate is governed by diffusion through pore networks that progressively evolve as the matrix undergoes degradation. After exposure to aqueous media, water absorption by the polymeric system occurs immediately. As time progresses, the absorbed water gradually induces the formation of water-filled pores within the matrix. These pores grow in both number and size until they become sufficiently large to permit medication release [122,123].

In the diffusion pathway illustrated in **Figure 3B**, therapeutic compounds are simply released via diffusion out of the polymeric matrices. This diffusion-controlled mechanism constitutes the dominant mode of therapeutic release in non-degradable polymeric systems. Medicinal release in such systems is typically characterized by a constant rate, which is not influenced by gradients in drug concentration but rather by the polymeric membrane's properties, such as the thickness and the permeability [122].

In osmotic pumping (**Figure 3C**), drug release is driven by osmotic pressure-induced convection. The elementary osmotic pump resembles a reservoir-type system but features an osmotic core composed of a drug-loaded formulation and an osmotically active compound, which together promote water influx across a semipermeable membrane from the external medium. This water influx is driven by osmotic pressure induced by solute concentration gradients across the membrane [124,125]. The imbibed water then generates internal pressure, which enables convective transport of the drug solution through a preformed delivery orifice [123,124]. A constant rate of drug release is maintained while undissolved solid drug remains within the formulation. Following complete dissolution, the solubilized drug continues to be released, but at a gradually reduced rate as the osmotic gradient diminishes. Once osmotic equilibrium is established between the internal and external environments, water influx ceases, and the release process terminates [125]. In osmotic systems, drug release is influenced by multiple parameters, including the osmotic pressure generated within the core, the concentration and solubility of the core constituents, the properties of the membrane, and the orifice size [124,125].

Erosion constitutes an additional pathway of drug release in polymeric systems. Erosion in polymeric drug delivery systems occurs via two primary modes: surface erosion, a heterogeneous process, and bulk erosion, a homogeneous process [126]. In surface erosion, degradation begins at the polymer surface, leading to a gradual reduction in matrix size from the exterior inward. Drug release proceeds progressively as the surface recedes. This mechanism dominates when the rate of water penetration into the polymer bulk is slower than the rate of erosion. Surface erosion can offer benefits for water-sensitive drug compounds, as the slow rate of water ingress helps maintain drug stability throughout the release process. Several drug delivery platforms can also benefit from this release mode, as its erosion kinetics are both reproducible and controllable, enabling predictable and controlled drug release [122]. In bulk erosion, water penetrates into the polymer bulk, initiating erosion and triggering uniform degradation across the whole polymeric matrix. Here, hydrolysis and erosion begin in the polymer bulk rather than at the surface, due to the relatively higher rate of water penetration compared to the erosion rate [122].

Drug release from responsive carrier systems with temporally and spatially control can also be achieved by exogenous stimuli applied from outside the biological system [122,127]. As illustrated in **Figure 3E**, these triggers can include ultrasound, light, heat, magnetic fields, and electric signals [122,126]. Upon stimulation, the carrier system may undergo structural or physicochemical changes—such as charge reversal, conformational rearrangement, shell cleavage, bond cleavage, or carrier degradation—or may be directed toward a specific target site, depending on the nature of the stimulus and the design of the delivery system [122,127]. This strategy is beneficial for achieving precise drug release at the site of stimulation, thereby minimizing adverse effects [126].

Moreover, as illustrated in **Figure 3F**, drug release can be achieved by exploiting endogenous stimuli, including enzyme overexpression at specific pathological sites, site-specific ionic and redox microenvironments, and pH shifts within cellular and tissue compartments [122,127]. For instance, by exploiting the lower pHe of cancer cells relative to normal cells, along with the acidic nature of intracellular endosomes and lysosomes, more selective drug release can be achieved within cancerous tissues [76,127]. Polymeric carrier systems can be designed to exhibit appropriate responsiveness to these acidic pH shifts, thereby facilitating drug release near the pathological site—either through the cleavage of the pH-sensitive linkages that conjugate the therapeutic payload to the carrier or via destabilization of the polymeric carrier itself [127]. Collectively, these endogenous stimuli can offer valuable opportunities for achieving enhanced and site-specific drug release.

2.7 Drug release mathematical models

Mathematical modelling of drug release provides predictive insights into release kinetics and the diffusional behaviour of drugs from their delivery systems. This knowledge facilitates the identification of the underlying mechanisms governing the release process and supports the optimization of formulation parameters to achieve desired release kinetics. Accurate prediction of drug release dynamics has the potential to enhance medication safety profiles and increase overall therapeutic efficacy [122]. The predictive capability of these mathematical models for both release mechanisms and kinetics can also assist researchers in designing systematic experiments, thereby reducing the need for extensive experimental trials and saving time and cost [122,128]. To assess the drug release mechanism and kinetics in this study, the *in vitro* release data were fitted to several well-established mathematical models (Equations 1–5).

Zero-order model:	$X_t = X_0 + k_0 t$	(1)
First-order model:	$\log Q_t = \log Q_0 - k_1 t / 2.303$	(2)
Higuchi model:	$X_t = X_0 + k_H t^{1/2}$	(3)
Hixson-Crowell model:	$Q_t^{1/3} = Q_0^{1/3} - k_{HC} t$	(4)
Korsmeyer–Peppas model:	$M_t / M_\infty = k_{KP} t^n$	(5)

In these models, X_0 denotes the initial amount of drug released, while X_t represents the released amount at time t . Q_0 refers to the initial drug content in the formulation, while Q_t indicates the residual amount remaining at time t . The ratio M_t / M_∞ expresses the fractional release of drug at time t , and n is the diffusional or release exponent. The parameters k_0 , k_1 , k_H , k_{HC} , and k_{KP} represent the release rate constants corresponding to the zero-order, first-order, Higuchi, Hixson–Crowell, and Korsmeyer–Peppas models, respectively. These kinetic models are widely applied in drug-release studies and have been extensively described across numerous publications [108,129–132].

2.8 Investigated drugs and their customized delivery approaches

2.8.1 Mitomycin C (MMC)

Mitomycin C (MMC) is a naturally produced compound that was first isolated in the late 1950s from the fermentation broth of *Streptomyces caespitosus*, an actinobacterial species widely distributed in soil [133–135]. This clinically active drug is known for its potent antibiotic, antifibrotic, and most importantly antineoplastic properties [133,136]. MMC’s antitumor effect stems from its ability to inhibit DNA synthesis by acting as an alkylating agent [135,136]. Its mechanism of action involves DNA alkylation as an initial step, followed by interstrand crosslink formation between DNA strands, which disrupts replication and ultimately induces tumor cell death [136]. Through this mechanism, MMC has demonstrated therapeutic potential across a broad spectrum of malignancies, including bladder, anal, colon, stomach, breast, head and neck cancers [137,138]. But, like most anticancer drugs, MMC exhibits some adverse effects, typically attributed to its high toxicity, absence of selectivity, and lack of specificity throughout its pathway [136,138]. For example, adverse effects such as lung toxicity, nephrotoxicity, and bone marrow suppression may arise and, in some cases, necessitate dose reduction or discontinuation of therapy [138,139]. Another challenge associated with this hydrophilic drug, which exhibits relatively good water solubility (~0.9 mg/mL), is its rapid clearance from the body when administered in free form, with no detectable levels observed in urine samples after 24 hours [3,140,141]. This pharmacokinetic limitation can compromise patient compliance, as it requires frequent dosing to maintain therapeutic relevance [142].

In response to these challenges, researchers have incorporated MMC into various drug delivery systems such as polymeric systems, aiming to minimize side effects and achieve prolonged release profiles, thereby enhancing therapeutic efficacy and supporting patient adherence [136,140,143]. In recent years, non-responsive PVA-based films and NPs have demonstrated promising potential for encapsulating MMC and prolonging its release. Notably, while some of these systems incorporate design features that could, in principle, support stimuli responsiveness within the cancer microenvironment and thus enhance selectivity, this potential functionality was not explored or discussed in the reported designs [88,136,140]. These observations suggest that stimuli-responsive PVA-based NPs—leveraging tumor-associated acidic pH for selective drug release or magnetic components for spatial targeting—could provide a means to prolong MMC release while improving its selectivity toward the cancer microenvironment.

2.8.2 Nimodipine (NIMO) and HC-067047 (HC)

Both nimodipine (NIMO), a calcium channel blocker, and HC-067047 (HC), a TRPV4 inhibitor, are regarded as key compounds in studies addressing brain ischemia-related complications [144,145]. As is well known, brain ischemia is a neurological disorder that typically arises from a sudden decline or interruption in the brain's blood flow, depriving brain tissue of oxygen and nutrients essential for cellular survival. Under these hypoxic conditions, the brain shifts to anaerobic glycolysis, a compensatory mechanism that generally causes the pH level to decline to 6.5–6.0 [146–148]. This brain ischemic state, along with its severe associated complications—such as blood–brain barrier disruption and malignant cerebral edema—is recognized as a significant contributor to global mortality and neurological disability [145,149,150]. Accordingly, pharmacological agents such as NIMO and HC have garnered considerable attention for their capacity to counteract ischemia-induced damage by inhibiting calcium influx and blocking TRPV4 channel activity, respectively [144,145].

Despite the therapeutic promise of these two compounds, their efficacy is significantly hindered by poor water solubility, which reduces their bioavailability and poses substantial formulation challenges [32,151–153]. For instance, HC requires organic solvents such as dimethyl sulfoxide (DMSO) or ethanol to improve solubility and enable administration via injectable routes [154,155]. Similarly, the commercial intravenous formulation of NIMO (Nimotop®), developed by Bayer, contains approximately 24% v/v ethanol as a solubilizing agent [156]. While these organic solvents are essential for solubilization, their evident high concentrations can induce local adverse effects, including inflammation and pain at the injection site [32,156]. Moreover, the intravenous infusion of ethanol-containing formulations may result in phlebitis and thrombosis [156–158]. Crystallization and precipitation of these poorly water-soluble

compounds may also occur when their injection solutions are diluted with saline solutions, which in turn poses additional risks to patient safety during intravenous administration [156,159]. In addition to their solubility-related issues, the nonspecific release of these drugs represents a further challenge, particularly for agents like NIMO, which has demonstrated poor selectivity and notable adverse effects on healthy tissues [108,160]. Together, these limitations highlight the need for suitable delivery systems for these drugs that can enhance their aqueous dispersibility, enable more selective release within ischemic microenvironments, and support sustained delivery. For NIMO, polymeric nanocarriers—such as those based Soluplus® [161], chitosan [108], and polyesters [121,162]—have shown encouraging potential in improving drug efficacy. However, some of these studies did not focus on enhancing selectivity toward the acidic pH of ischemic tissues, and others reported rapid drug release under such conditions, which may compromise sustained therapeutic action and reduce overall effectiveness. In contrast, for HC, no prior research has explored its encapsulation within polymeric NPs, making this study the first to investigate such an approach.

2.8.3 Methylprednisolone Acetate (MPA)

Methylprednisolone acetate (MPA) is a synthetically derived glucocorticoid widely renowned for its powerful immunosuppressive and anti-inflammatory effects, positioning it as a pivotal therapeutic agent in treating various autoimmune and inflammatory diseases. Mechanistically, MPA achieves its therapeutic outcomes by downregulating the expression of genes responsible for the synthesis of major inflammatory mediators, such as prostaglandins, thereby attenuating inflammation [163–165].

Despite its clinical significance, MPA faces considerable physiochemical and pharmacokinetic challenges that compromise its therapeutic efficacy when administered orally [164,166]. A primary challenge is its poor aqueous solubility (~0.02 mg/mL), which compromises the stability of aqueous suspensions and adversely affects the dissolution rate in aqueous media, thereby reducing bioavailability [164]. Furthermore, MPA undergoes extensive first-pass hepatic metabolism, whereby a considerable fraction of the administered dose is biotransformed in the liver prior to reaching systemic circulation. This metabolic barrier significantly diminishes the drug's availability, as only about 50% of the given dosage enters systemic circulation [166–168]. These limitations highlight the need for drug delivery systems that can enhance aqueous stability and dissolution of MPA, while supporting enhanced oral bioavailability.

One promising strategy involves the use of mucoadhesive polymeric nanoparticulate systems. Mucoadhesive polymeric drug delivery systems can adhere to mucosal surfaces in the gastrointestinal (GI) tract, thereby prolonging the residence time of the formulation at the

absorption site [169,115,170,171]. This prolonged retention and closer contact with the mucosal surface facilitate higher drug flux at the absorption site and thus provide a promising approach to improving bioavailability through maximized drug absorption [169,115,170,171]. Literature reports indicate that drugs encapsulated in more mucoadhesive NP types exhibit higher oral bioavailability compared to those encapsulated in less mucoadhesive ones. Mucoadhesion helps maintain the dosage form at the absorption site, counteracting its rapid GI transit, while the concurrent water flow promotes NP passage through the mucus layer from the lumen to the epithelium, where NPs can be taken up by cells [171,172]. Consequently, mucoadhesive polymeric NPs can improve systemic exposure for drugs suffering from low oral bioavailability, such as MPA. Moreover, extended contact time at the mucosal surface can increase drug concentration at the site of action, thereby improving local bioavailability [115,169]. This localized effect is especially advantageous in treating inflammatory conditions such as Crohn's disease, which primarily affects the small intestine [115,173], where MPA is used to reduce inflammation and alleviate symptoms [174–176]. Beyond these benefits, the small size and the large surface area of these NPs can facilitate the dissolution and enhance the stability of poorly water-soluble drugs such as MPA in aqueous media, thereby contributing to improved bioavailability and overall therapeutic efficacy.

The focus on developing mucoadhesive polymeric NPs for MPA encapsulation to improve its oral delivery remains largely unexplored. This gap presents an opportunity for further investigation, especially following the promising outcomes reported with mucoadhesive polymeric microparticles developed for methylprednisolone — the non-esterified form of MPA — which were specifically designed to prolong the residence time of the formulation at the mucosal surfaces [167]. Building on these findings, the development of mucoadhesive polymeric NPs represents a promising strategy for improving the aqueous stability and enhancing the dissolution of MPA and for contributing to prolonged gastrointestinal residence time, thereby supporting the potential for improved oral delivery.

3. Objectives

Throughout my doctoral research, we developed polymer-based drug delivery systems to potentially overcome critical barriers associated with different pharmacological agents, including the hydrophilic drug Mitomycin C (MMC) and the hydrophobic compounds Nimodipine (NIMO), HC-067047 (HC), and Methylprednisolone Acetate (MPA). Specifically, four distinct polymeric platforms—each exhibiting unique structural and functional features—were constructed by synthesizing or modifying biocompatible polymers to enhance therapeutic applicability and overcome limitations inherent to conventional drug-delivery approaches.

I. Development of multifunctional pH-responsive polymeric NPs for preferential release of MMC in the tumor microenvironment and for bioimaging applications

As previously discussed, MMC is a potent hydrophilic anticancer agent; however, its clinical effectiveness is constrained by rapid systemic clearance and non-specific release, which necessitate frequent dosing and increase the risk of cytotoxicity in healthy cells. To reduce the effect of these issues, this work aimed to develop multifunctional, pH-responsive PVA-based particles capable of encapsulating MMC, prolonging its release, and enabling preferential release in the cancer microenvironment, while simultaneously enabling fluorescence-based imaging. To realize this objective, we planned to synthesize a partially succinylated polyvinyl alcohol derivative (PVA-SA) that can enable pH-sensitive amide bonding with MMC, facilitate electrostatic interactions with the fluorescent probe Rhodamine B (RB), and promote spontaneous self-assembly into well-defined NPs. Prior to drug encapsulation, we aimed to optimize the RB content of the NPs via a one-pot synthesis approach. We also intended to characterize the resulting NPs using spectroscopic, microscopic, and dynamic light scattering techniques to evaluate conjugation efficiency, photophysical behaviour, and nanostructure formation. Moreover, we sought to evaluate the pH-responsiveness of the system through *in vitro* drug release studies under physiologically neutral (pH 7.4) and acidic (pH 5.8) conditions. Finally, we planned to perform cytotoxicity and cellular uptake experiments to evaluate selective bioactivity and the platform's potential for image-guided drug delivery.

II. Development of magnetically responsive polymeric NPs for spatially targeted delivery of MMC

While the first system aimed to enhance tumor selectivity primarily through pH-responsive drug release, the second aimed to improve spatial precision through the synthesis of a magnetically responsive, MMC-loaded polymeric prodrug intended to support field-guided accumulation of drug-loaded carriers at tumor sites. To achieve this, we planned to conjugate MMC and iron oxide magnetic nanoparticles (MNPs) to the same developed PVA-SA matrix. Specifically, we first aimed to synthesize MNPs-PVA-SA NPs at varying MNP loading to determine the

maximum MNP content that could be incorporated while maintaining appropriately small particle size, colloidal stability and magnetic responsiveness. Following this, we aimed to load MMC into the system via a one-pot approach to produce drug-loaded NPs with combined therapeutic and magnetic functionalities. We also intended to study the physicochemical behaviour of the resulting NPs to assess their applicability. Moreover, we planned to investigate *in vitro* drug release under pH conditions representative of normal physiological (7.4) and tumor-like environments (5.8) to assess the system's potential for pH-responsive drug release. Finally, we sought to evaluate the magnetic responsiveness of the resulting NPs to assess their suitability for field-induced localization in spatially controlled cancer therapy.

III. Development of hydrophobically modified Chitosan-Pluronic NPs for pH-responsive and sustained release of NIMO and HC in ischemic brain tissues, and for supporting intravenous administration without using organic solvents

As discussed, the efficacy of NIMO and HC is significantly hindered by their poor aqueous solubility, which often necessitates formulation strategies with tolerability concerns, and by their nonspecific release, which increases off-target exposure. Therefore, this point aimed to develop a multifunctional polymeric drug delivery system that can enable pH-responsive and sustained drug release within ischemic brain regions characterized by localized acidosis (pH 6.5–6.0), while also improving the aqueous dispersibility of such compounds. To achieve the desired pH-responsive behaviour, chitosan (Chit) was chemically modified via reductive amination with hydrophobic aldehydes of varying alkyl chain lengths, yielding N-alkylated Chit derivatives. This modification was expected to influence aqueous solubility and shift the pH threshold required for NP formation. The study therefore aimed to assess the hydration behaviour and the pH-dependent solubility of the synthesized derivatives, and to identify a candidate capable of forming colloiddally stable NPs at pH 7.4 while undergoing disintegration under ischemic pH conditions (pH 6.5–6.0). Research also aimed to synthesize NPs from the selected polymer (C₆-chit) using desolvation method and to systematically optimize the formulation parameters to obtain particles with favourable physicochemical properties. We also aimed to achieve not only targeted but also sustained drug release at ischemic sites by incorporating varying contents of Pluronic® F-127 (PF). Finally, we planned to encapsulate NIMO and HC via a one-pot synthesis approach and to evaluate the impact of encapsulation on drug crystallinity, aqueous dispersibility, colloidal stability, and *in vitro* drug release behaviour under simulated physiological and ischemic pH conditions.

IV. Development of mucoadhesive polymeric NPs for improved oral delivery of MPA

The poor aqueous solubility of the anti-inflammatory corticosteroid MPA compromises suspension stability and lowers the dissolution rate, thereby reducing oral bioavailability. Therefore, the work aimed to develop a mucoadhesive polymeric nanocarrier system for MPA to enhance its aqueous stability and dissolution, while also leveraging the mucoadhesive properties of the carrier to prolong gastrointestinal residence time and thereby support its potential for improved oral bioavailability. To achieve this, we first planned to synthesize poly(ethylene thiomalate) (PET), a biodegradable thiolated polyester, through catalyst-free polycondensation reaction. The study aimed to tailor the polymer molecular weight, and thereby modulate drug release, by systematically varying the polycondensation time. In view of the limited data available on PET's physicochemical behaviour, the work further aimed to conduct a detailed investigation of its solubility and precipitation characteristics. These findings guided the establishment of an optimized precipitation-based encapsulation strategy for MPA. The resulting drug-loaded NPs were characterized to evaluate the impact of encapsulation on drug properties, and *in vitro* release studies were conducted to assess how polyester NPs of different molecular weights influence MPA dissolution. The mucoadhesive properties of PET-based NPs were also investigated to determine their capacity for mucosal adhesion.

Table 1. Overview of the targeted pharmacological agents and the developed delivery systems

Drug	MMC		NIMO/HC	MPA
Therapeutic use	Anticancer agent		Pharmacological agents for brain ischemia-related complications	Anti-inflammatory and immunosuppressive agent
Intended administration route	Intravenous administration			Oral administration
Key challenges addressed	-High toxicity and lack of selectivity - Rapid systemic clearance		-Poor aqueous solubility -Nonspecific release	-Poor aqueous solubility -Slow dissolution in aqueous medium -Low bioavailability
Developed polymeric system	PVA-SA with RB	PVA-SA with MNPs	N-alkylated Chit with PF	PET polyester

MMC: mitomycin C; NIMO: nimodipine; HC: HC-067047; MPA: methylprednisolone acetate; PVA-SA: partially succinylated polyvinyl alcohol derivative; RB: rhodamine B; MNPs: iron oxide magnetic nanoparticles; Chit: chitosan; PF: Pluronic® F-127; PET: poly(ethylene thiomalate).

4. Material and methods

4.1 Materials

4.1.1 Modification of Polyvinyl Alcohol (PVA)

Materials used for synthesis of PVA-SA

- Succinic acid ($C_4H_6O_4$), Reanal.
- Acetic anhydride ($C_4H_6O_3$), Molar Chemicals Kft.
- Polyvinyl alcohol (PVA), Mw ~47 kDa, 86–89% degree of hydrolysis, Nagart Kft.
- Sodium acetate ($C_2H_3NaO_2$), Molar Chemicals Kft.
- N,N-Dimethylformamide (DMF), Merck KGaA.
- Diethyl ether ($C_4H_{10}O$), VWR Chemicals BDH®.

Materials used for synthesis and characterization of RB-PVA-SA and RB-PVA-SA-MMC NPs

- Rhodamine B (RB, $C_{28}H_{31}ClN_2O_3$), Sigma-Aldrich.
- 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, $C_8H_{17}N_3.HCl$), Sigma-Aldrich.
- Mitomycin C (MMC), 1Pluschem LLC.
- Sodium dodecyl sulfate (SDS, $NaC_{12}H_{25}SO_4$), Sigma-Aldrich.
- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Sigma Aldrich.
- Ethanol (C_2H_6O), VWR Chemicals BDH®.
- Acetone (C_3H_6O), VWR Chemicals BDH®.

Materials used for synthesis and characterization of MNPs-PVA-SA and MNPs-PVA-SA-MMC NPs

- Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), Molar Chemicals Kft.
- Ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), Sigma-Aldrich.
- Sodium hydroxide (NaOH), Merck KGaA.
- 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, $C_8H_{17}N_3.HCl$), Sigma-Aldrich.
- Mitomycin C (MMC), 1Pluschem LLC.
- Ethanol (C_2H_6O), VWR Chemicals BDH®.
- Acetone (C_3H_6O), VWR Chemicals BDH®.

All purchased materials were utilized with no additional purification.

4.1.2 Modification of Chitosan (Chit)

Materials used for synthesis and characterization of N-alkylated Chit products (C_6 -chit and C_{12} -chit), C_6 -chit NPs, PF- C_6 -chit NPs and the NIMO- and HC-loaded polymeric NPs

- Chitosan (Chit), Mw 310-375 kDa, deacetylation degree: >75%, Sigma-Aldrich.
- Hexyl aldehyde ($C_6H_{12}O$), Sigma-Aldrich.
- Dodecyl aldehyde ($C_{12}H_{24}O$), Sigma-Aldrich.
- Sodium cyanoborohydride ($NaCNBH_3$), Sigma-Aldrich.
- Pluronic F127 (PF, $(C_3H_6O \cdot C_2H_4O)_x$), Mw ~12600 Da, Sigma-Aldrich.

- Sodium hydroxide (NaOH), Merck KGaA.
- HC-067047 (HC, $C_{26}H_{28}F_3N_3O_2$), $\geq 98\%$, Sigma-Aldrich.
- Nimodipine (NIMO, $C_{21}H_{26}N_2O_7$), $\geq 98\%$, Alpha Aesar.
- Sodium tripolyphosphate (TPP, $Na_5P_3O_{10}$), Sigma-Aldrich.
- Iodine (I_2), Sigma-Aldrich.
- Potassium iodide (KI), Molar Chemicals Kft.
- Sodium dodecyl sulfate (SDS, $NaC_{12}H_{25}SO_4$), Molar Chemicals Kft.
- Toluene (C_7H_8), Merck KGaA.
- Acetic acid ($C_2H_4O_2$) Merck KGaA.
- Methanol (CH_4O), VWR Chemicals BDH®.
- Ethanol (C_2H_6O), VWR Chemicals BDH®.
- Acetone (C_3H_6O), VWR Chemicals BDH®.

All reagents were used as received without further purification.

4.1.3 Synthesis of Poly(ethylene thiomalate) (PET)

Materials used for synthesis and characterization of PET and MPA-loaded PET NPs

- Thiomalic acid (TA, $C_4H_6O_4S$), 97%, Sigma-Aldrich.
- Ethylene glycol (EG, $C_2H_6O_2$) 99.99% Molar Chemicals Kft.
- Methylprednisolone Acetate (MPA, $C_{24}H_{32}O_6$), $> 98\%$, TCI EUROPE N.V.
- Sodium hydroxide (NaOH), Merck KGaA.
- Potassium thiocyanate (KSCN), Reanal.
- Silver nitrate ($AgNO_3$), Molar Chemicals Kft.
- Ferric nitrate ($Fe(NO_3)_3$), VWR Chemicals BDH®.
- Mucin from porcine stomach, Sigma-Aldrich.
- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Sigma Aldrich.
- Sodium dodecyl sulfate (SDS, $NaC_{12}H_{25}SO_4$), Sigma-Aldrich and Molar Chemicals Kft.
- Dimethyl sulfoxide (DMSO), Molar Chemicals Kft.
- Methanol (CH_4O), VWR Chemicals BDH®.
- Tetrahydrofuran (THF), Reanal.

All chemicals were used as received without further purification.

Materials used to prepare phosphate-buffered saline (PBS, pH 5.8-7.4) solution.

- Di-sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$), Molar Chemicals Kft.
- Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) from Sigma-Aldrich.
- Sodium chloride (NaCl), Molar Chemicals Kft.

4.2 RB-PVA-SA and RB-PVA-SA-MMC polymeric prodrug

4.2.1 Synthetic procedures of succinic anhydride precursor

Succinic anhydride was prepared according to the following protocol [177]. In brief, 25 mL of acetic anhydride were added to 15 g of succinic acid under a nitrogen atmosphere. This mixture was placed in a round-bottom flask, which was fitted with a reflux condenser and an anhydrous calcium chloride drying tube. Next, a steam bath was used to gently heat the reaction until it reached a clear solution. After that, the reaction was heated for one hour to ensure completion. Then, the white succinic anhydride crystals were produced by cooling down the mixture in an ice bath and obtained by filtration. To wash these crystals, a small quantity of diethyl ether was utilized, followed by drying under vacuum. These synthetic procedures end up with almost 10.5 g of white crystals.

4.2.2 Synthetic procedures of succinylated PVA (PVA-SA)

The prepared succinic anhydride was used to modify PVA in accordance with the modified protocol reported [178]. Briefly, 9 mL of DMF was used to dissolve 1 g of PVA, after which 0.5 g of succinic anhydride was added. Anhydrous sodium acetate (0.05 g) was also introduced as a catalyst. The mixture was then stirred at 40 °C for 24 h. The reaction product was then precipitated by adding an excess of diethyl ether. This crude product was purified by repeated dissolution in DMF followed by precipitation with excess ether, then washed with ether and dried under vacuum.

4.2.3 Synthesis of RB-PVA-SA and RB-PVA-SA-MMC NPs

RB-PVA-SA NPs were prepared using the subsequent steps: 100 mg of PVA-SA (COOH content = 2.5 mmol/g polymer) was dissolved in 10 ml of MQ water. Next, various RB contents (0.5, 1.0, 1.5, 3.0 and 6.0 wt%, referring to PVA-SA) were added to the prepared solutions. After that, approximately 125 mg of EDC carboxyl group activator (2.6 equivalents to the COOH content) were introduced to the reaction mixture. The resulting samples were then stirred at room temperature for almost 1 h. The self-assembled RB-PVA-SA NPs were then precipitated with an excess amount of acetone and centrifuged to remove the solvent. After that, the resulting NPs were washed several times with ethanol and acetone to ensure their purity. The purified NPs were then lyophilized using the freeze-dryer apparatus (Christ Alpha 1–2 LD plus). The synthesis of drug-loaded NPs (RB-PVA-SA-MMC) was executed as per the above-stated procedures. The only exception was the addition of 4 mg MMC or 12 mg MMC to the prepared mixtures just before EDC addition. After the synthesis and centrifugation of NPs, supernatants were collected. The absorbance was then determined using a UV-VIS spectrophotometer, either at 554 nm to determine the RB content or at 364 nm to determine the MMC content in the collected supernatants. Then, the amounts of unconjugated dye and

unconjugated drug were quantified using the predetermined RB calibration curve and the MMC calibration curve prepared in MQ water. Subsequently, equations 6 and 7 were used to calculate the binding efficiency as well as the percentages of dye or drug loading.

$$\text{Binding Efficiency (\%)} = \frac{\text{Initial RB or MMC weight} - \text{RB or MMC weight in supernatant}}{\text{Initial RB or MMC weight}} \times 100 \quad (6)$$

$$\text{Loading (\%)} = \frac{\text{Weight of RB or MMC in NPs}}{\text{Total weight of NPs after synthesis}} \times 100 \quad (7)$$

4.3 MNPs-PVA-SA and MNPs-PVA-SA-MMC polymeric prodrug

4.3.1 Synthetic procedures of iron oxide magnetic nanoparticles (MNPs)

MNPs were prepared by using $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ salts via co-precipitation synthesis method [179,180]. In summary, 5.5 g of Fe^{2+} and 13.5 g of Fe^{3+} salts were each dissolved in 100 mL of distilled water and then mixed intensely with the use of a magnetic stirrer. The double iron salt solution was diluted by adding another 200 mL of MQ water and then precipitated by using 100 mL of 2.05 M NaOH solution. The alkaline solution was added in two portions: the first 50 mL was added slowly, and the second was added all at once, resulting in a black suspension having a pH of ~11. After a few minutes of vigorous stirring, the black iron oxide precipitate was magnetically collected and washed repeatedly with MQ water until the suspension reached a pH of ~7.3. After the final wash, the MNPs were collected and dried in an oven at nearly 50 °C.

4.3.2 Synthesis of MNPs-PVA-SA and MNPs-PVA-SA-MMC NPs

PVA-SA was first prepared as described in Section 4.2.2, and the MNPs-PVA-SA samples were then synthesized according to the following procedures: 130 mg of PVA-SA with 2.5 mmol/g carboxylic group content was dissolved in 10 mL of MQ water. After that, different weight ratios of MNPs (5, 10, 15, and 20 wt%, equivalent to 6.5, 14, 23, and 33 mg, respectively) were added to the aqueous solutions. Then, approximately 125 mg of EDC carboxyl group activator was introduced to the reaction mixture. The resulting mixtures were then shaken at room temperature for almost an hour. After MNP conjugation and self-particle formation, composite particles were precipitated by adding an excess of acetone and then centrifugated at 5000 rpm for 10 min. After that, the resulting particles were washed several times with ethanol and acetone to ensure their purity. The purified particles were then freeze-dried using Christ Alpha 1–2 LD plus instrument. A predetermined calibration curve with known concentrations of MNPs in PBS (pH 7.4, 0.9% NaCl) was also used to figure out the amount of MNPs present in the collected supernatants. The preparation of drug-loaded NPs followed the same steps as described above, except that 4 mg of MMC was added to the reaction mixture prior to the addition of EDC. Unbound MMC was determined by measuring the absorbance of the

supernatant at 364 nm via UV-VIS spectroscopy. After that, MMC binding efficiency and its loading percentage were determined using equations 6 and 7.

4.4 N-alkylated Chit products, PF-C₆-chit NPs and the NIMO- and HC-loaded NPs

4.4.1 Synthesis of hexyl and dodecyl-modified chitosan (C₆- and C₁₂-chit)

To synthesize the C₆-chit and the C₁₂-chit samples, reductive amination of Chit with either hexyl or dodecyl aldehydes was performed. The synthetic procedures were carried out in accordance with our previous research [108], with some modifications. In short, 1 g of Chit was dissolved into 250 or 500 ml of a 5% aqueous acetic acid solution to produce either 0.4 or 0.2 wt/v% Chit solutions. Subsequently, around 4 ml of hexyl aldehyde solution in ethanol and 8 ml of dodecyl aldehyde solution in ethanol were gradually introduced into the 0.4% and 0.2% Chit solutions, respectively. Based on the used quantities, the aldehyde feed corresponded to approximately 60% of the nominally available amine groups of Chit. After 30 minutes of stirring, approximately 18 ml of sodium cyanoborohydride solution (1 M) was introduced into each mixture to complete the reductive amination reaction. The reactions were then left to stir for 24 h. Following this, the mixtures were dialyzed for four days against distilled water. The products were then isolated from the cellulose dialysis bags (Sigma Aldrich, M_w cut-off 14000 Da), precipitated and washed with ethanol, centrifuged to remove unreacted free aldehydes. Next, they were washed with water, rinsed twice more with ethanol, centrifuged and finally freeze-dried.

4.4.2 Synthesis of C₆-chit NPs

C₆-chit NPs were synthesized utilizing the desolvation method. In general, the modified polymer was dissolved in 4.5 ml of distilled water containing 5% acetic acid, and the pH was adjusted to 6.0 using a 10 M NaOH solution. NPs were then produced at room temperature by the dropwise addition of the desolvating agent. The particles were also stabilized by adding 100 μ l of TPP crosslinker aqueous solution. After 30 min of stirring, the resulted NPs were centrifuged at 5000 rpm for 30 min. Then the supernatant was removed, and the NPs were dispersed in MQ water and centrifuged again. These washing steps were repeated several times to ensure NPs purification. The obtained particles were then lyophilized.

During the synthesis, various parameters were systematically altered to evaluate their effects on the colloidal properties of the produced NPs. These parameters included the concentration of the C₆-chit solution (0.2-1.2 wt/v%), the type and the amount of the applied desolvating agent (methanol, ethanol, and acetone), and the amount of TPP crosslinker (10-40 wt/wt%, referring to C₆-chit).

4.4.3 Preparation of Pluronic® F-127-loaded C₆-chit NPs (PF-C₆-chit NPs)

Initially, 9 mg of the powdered C₆-chit was dissolved in 4.5 ml of 5% acetic acid aqueous solution to produce a 0.2 wt/v% homogeneous polymeric solution. The prepared solution was then exposed to varying amounts of PF copolymer (15, 20, and 25 wt/wt% relative to C₆-chit). Subsequently, the pH was adjusted to 6.0 using 10 M NaOH solution. The PF-C₆-chit NPs were then produced by adding 1.8 ml of ethanol precipitant dropwise. Thereafter, 100 µl of TPP crosslinker solution (10 wt/wt %, referring to C₆-chit) was added dropwise to the resulting NPs. After 30 min of stirring, the PF-loaded NPs were centrifuged and purified using the same procedures as those employed for the C₆-chit NPs. A schematic diagram for the entire synthesis process can be seen in the supplementary material (**Figure A1**).

4.4.4 Preparation of NIMO- and HC-loaded polymeric NPs

The drug-loaded NPs were prepared in a very similar manner to the unloaded ones but with some modifications. Briefly, 1 mg of either NIMO or HC was dissolved in 1.8 ml of ethanol to prepare a homogenous solution. Next, a 0.2% C₆-chit solution (4.5 ml) containing varied amounts of PF copolymer (0, 15, 20, and 25 wt/wt% relative to C₆-chit) was prepared, and its pH was adjusted to 6.0 using 10 M NaOH solution. After that, the prepared polymeric solution was added dropwise to the NIMO or the HC solution under vigorous stirring at room temperature. Then, for the NP stabilization, 100 µl of TPP crosslinker solution (10 wt/wt %, referring to C₆-chit) was added. Finally, the NIMO-loaded NPs as well as the HC-loaded NPs were centrifuged and purified exactly like the unloaded ones. During the particle purification process, the supernatants were collected, and the remaining sediments were dissolved in PBS (pH 7.4, containing 0.9% NaCl and 0.5% SDS). This was done to determine the amount of drug present in the supernatant. For that, the absorbance value was first determined using a UV-VIS spectrophotometer either at 360 nm for NIMO or at 281 nm for HC. Following this, the unloaded drug content was calculated using the predetermined NIMO calibration curve or the HC calibration curve in PBS. Then, equations 8 and 9 were employed to determine the encapsulation efficiency and drug loading, respectively.

$$\text{Encapsulation Efficiency (\%)} = \frac{\text{Initial NIMO or HC weight} - \text{NIMO or HC weight in supernatant}}{\text{Initial NIMO or HC weight}} \times 100 \quad (8)$$

$$\text{Drug Loading (\%)} = \frac{\text{Weight of NIMO or HC in NPs}}{\text{Total weight of NPs}} \times 100 \quad (9)$$

4.5 Poly(ethylene thiomalate) PET polyester

4.5.1 Synthesis of PET polyester with varying molecular weights

The synthesis of PET polyester was achieved through a direct melt polycondensation reaction using equimolar TA (17.91 mmol, 2.77 g) and EG (17.91 mmol, 1 mL) as reactive

monomers [112,120]. The reactants were heated in an oil bath at approximately 165°C with continuous magnetic stirring. Neither acid nor metal catalysts were employed in the polycondensation process. At specific time periods (40, 60, 80, 100, 120, and 140 minutes), the process was halted by swiftly cooling the mixture in an ice bath. After 5 minutes of cooling, the resulting crude product was fully dissolved in 2 ml of THF and precipitated with a 20-fold excess of methanol. The polymeric products were then collected through centrifugation at 5000 rpm for 30 minutes and subsequently washed multiple times with methanol. Finally, the purified viscous PET samples were allowed to dry for 24 hours at 40°C.

4.5.2 Preparation of PET NPs & MPA-Loaded PET NPs

The encapsulation of MPA within the synthesized PET polyesters was achieved using the precipitation method. Specifically, 128 mg of PET (using separate PET samples with different molecular weight) and 16 mg of MPA (11 w/w% nominal MPA content) were dissolved in 2 mL of DMSO. The prepared solution was then added dropwise to 2.5 mL of distilled water under continuous magnetic stirring to precipitate the encapsulated form. The resultant product was obtained through centrifugation at 5000 rpm for 30 minutes, followed by washing with distilled water and lyophilization. The same procedure was conducted without the addition of MPA to produce empty NPs. The encapsulation efficiency of MPA within the PET NPs was determined using equation 10.

$$\text{Encapsulation Efficiency (\%)} = \frac{\text{Amount of MPA in the NPs}}{\text{Total amount of MPA used during encapsulation}} \times 100 \quad (10)$$

where the amount of MPA encapsulated in the NPs was indirectly estimated by measuring the drug concentration in the supernatant using UV-VIS spectroscopy.

4.6 Methods of characterization

4.6.1 Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was performed to characterize the chemical structure of both synthesized and modified polymers and to confirm the success of the polymerization or modification processes. The measurements were performed using a Jasco FT/IR-4700 spectrometer. The FTIR spectra were collected over a range of 4000 to 500 cm⁻¹, with 128 scans accumulated at a resolution of 1 cm⁻¹. All samples were analyzed in their dry solid form.

4.6.2 Proton nuclear magnetic resonance (¹H-NMR)

¹H-NMR spectroscopy measurements were carried out to study the chemical structure of the synthesized polyesters. The NMR spectra were recorded at room temperature using a Bruker BioSpin AG spectrometer operating at 400 MHz. The measurements were done in DMSO-d₆ with a drop of D₂O, using tetramethylsilane (TMS) as the internal standard. Chemical shifts

were reported in ppm (δ scale). The number of repeating units (n) and the number-average molecular weights (M_n) of the synthesized polyesters were determined following the methodology described by Izunobi and Higginbotham [181].

4.6.3 Raman Spectroscopy

Raman spectra were recorded using a Bruker Sunterra II Raman microscope to verify the coupling reaction between PVA-SA and MMC. A near-infrared excitation laser (785 nm) operating at 100 mW was used for spectral acquisition. Each spectrum was obtained by averaging five scans to improve signal quality. All measurements were performed on the purified powder form of the resulting products.

4.6.4 X-ray diffraction (XRD) measurements

X-ray diffractograms were recorded using a Philips X-ray diffractometer equipped with a Cu-K α radiation source ($\lambda = 1.5418 \text{ \AA}$). Measurements were performed at ambient temperature. The 2θ scanning ranges were set as follows: 20° – 80° for the magnetic samples; 2° – 35° for the C₆-chit polymer, PF-C₆-chit NPs, NIMO- and HC-loaded NPs, and the bare NIMO and HC; and 2° – 50° for MPA, PET, and MPA-PET. A step size of 0.02° was applied for all measurements.

4.6.5 Energy dispersive X-ray (EDX) measurements

EDX measurements were performed using a Röntec EDX detector for elemental composition analysis of the modified polymers. Morphology was examined using a field emission scanning electron microscopy (SEM-Hitachi S-4700 microscope) at an accelerating voltage of 10 kV. Dried powder samples were used for all measurements.

4.6.6 Thermogravimetric (TG) and differential scanning calorimetry (DSC) analyses

TG and DSC analyses were performed on the original Chit polymer and its hydrophobically modified forms to evaluate the effect of hydrophobic modification. Before the measurements, each polymeric sample was swelled in a 5% acetic acid solution to obtain a 1 wt% concentration. Samples were left to stir for 2 days to ensure reaching the swelling/dissolution equilibrium. Afterward, the samples were exposed to centrifugation for 15 min at 14000 rpm. The supernatants were then removed, and the obtained sediments (concentrated polymer gels) were used for analysis. The water content in the concentrated polymeric sediments was determined using TG analysis on a Mettler-Toledo TGA/SDTA 851^e instrument. During the analysis, samples were heated from 30 to 130 °C at a rate of 2.5 °C/min. DSC measurements were also performed to determine the desorption enthalpy, using a Mettler-Toledo 822^e DSC under the same temperature range and heating rate. All measurements were performed in triplicate, and outcomes were reported as mean values.

DSC measurements were also conducted on the initial monomers and the synthesized polyester samples using the same Mettler-Toledo 822e instrument. These samples were heated from 25 to 420 °C at a rate of 5 °C/min. In addition, TG analysis was performed on PET-120 min sample using a TGA Q500 V20.13 Build 39 instrument to assess its thermal behaviour and degradation. The sample was heated in a nitrogen atmosphere from ambient conditions to 420 °C at a rate of 5 °C/min.

4.6.7 Dynamic light scattering (DLS) measurements

DLS was used to determine the hydrodynamic diameter (DH), polydispersity index (PDI), and ζ -potential values of the samples. Measurements were performed using a Horiba SZ-100 Nanoparticle Analyzer at 25 °C with a 90° scattering angle. Before analysis, the purified particles were dispersed in PBS (pH 7.4 or 5.8, 0.9% NaCl) and sonicated to obtain homogeneous dispersion. The measurements were performed in triplicate, and the values are presented as mean $\pm$ SD.

DLS was also used to determine the weight-average molecular weight (M_w) of the initial Chit, its hydrophobically modified derivatives, and the synthesized polyesters. All measurements were performed at a fixed scattering angle of 90° using polymer concentrations in the range of 0–5 mg/ml. For Chit and its derivatives, samples were dissolved in aqueous acetic acid solutions and filtered through 0.22 μ m Millipore membrane filters prior to measurement, whereas the polyester samples were dissolved in DMSO before analysis. According to the Rayleigh equation (equation 11) and Debye plot formulation, the M_w was calculated from the concentration (C) dependence of KC/R_θ .

$$\frac{KC}{R_\theta} = \left(\frac{1}{M_w} + 2A_2C \right) \quad (11)$$

where K denotes the optical constant, C means the sample concentration, R_θ indicates the Rayleigh ratio of the scattered to incident light intensity, and A_2 indicates the second virial coefficient. For the determination of K, the refractive index increment (dn/dc) values were determined across the specified concentration range using a Mettler Toledo RM50 LiquiPhysics Excellence refractometer. Toluene served as a standard for Rayleigh ratio determination. For the M_w determination, three measurements were conducted.

4.6.8 Transmission electron microscope (TEM) measurements

TEM measurements were performed to investigate the particle size and morphology of the prepared particles. The measurements were carried out using a Jeol JEM-1400Plus transmission electron microscope operated at 120 kV and a FEI TECNAI G2 20 X-Twin high-resolution transmission electron microscope operated at 200 kV. For TEM investigations, one drop of the

diluted, dispersed samples was deposited onto carbon film-coated copper grids and allowed to dry overnight. ImageJ software was used for the analysis of the TEM images.

4.6.9 Water contact angle (θ_w) measurements

The Easy Drop system (Krüss GmbH) was used to determine the water contact angles (θ_w) of the prepared PET films. The device was equipped with a 0.5 mm diameter steel syringe needle, which was used to dispense a droplet of distilled water onto the film surface. Side-view images of the sessile drop were recorded using the CCD camera integrated into the goniometer. The droplet profiles were then analyzed using the Young–Laplace fitting method in the DSA100 software, which determines the contact angle by fitting the entire droplet shape. For the measurements, PET films were prepared by melting polyester samples of different molecular weights on glass substrates and allowing them to cool to room temperature.

4.6.10 Fluorescence emission and decay properties of RB-PVA-SA NPs

The emission of NPs was checked using a Leica DM IL LED inverted laboratory fluorescence microscope. For the microscopic study, a dispersion of RB-PVA-SA NPs ($C_{RB} = 1.5 \mu\text{g/ml}$) was prepared. The sample was then dropped on a glass slide and examined. Moreover, the maximum emission bands and the photostability of the free and coupled RB were investigated. For the photostability measurements, RB and RB-PVA-SA samples were first prepared, each containing RB at a concentration of $1.5 \mu\text{g/ml}$. Following this, samples were repeatedly excited at 554 nm for 2 h using a JASCO FP-8500 fluorimeter equipped with a Xe lamp light source. To assess the fluorescence intensity stability, the emissions were observed at the peak maxima after each excitation. The measurements were performed with a 4-sided quartz cuvette of 1 cm optical length. The spectra were recorded using a 2.5–2.5 nm bandwidth, a scanning speed of 200 nm/min, and a resolution of 1 nm. The absolute quantum yield was also determined by analyzing the incident light spectra and the indirect and direct excitation spectra of the samples. For the measurements, the same JASCO fluorimeter was used, equipped with the JASCOILF-835 integrating sphere ($d = 100 \text{ mm}$). For the calibration of the instrument, a calibrated JASCOESC-842 WI lamp was utilized; thus, no other references were required. For the quantum yield calculations, the Spectra Manager 2.0 software of the apparatus was applied. The time-correlated single photon counting method was also used to figure out the emission lifetime (ns) of both the free RB and the RB-loaded PVA-SA NPs. The measurement was conducted using a Horiba Delta Flex fluorometer. A delta-diode pulsed laser operating at a wavelength of 467 nm served as an excitation light source. The emissions were detected at a wavelength of 580 nm. During the measurement, a 1 nm band width was chosen, and 10,000 counts were recorded on the peak channel. The instrument response function (IRF) was generated using a standard LUDOX[®] colloidal silica dispersion. To determine the essential

lifetime components, a two-exponential fit was applied to the resulting curves, and Chi square (χ^2) values were used to assess the goodness of the fit.

4.6.11 Magnetic properties of the prepared magnetic samples

The magnetic behavior of the unmodified MNPs, the MNPs–PVA–SA, and the MNPs–PVA–SA–MMC dispersions was evaluated using an external magnet with a flux density of 0.35 T. For each measurement, the solid magnetic powders (equivalent to ~ 7 mg MNPs) were dispersed in 10 mL of PBS (pH 7.4, 0.9% NaCl). After 60 s of magnetic exposure, the liquid was carefully removed while the magnetically attracted particles were left to dry before being weighed. The magnetic collection efficiency was calculated as the ratio of the dried mass of the collected material to the initial mass of the sample. A second set of nanosuspensions was prepared to check the magnetic behavior after a storage time of 28 days. All experiments were performed in triplicate, and the reported values represent the corresponding averages.

Magnetic properties of the solid magnetic particles were also measured at room temperature using a vibrating sample magnetometer (VSM, Lakeshore Model 7410). Magnetization (M) was recorded as a function of the applied magnetic field (H) up to a maximum field of 20 kOe, and the saturation magnetization (M_s), coercivity (H_c), and remanent magnetization (M_r) were obtained from the resulting hysteresis loops.

4.6.12 Mucoadhesive properties of the prepared PET NPs

The mucoadhesive properties of the synthesized PET NPs were ensured by the presence of thiol groups, which were quantified using Volhard's method [182]. In this process, 40 mg of thiolated polyester NPs (prepared from PET with various molecular weights) was dispersed in 25 mL of 0.01 M AgNO_3 solution. The dispersion was then carefully covered to prevent light exposure and stirred for 3 hours to ensure sufficient interaction between the thiol groups and AgNO_3 . Afterward, the mixture was titrated against a standardized 0.01 M KSCN solution to determine the unreacted AgNO_3 , using $\text{Fe}(\text{NO}_3)_3$ as an indicator. The endpoint was identified by the formation of a red complex $[\text{Fe}(\text{SCN})_6]^{3-}$, which resulted from the reaction between the excess thiocyanate ions (SCN^-) supplied by the titrant and the ferric ions (Fe^{3+}) provided by the indicator. Finally, the polyester thiol content (mmol/g) was calculated using equation 12, based on the added volume (V, mL) and the concentration (C, M) of the KSCN solution [182].

$$\text{Thiol content (mmol/g)} = \frac{0.25 - (C \times V)}{\text{Weight of PET}} \quad (12)$$

The mucoadhesive properties were further evaluated through oscillatory rheology measurements using a Physica MCR 302 rheometer (Anton Paar, Graz, Austria). For the analysis, aqueous dispersions of mucin and PET NPs (synthesized from PET with various

molecular weights) were prepared separately at a concentration of 0.5 w/v% to serve as controls for comparison. To assess interactions, mucin and PET dispersions were first prepared at 1 w/v%, then mixed in equal volumes, yielding a final concentration of 0.5 w/v% for both components. Following overnight incubation, gelation behavior was examined under controlled conditions at 25°C. The storage modulus (G') and the loss modulus (G'') were measured across a strain range of 0.1 to 10% and at an angular frequency of 10 Hz, providing valuable insights into the viscoelastic characteristics of the samples.

The mucoadhesive characteristics of the polyester NPs were further demonstrated through a simple adsorption experiment. First, the PET NPs obtained from PET polymers of different molecular weights were dispersed separately in 10 mL of PBS (pH 7.4, 0.9% NaCl) at a final concentration of 0.025 wt/v%. Subsequently, an 8 cm section of pre-moistened pig intestine, sourced from a local slaughterhouse, was submerged in each turbid dispersion. Each dispersion was stirred at 300 rpm, and the reduction in turbidity, resulting from particle adsorption onto the intestinal membrane surface, was monitored over time using a turbidity meter. Control experiments were conducted under identical conditions in the absence of the pig intestine to isolate the effect of adsorption.

4.6.13 Special analytical methods

Carboxyl content determination of PVA-SA

The available carboxyl groups in the succinylated PVA (PVA-SA) were quantified using acid–base titration. For each measurement, 0.1 g of the polymer was dissolved in 10 mL of distilled water, and the resulting solution was titrated against 0.1 M NaOH using phenolphthalein as the indicator. The titration was performed in triplicate, and the reported value corresponds to the average of the three measurements.

Study of self-assembled particle formation of PVA-SA, RB-PVA-SA and MNPs-PVA-SA

The formation of self-assembled NPs from linear PVA-SA macromolecules via ester bond formation between carboxyl and hydroxyl groups was examined by turbidity measurements at 25 °C using a turbidity meter (HI98703, Hanna Instruments). In this study, 125 mg of EDC was added to two aqueous samples (10 mL), the first containing 1.3% PVA-SA without RB and the second containing 0.65 mg of RB, and the change in turbidity was recorded over time. Baseline turbidity was measured prior to EDC addition and subtracted from all subsequent values. Each experiment was performed in triplicate, and data are reported as mean $\pm$ standard deviation. Similar procedures were applied to evaluate the effect of MNP incorporation on self-assembly. For this purpose, 125 mg of EDC was added to 10 mL of a 1.3% PVA-SA solution containing 14 mg of MNPs, and turbidity was monitored over time.

Determination of deacetylation degree (DD%) of the initial Chit

The DD of the polymer was determined using potentiometric titration [183,184]. The titration technique was used by dissolving ~0.2 g of the powdered polymer in a known excess of acid (20 ml of 0.1 M HCl) with continuous stirring for 30 min. Following that, 25 ml of distilled water was added to the solution, and stirring continued for another 30 min. From the titration of the solution with a 0.1 M NaOH solution, a potentiometric curve with two inflection points was obtained. The difference of the volumes of these two points was corresponding to the acid consumed for the acidification of amine groups and allows the determination of the DD. For DD% calculation, equation 13 was used.

$$DD\% = \frac{\Delta V \times [NaOH] \times 203}{(42 \times \Delta V \times [NaOH]) + m} \times 100 \quad (13)$$

where ΔV ($V_2 - V_1$) is the volume of NaOH solution required to neutralize the ammonium functionalities (L), $[NaOH]$ is the concentration of the NaOH solution used for titration (mol/L), 203 is the molar mass of the N-acetyl-D-glucosamine monomer (g/mol), 42 is the difference between the molar mass of the N-acetyl-D-glucosamine monomer and that of the D-glucosamine monomer (g/mol), and m is the mass of the sample in the dry state before titration (g).

Determination of N-Substitution Degree (SD%) in Chit derivatives

The SD% of Chit with hexyl or dodecyl chains was determined by assessing the total number of accessible reaction sites in the Chit chains prior to functionalization and the reacted number of moles of aldehyde (N_{react}). To calculate N_{react} , the filtrate obtained during the dialysis and centrifugation processes was collected and left to dry. The remaining residue was then dissolved in 100 ml of ethanol to measure the absorbance value using UV-VIS spectroscopy (SHIMADZU UV-1800), either at 291 nm for hexyl aldehyde or at 284 nm for dodecyl aldehyde. The unreacted number of moles of each aldehyde was then determined using predetermined calibration curves. Since the initial number of moles of each aldehyde and the unreacted moles are known, N_{react} can be determined. Finally, equation 14 [185] was used to calculate the SD%.

$$SD\% = \frac{N_{\text{react}} \times 161}{W_{\text{Chit}} \times DD} \times 100 \quad (14)$$

where N_{react} is the reacted number of moles of aldehyde (mol), 161 is the molar mass of the D-glucosamine units of Chit (g/mol), W_{Chit} is the used Chit mass (g) and DD is the degree of deacetylation of Chit (as a fraction).

Study of pH dependent solubility characteristics of the initial Chit and its derivatives

The pH-dependent solubility characteristics of the initial Chit, the C₆-chit, and the C₁₂-chit were studied using a diode array spectrophotometer (Ocean Optics USB2000). Each polymer was first dissolved in 5 ml of aqueous acetic acid to produce a 1 wt% polymer solution. Then, every solution received a dropwise addition of 1 M NaOH solution while being continuously stirred. During these additions, both the pH and the absorbance of the polymer solution at 500 nm were recorded to obtain the displayed pH–absorbance curves.

Determination of PF critical micelle concentration (PF CMC)

To determine the CMC value of the PF copolymer under the applied synthetic conditions, the iodine UV-VIS spectroscopy method [186] was utilized. First, 2 g of potassium iodide (KI) and 1 g of iodine (I₂) were dissolved in 100 ml of distilled water to prepare a standard solution of KI/I₂. Next, a set of 0.2% C₆-chit solutions was prepared. All these C₆-chit solutions contained the same amount of KI/I₂ standard solution (25 µl) but various PF concentrations ranging from 0.0001 to 0.1 wt/v%. After the solution preparation step, the final mixtures were left in the dark at room temperature for 12 h. A UV-VIS spectrometer was then used to measure the absorbance value at a λ of 366 nm for each solution. These absorbance values were then plotted against the logarithm of PF concentrations. The CMC value was finally determined by identifying the intersection point of the two extrapolated linear fits. The experiment was then repeated twice to calculate the average value.

Determination of degree of polymerization (X_n) for synthesized PET polyesters

The degree of polymerization was assessed by quantifying the unreacted carboxyl (COOH) groups of thiomalic acid (TA) through an acid-base titration method. To achieve this, 100 mg of the unpurified polyesters were well-dispersed in 20 mL of distilled water. The resulting dispersions were then titrated with 0.1 M NaOH using phenolphthalein as an indicator. The same steps were repeated with the initial reaction mixture (0 min of reaction time) to serve as a reference. Using equation 15, the polymerization percent (P %) could be calculated. Subsequently, the monomer conversion (P) was used to determine the degree of polymerization (X_n) by applying equation 16, which is known as the Carothers equation [120].

$$\text{Polymerization percent (P\%)} = \frac{\text{Initial COOH content} - \text{unreacted COOH content}}{\text{Initial COOH content}} \times 100 \quad (15)$$

$$\text{Degree of polymerization (X}_n\text{)} = \frac{1}{1 - P} \quad (16)$$

Solubility/precipitation measurements of the synthesized PET Polyesters

The solubility/precipitation behaviour of the synthesized polyesters were assessed using biocompatible DMSO as the solvent and distilled water as the precipitant. To determine the theta composition (Θ composition) of these solvents for the polyester, the turbidimetric titration technique, originally developed by Elias and later refined by van Ballegooijen and Cornet, was employed [187]. During the experiment, distilled water, serving as a poor solvent, was added drop by drop to the PET-120 min DMSO-based solution (good solvent) under constant magnetic stirring until precipitation commenced. The onset of precipitation was identified through turbidity measurements, utilizing the Turbidity Meter-HI98703 from Hanna Instruments. Various concentrations of PET-120 min, ranging from 0.2 to 1 w/v%, were tested during the experiment. The Θ composition was ultimately derived by plotting the logarithmic values of the volume fraction of water (Φ_{water}) required to initiate precipitation against the logarithmic values of the corresponding volume fraction of polyester (Φ_{PET}), then extrapolating to a pure PET system ($\Phi_{PET}=1$) [187].

After determining the Θ -composition, the solubility/precipitation behaviour of the PET polyester products with various molecular weights was also studied. In this investigation, 20 mg of each polyester sample was dissolved in 10 mL of DMSO, which acted as a good solvent. Turbidity measurements were then performed during the dropwise addition of distilled water to the polyester solutions, which were subjected to continuous magnetic stirring at 25°C.

After determining the volume fraction of water (Φ_{water}) required to initiate the precipitation of PET samples (with varying M_n), the Schulz equation (equation 17) was employed to calculate the A and B coefficients. These coefficients are indicative of the interactions within the solvent–polymer–precipitant system [120,188]. According to the Schulz theory, identifying these coefficients enables molecular weight determination. To determine these coefficients, the reciprocal of the M_n of each PET sample (determined via $^1\text{H-NMR}$) was plotted against the corresponding Φ_{water} required to initiate the precipitation. A straight line was obtained, and from its slope, the B coefficient was derived and then the A coefficient was calculated from the intercept using B [120,188].

$$\frac{1}{M_n} = \frac{100}{B} \Phi_{water} - \frac{A}{B} \quad (17)$$

Investigating the precipitation properties of MPA and PET in DMSO/Water system

Since the nanoprecipitation method was employed to encapsulate the MPA drug within PET, it was essential to first examine the solubilities of both components in a DMSO/water system, which served as an effective solvent-poor solvent system for both [121,166,189]. To achieve

this, 2.5 mL of 0.2 w/v% MPA and 0.1 w/v% PET-120 min solutions were prepared using DMSO. After that, about 3 mL of distilled water was added dropwise to the prepared solutions while being subjected to vigorous magnetic stirring. The change in absorbance at $\lambda = 550$ nm was then determined using an Ocean Optics USB2000 UV-VIS Spectrometer to monitor the precipitation of the drug and the formation of the polyester particles.

The precipitation of PET-120 min and MPA solutions was then evaluated in a more concentrated polyester system to identify the ideal volume fraction of DMSO needed for the simultaneous precipitation of both polyester and MPA drug, thus producing MPA-loaded PET particles. Prior to the measurements, 5 mL of 1.6% w/v PET and 0.2% w/v MPA solutions were prepared in DMSO. The PET or MPA DMSO-based solution was then gradually introduced into 2.5 mL of distilled water under vigorous magnetic stirring. To monitor the precipitation process, the absorbance at 550 nm was continuously measured.

Stability studies

Colloidal stability of magnetic samples

The stability of the (colloid) samples with varying MNP contents (see chapter 4.3.2) was assessed by recording the change in the absorbance value at 312 nm as a function of time (over 28 days) using a UV-VIS spectrophotometer (SHIMADZU UV-1800). For measurement, the purified dried powders (equivalent to ~ 14 mg MNPs) were dispersed in 10 mL of PBS (pH 7.4, 0.9% NaCl) and sonicated for 10 min. After that, 0.4 mL was taken from the suspensions at certain times, diluted to 3 mL, and then introduced into a quartz cuvette. The absorbance of each sample was measured in the wavelength range of 250 to 600 nm. After 360 min of measuring, all suspensions were stored in the fridge at 4 °C and allowed to equilibrate at room temperature before any further measurement. The final stability curves show the change in the normalized absorbance as a function of time. Furthermore, the size of both MMC-free and MMC-loaded magnetic NPs (dispersed in PBS) was measured after 1 day, 14 days, and 28 days of being stored in the fridge. Samples are allowed to equilibrate at room temperature before any measurement. All stability experiments were performed in triplicate.

Stability study of bare NIMO and HC drugs and drug-loaded NPs in aqueous medium

The colloidal stability of drugs (NIMO and HC) and drug-loaded NPs (C₆-chit/NIMO NPs, (25 wt/wt% PF)-C₆-chit/NIMO NPs, C₆-chit/HC NPs and (25 wt/wt% PF)-C₆-chit/HC NPs) in aqueous PBS medium (pH 7.4, 0.9% NaCl) was examined through sedimentation measurement. NIMO-loaded NPs and HC-loaded NPs (equivalent to 2 mg of NIMO or HC) were dispersed in 10 ml of PBS. These dispersions were sonicated for 10 minutes and promptly transferred to an ISO-HI98703 Hanna turbidimeter. Their sedimentation was subsequently monitored for 8 hours at room temperature under steady-state conditions without stirring. Similar procedures

were carried out with free HC and free NIMO. For measuring stability during storage conditions, all dispersions (NIMO-loaded NPs & HC-loaded NPs) were prepared as mentioned above and stored in the refrigerator at 4°C. Before any further measurements, the dispersions were allowed to equilibrate at room temperature. Stability was monitored over approximately one month, with periodic measurements of changes in turbidity, particle size, and ζ -potential. All measurements were conducted in triplicate.

Stability study of bare MPA and MPA-loaded PET NPs in aqueous medium

The colloidal stability of MPA and MPA-loaded PET NPs in aqueous PBS medium (pH 7.4, 0.9% NaCl) was assessed through sedimentation analysis. MPA-loaded PET NPs dispersions (0.01 w/v% MPA) were prepared using 10 ml of PBS. The dispersions were sonicated for 10 minutes and promptly transferred to the turbidimeter for analysis. Sedimentation behavior was observed for 8 hours under steady-state conditions without stirring. For comparison, the same procedure was applied to the uncoated MPA. The particle size and ζ -potential of the MPA-loaded NPs were also monitored after 24 hours of dispersion in PBS to assess their colloidal stability. All measurements were conducted in triplicate.

In vitro drug-release studies

In vitro release of MMC from RB-PVA-SA particles

The *in vitro* drug release study was conducted on free MMC, ((0.5% RB)-PVA-SA)-MMC NPs, and ((1.5% RB)-PVA-SA)-MMC NPs. The unconjugated MMC was used in its purchased powder form, while the conjugated ones were used in their purified lyophilized state. The study was performed in PBS medium (pH = 5.8 or 7.4, 0.9% NaCl) at 37 °C over 221 h. The powder samples with 2 mL of PBS medium were inserted into cellulose membranes (Sigma Aldrich, M_w Cut-Off of 14000 Da) and then immersed in 48 mL of PBS. At certain time intervals, 3 mL was withdrawn from the dissolution medium to measure spectrophotometrically the MMC absorbance value at a wavelength of 364 nm. Then, the extracted volume was reintroduced into the release medium. The released MMC concentration was then calculated using the calibration plots presented in **Figure A2**. Following that, the MMC release percentage was calculated by dividing the released concentrations at each time point by the maximal concentration (0.01 mg/mL). These release experiments were repeated in triplicate, and the mean release percentage values were plotted as a function of time. Moreover, the kinetics of the resulting release profiles were evaluated via various kinds of mathematical models (zero- and first-order models, as well as Higuchi-, Hixson-Crowell- and Korsmeyer-Peppas models).

In vitro release of MMC from MNPs-PVA-SA particles

The *in vitro* release experiments were carried out on bare MMC in its purchased powder form and on the conjugated MMC ((10 wt% MNPs)-PVA-SA-MMC) in its purified lyophilized state. The studies were conducted in PBS (pH 5.8 or 7.4, 0.9% NaCl) at 37 °C for 173 h. As before, MMC samples dispersed in 2 mL of PBS were placed in cellulose membranes (M_w cut-off 14 000 Da) and immersed in 48 mL of dissolution medium. At certain time intervals, 3 mL aliquots were withdrawn to measure MMC absorbance at 364 nm and then returned to the medium. The released MMC concentration was then calculated using the calibration equations obtained from the concentration–absorbance relationship shown in **Figure A2** and expressed as release percentage relative to the maximum MMC concentration (0.01 mg/mL). All experiments were performed in triplicate, and the mean release percentage values were plotted as a function of time. After that, the release kinetics were assessed using the same mathematical models mentioned in the previous subsection.

In vitro release of NIMO and HC from the developed Chit-based NPs

The *in vitro* release study was conducted on the free NIMO and the NIMO-loaded NPs (C₆-chit/NIMO, (15 wt/wt% PF)-C₆-chit/NIMO, and (25 wt/wt% PF)-C₆-chit/NIMO). Moreover, it was also performed on the free HC and the HC-loaded NPs (C₆-chit/HC, (15 wt/wt% PF)-C₆-chit/HC, and (25 wt/wt% PF)-C₆-chit/HC). The drug-loaded samples were used in their lyophilized form. The experiments were performed at 37 °C in PBS medium (pH = 6.0 or 7.4, containing 0.9% NaCl and -due to the poor water solubility of the drugs-0.5% sodium dodecyl sulfate (SDS)) over 8 h. For measuring, each sample was first placed inside a dialysis cellulose membrane (Sigma Aldrich, M_w cut-off 12000 Da) and then submerged into 20 mL of the dissolution medium. At specified time intervals, the intensity of the characteristic absorbance peak of HC (at λ = 281 nm) or NIMO (at λ = 360 nm) was spectrophotometrically recorded by extracting 3 mL from the release medium. Then, the drug released concentration was determined by utilizing NIMO calibration curves (**Figure A3**) or HC calibration curves (**Figure A4**). After that, the release percentage was determined by dividing the concentrations released at each time by the maximum concentration values for NIMO (0.02 mg/ml) or for HC (0.03 mg/ml). These values were then averaged by repeating each experiment three times. Additionally, the release kinetics were studied using a variety of release kinetic models.

In vitro release of MPA from the synthesized PET NPs

The *in vitro* drug release study was conducted on the uncoated MPA and the MPA-loaded PET NPs. The MPA-loaded polyester samples were utilized in their lyophilized state. The experiments were carried out using PBS medium (pH = 7.4, containing 0.9% NaCl and -due to the poor water solubility of the MPA-0.5% SDS surfactant) at 37 °C. Initially, each sample was

carefully placed inside a dialysis membrane (Spectra/Por® Biotech CE, Repligen, M_w cut-off 500 Da). The membranes were then immersed in 20 mL of PBS medium. At predefined time intervals, 3 mL aliquots were withdrawn from the release medium, and the absorbance of MPA at its characteristic wavelength ($\lambda = 247$ nm) was measured spectrophotometrically. Then, the released concentration of MPA was calculated using its calibration curve (**Figure A5**). Subsequently, the percentage of drug release was determined by dividing the released concentration at each time interval by the maximum theoretical release concentration of MPA (0.025 mg/mL). To ensure the reproducibility, all experiments were conducted in triplicate, and the average values were reported. The release kinetics were evaluated by applying various kinetic models. Moreover, dissolution efficiencies ($DE_{8\text{ h}}$ and $DE_{30\text{ h}}$) were calculated based on the area under the dissolution curve from 0 to 8 and 30 h, respectively, measured by trapezoidal rule, and expressed as a percentage of the area at maximum dissolution [190]. Furthermore, the dissolution data were used to determine the mean dissolution time (MDT), calculated as the sum of different release fraction intervals during dissolution studies divided by the initial loading dose [191,192].

4.6.14 Biological methods

In vitro cytotoxicity assessment of RB-PVA-SA, RB-PVA-SA-MMC, and MMC

Cytotoxicity of RB-PVA-SA, RB-PVA-SA-MMC, and MMC was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. To perform this assay, normal CCD-19Lu human lung fibroblast cells and HEp-2 human epithelial cancer cells were used. The cell lines were originally obtained from the American Type Culture Collection (ATCC). The adherent normal and cancer cells were cultured in 96-well flat-bottomed microtiter plates using Eagle's Minimal Essential Medium (EMEM). The pH of the culture medium was adjusted to reach 7.4 or 5.8 through the addition of hydrochloric acid solution. The density of the cells was adjusted to 6×10^3 cells in 100 μL per well. The cells were seeded for 24 h at 37 °C with 5% CO_2 before the assay. Samples were diluted in EMEM (with the desired pH value) in a separate plate, and 100 μL of each dilution was transferred into the designated sample wells after the 24 h incubation period. The final concentrations for the labeled carrier (1.5% RB-PVA-SA NPs) were about 1, 2, 4, 8, 16, 31, 63, 125, and 250 $\mu\text{g/mL}$. However, for the free MMC and the MMC-loaded NPs, the MMC final concentrations were 0.2, 0.4, 0.8, 1.6, 3, 6, 13, and 25 $\mu\text{g/mL}$. Following a 72 h incubation period at 37 °C, 20 μL of MTT stock solution (5 mg/mL; Sigma-Aldrich) was added to each well. After a further incubation at 37 °C for 4 h, 100 μL of a 10% SDS solution was added to each well to solubilize the formazan crystals, and the plates were further incubated at 37 °C overnight. Cell viability % was determined by measuring the optical density (OD) at 540 nm (ref. 630 nm) using a Multiscan

EX ELISA reader (Thermo Lab Systems) and by applying equation 18. Mortality % was also calculated according to equation 19. Moreover, the inhibitory concentration (IC₅₀) values was determined by applying the nonlinear regression dose–response curve fitting using GraphPad Prism 10.0.2. software. Results are presented as the mean ± standard deviation of triplicate trials.

$$\text{Cell viability \%} = \left[\frac{\text{OD}_{\text{treatment}} - \text{OD}_{\text{cell-free blank, with sample}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{cell-free blank, without sample}}} \right] \times 100 \quad (18)$$

$$\text{Mortality \%} = 100 - \left[\frac{\text{OD}_{\text{treatment}} - \text{OD}_{\text{cell-free blank, with sample}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{cell-free blank, without sample}}} \right] \times 100 \quad (19)$$

In vitro cellular uptake of RB-PVA-SA-MMC NPs

HEp-2 carcinoma cells obtained from ATCC were seeded at a density of 2×10^5 cells/well and cultured at 37 °C in a humidified atmosphere containing 5% CO₂ for 24 h. Cells were then incubated with either ((0.5% RB)-PVA-SA)-MMC NPs or ((1.5% RB)-PVA-SA)-MMC NPs at a concentration of 100 µg/ml at 37 °C for 12 h. Following the incubation period, cells were washed and stained with BioTracker 488 Green Nuclear Dye (Sigma-Aldrich) for 20 min at room temperature. The cells were subsequently rinsed and supplied with fresh culture medium. Finally, a Leica DM IL LED inverted laboratory fluorescence microscope was used to investigate the cellular uptake of NPs.

In vitro cytotoxicity assessment of PET NPs

Cytotoxicity of catalyst-free PET NPs (synthesized from PET with M_w of 908 or 1652 Da) was evaluated using the MTT assay. MRC-5 cells (human embryonic lung fibroblasts; obtained from ATCC) were cultured in 96-well flat-bottomed microtiter plates with EMEM (pH ≈ 7.4). Cells were seeded at a density of 1×10^4 cells in 100 µL per well and incubated for 24 h at 37 °C, with 5% CO₂ before the assay. PET samples were diluted in EMEM in a separate plate, and 100 µL of each dilution was transferred into the designated sample wells after the 24 h incubation period. The cells were exposed to PET NPs at final concentrations ranging from 1 to 150 µg/mL. After 24 h of incubation at 37 °C, each well received 20 µL of MTT solution (5 mg/mL) and was incubated for 4 h at 37 °C. Subsequently, 100 µL of 10% SDS was added to solubilize the formazan crystals, and plates were further incubated overnight at 37 °C. Cell viability (%) was calculated by measuring the optical density (OD) at 540 nm (ref. 630 nm) and by applying equation 18. Results are presented as the mean ± standard deviation of three independent experiments.

4.6.15 Statistical analysis

Data were statistically analyzed using a one-way ANOVA or a t-test. The following designation was used for the level of significance: *p < 0.05; **p < 0.01; ***p < 0.001.

5. Results and Discussion

This chapter presents the outcomes of the four polymer-based drug delivery systems developed during my doctoral research. These systems were designed in response to the distinct pharmacological and biopharmaceutical limitations of MMC, NIMO, HC, and MPA.

For the anticancer hydrophilic drug MMC, two delivery platforms were developed using partially succinylated PVA (PVA-SA), a derivative containing both OH and COOH groups suitable for pH-sensitive amide bond formation with MMC. In the first platform (I.), PVA-SA was conjugated to MMC and RB dye to obtain multifunctional NPs suitable for pH-responsive sustained MMC release under tumor-like acidic conditions and for fluorescence-based imaging applications. In the second system (II.), MNPs replaced RB to produce magnetically responsive composite nanocarriers intended to enable spatially targeted delivery of MMC. For the neuroprotective hydrophobic compounds NIMO and HC, which are used in ischemia-related complications, Chit as initial biopolymer was hydrophobically modified using aliphatic hydrophobic aldehydes via reductive amination reaction to modulate its pH-dependent solubility, and the suitable N-alkylated Chit derivative was formulated with PF copolymer to produce NPs capable of providing sustained, pH-triggered release under ischemic brain conditions while also improving the dispersibility and stability of these hydrophobic compounds in aqueous media (III). For the anti-inflammatory hydrophobic drug MPA, thiolated aliphatic polyester (PET polyester) with different molecular weights was synthesized (IV) via catalyst-free polycondensation, and its solubility and precipitation behaviour were investigated to establish an encapsulation strategy aimed at improving the oral delivery of the drug through enhanced formulation stability, dissolution, controlled release, and mucoadhesivity. The detailed results of each system are presented in the following four chapters, in the same order outlined above.

5.1 Development of multifunctional pH-responsive polymeric NPs for preferential release of MMC in the tumor microenvironment and for bioimaging applications

As previously discussed, MMC is a potent hydrophilic anticancer agent, yet its clinical utility is limited by rapid systemic clearance and non-specific release, which necessitate frequent dosing and increase the risk of cytotoxicity in healthy tissues. To reduce the effect of these issues, this chapter focused on developing multifunctional, pH-responsive PVA-based NPs capable of encapsulating MMC, prolonging its release, and enabling preferential drug release under acidic tumour-like conditions, while also providing fluorescence-based imaging. A partially succinylated PVA derivative (PVA-SA) was synthesized to enable pH-sensitive amide bonding with MMC, support interactions with RB, and promote spontaneous nanoparticle formation (**Figure 4**). Fluorescent RB-PVA-SA NPs were first prepared via a one-pot approach

to optimize RB content, followed by the one-pot synthesis of fluorescent MMC-loaded NPs (RB-PVA-SA-MMC). The systems were characterized using spectroscopic, microscopic, and dynamic light scattering techniques to evaluate conjugation efficiency, photophysical behaviour, and nanostructure formation. Their pH-responsive behaviour was examined through *in vitro* release studies at pH 7.4 and 5.8. Finally, cytotoxicity and cellular uptake experiments were conducted to assess selective bioactivity and the potential of the platform for image-guided drug delivery.

5.1.1 Structural characterization of the synthesized samples

The modification of the parent PVA was carried out by reacting it with succinic anhydride at a proportion corresponding to 32 mol% of its available hydroxyl moieties (~16 mmol/g, quantified via acetic anhydride/pyridine titration [140,193]). Acid–base titration of the resulting PVA-SA revealed that the polymer contains 2.5 mmol/g of available carboxyl groups, which corresponds to nearly 16% of the initial hydroxyl content. These introduced COOH functionalities enabled PVA-SA to form amide bonds with MMC and facilitated its interaction with RB through hydrogen-bonding and electrostatic forces [88,98] in the presence of the EDC carboxyl-activating agent. In addition, the remaining unreacted carboxyl groups can couple with pendant hydroxyl moieties along the PVA-SA chain, forming ester linkages that promote self-assembled particle formation (**Figure 4**) [194].

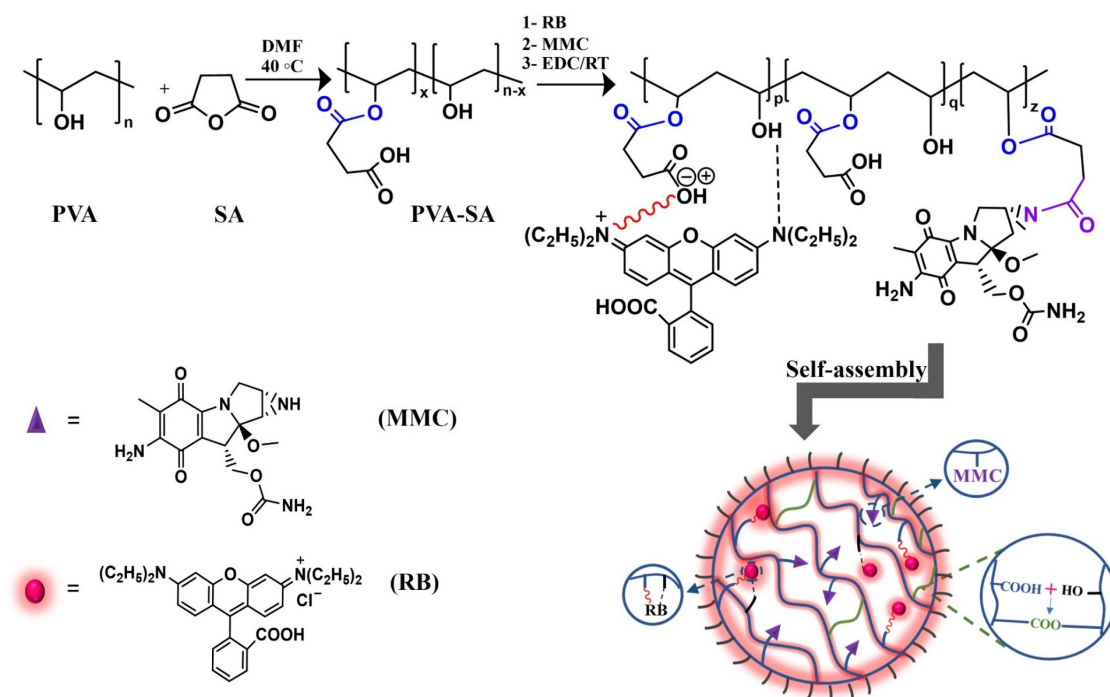


Figure 4. The synthesis process of succinylated PVA, the coupling reactions of RB (via electrostatic interaction presented in a red zigzag line and hydrogen bond presented in a black dashed line), and MMC (through amide bonds highlighted in purple) with PVA-SA, and the crosslinking of carboxyl and hydroxyl groups to form self-assembled NPs [195].

The success of the reactions was verified through FTIR analysis by evaluating the chemical structure of the native PVA, its PVA-SA derivative, and the resulting RB dye-loaded and MMC drug-loaded fluorescent NPs. As demonstrated in **Figure 5**, the FTIR profile of native PVA exhibits the characteristic vibrational features of the polymer backbone. In particular, the broad absorption centered around 3275 cm^{-1} is attributed to the stretching vibration of hydroxyl groups, while the peaks at 2934 and 2910 cm^{-1} are related to the stretching modes of the methylene units. Moreover, the absorption band appearing at 1725 cm^{-1} is due to the C=O stretching of the unhydrolyzed acetate groups. In addition, the signals detected at 1425 , 1370 , and 1318 cm^{-1} are associated with the bending vibrations of the CH and CH₂ groups, whereas the absorptions observed at 1238 , 1080 , and 833 cm^{-1} correspond to the stretching modes of the C–O–C, C–O and C–C linkages, respectively [140,196].

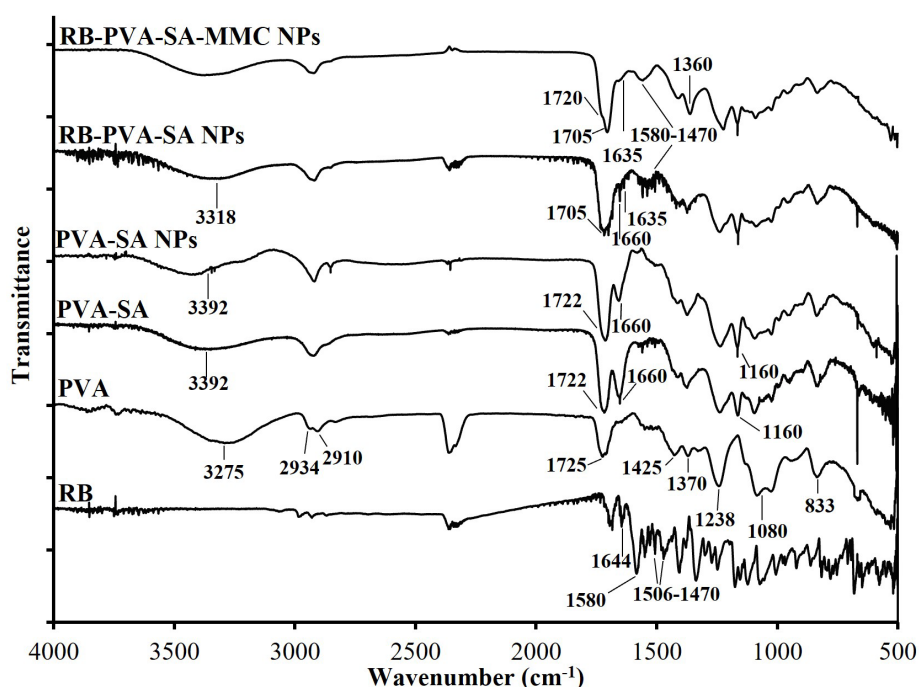


Figure 5. FTIR spectra of RB, PVA, PVA-SA, PVA-SA NPs, RB-PVA-SA NPs and RB-PVA-SA-MMC NPs [195].

The succinylation of PVA and the successful formation of the PVA–SA derivative were further confirmed by the reduction in the intensity of the broad O–H stretching band together with the pronounced enhancement of the ester-associated C=O vibration at 1725 cm^{-1} . Additionally, the two new bands observed at 1660 cm^{-1} and 1160 cm^{-1} provided further evidence of this structural modification, with the former corresponding to vibrations of the carboxylate moiety (RCOO[−]) and the latter attributed to C–O stretching vibrations of the newly introduced ester groups [88,197,198].

Compared to PVA-SA, the NPs exhibited an enhancement in the 1160 cm^{-1} band corresponding to the C–O stretching vibrations of the ester groups and a strong reduction in the 1660 cm^{-1} band associated with the carboxylate moiety of succinate [3,198], verifying ester-related cross-linking and supporting the self-assembled particle formation (see later) through reactions between the accessible OH and COOH moieties along the polymeric backbone [194].

The synthesis of RB-loaded polymeric particles was further confirmed by analysing the recorded RB-PVA-SA spectrum. The observed shift in the OH vibrational band from 3392 to 3318 cm^{-1} verified the formation of hydrogen-bonding interactions between PVA-SA and RB [199]. In addition, the decrease in the C=O stretching wavenumber from 1722 to 1705 cm^{-1} supported the presence of electrostatic interactions between the two components. Moreover, the IR profile exhibited absorptions within the $1580\text{--}1470\text{ cm}^{-1}$ region, which were consistent with those observed for free RB and can be attributed to the aromatic C=C stretching modes [200,201]. Furthermore, the band related to the stretching vibrations of the conjugated C=C groups within the xanthene ring, recorded at 1644 cm^{-1} for free RB [202], was observed at 1635 cm^{-1} in the RB-PVA-SA spectrum. This change can be ascribed to the electrostatic and hydrogen-bonding interactions between the PVA-SA matrix and the dye.

The binding between MMC and PVA-SA was further confirmed by the absence of the NH band of MMC at 3440 cm^{-1} , along with the emergence of the C=O amide band at 1720 cm^{-1} and the emergence of the C–N stretching band at 1360 cm^{-1} [3,88]. These absorptions verified the formation of amide linkages between the NH moieties of the drug and the COOH moieties of the polymer.

Raman spectroscopy provided further confirmation of the successful loading of MMC (**Figure A6**). Relative to PVA-SA, the raman spectrum of PVA-SA-MMC displayed additional bands at 1011 , 1570 , and 1660 cm^{-1} , consistent with those existing in the MMC spectrum [203]. Moreover, the formation of amide linkages between the NH groups of the drug and the COOH moieties of the polymer was supported by the appearance of the amide-specific bands in the PVA-SA-MMC spectrum, namely the amide I (C=O) band at 1717 cm^{-1} [204,205], and the amide III (C–N) bands at 1327 and 1366 cm^{-1} [205,206].

The existence of the elements in the resulting conjugates was further detected through EDX analysis. In contrast to PVA-SA, which shows no detectable nitrogen, both RB-PVA-SA and RB-PVA-SA-MMC particles exhibited nitrogen in their EDX elemental maps (**Figure A7**), supporting the conjugation of RB and MMC to PVA-SA.

5.1.2 Self-assembly of polymeric chains and formation of RB-PVA-SA NPs

Because the PVA-SA backbone contains both OH (13.3 mmol/g) and COOH (2.5 mmol/g) functional groups, the addition of EDC (the carboxyl-group activator) can facilitate ester bond

formation between these groups, thereby promoting the spontaneous formation of self-assembled nanoparticles. To verify this self-assembly behavior, two aqueous PVA-SA solutions (1.3 wt/v%) were prepared. In one of these solutions, RB was added at 0.5 wt% relative to the polymer. Following the introduction of EDC to both formulations, the change in turbidity was followed as a function of time, as demonstrated in **Figure 6A**. Within the first minute of measurement, both macromolecular formulations exhibited a turbidity value of approximately 15 NTU. This initial rise in turbidity can be attributed to the activation of the COOH groups triggered by EDC through O-acylisourea formation [88]. The turbidity then continued to increase significantly as self-assembled structures formed through crosslinking between the OH groups and the COOH groups of PVA-SA, eventually reaching a steady plateau after about 10 minutes, with final values of ~95 NTU for the PVA-SA sample and ~89 NTU for the RB-containing sample. These comparable final turbidity values indicate that the presence of RB does not impair the ability of PVA-SA to produce self-assembled particles.

To further confirm the formation of the self-assembled particles and examine their size and morphological characteristics, TEM analysis was performed after sample purification. As shown in **Figure 6B**, both samples exhibited a spherical morphology, with average particle sizes of 12 ± 3 nm for the PVA-SA NPs and 49 ± 15 nm for the RB-PVA-SA NPs. Based on the above observations, the production of self-assembled polymer NPs can indeed be confirmed.

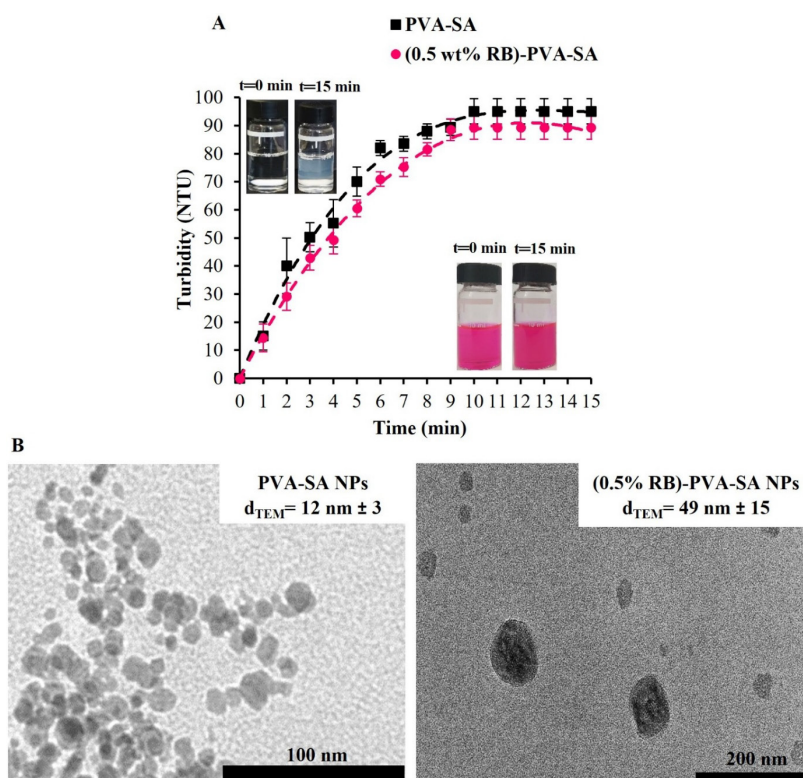


Figure 6. (A) The variation in the turbidity of PVA-SA and RB-PVA-SA solutions ($c = 1.3$ wt/v%) with EDC over time. (B) TEM images of the produced NPs. The nominal concentration of RB in the RB-PVA-SA sample is 0.5 wt% [195].

5.1.3 Optimization of RB loading in the composite NPs

Subsequently, to assess the feasibility of introducing larger quantities of the dye into the system, various RB concentrations (0.5–6.0 wt% with respect to PVA-SA) were applied during RB-PVA-SA NP synthesis. After determining the dye amount in the supernatants, the binding efficiency was calculated. As demonstrated in **Figure 7A**, raising the initially added dye amount from 0.5 to 6.0 wt% resulted in a marked increase in both binding efficiency (27.35% → 40.37%) and loading percentage (0.13% → 2.42%). The hydrodynamic diameter (DH) and the ζ -potential of the resulting NPs, dispersed in PBS (pH 7.4, 0.9% NaCl), were also determined. As illustrated in **Figure 7B**, increasing the nominal RB concentration from 0.5 to 6.0 wt% led to a considerable increase in particle size, with the DH rising from 80 ± 13 nm to 278 ± 28 nm, confirming the higher dye loading. Moreover, the polydispersity index (PDI) values varied between 0.206 ± 0.18 and 0.279 ± 0.09 (**Figure 7C**), indicating that all samples maintained good size uniformity [207,208]. A corresponding increase in ζ -potential was also observed, rising from -28.5 ± 2.6 mV to -60.7 ± 2.5 mV (**Figure 7D**), which can be attributed to the presence of COOH groups in the RB fluorescent dye, which are predominantly present in their deprotonated (COO^-) state at pH values above 4.2. Thus, by increasing the dye loading, more ionized carboxyl groups are introduced into the system, and this will result in a more negative ζ -potential [209,210].

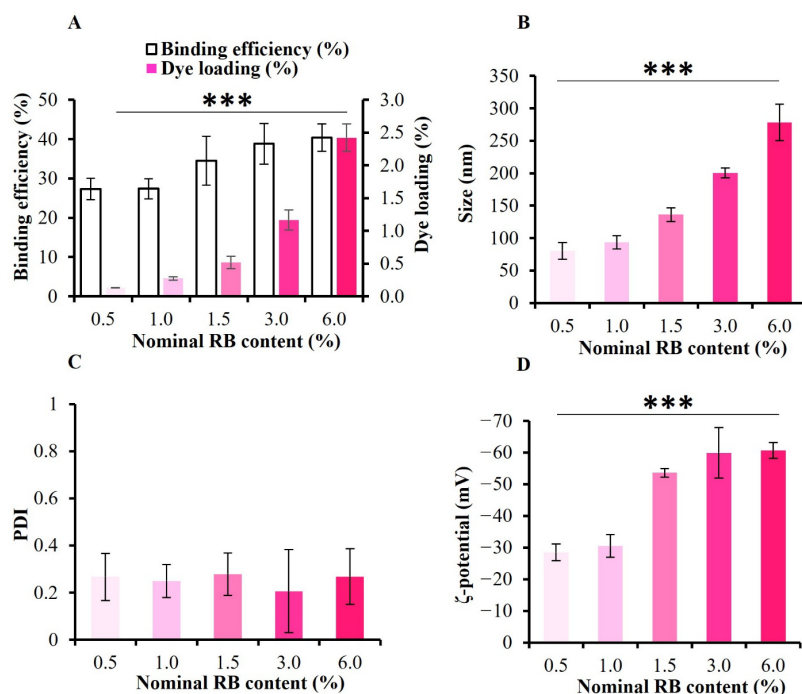


Figure 7. (A) The binding efficiency results and the dye loading percentage for the prepared NPs as a function of increasing the nominal RB content. (B) The average particle sizes, (C) the PDI values, and (D) the ζ -potential values of the produced RB-loaded NPs. Statistical analysis of the results: one-way ANOVA with Tukey's HSD post-hoc analysis. The added asterisks in the figure represent the summarized p value obtained from the analysis. *** $p < 0.001$ [195].

The size and zeta-potential values of the NPs were also determined at pH 5.8, as this pH resembles the extracellular environment of cancer cells [73,211,212] and the acidic conditions of lysosomal and endosomal compartments [75,195], showing DH and ζ -potential values (**Figure A8**) comparable to those measured at pH 7.4 (**Figure 7**). These results confirmed that our polymeric NPs are capable of accommodating higher RB loadings. Nevertheless, for effective cancer treatment, the literature always recommends the use of particles smaller than 200 nm, as they can evade immune system and penetrate cancer tumor tissues more effectively [213–215]. Therefore, the (0.5% RB)-PVA-SA NPs and (1.5% RB)-PVA-SA NPs formulations were selected for subsequent investigations.

5.1.4 Photophysical characteristics of RB-loaded particles

To determine whether the RB-loaded NPs are appropriate for imaging applications, their emission characteristics were investigated. The emission capability of the NPs was initially examined using a fluorescence microscope, which verified the ability of the particles to emit fluorescence (**Figure 8A**). With respect to the spectrofluorimetric measurements, the free dye exhibited its emission maximum at $\lambda_{\text{max}} = 576$ nm, whereas the RB-loaded particles showed a red-shifted maximum at $\lambda_{\text{max}} = 589$ nm (**Figure 8B**). This noticed shift supports the interaction between the dye molecules and the polymer particles, as it has been previously established that Rhodamine B undergoes a red shift when it exists in a bound state [216,217].

In addition, in comparison to the free dye, our RB-loaded polymeric particles demonstrated enhanced emission stability when subjected to continuous excitation at 554 nm for 2 hours. As can be seen in **Figure 8C**, the emission intensity for the free RB progressively decreased over time, reaching 78% after 120 minutes, whereas the RB-loaded NPs exhibited a nearly constant emission intensity throughout the entire excitation period. Besides emission stability, the synthesized NPs—(0.5% RB)-PVA-SA and (1.5% RB)-PVA-SA—also exhibited high quantum yields of 43.7% and 42.7%, respectively, as presented in **Figure 8D**. These values are markedly higher than the quantum yield of the free dye, which was determined to be 29.8% and is consistent with literature data [218]. The observed increase in quantum yield can be ascribed to the ability of the polymer chains to wrap the fluorescence centers and restrict the motion freedoms [219], thereby suppressing non-radiative relaxation pathways.

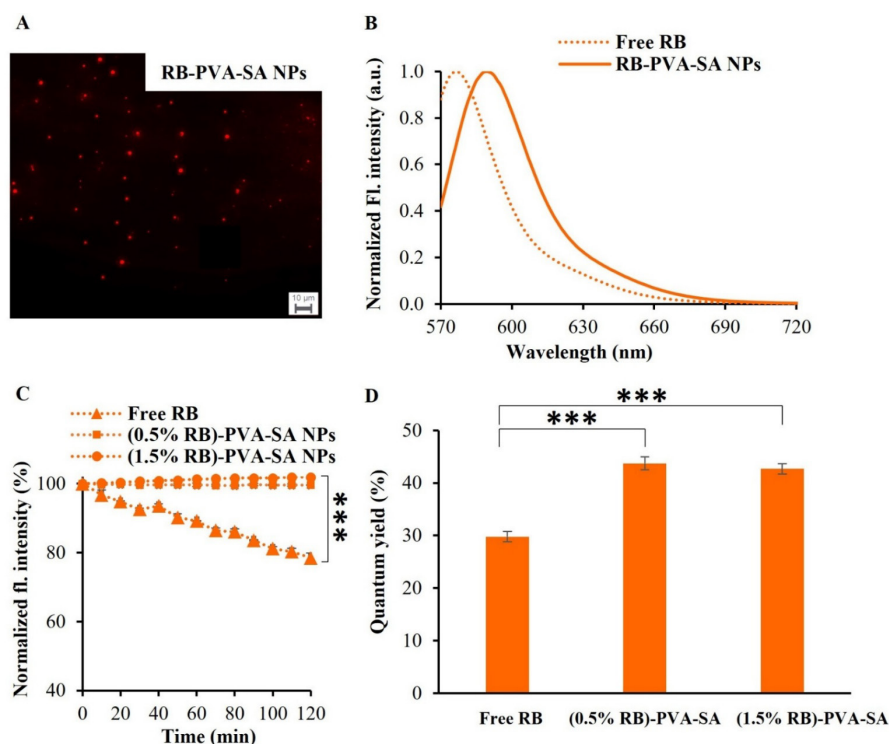


Figure 8. (A) The fluorescence microscopy image of RB-loaded NPs ($C_{RB} = 1.5 \mu\text{g/mL}$). (B) The normalized emission spectra of the free dye and the dye-loaded NPs dispersed in PBS (pH 7.4, 0.9% NaCl). Samples were excited at their peak maxima (RB at 554 nm and RB-PVA-SA at 565 nm), and their emission was observed from 570 to 850 nm. (C) The fluorescence intensity stability of unbound RB and RB-loaded NPs ($C_{RB} = 1.5 \mu\text{g/mL}$) upon repeated excitation at 554 nm for 120 min using a Xe arc lamp. (D) The quantum yield of free RB and RB-PVA-SA NPs dispersed in MQ water. Statistical analysis of the difference in photostability and quantum yield results was performed using one-way ANOVA with Dunnett's post hoc test (control group: free RB). *** $p < 0.001$ [195].

Figures 9A, 9B, and A9 also present the decay profiles of free RB, (0.5% RB)-PVA-SA NPs, and (1.5% RB)-PVA-SA NPs, respectively. The fitting of all decay profiles revealed two distinct lifetime components; and Figure 9C presents their lifetime values together with the ratios of their contributions. The short lifetime (τ_1) originates from the RB dimeric species, while the long lifetime (τ_2) arises from the monomeric RB species [220]. For both the free dye and the dye-loaded NPs, the decay component associated with the longest lifetime is the dominant contributor, representing over 80% of the total fluorescence emission. Notably, the dye-loaded polymeric NPs showed a minimum of a 2.4-fold increase in τ_1 and a 1.7-fold increase in τ_2 . This improvement can be clearly ascribed to the polymeric carrier, which wraps the dye molecules and maintains them in a highly dispersed state. This aligns with earlier research, which indicated that free RB molecules in solution can readily collide with one another, leading to weak molecular separation and thereby shortening the lifetime. However, confining RB molecules through encapsulation or chemical conjugation can potentially enhance their dispersion and, thus increase the excited-state lifetime [99,195,221].

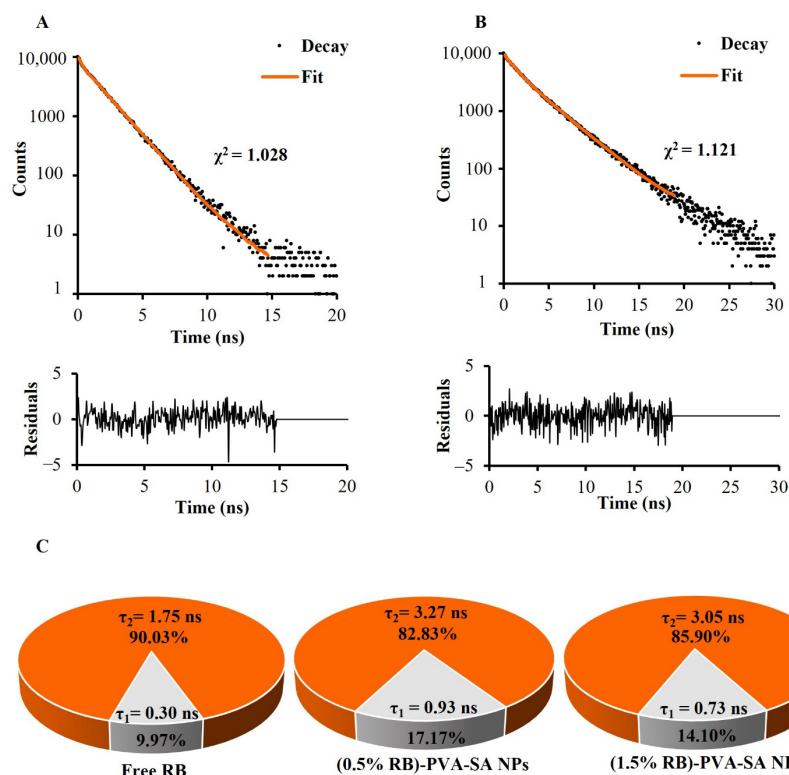


Figure 9. The typical fluorescence decay profiles with the fittings for (A) free RB and (B) (0.5% RB)-PVA-SA NPs. (C) The ratio of the determined two dominant lifetime components and the lifetime values for free RB as well as (0.5% RB)-PVA-SA NPs and (1.5% RB)-PVA-SA NPs [195].

5.1.5 One-pot preparation of RB-PVA-SA-MMC particles

Two amounts of MMC (4 mg and 12 mg) were used to synthesize the fluorescent drug-loaded PVASA NPs in a single-pot process. The 4 mg amount was selected because it lies within the dosing range commonly reported for clinically used MMC formulations and has also been employed in previous research, making it a relevant and representative loading level [88,222]. The 12 mg amount was included to assess whether the formulation process can accommodate a higher MMC content. **Figure 10** shows the results obtained using the lower MMC dose in the presence of the two RB contents (0.5 and 1.5 wt%).

As illustrated in **Figure 10A**, the MMC binding efficiency was markedly high, reaching nearly 96.9% when 0.5 wt% RB was used and approximately 92.2% when 1.5 wt% RB was applied. RB binding efficiency was also redetermined to find out whether the previously established values were influenced by the incorporation of MMC or not. As illustrated, values obtained were in very close agreement with those previously reported in **Figure 7A**, demonstrating that MMC has no effect on the incorporated amount of RB.

Additionally, the DLS results at pH 7.4 (**Figure 10B**) and at pH 5.8 (**Table A1**) showed that the DH values of MMC-loaded particles were greater than those of unloaded particles. For example, at pH 7.4, the mean DH values for ((0.5% RB)-PVA-SA)-MMC and ((1.5% RB)-PVA-SA)-MMC were approximately 118 ± 30 nm and 164 ± 29 nm, respectively, compared to 80 ± 13 nm and 136 ± 11 nm for the corresponding unloaded particles. These findings further support the successful incorporation of MMC.

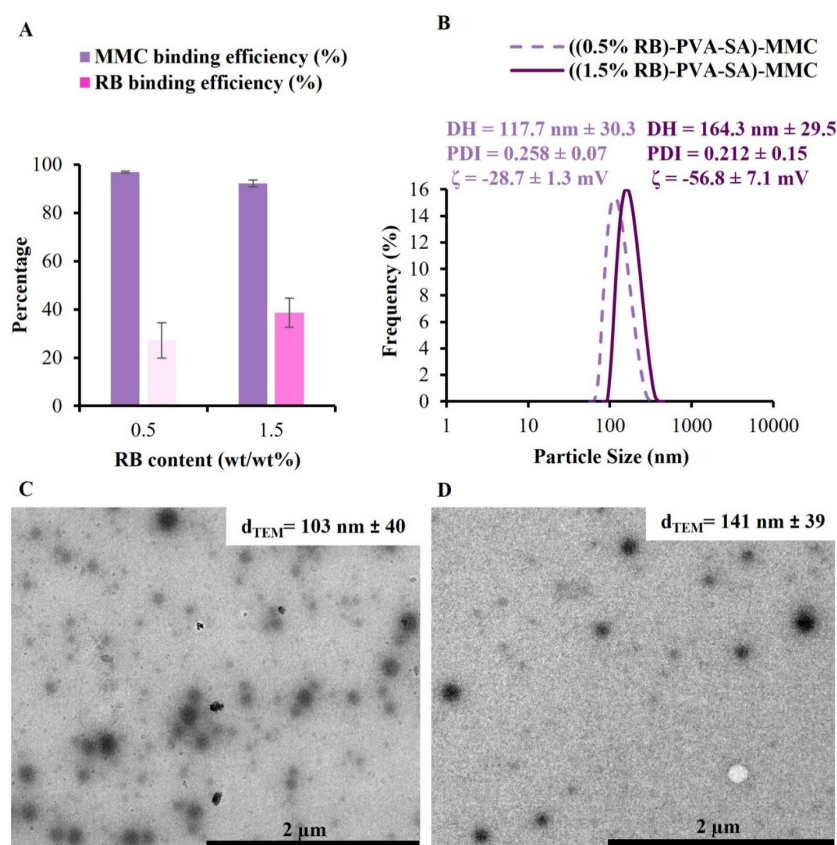


Figure 10. (A) The MMC and RB binding efficiency results as well as (B) the DLS data at pH 7.4 for the two samples obtained from applying the first MMC amount (4 mg) either with a 0.5% or 1.5% RB. (D) TEM images of the produced ((0.5% RB)-PVA-SA)-MMC NPs (C) and ((1.5% RB)-PVA-SA)-MMC NPs [195].

Despite the increase in DH, the MMC-loaded particles maintained ζ -potential values similar to those of the unloaded ones, suggesting that most of the incorporated MMC resides within the nanoparticle core. The drug-loaded NPs also exhibited a spherical morphology, as indicated by the TEM images (**Figures 10C and 10D**) and the NP sizes were determined as 103 ± 40 nm for ((0.5% RB)-PVA-SA)-MMC and 141 ± 39 nm for ((1.5% RB)-PVA-SA)-MMC.

With respect to the higher applied MMC amount (12 mg), the data in **Figure A10** show that the drug-binding efficiency consistently surpassed 94% for both RB contents. Taken together, these findings demonstrate that the fluorescent PVA-SA particles can incorporate the recommended therapeutic dose and also accommodate higher MMC levels.

5.1.6 In vitro drug release study

The release profiles of MMC, either in its free form or in its conjugated state, can be shown in **Figure 11**. The dissolution studies were carried out in PBS at 37 °C under two different pH settings: pH 7.4 to represent the typical physiological environment and pH 5.8 to represent the acidic tumor microenvironment of cancer cells [73,211,212] and the acidic conditions of lysosomal and endosomal compartments [75,195].

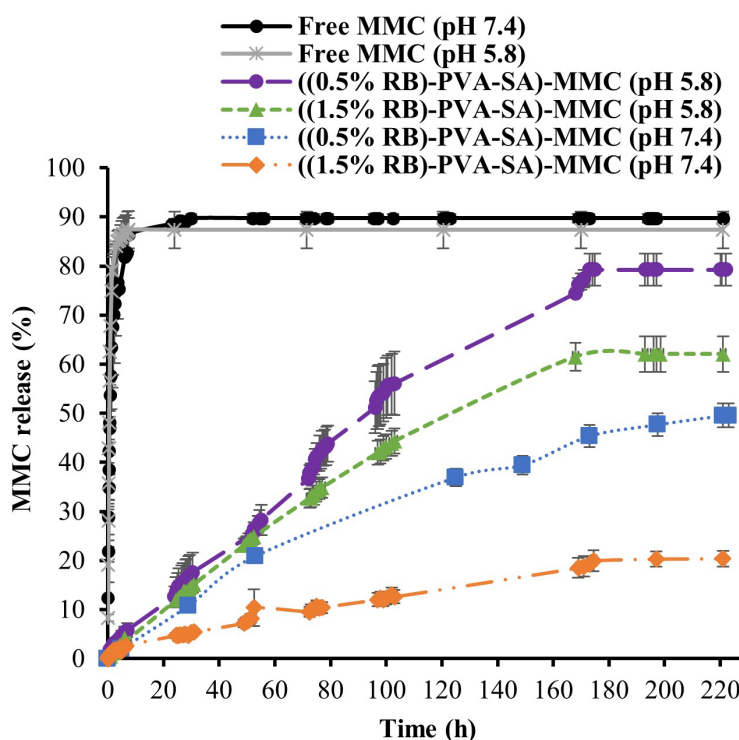


Figure 11. The in vitro release profiles of free MMC and MMC-loaded NPs (drug loading $\approx$ 10%, equivalent to 0.01 mg/mL MMC) at 37 °C in PBS buffer with pH = 5.8 and 7.4 [195].

For both pH conditions, the release data showed that conjugating MMC to the polymeric matrix markedly prolonged its release; whereas bare MMC reached $\sim$ 90% cumulative release within $\leq$ 30 h, the conjugated formulations required 7–9 days to reach their highest measured release ($\sim$ 20–79%). This extended-release behavior can be ascribed to the amide linkages connecting MMC to the polymer particles, as these bonds are known to hydrolyze very slowly [223,224]. From the release patterns, it can also be seen that all NP formulations exhibited a markedly higher drug release under acidic conditions compared to the neutral environment. For example, after 9 days, the ((0.5% RB)-PVA-SA)-MMC and ((1.5% RB)-PVA-SA)-MMC NPs released approximately 79% and 62% MMC at pH 5.8, compared to $\sim$ 50% and $\sim$ 20% at pH 7.4. The observed behaviour was anticipated because amide bonds are more prone to undergo hydrolysis under acidic pH conditions than at neutral ones [72,224].

Furthermore, the data demonstrated that the ((0.5% RB)-PVA-SA)-MMC particles released MMC at a faster rate and reached a higher cumulative release under both pH conditions compared with the ((1.5% RB)-PVA-SA)-MMC formulation. This difference can be explained by variations in particle size and ζ -potential. In the smaller composite NPs (((0.5% RB)-PVA-SA)-MMC), the MMC molecules would be closer to the nanoparticle surface, and this would facilitate quicker dissolution and outward diffusion. In contrast, NPs with larger sizes (((1.5% RB)-PVA-SA)-MMC) possess larger cores, which can contribute to much slower drug diffusion. Additionally, these larger particles exhibit more negative ζ -potential values than the smaller ones, indicating greater colloidal stability, which could further contribute to the slower MMC release [49,225].

The release profiles were further analyzed with several kinetic models to gain insight into the underlying release mechanisms. As shown in **Table A2**, the Korsmeyer–Peppas model (equation 5) offered the best fit to both the bare MMC and the encapsulated MMC release profiles. In this model, an exponent value (n) ≤ 0.43 indicates that the release is governed predominantly by Fickian diffusion, whereas values between 0.43 and 0.85 are characteristic of a non-Fickian diffusion mechanism [88,226]. Given that the n values of free MMC are ≤ 0.2 , one could say that the release follows a Fickian diffusion process. Conversely, all conjugated MMC formulations exhibited n values within the 0.43–0.85 range, suggesting that their release proceeds through a non-Fickian process involving both erosion-controlled and diffusion transport. This can imply that the MMC release process relies on both the hydrolysis of the amide bond as well as the diffusion of the drug.

In light of these findings, it can be concluded that the developed polymeric drug-delivery system has the potential to prolong MMC release. Moreover, the inclusion of the amide linkage has the potential to enable pH-responsive release, promoting drug release under the acidic conditions typical of tumor microenvironments.

5.1.7 Cytotoxicity of fluorescent polymeric NPs (RB-PVA-SA NPs)

Because biocompatibility is essential for materials intended for biological applications, the cytotoxicity of the synthesized fluorescent polymeric particles was evaluated. The (1.5% RB)-PVA-SA formulation was selected as a representative sample, and its effects on normal human lung fibroblast cells (CCD-19Lu) were assessed. **Figure A11** presents the cell viability (%) after 72 h of incubation with the nanoparticles at concentrations ranging from 1 to 250 $\mu\text{g/mL}$. No noticeable reduction in viability (%) was observed at the lower concentrations. A significant decrease in cell viability (%) became evident starting from 63 $\mu\text{g/mL}$; however, even at the highest tested dose (250 $\mu\text{g/mL}$), the cells maintained approximately 80% viability. According to the literature, samples that show above 70% cell viability are considered nontoxic [227,228].

These findings confirm that RB-PVA-SA NPs exhibit good biocompatibility, supporting their potential for safe and effective bioimaging applications.

5.1.8 Cytotoxicity of bare MMC and MMC-loaded NPs

The cytotoxic effects of bare MMC and MMC-loaded NPs (containing ~10% MMC) were tested using HEP-2 human epithelial cancer cells. The cells were incubated with a series of MMC concentrations (0.2–25 $\mu\text{g/mL}$) for 3 days under two distinct pH environments (pH 7.4 and pH 5.8). **Figures 12A** and **12B** present, respectively, the mortality percentages and the half-maximal inhibitory concentration (IC_{50}) values of MMC for the examined formulations.

As demonstrated, at pH 7.4, the MMC-loaded NPs produced substantially lower cell mortality than free MMC across all tested concentrations. For example, at a concentration of 25 $\mu\text{g/mL}$ MMC, the free drug caused 93.3% cell death, whereas the ((0.5% RB)-PVA-SA)-MMC NPs and ((1.5% RB)-PVA-SA)-MMC NPs resulted in 63.9% and 54.0% cell death, respectively. This was anticipated given the nature of the prodrug as well as the minimal cumulative release of MMC from the particles at pH 7.4. However, when the culture medium pH was set to 5.8, a condition that mimics the acidic tumor microenvironment, a dramatic increase in cell death was observed for both NPs across all MMC concentrations. For instance, at an MMC concentration of 25 $\mu\text{g/mL}$, the ((0.5% RB)-PVA-SA)-MMC NPs and ((1.5% RB)-PVA-SA)-MMC NPs produced 88.8% and 88.7% cell death, respectively, while the free drug caused 90.2% cell death. This pronounced pH-dependent enhancement in potency for the NP formulations is consistent with their pH-responsive MMC release behavior.

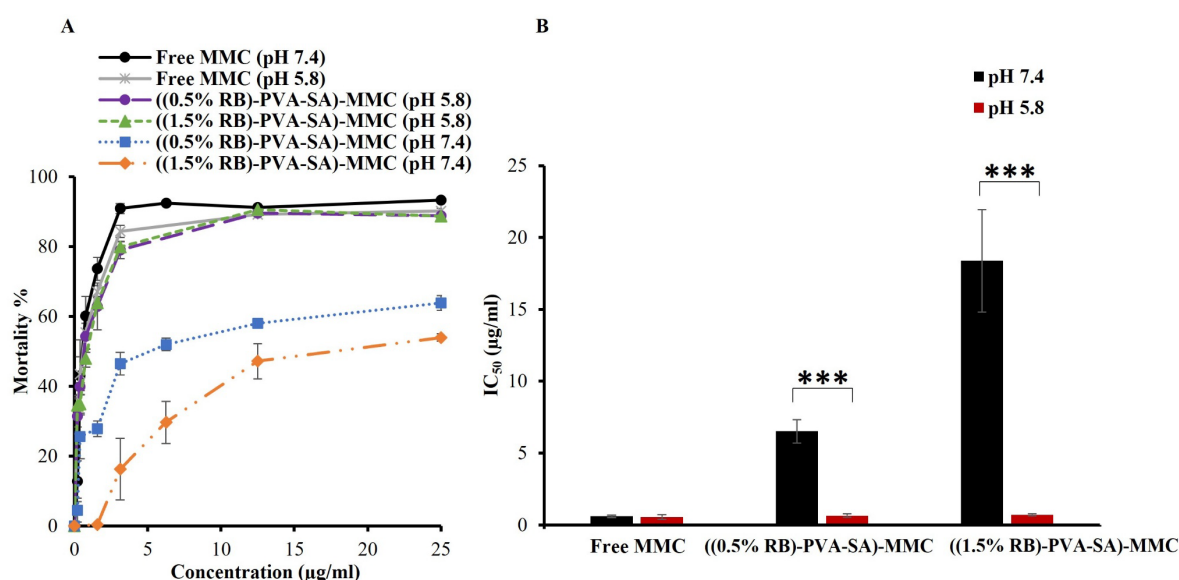


Figure 12. (A) The mortality of HEP-2 cells incubated for 72 h with free MMC, ((0.5% RB)-PVA-SA)-MMC NPs, and ((1.5% RB)-PVA-SA)-MMC NPs, at varying MMC concentrations in a culture medium with a pH of 7.4 or 5.8. (B) The determined IC_{50} values for all samples at both pH conditions. Statistical analysis was done using a 2-sample t-test. *** $p < 0.001$ [195].

Figure 12B further demonstrates that the IC_{50} values of MMC delivered via both NP formulations were markedly reduced at pH 5.8 compared with pH 7.4. At pH 7.4, the values were 6.5 ± 0.8 and 18.4 ± 3.6 $\mu\text{g/mL}$ for the ((0.5% RB)-PVA-SA)-MMC NPs and ((1.5% RB)-PVA-SA)-MMC NPs, respectively, whereas at pH 5.8 these values decreased to 0.6 ± 0.1 and 0.7 ± 0.1 $\mu\text{g/mL}$. In contrast, the IC_{50} of free MMC remained nearly unchanged (0.6 ± 0.1 $\mu\text{g/mL}$ at pH 7.4 vs. 0.5 ± 0.2 $\mu\text{g/mL}$ at pH 5.8). Based on these results, one could say that a markedly greater NP concentration is required to achieve a 50% mortality in cells with an extracellular pH of 7.4 versus those with an extracellular pH of 5.8, a trend that is not observed with bare MMC. These findings supported the NPs' pH-responsive behaviour, demonstrating that their cytotoxicity is higher under tumour-like acidic conditions compared to physiological pH.

5.1.9 *In vitro* cellular uptake of MMC-loaded NPs

Fluorescence microscopy was used for evaluating the uptake of the nanoparticles by HEP-2 carcinoma cells and for visualizing their intracellular localization. Cells were treated with ((0.5% RB)-PVA-SA)-MMC or ((1.5% RB)-PVA-SA)-MMC particles (100 $\mu\text{g/mL}$) for 12 h, followed by treatment with BioTracker 488 Green Nuclear Dye, which can selectively stain the nucleus. As demonstrated in **Figure 13**, a clear red fluorescence was observed in the red channel during the cell imaging experiments even at the lower nominal dye loading (0.5 wt%), demonstrating both the uptake of the NPs by the cells and their suitability for fluorescent cell imaging.

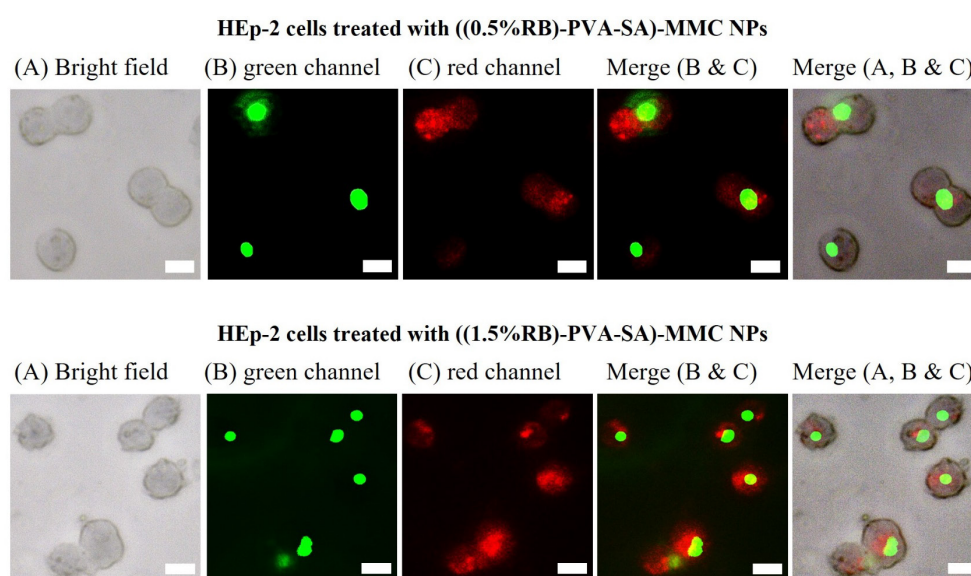


Figure 13. Fluorescence microscopy images of HEP-2 cancer cells after being treated with ((0.5% RB)-PVA-SA)-MMC NPs or ((1.5% RB)-PVA-SA)-MMC NPs for 12 h. The concentration of NPs is 100 $\mu\text{g/mL}$. The green fluorescence signals correspond to the BioTracker 488 Green Nuclear Dye. The red fluorescence signals correspond to the NPs accumulation. The scale bar represents 10 μm [195].

Furthermore, the merged images showed that RB-PVA-SA-MMC NPs were internalized within HEP-2 cancer cells and accumulated in the cytoplasm and perinuclear regions. The high accumulation of particles in the cytoplasmic areas, rather than in the nucleus, was expected, as nuclear membrane pores typically permit the entry of very small NPs, generally below about 9 nm [229]. However, the collective findings demonstrate that both nanoparticle formulations are highly promising for cellular imaging applications.

It is important to mention that the biological tests described in Sections 5.1.7 to 5.1.9 were a collaborative effort with the Department of Oto-Rhino-Laryngology and Head & Neck Surgery and the Department of Medical Microbiology. While the experimental execution and final IC₅₀ calculations were performed by the collaborating departments, all other aspects of the study—including project conceptualization, experimental design, participation in cell viability calculations, and final data interpretation—were undertaken jointly by both parties.

In summary, we have successfully developed multifunctional, pH-responsive PVA-based nanoparticles capable of encapsulating MMC, prolonging its release, and enabling preferential drug release under tumor acidic microenvironment while also providing fluorescence-based imaging. The synthesized PVA-SA polymer, with its carboxyl (2.5 mmol/g) and hydroxyl (13.3 mmol/g) moieties, enabled efficient RB incorporation (EE= 27–40% across the applied RB feed range) and high MMC encapsulation (EE= 92–97% across the prepared RB/MMC formulations) through spontaneous self-assembly. The selected RB-loaded NPs enhanced the photophysical performance of RB by increasing its quantum yield from 29.8 % to ≥ 42.7 %, maintaining emission stability under continuous excitation for 2 h, and increasing its fluorescence lifetime through dye–polymer interactions. More importantly, the MMC-loaded fluorescent NPs (DH: ~ 118 – 194 nm; ζ -potential: ~ -24 to -58 mV) showed prolonged, pH-responsive release with consistently higher MMC release at pH 5.8 than at pH 7.4. The drug-loaded NPs also exhibited potent toxicity under tumor-like acidic conditions (IC₅₀ = 0.6–0.7 $\mu\text{g/mL}$ at pH 5.8) but markedly reduced activity at physiological pH (IC₅₀ = 6.5–18.4 $\mu\text{g/mL}$ at pH 7.4), unlike free MMC. Overall, these drug-loaded fluorescent NPs, with their ability to self-assemble, their favourable nanoscale size for prolonged circulation, their sustained pH-responsive drug-release behaviour, their potent cytotoxicity under tumour-like acidic conditions compared to physiological conditions, and their clear fluorescence emission with efficient cellular uptake, offer a versatile platform for controlled MMC release with integrated bioimaging capability, which could be helpful in supporting safer and more efficient cancer-related therapy.

5.2 Development of magnetically responsive polymeric NPs for spatially targeted delivery of MMC

While the first chapter focused on enhancing tumour selectivity primarily through pH-responsive drug release, this chapter aimed to improve spatial precision by developing a magnetically responsive, MMC-loaded polymeric prodrug designed to support field-guided accumulation of drug-loaded carriers at tumour sites. To achieve this, MMC and iron oxide magnetic nanoparticles (MNPs) were conjugated to the previously developed PVA-SA matrix, which promotes spontaneous nanoparticle formation (**Figure 14**). MNPs-PVA-SA NPs were first prepared at varying MNP loading to determine the maximum magnetic content that could be incorporated while maintaining suitably small particle size and colloidal stability. This was followed by the one-pot synthesis of the magnetic MMC-loaded polymeric nanoparticles (MNPs-PVA-SA-MMC). The relevant physicochemical parameters of the resulting NPs were characterized to assess their applicability, and their pH-responsive behaviour was evaluated through *in vitro* release studies at pH 7.4 and 5.8. Finally, the magnetic responsiveness of the NPs was examined to determine their suitability for field-induced localisation in spatially controlled cancer therapy.

5.2.1 Structural characterization of the synthesized samples

The presence of both hydroxyl (13.3 mmol/g) and carboxyl groups (2.5 mmol/g) along the PVA-SA backbone provided the functional handles required for constructing the magnetic MMC-loaded system (**Figure 14**).

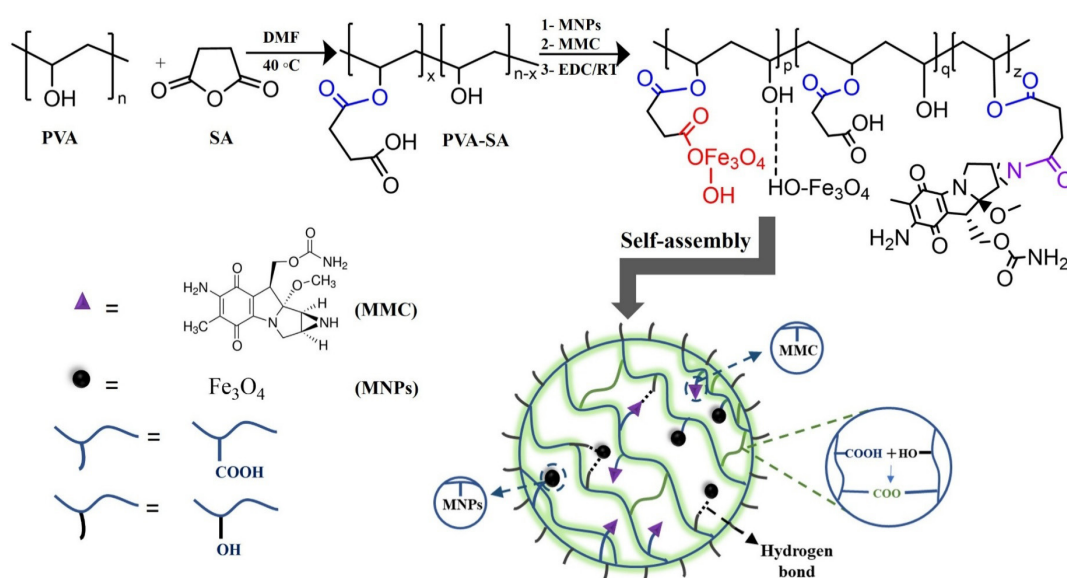


Figure 14. The scheme of synthesis of PVA-SA and the conjugation reactions of MNPs (through metal-carboxylate bonds highlighted in red and hydrogen bonds presented in dotted lines) and MMC (through amide bonds highlighted in purple) with PVA-SA, as well as the formation of self-assembled NPs by crosslinking of the carboxyl groups and the hydroxyl groups [193].

The hydroxyl groups enabled hydrogen-bonding interactions with the MNP surface, while the introduced COOH functionalities, under EDC activation, facilitated metal–carboxylate interactions with the iron oxide MNPs alongside amide bond formation with MMC [88,230,231]. Moreover, as previously described for the PVA-SA system, the carboxyl groups enabled coupling with pendant hydroxyl moieties along the PVA-SA chain, forming ester linkages that promote self-assembled particle formation (**Figure 14**).

FTIR analysis was performed to confirm the interactions between the PVA-SA polymer, the MNPs, and MMC in the magnetic formulation (**Figure 15**). Since the characteristic bands of native PVA and the spectral features confirming PVA succinylation were already detailed in the previous chapter, the present section focuses on the additional structural changes associated with MNP coordination and drug conjugation within the magnetic system.

Compared to PVA-SA, the NPs exhibited a strong reduction in the 1660 cm^{-1} band associated with the carboxylate moiety of succinate and an increase in the 1160 cm^{-1} band corresponding to the C–O stretching vibrations of the ester groups [3,198], verifying ester-related cross-linking and supporting the self-assembled particle formation through reactions between the accessible OH and COOH moieties along the polymeric backbone [194].

One can also see that the spectrum of the MNPs-PVA-SA particles exhibited a shift in the OH vibrational band from 3392 to 3288 cm^{-1} , confirming the formation of hydrogen-bonding interactions between PVA–SA and MNPs [199]. In addition, the carboxylate band at 1660 cm^{-1} showed a noticeable decrease in intensity compared to PVA-SA NPs, supporting the presence of metal–carboxylate interactions between the polymer matrix and the MNPs. Furthermore, the shift in the stretching frequency of the carbonyl group toward a lower wavenumber (from 1722 to 1705 cm^{-1}), together with the shift of the carboxylate band from 1660 to 1650 cm^{-1} , was consistent with metal–carboxylate interaction, in which electron density is withdrawn from the carboxylate oxygen toward the metal center [232]. Moreover, compared to the IR profile of the free MNPs (537 , 1547 , 1640 , and 3290 cm^{-1}), the MNPs–PVA–SA sample exhibits absorption bands at 530 and 1550 cm^{-1} , which can be ascribed to Fe–O stretching modes and vibrations associated with adsorbed water on the Fe_3O_4 surface [233].

The binding between MMC and PVA-SA was further confirmed by observing features similar to those observed in the first system, specifically the emergence of the C–N stretching band at 1360 cm^{-1} and the C=O amide band at 1720 cm^{-1} . Overall, these spectral changes collectively confirm the successful incorporation of both MNPs and MMC within the PVA–SA matrix.

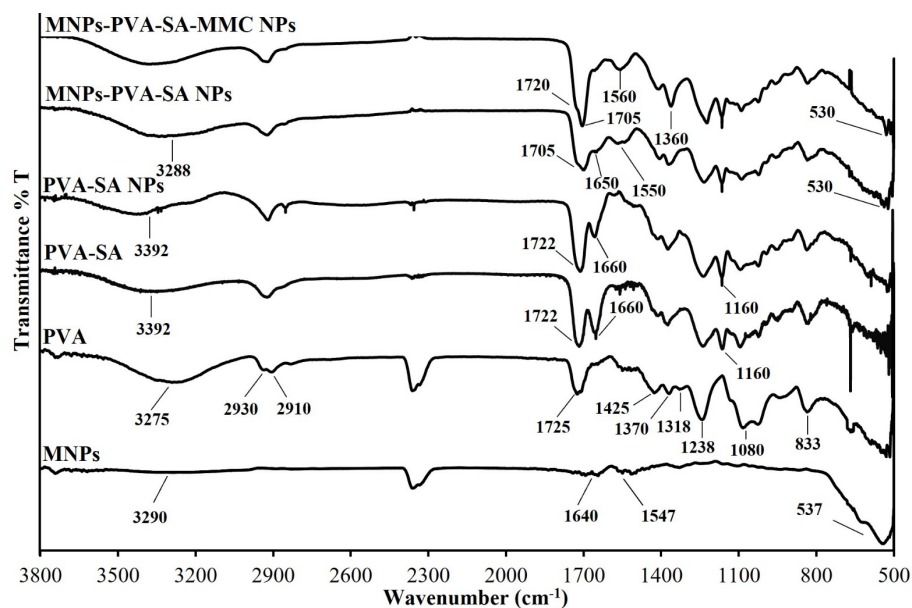


Figure 15. FTIR spectra of MNPs, PVA, PVA-SA, PVA-SA NPs, MNPs-PVA-SA NPs and MNPs-PVA-SA-MMC.

XRD analysis was also carried out to gain deeper insight into the magnetic phase type. As illustrated in **Figure A12**, the diffraction pattern of the free MNPs displayed peaks at $2\theta = 30.16^\circ$, 35.34° , 43.18° , 57.04° , and 62.94° , corresponding respectively to the (220), (311), (400), (511), and (440) lattice planes, which are consistent with the standard magnetite phase pattern [223,234]. In the presence of the polymer and the drug, the reduced peak intensities and the broadening or disappearance of some MNP peaks were expected and aligned with previous studies that used amorphous or semi-amorphous polymers to coat MNPs [234,235]. The XRD results were also in line with earlier findings showing that PVA coating can reduce the crystallinity of Fe_3O_4 nanoparticles [234,236].

5.2.2 Self-assembly of polymeric chains and formation of MNPs-PVA-SA NPs

To investigate the self-assembly behavior of PVA-SA with MNPs present, two aqueous PVA-SA solutions (1.3 wt/v%) were formulated, one of which contained 10 wt% MNPs. Following EDC introduction, turbidity was tracked over time (**Figure 16A**). As demonstrated, both formulations exhibited an immediate rise in turbidity during the first minute (~ 15 NTU for PVA-SA and ~ 10 NTU for the MNP-containing sample), reflecting the activation of the COOH groups by EDC [88]. Turbidity then continued to increase as crosslinking between the OH groups and the COOH groups of PVA-SA promoted the formation of self-assembled structures, eventually reaching a steady plateau after about 10 minutes. The final turbidity values (~ 95 NTU for PVA-SA and ~ 85 NTU for the MNP-containing sample) indicate that the presence of MNPs does not hinder the ability of PVA-SA to produce self-assembled particles.

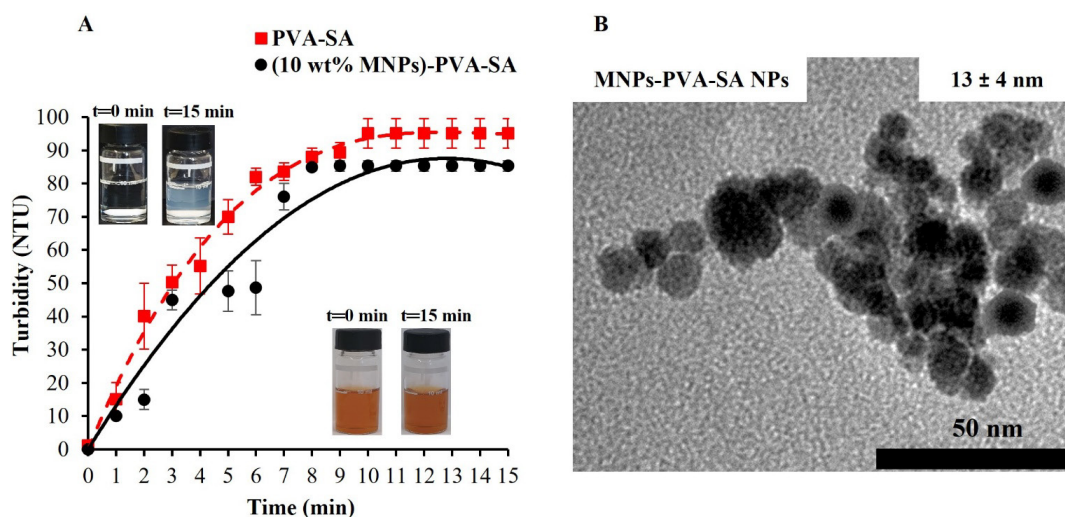


Figure 16. (A) The change in the turbidity of PVA-SA and MNPs-PVA-SA in the presence of EDC as a function of time. (B) TEM image of the produced MNPs-PVA-SA particles [193].

To further confirm particle formation, TEM analysis was performed after purification. As shown in **Figure 16B**, the MNPs–PVA–SA formulation produced spherical NPs with an average diameter of 13 ± 4 nm. These findings demonstrate that PVA–SA successfully forms self-assembled NPs even when MNPs are incorporated into the system.

5.2.3 Maximization of MNP content in the composite NPs

To determine the maximum MNP content that could be incorporated into the particles while maintaining suitably small particle size and colloidal stability, various MNP weight ratios (5–20 wt%) were applied during MNPs-PVA-SA NPs synthesis. Quantification of residual MNPs in the supernatant showed that more than 94% of the starting amount had been incorporated. However, it was still necessary to investigate the effect of increasing the MNP content on the size and ζ -potential of the resulting particles, and to assess their colloidal stability.

Figure 17A presents the mean hydrodynamic diameters of the particles dispersed in PBS (pH 7.4, 0.9% NaCl). As can be seen, increasing the MNP content in the composite from 5 wt% to 20 wt% led to a noticeable rise in particle size, from about 84 ± 6 nm to 149 ± 4 nm. These findings are consistent with the results reported by Zhou et al., who observed that higher MNP dosage produced larger drug-loaded magnetic PLGA/PLA microparticles [237]. The DLS measurements also revealed PDI values ranging from 0.055 to 0.146. Based on **Figure 17B**, one can say that all formulations exhibited relatively narrow size-distribution profiles, with the exception of the sample containing 20 wt% MNPs, which showed a broader size distribution.

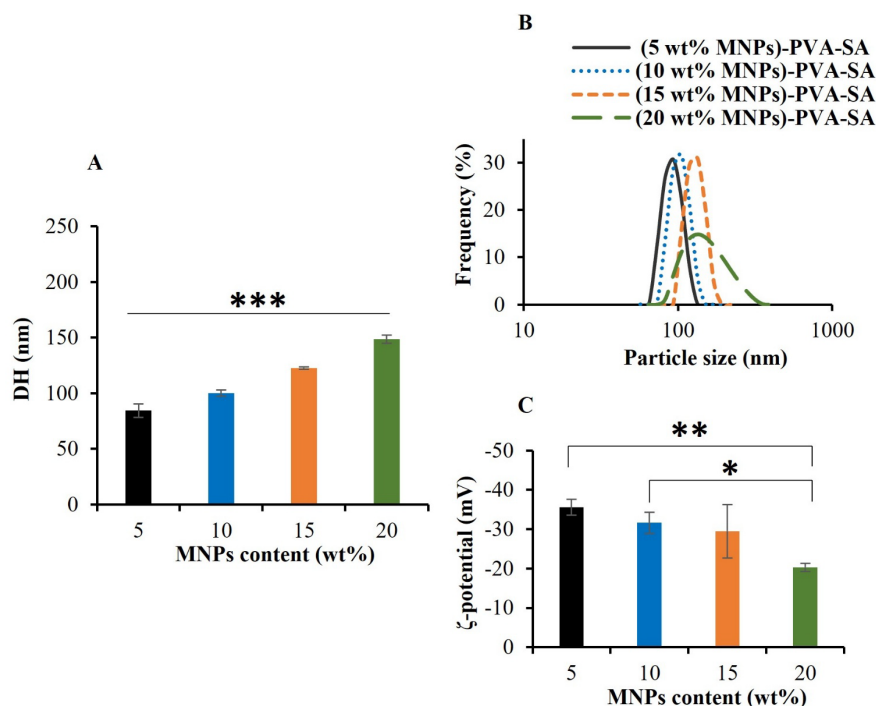


Figure 17. (A) The average sizes, (B) the corresponding size distribution curves, and (C) the ζ -potential values of the composite NPs as a function of increasing MNP content. Particles were dispersed in PBS (0.9% NaCl, pH 7.4). Statistical analysis of the difference in both DH and ζ -potential: one-way ANOVA with Tukey's HSD post-hoc analysis. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ [193].

Regarding the ζ -potential measurements, as shown in **Figure 17C**, increasing the MNP content shifted the surface charge from about -35.6 ± 2.0 mV to -20.3 ± 1.1 mV. This ζ -potential reduction may be ascribed to the decreased amount of ionized hydroxyl moieties on the PVA-SA nanoparticle surface, as more magnetite particles can bind to them. These observations align with previous work [238], which indicated that at lower iron oxide nanoparticle molar ratios, the negatively charged hydroxyl moieties on the PVA surface played a key role in reducing MNP agglomeration. Nevertheless, even at 15 wt% MNPs content, the dispersion maintained a relatively high ζ -potential value (-29.5 ± 6.7 mV), indicating that sufficient electrostatic repulsion was still present to preserve colloidal stability.

Since nanoparticles with adequate colloidal stability are crucial for drug-delivery applications, the stability of the resulting dispersions was examined in a more quantitative manner through UV–Vis spectroscopic measurements. As illustrated in Figures 18A and 18B, the variation in the normalized absorbance at 312 nm was monitored over time. Examination of the first six hours of measurements (**Figure 18A**) clearly indicates that the PVA-SA-coated MNP dispersions exhibit markedly higher stability compared to the bare MNPs dispersion. This enhanced stability reflects the ability of the PVA–SA polymer to suppress the aggregation and the sedimentation of the coated MNPs. These results indicate that PVA–SA can enhance the

stability of the MNP dispersion as unmodified PVA has already been reported to do [239]. The stability of the dispersions was further evaluated over a 28-day period (**Figure 18B**). As presented, samples containing up to 10 wt% MNPs demonstrated more than 90% retention of their initial absorbance, indicating good colloidal stability. In the meantime, the samples containing 15 and 20 wt% MNPs showed a decrease in normalized absorbance to approximately 85% and 75%, respectively. The pronounced reduction observed in these samples, compared with the formulations containing up to 10 wt% MNPs, can be ascribed to the higher MNP amount relative to the polymer content. The size stability of the NPs was also examined throughout the 28-day period (**Figures 18C and 18D**). While all samples except the 5 wt% MNP-containing sample exhibited significant variation in particle size over time, the level of significance was substantially higher in the 15 and 20 wt% MNP-containing samples, which both also showed increases in PDI, indicating a loss of uniformity at elevated MNP contents during storage.

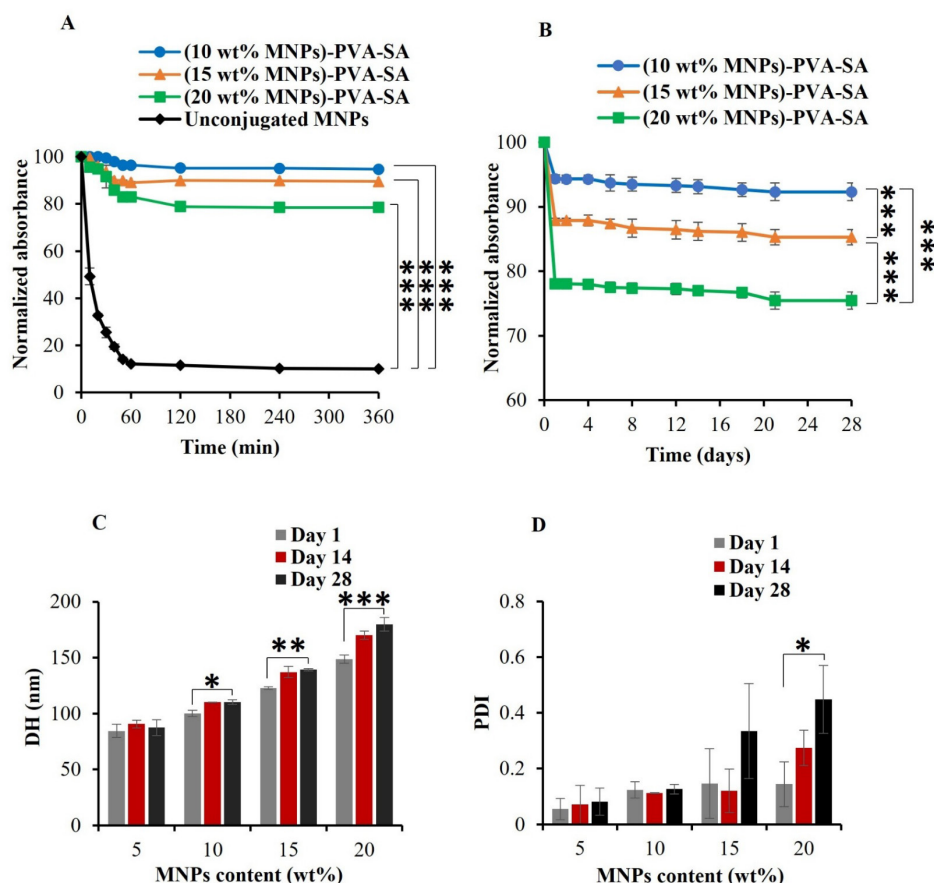


Figure 18. (A & B) The change in the normalized absorbance at 312 nm for MNPs and MNPs-PVA-SA suspensions as a function of time. (C) The change in particle size and (D) the PDI as a function of time. Particles were dispersed in PBS (0.9% NaCl, pH 7.4). Statistical analysis of the difference in colloidal stability results after 360 min was performed using one-way ANOVA with Dunnett's post-hoc test (control group: MNPs). However, results after 28 days were analyzed using one-way ANOVA with Tukey's HSD post-hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ [193].

Taken together, these findings indicated that 10 wt% is the maximum amount of MNPs that can be used to synthesize magnetic PVA–SA NPs while still maintaining suitably small particle size (~100 nm) and acceptable colloidal stability (>90%) over prolonged time. Accordingly, we used approximately 14 mg MNPs, which is equivalent to about 10 wt% relative to the total polymer and MNPs content, for the synthesis of the drug-loaded NPs.

5.2.4 One-pot preparation of MNPs-PVA-SA-MMC particles

As discussed in the methods section, 4 mg MMC was employed during the single-pot synthesis of the magnetic MMC-loaded polymeric NPs. One can see in **Table A3** that MMC exhibited a high binding efficiency of approximately 97.9%. TEM measurements revealed that the particle size increased from about 13 ± 4 nm to 23 ± 8 nm when MMC was included during nanoparticle formation. Additionally, the DLS measurements for the particles dispersed in PBS at pH 7.4 or pH 5.8 (**Table A3**) revealed that the hydrodynamic diameter values of the MMC-loaded particles were significantly greater than those of unloaded counterparts. These findings support the successful incorporation of MMC. The PDI values of the particles (0.284 ± 0.03 at pH 7.4 and 0.307 ± 0.04 at pH 5.8) also reflected a homogeneous particle population. Compared with the drug-free NPs, the drug-loaded particles showed a clear reduction in ζ -potential values, resulting in a less anionic surface charge. For example, at pH 7.4, the ζ -potential decreased from -31.6 ± 2.7 mV to -18.5 ± 1.0 mV. This indicates that MMC may present not only in the core but also at the particle surface [240], where it can interact with surface functional groups, reducing ionization and contributing to the observed decrease in ζ -potential. Despite this observation, the MMC-loaded NPs exhibited high colloidal stability. As shown in **Figure A13**, the average values of hydrodynamic diameter, PDI, and ζ -potential determined at pH 7.4 or pH 5.8 were comparable over the storage period, and no significant differences were detected.

5.2.5 In vitro release of MMC from polymeric nanocomposite particles

The release profiles of MMC from the MNPs-PVA-SA-MMC formulation can be shown in **Figure 19**. As in the previously developed formulations, the experiments were carried out in PBS at 37 °C under pH 7.4 and pH 5.8. The results demonstrate that the magnetic polymeric NPs are capable of sustaining and modulating MMC release in a pH-dependent manner. As illustrated, free MMC reached ~90% cumulative release within ≤ 30 h, whereas the MMC-loaded NPs released the drug more gradually, reaching about 50% at pH 7.4 and 80% at pH 5.8 after nearly 7 days. This sustained and pH-dependent release behaviour is consistent with that observed for the fluorescent MMC-loaded NPs and can be ascribed to the amide linkages anchoring MMC to the polymer chains—bonds that hydrolyze slowly and exhibit greater susceptibility to cleavage under acidic pH conditions than at neutral ones [72,224]. Moreover, similarly to the fluorescent MMC-loaded NPs, the release profiles of MMC from the magnetic

polymeric NPs were also best fitted by the Korsmeyer–Peppas model (**Table A4**), where the release-exponent (n) values fell within the 0.43–0.85 range, suggesting that a non-Fickian diffusion mechanism involving both erosion and diffusion-controlled transport [88,226]. Overall, the findings indicate that the presence of MNPs does not compromise the system's performance; the magnetic polymeric NPs retain the ability to sustain MMC release and to provide pH-responsive drug release, with higher release occurring under the acidic conditions typical of tumor microenvironments.

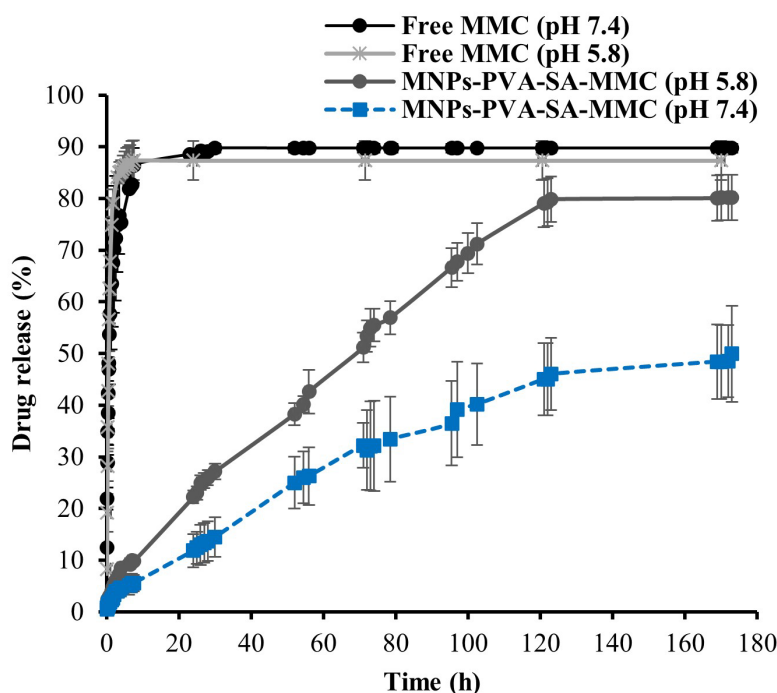


Figure 19. The *in vitro* release profiles of free MMC and MNPs-PVA-SA-MMC (equivalent to 0.01 mg/mL MMC) at 37 °C in PBS buffer (0.9% NaCl, pH 7.4 or 5.8) [193].

5.2.6 Magnetic properties of the prepared magnetic samples

The magnetic properties of the NPs were characterized using a vibrating sample magnetometer (VSM). **Figure 20** shows the hysteresis curves of the samples. For all formulations, the magnetization (M) increased with increasing applied magnetic field (H) and eventually reached a maximum value, known as the saturation magnetization (M_s). The uncoated MNPs exhibited an M_s of 69.940 emu/g, which is comparable to the reported values for neat Fe_3O_4 NPs prepared by the co-precipitation method [241,242]. Compared to MNPs, the MNPs-PVA-SA and MNPs-PVA-SA-MMC samples showed much lower M_s values of 8.649 emu/g and 8.541 emu/g, respectively. This reduction was expected and is consistent with numerous studies showing that incorporation of magnetite NPs into a non-magnetic polymer matrix can significantly reduce the measured M_s [236,243,244]. The decrease can be attributed to the dilution of the magnetic material by the non-magnetic PVA–SA matrix, as well as the possibility

of surface spin disorder at the MNPs–polymer interface, which may hinder the full alignment of magnetic moments. Despite this reduction, the M_s values of MNPs–PVA–SA and MNPs–PVA–SA–MMC remain higher than several previously reported values for magnetite-based systems, including 0.52 emu/g for PVA-coated magnetite and 0.78 emu/g for uncoated magnetite NPs reported by Wang et al. at an applied field of 20 kOe [245], as well as the 8.1 emu/g reported by Badawy et al. for a magnetite/PVA nanocomposite [243]. Moreover, according to previous reports, M_s values in the range of ~ 7 –19 emu/g are considered adequate for potential applications in magnetic targeting and magnetic hyperthermia [246–248]. Therefore, based on the VSM results, the synthesized NPs fall within this suitable range.

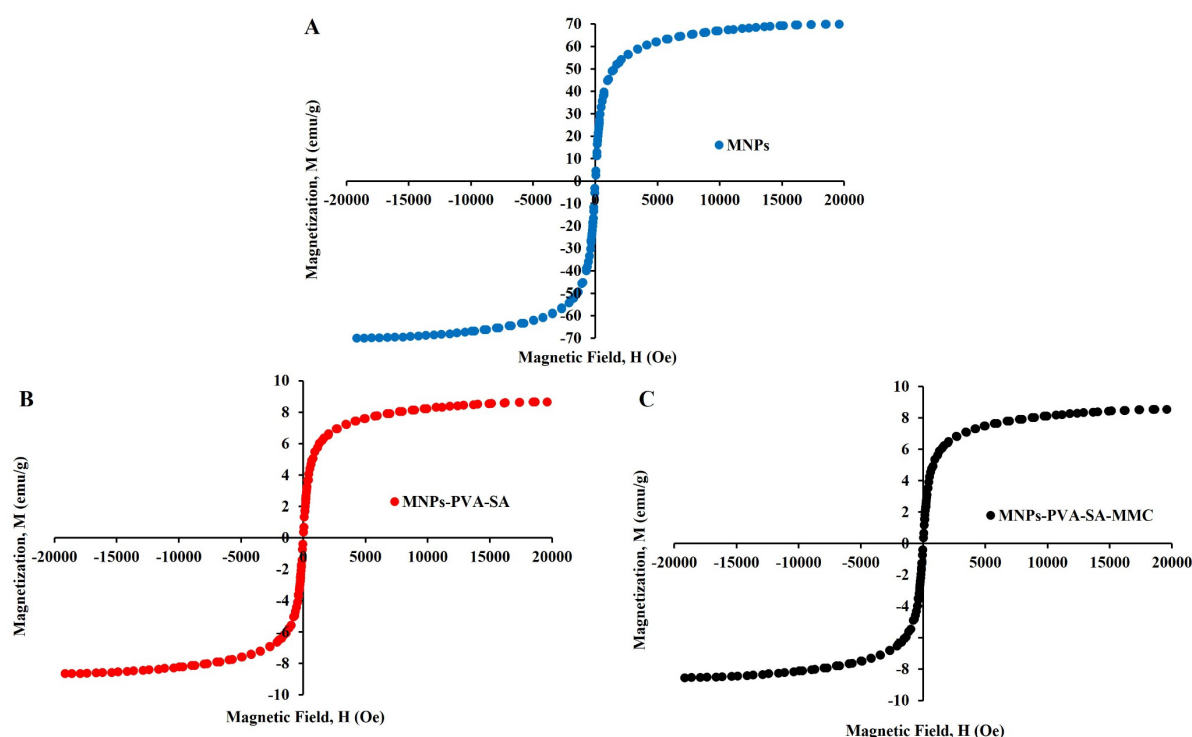


Figure 20. Field-dependent magnetization (M versus H) curves of (A) MNPs, (B) MNPs-PVA-SA and (C) MNPs-PVA-SA-MMC. Samples contain 10 wt% MNPs and 2.6% MMC.

In addition to M_s values, all samples exhibited very low coercivity (H_c , the reverse field required to reduce the magnetization to zero) and very low remanent magnetization (M_r , the magnetization retained after the external field was removed). The coercivity values were 8.251 Oe for MNPs, 8.023 Oe for MNPs-PVA-SA, and 7.930 Oe for MNPs-PVA-SA-MMC, while the corresponding remanent magnetization values were 0.8027, 0.1086, and 0.1060 emu/g, respectively. These low H_c and M_r values, together with the low M_r/M_s ratios of 0.011, 0.013, and 0.012, are characteristic of superparamagnetic NPs [249]. This superparamagnetic behaviour is particularly advantageous for drug-delivery applications, as it enables the NPs to be guided and controlled by an external magnetic field while not retaining

magnetization before or after field exposure, thereby minimizing the risk of particle aggregation due to magnetic dipole interactions [236]. Overall, these results demonstrate that the drug-loaded composite NPs can be easily manipulated using an external magnet, supporting their potential use in magnetic drug-targeting applications, particularly in regions close to the skin surface (e.g., head and neck).

The magnetic responsiveness of the particles was also investigated utilizing an external magnet that provides a 0.35 T flux density. As presented in **Figure 21**, the application of the magnet caused the suspended particles to move rapidly toward the magnet, accumulating on the wall.

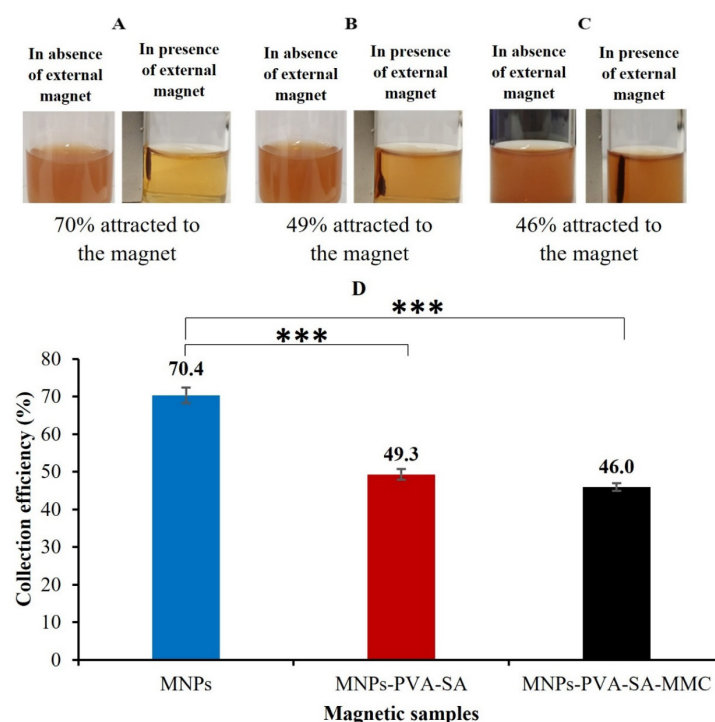


Figure 21. The effect of a 0.35 T magnetic field on suspensions of (A) MNPs, (B) MNPs-PVA-SA, and (C) MNPs-PVA-SA-MMC, and (D) a column chart showing the collection efficiency of the magnetic samples after suspension in PBS (pH 7.4, 0.9% NaCl) and exposure to the magnetic field for approximately 60 s. Samples contain 10 wt% MNPs and 2.6% MMC [193]. Statistical analysis of differences in magnetic collection efficiency was performed using one-way ANOVA followed by Tukey’s HSD post-hoc analysis. *** $p < 0.001$.

To compare the magnetic responsiveness of the different samples, the magnetic collection efficiency after approximately 60 s of magnetic exposure was determined as described in the Methods section. In all cases, the samples showed clear magnetic responsiveness, as reflected by the amount of material collected after magnetic exposure. Nevertheless, the collection efficiency of the bare MNPs was found to be markedly higher (approximately 70%), whereas the efficiencies for MNPs-PVA-SA and MNPs-PVA-SA-MMC were 49 and 46%, respectively. This reduction can be ascribed to the polymer coating masking the magnetic core, which reduces how directly the applied field can act on the MNPs, and also keeps the MNPs

sufficiently apart from each other, minimizing the formation of larger magnetic aggregates that would otherwise enhance magnetic collection. The magnetic behaviour was further examined after 28 days of storage. All dispersions exhibited a good response to the magnetic field, and nearly identical magnetic collection efficiencies were observed [193].

In summary, this section focused on the development of magnetically responsive, MMC-loaded polymeric NPs to support field-guided accumulation of drug-loaded carriers at tumour sites, thereby potentially reducing off-target exposure. As a summary, we can state that the PVA–SA matrix enabled efficient loading of MNPs (5–20 wt%), with 10 wt% identified as the maximum MNP content that can maintain high colloidal stability (>90%) and preserve a uniform particle-size distribution over time. Using this MNP composition, MMC-loaded polymeric magnetic NPs (hydrodynamic size ~128 nm at pH 7.4 and ~134 nm at pH 5.8) with very high drug-binding efficiency (~98%) could be produced, exhibiting stable physicochemical properties, sustained and pH-responsive MMC release, and clear magnetic responsiveness (M_s ~ 9 emu/g). Overall, these findings highlight the potential of the developed composite NPs as a magnetically guidable platform for spatially controlled MMC delivery in cancer therapy.

5.3 Hydrophobically modified Chitosan-Pluronic NPs for pH-responsive and sustained release of NIMO and HC

As previously outlined, NIMO and HC are neuroprotective compounds commonly applied in studies addressing ischemia-related complications, but their effectiveness is limited by poor aqueous solubility and nonspecific release. This chapter therefore focused on developing a polymeric drug-delivery system capable of improving the aqueous dispersibility of these compounds while enabling pH-responsive and sustained drug release within ischemic brain regions characterized by localized acidosis (pH 6.5–6.0). To achieve this, Chit was hydrophobically modified via reductive amination with aldehydes of different alkyl chain lengths to tune polymer hydration and thereby modulate its pH-dependent colloidal stability. The derivative capable of forming stable particles at physiological pH while disintegrating under ischemic pH (C₆-chit) was then used to fabricate NPs via the desolvation method, followed by optimisation of formulation parameters and incorporation of varying amounts of PF copolymer to achieve prolonged drug release. Finally, NIMO- and HC-loaded NPs were prepared through a one-pot synthesis approach, and the effects of encapsulation on drug crystallinity, aqueous dispersibility, and colloidal stability were evaluated together with in vitro drug-release behaviour under simulated physiological and ischemic pH conditions.

5.3.1 Structural characterization of the hydrophobically modified Chit samples

To obtain the required pH-responsive characteristics as well as the desired cut-off pH, the native Chit (deacetylation degree 79.4%, calculated using **equation 13** after evaluating the potentiometric titration curve shown in **Figure A14**) was subjected to a reductive amination process [108,250]. The overall reaction (**Figure 22**) involved two consecutive steps. In the first step, Chit was reacted with aldehydes of different alkyl chain lengths, forming intermediate chitosan-imine structures. Then, in the second step, the imine functionalities were reduced, yielding the corresponding N-alkylated Chit derivatives (C₆-chit and C₁₂-chit).

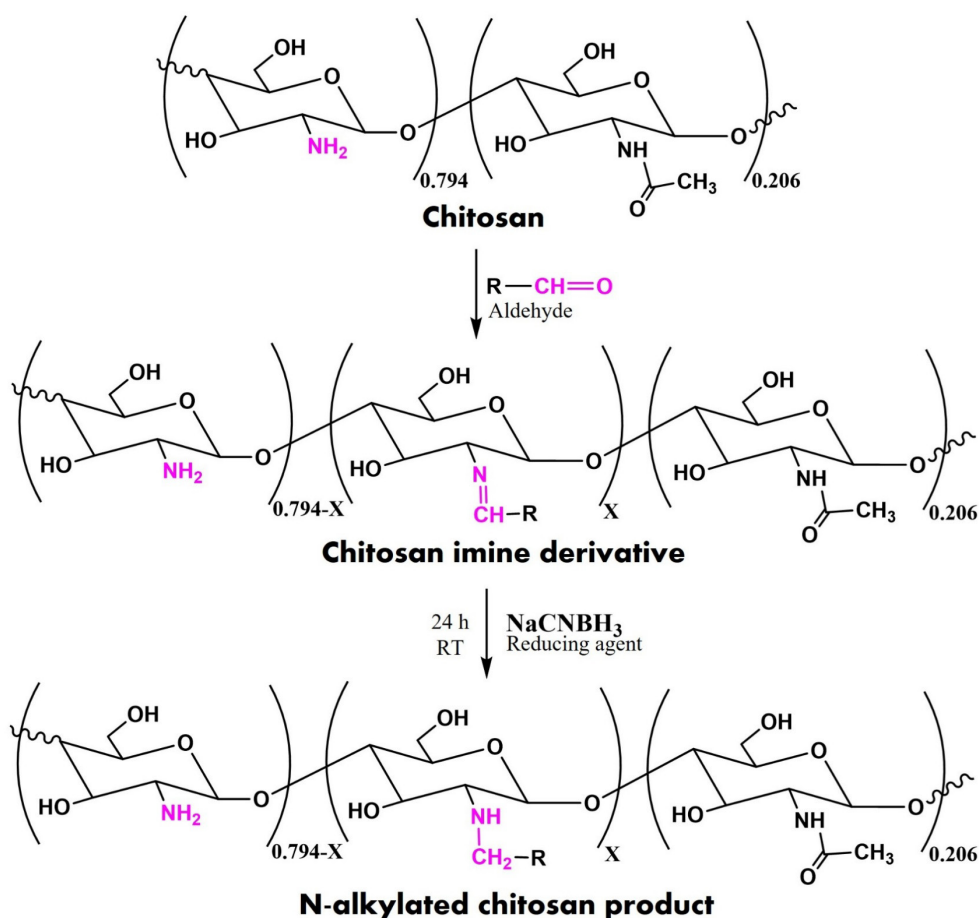


Figure 22. Reductive amination of hydrophilic Chit using hexyl aldehyde (R= CH₃(CH₂)₄) and dodecyl aldehyde (R= CH₃(CH₂)₁₀) [251].

FTIR spectral analysis of both the native and the modified (hydrophobized) Chit samples (**Figure 23**) confirmed the success of the modification. Compared with the unmodified polymer, the C₆-chit derivative showed more intensified CH₂ stretching absorption bands at 2920 and 2855 cm⁻¹. This increase in band intensity indicated the presence of additional CH₂ groups and thus the existence of alkyl chains within the hydrophobically modified chitosan product [252,253]. In C₁₂-chit, these CH₂ vibrations were even more intense, indicating its

higher methylene group content [108,253]. Moreover, the non-appearance of the aldehydic C–H stretching absorptions in the 2830–2705 cm^{-1} region and the aldehydic C=O stretching band in the 1740–1720 cm^{-1} region [252], indicated that both products contained no free aldehydes. Furthermore, the band corresponding to the N–H deformation vibrations, which was observed for the native chitosan at 1590 cm^{-1} , was detected in the C₆-chit spectrum at 1555 cm^{-1} and in the C₁₂-chit spectrum at 1557 cm^{-1} . This downward shift confirmed the establishment of secondary amine groups, whose N–H deformation vibrations typically occur within the 1650–1550 cm^{-1} region [252]. In addition to the above observations, which demonstrate the reaction's success, the spectra of the synthesized products showed a new absorption feature at about 1400 cm^{-1} . This signal, which is characteristic of C–N stretching vibrations in aliphatic amines [252], signifies that C–N bonds were successfully established between the polymer and the aldehyde moieties. Typical Chit bands were also observed in all spectra, including the broad O–H stretching band that overlapped with the N–H stretching band and exhibited a band maximum at $\sim 3312 \text{ cm}^{-1}$ [252,253], as well as the amide C=O absorption at $\sim 1630 \text{ cm}^{-1}$ and the C–O–C deformation band at $\sim 1040 \text{ cm}^{-1}$ [108,252].

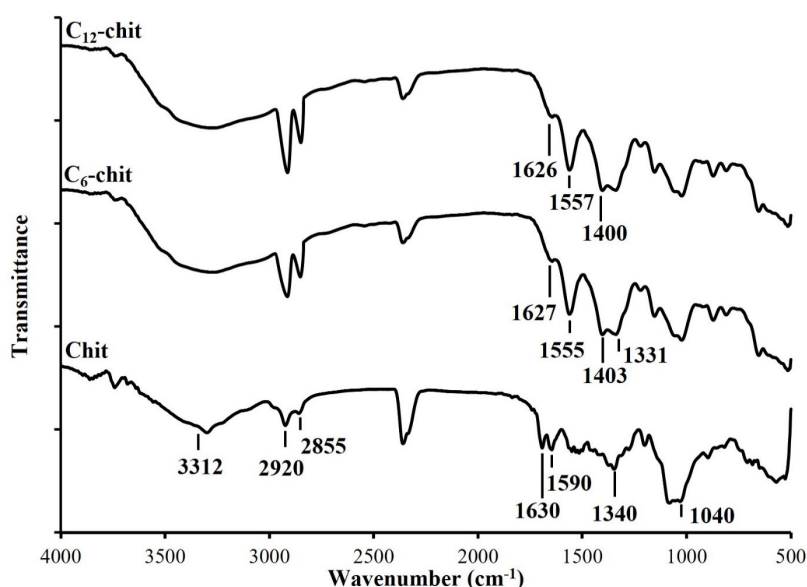


Figure 23. FTIR spectra of the original chitosan and its modified versions [251].

5.3.2 Determination of substitution degree (SD%) and molecular weight (M_w) of Chit, C₆-chit and C₁₂-chit

The SD% values, which represent the fraction of chitosan amino groups modified with hexyl or dodecyl chains by reductive amination, were determined as described in the Methods section. As shown in **Table A5**, the SD was about 30% for the hexyl-modified sample and 19% for the dodecyl-modified sample. These values were lower than the maximum theoretical value (60%), which is consistent with previous reports on the reductive amination of Chit [253,254]. The

lower SD% of the dodecyl derivative is also in agreement with reports showing that longer alkyl chains lead to lower substitution degrees because they create greater steric hindrance, making it more difficult for the aldehyde to access the Chit amino groups and thereby reducing the extent of substitution [254].

The molecular weights of the native Chit, C₆-chit and C₁₂-chit were also determined from the corresponding Debye plots (**Figure A15**) constructed from DLS data by applying **equation 11**. The M_W values were found to be 315, 352, and 362 kDa, respectively.

5.3.3 Thermal behaviour characteristics of Chit, C₆-chit and C₁₂-chit

The swelling of the native Chit and its hydrophobically modified derivatives was further examined to quantify, via thermoanalytical measurements, the differences in their hydrophilicity. **Figure 24** shows the TG and DSC profiles of the polymer-based hydrogels with equilibrium water content. Before TG analyses, the diluted polymer solutions (1 wt%) were concentrated via centrifugation. Then, after the removal of the supernatants, the water content of the collected hydrogel sediments was determined. As evident from the TG curves (**Figure 24A**), all polymers showed a clear weight-loss event between 30 and 130 °C, corresponding to the evaporation of water present within the materials [108]. The accompanying column chart displays the water-content values derived from these water-related mass losses. As shown, the native hydrophilic chitosan possesses the highest water content (78.5 wt%), whereas the hydrophobic modification reduced this value to 74.2 wt% for C₆-chit and 47.1 wt% for C₁₂-chit. This decrease in equilibrium water content confirms the higher hydrophobicity of the resulting derivatives relative to the native polymer.

The DSC thermograms (**Figure 24B**) also showed the variation in the hydrophilicity of the polymers swollen under similar circumstances. As can be seen, all DSC curves exhibited an endothermic peak associated with water evaporation, which decreased in intensity with the increase in hydrophobicity. Additionally, decreasing hydrophilicity shifted the endothermic peak minimum toward lower temperatures, from 112.8 °C in unmodified Chit to 96.3 °C in C₁₂-chit. Moreover, the vaporization enthalpy (ΔH_w) was derived through the integration of the areas under the endothermic peaks and the subsequent normalization to the determined water contents [108]. As presented in **Figure 24B**, the unmodified biopolymer showed the highest value (32.85 kJ/mol), followed by C₆-chit (30.15 kJ/mol) and C₁₂-chit (24.39 kJ/mol). Collectively, these findings indicate that the hydrophobicity of native chitosan can be tuned through modification with aldehydes of varying alkyl chain lengths.

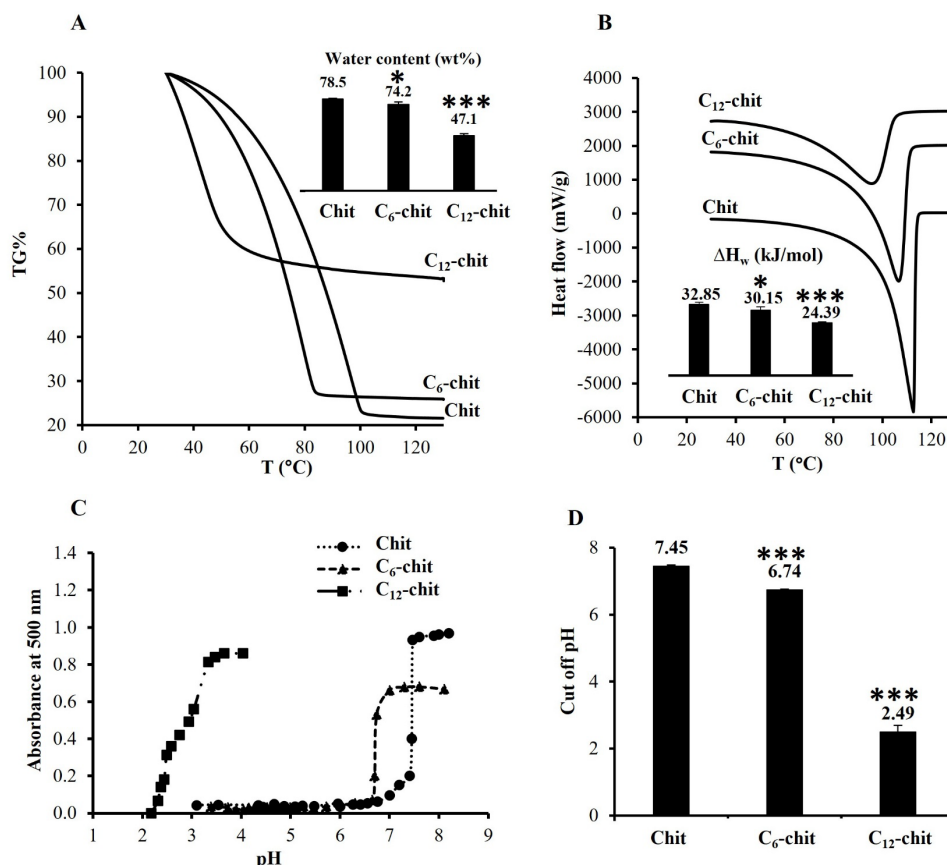


Figure 24. (A) The recorded TG profiles for both the unmodified and modified Chit hydrogels (with the equilibrium water content), accompanied by a column chart displaying the determined water content percentages. (B) The DSC profiles of the hydrogels, with an inset column chart illustrating the determined water desorption enthalpy (ΔH_w) values. (C) The effect of pH on the solubility behaviour of the resulting polymers. (D) The cut-off pH values of all polymer forms. Statistical analysis: one-way ANOVA with Dunnett's post hoc test (control group: Chit). * $p < 0.05$; *** $p < 0.001$ [251].

5.3.4 pH-dependent solubility characteristics of Chit, C₆-chit and C₁₂-chit

Studying the pH-solubility behaviour of the samples was essential for identifying the polymer most capable of releasing the bioactive agents preferentially to ischemic areas. **Figure 24C** shows the solvation characteristics of the polymers as a function of pH. At low pH levels, the biopolymer solutions were transparent and showed minimal absorbance values. However, elevated pH levels led to turbidity, suggesting the development of polymeric particles. These observations were consistent with the pH-dependent solubility of chitosan reported in the literature, arising from its polycationic nature. At low pH, the amine moieties of chitosan ($pK_a \approx 6.5$) are protonated, which makes the biopolymer highly soluble in aqueous media. At higher pH, these groups become progressively deprotonated and uncharged, lowering the biopolymer's solubility and leading to particle formation [255,256]. It is also documented that incorporating a hydrophobic agent and changing the length of its alkyl chain can markedly influence the solubility behaviour of chitosan [250].

As shown in **Figure 24D**, the cut-off pH values—each derived from the inflection point of the corresponding precipitation curve—exhibited a downward trend with increasing polymer hydrophobicity. For example, while the unmodified Chit displayed a cut-off pH of 7.45, the C₆-chit and the C₁₂-chit derivatives showed markedly lower values of 6.74 and 2.49, respectively. The explanation behind this behaviour is that, in the hydrophobically modified derivatives, three-dimensional networks are generated because of the associative interactions among the hydrophobic moieties, which in turn facilitate nanoparticle formation at reduced pH levels [108,257]. This pH-driven nanoparticle formation or disintegration can be exploited to achieve pH-responsive drug release. For example, since our work intended to release the bioactive agents within the ischemic regions (pH 6.5–6.0) rather than in normal tissues (pH 7.4), it was necessary to have particles that remain closed at physiological pH but disintegrate under ischemic pH conditions. For this reason, the C₆-chit polymer was chosen for nanoparticle production.

5.3.5 Synthesis of C₆-chit NPs

Because C₆-chit polymer is capable of dissolving in dilute aqueous acid solutions and the bioactive agents intended for encapsulation are readily soluble in solvents like absolute alcohols [154,258] and acetone [259], the desolvation method was selected for preparing the drug-loaded NPs. However, the physicochemical characteristics of NPs produced through this technique can be substantially influenced by the preparation conditions applied during synthesis [60,61]. Therefore, before incorporating PF or loading the therapeutic agents, key formulation parameters—such as the desolvating agent type, the polymer concentration, and the crosslinker concentration—were varied to assess their impact on the colloidal characteristics of the unloaded C₆-chit particles.

Effect of different desolvating agents on NP formation

To identify the most effective desolvating agent for nanoparticle development, the effects of various agents, including acetone, methanol, and ethanol, were evaluated with particular emphasis on their influence on relative yield and particle size distribution. As illustrated in **Figure 25A**, the 0.24 wt/v% C₆-chit solution exhibited its maximum turbidity upon the introduction of 1.8 mL (0.29 v/v) of each desolvating agent. One can also observe that using ethanol produced a markedly higher turbidity compared to methanol or acetone, which may imply that a higher quantity of nanoparticles was produced [60]. To verify this interpretation, the nanoparticle yield was calculated after determining the solid content in the dispersions (**Figure 25B**). As anticipated, the use of ethanol resulted in a considerable NP yield ($95.5 \pm 4.0\%$), exceeding the yields obtained with acetone ($74.8 \pm 3.7\%$) and methanol ($49.7 \pm 3.8\%$).

Particle size and polydispersity were further assessed (**Figure 25C**) after dispersing the NPs in PBS (pH 7.4, 0.9% NaCl). As can be seen, particles prepared using ethanol displayed the smallest size (170 ± 19 nm), whereas those obtained with acetone and methanol measured 271 ± 93 nm and 300 ± 36 nm, respectively. Furthermore, compared to methanol and acetone, the utilization of ethanol produced NPs with the lowest PDI (0.226 ± 0.071), implying higher size uniformity.

Examination of the TEM micrographs also revealed that particles synthesized using ethanol exhibited a more defined spherical morphology (**Figure 25D**) in comparison to those obtained with acetone or methanol (**Figure A16**). Considering the collective results, ethanol appeared to be the most effective desolvating agent for producing C₆-chit NPs with relatively small sizes, good uniformity, and satisfactory yields. Moreover, a 0.29 ethanol volume fraction was determined to be sufficient for C₆-chit NPs preparation. Lower volume fractions resulted in similarly sized NPs but with diminished yield, whereas increasing the volume fraction to 0.31 did not produce any noticeable improvement in either yield or size [251].

The results are in line with previous studies showing that ethanol generally functions as a more effective nonsolvent than acetone or methanol in nanoprecipitation, yielding smaller, more uniform polymeric NPs with high yield [60,260]. Compared to methanol, ethanol has a longer carbon chain and therefore provides stronger hydrophobic interactions with the polymer, which promotes more efficient desolvation and higher yield [60,261]. Compared to acetone, which is an aprotic solvent, ethanol is a protic solvent that can donate and accept hydrogen bonds. This property, together with its relatively higher polarity compared to acetone, enables ethanol to disrupt the polymer's hydration shell in a more controlled manner, leading to better nanoformulation and smaller particle sizes, which is consistent with findings reported in many studies [260,262]. Furthermore, the outcomes were consistent with earlier studies, demonstrating that ethanol can effectively function as a nonsolvent for chitosan biopolymer, yielding stable dispersions at ethanol volume fractions of approximately 30% [108] or 40% [263].

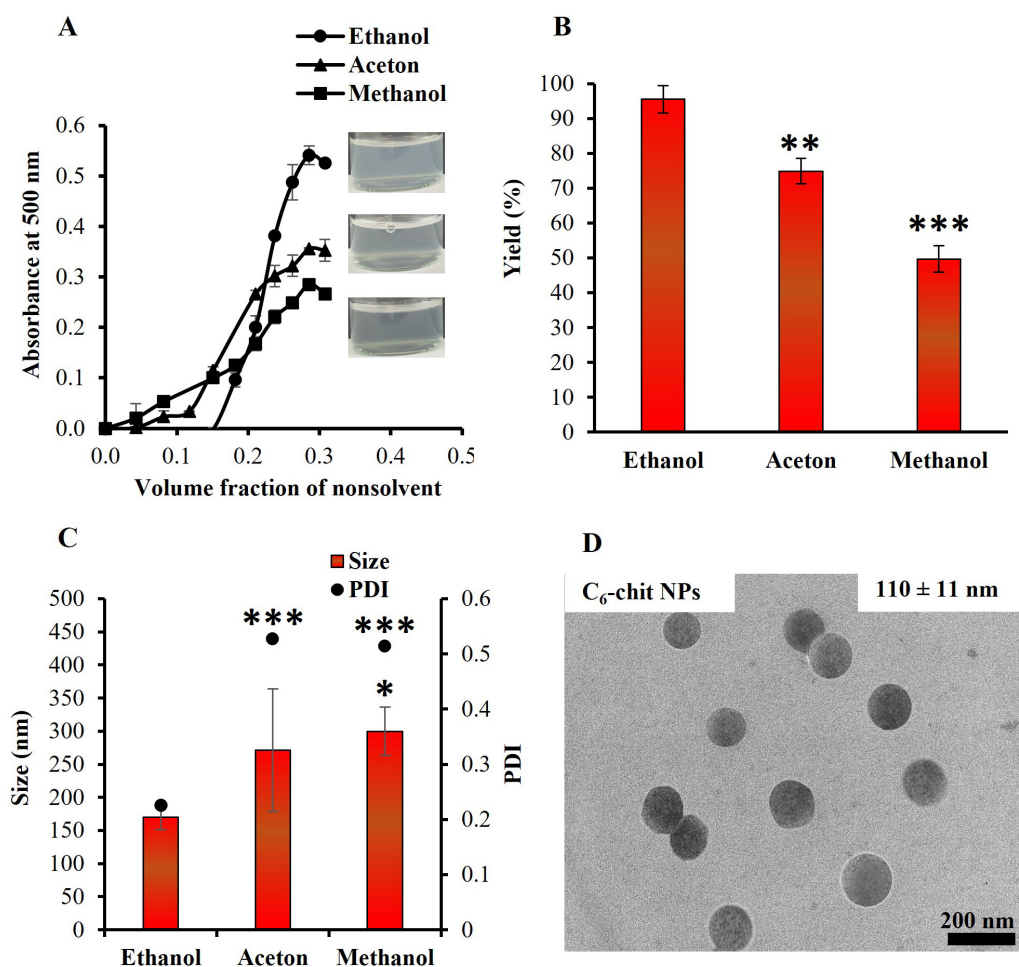


Figure 25. (A) The absorbance of C₆-chit solution in relation to acetone, methanol, and ethanol additions. (B) The percentage yield; (C) the particle sizes and the PDI values of the C₆-chit NPs produced using various precipitating agents and dispersed in PBS (0.9% NaCl, pH 7.4). (D) TEM image of C₆-chit NPs produced by using a 0.24 wt/v% C₆-chit solution and a 0.29 ethanol volume fraction. Statistical analysis: one-way ANOVA with Dunnett's post hoc test (control group: ethanol-containing sample). * p<0.05; ** p<0.01; *** p<0.001 [251].

Effect of polymer concentration on NP properties

Because several studies have demonstrated that polymer concentration plays a critical role in shaping the nanoparticle features [60,61], a range of initial C₆-chit concentrations was examined to determine the optimal concentration window capable of producing NPs with appropriate size and sufficient stability. In all formulations, ethanol was employed as the desolvating agent at a volume fraction of 0.29, while Sodium tripolyphosphate (TPP) was incorporated as a stabilizing agent at 10 wt/wt% relative to the biopolymer. The characteristics of the NPs dispersed in PBS (pH 7.4, 0.9% NaCl) can be shown in **Figure 26** and **Table A6**.

As illustrated in **Figure 26**, no marked variation in particle size was observed across the 0.2–0.6 wt/v% concentration range. Specifically, when a C₆-chit concentration of 0.2 wt/v% was applied, the particles exhibited an average size of about 161 ± 13 nm, which was comparable to

the 163 ± 9 nm measured at 0.6 wt/v%. In contrast, increasing the C₆-chit concentration beyond 0.6 wt/v% led to a marked rise in average particle size, reaching approximately 227 nm at 0.8 wt/v% and about 266 nm at 1.2 wt/v%. Such growth aligns with earlier reports and is typically attributed to the higher degree of supersaturation [60,264].

With respect to the PDI values (**Table A6**), no notable variation was detected. All NP samples displayed an average PDI of about 0.3, suggesting size homogeneity across all formulations [207,208]. On the other hand, increasing the C₆-chit concentration led to a gradual rise in ζ -potential, from about 24.1 mV at 0.2 wt/v% to 27.2 mV at 1.2 wt/v%. This positive surface charge, arising from the cationic amino functionalities [265], facilitated interparticle repulsion and resulted in stable dispersions across all formulations. Overall, the results indicate that a C₆-chit concentration in the range of 0.2–0.6% is appropriate for obtaining NPs with both small sizes and adequate stability.

Effect of TPP concentration on NP properties

Cross-linking agents are important for ensuring the stability of many nanoparticle systems. Within Chit-based nanoparticle systems, TPP is the most widely employed cross-linker. As an anionic molecule, TPP can interact electrostatically with the cationic amino groups of Chit, forming ionically cross-linked networks that stabilize the nanoparticle structure. It is further recognized as a safer alternative to other chemical cross-linkers (e.g., glutaraldehyde), which have a greater potential to cause toxic effects [109,266].

For unmodified chitosan, earlier investigations have indicated that a TPP concentration of 10 wt/wt% (with respect to chitosan) is effective for yielding small-sized particles, with adequate stability [267]. Further studies, however, have applied greater weight ratios [268]. In view of these discrepancies, a range of TPP concentrations (10–40 wt/wt%, relative to C₆-chit) was examined during NP preparation to identify the most appropriate cross-linker level. **Figure 26** presents the influence of varying TPP concentrations on the size and ζ -potential of the particles at a fixed polymer concentration (0.2 wt/v%).

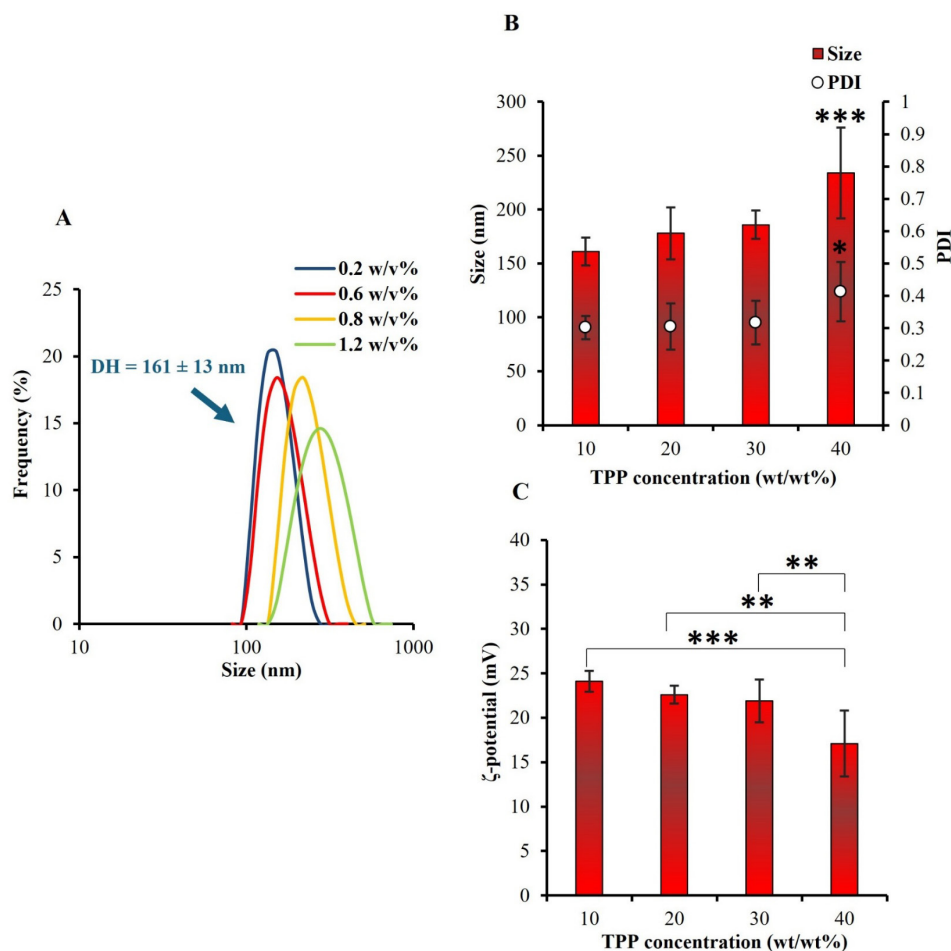


Figure 26. (A) DLS size distribution curves of the NPs produced using different initial C₆-chit concentrations (0.2-1.2 wt/v%). The impact of different TPP contents (10-40 wt/wt% with respect to C₆-chit, applied in a fixed volume of 100 μL) on (B) the particle size and (C) the ζ-potential values of the NPs produced at a constant C₆-chit concentration (0.2 wt/v%). Particles were dispersed in PBS (0.9% NaCl, pH 7.4). Statistical analysis: one-way ANOVA with Tukey's HSD post-hoc analysis. * p<0.05; ** p<0.01; *** p<0.001 [251].

As can be seen in **Figure 26B**, applying TPP concentrations up to 30 wt/wt% did not lead to a significant increase in particle size, whereas increasing the concentration to 40 wt/wt% resulted in a marked rise in both DH, which reached 234 ± 42 nm, and PDI, which increased to 0.413 ± 0.092 . Additionally, applying this concentration decreased the ζ-potential significantly to 17.1 ± 3.7 mV (**Figure 26C**). These findings are consistent with previous research showing that a TPP-to-polymer mass ratio of about 0.5 (50 wt/wt%) can result in larger particle sizes and aggregation [268]. This can be ascribed to the heightened presence of anionic groups in the medium, which led to more electrostatic interactions with the cationic amino groups of the polymer. This, subsequently, reduces the net positive charge on the particle surface and diminishes the interparticle repulsion, ultimately resulting in larger particle sizes [109]. Building on these observations, one may conclude that applying TPP at 10 wt/wt% relative to C₆-chit is sufficient to obtain small-sized NPs with adequate stability.

5.3.6 Synthesis of PF-loaded C₆-chit NPs (PF-C₆-chit NPs)

The synthesis of PF-C₆-chit NPs was performed as described in the methods section, and FTIR analysis of the resulting particles (**Figure A17**) confirmed the successful incorporation of Pluronic (PF) into the nanoparticle matrix. Compared with the C₆-chit, the PF-loaded sample showed two additional absorption peaks at 947 and 1100 cm⁻¹, which were further noted in the spectrum of free PF, and can be assigned to the C-O-C stretching vibrations. Moreover, the band at 2855 cm⁻¹, which corresponds to the CH₂ stretching vibrations, exhibited an increase in intensity relative to C₆-chit, further supporting PF incorporation [109]. In addition, upon PF incorporation, a shift of the NH/OH stretching band from 3312 to 3230 cm⁻¹ was observed. This could be ascribed to hydrogen-bonding interactions between the NH and OH groups of C₆-chit and the OH functionalities of PF.

During nanoparticle preparation, various PF copolymer amounts (15, 20, and 25 wt/wt% relative to C₆-chit) were employed to produce NPs that are potentially capable of exhibiting prolonged-drug release profiles. As can be seen in **Figure 27A**, the size of the particles synthesized in the presence of PF was markedly smaller than the size of PF-free NPs. For example, the presence of 25 wt/wt% PF reduced the DH from about 161 ± 13 nm to 124 ± 10 nm. These findings are in line with earlier studies, which have demonstrated that PF can reduce nanoparticle swelling, leading to smaller hydrodynamic diameters in aqueous media [109,269]. This decrease in swelling is probably attributable to the hydrophobic PPO segments present within the PF copolymer [270]. Regarding the ζ-potential measurements, as shown in **Figure 27B**, the ζ-potential of the PF-containing samples did not differ significantly from that of the PF-free sample (24.1 ± 1.2 mV). It is also important to mention that the PF concentrations applied in this work (0.3–0.5 mg/mL) were well above the polymer's CMC (0.043 mg/mL; **Figure 27C**). Under these conditions, the copolymer can create tightly packed micellar structures or larger multimolecular aggregates, which can all be trapped within the nanoparticle matrix [109].

The presence of PF within the nanoparticle matrix was further supported via TEM measurements. As shown in **Figure 27D**, the TEM micrograph of the PF-loaded particles displayed dark spots inside the spherical matrices. These darker spots can be ascribed to the incorporation of PF, as they were not observed in the PF-free C₆-chit particles (**Figure 25D**).

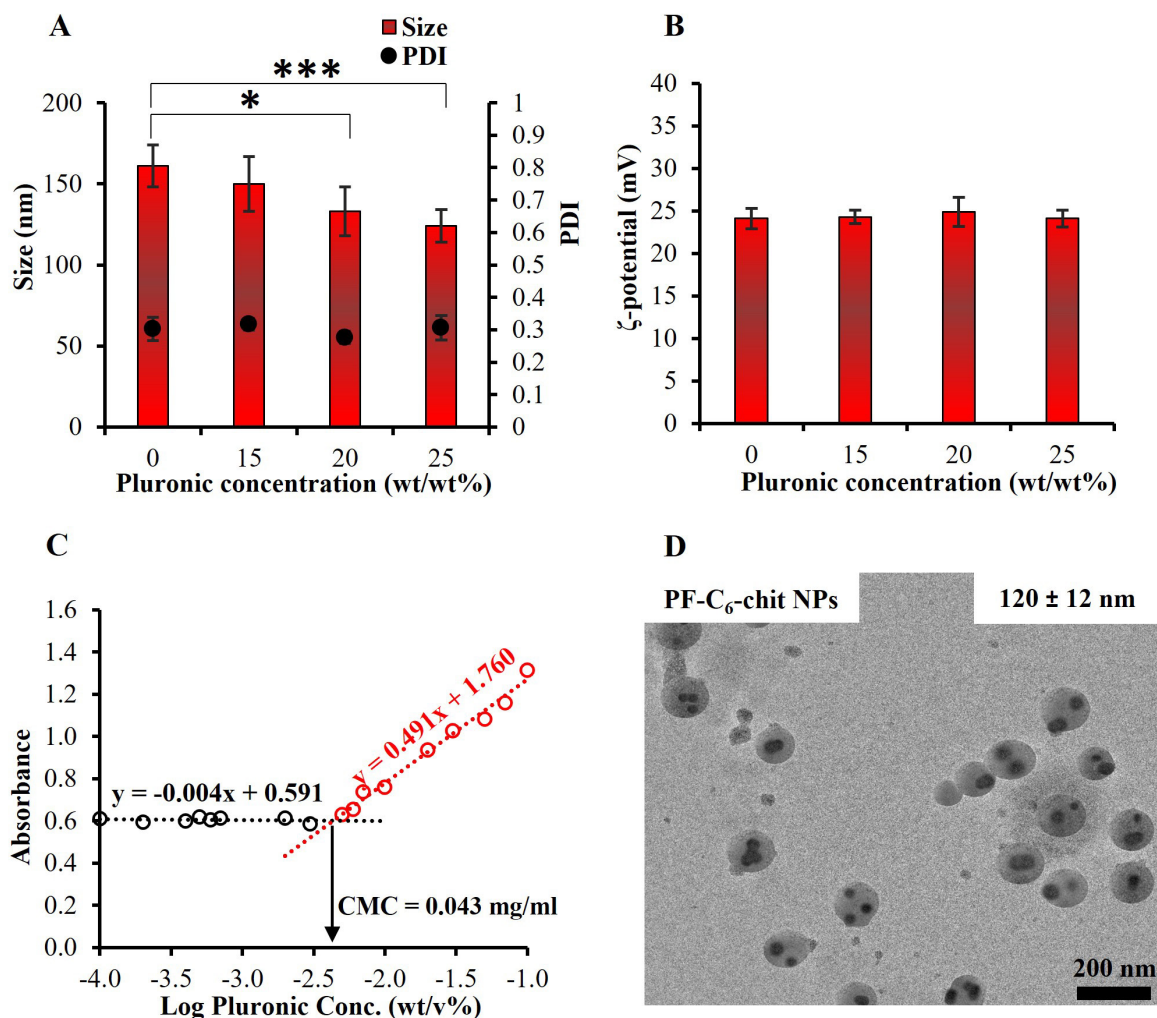


Figure 27. (A) DH and PDI values, and (B) ζ -potential values of the NPs produced using different initial Pluronic (PF) contents (0-25 wt/wt%, referring to C₆-chit). Particles were dispersed in PBS (0.9% NaCl, pH 7.4). (C) The UV absorbance intensity plot of I₂ against the logarithm of various PF concentrations with the determined CMC value at 25°C. (D) TEM image of PF-C₆-chit NPs produced using a PF content of 25 wt/wt%. Statistical analysis: one-way ANOVA with Dunnett's post hoc test (control group: PF-free sample). * $p < 0.05$; *** $p < 0.001$ [251].

5.3.7 NIMO and HC encapsulation

For the preparation of the drug-loaded nanoparticle formulations, 1 mg of either HC or NIMO was incorporated during the encapsulation process. The selected drug mass was guided by established findings in the literature. For HC, concentrations of 0.1 μ M (0.047 μ g/mL) or 1 μ M (0.47 μ g/mL), as well as a dose of 9 μ g/kg, have been reported to effectively inhibit TRPV4 activity [154,271]. Accordingly, 1 mg of HC was incorporated to allow the preparation of therapeutic and supra-therapeutic concentrations through the dispersion of the lyophilized particles in a suitable volume of dispersion medium. For NIMO, 1 mg was incorporated in alignment with the earlier studies and the 2011 Stroke Guidelines, which recommend

administering NIMO intravenously at an initial dose of 1-2 mg/h [272,273]. This reinforces the safety and efficacy of the therapeutic regimen for stroke management.

The encapsulation of the hydrophobic agents was verified via FTIR analysis (**Figure A18**). The distinctive IR peaks of nimodipine, specifically the carbonyl-group stretching band at 1720 cm^{-1} and the nitro-group vibrational band at 1519 cm^{-1} [162], were still detectable in the NIMO-loaded samples, but showed slight shifts to 1709 cm^{-1} and 1490 cm^{-1} , respectively. Likewise, the characteristic HC bands, particularly the amide carbonyl stretching band at 1678 cm^{-1} and the amide NH deformation band at 1549 cm^{-1} [252], were also present in the HC-loaded samples, but appeared at lower wavenumbers of 1650 cm^{-1} and 1530 cm^{-1} , respectively. Collectively, these findings verified drug incorporation into the polymeric particles, and the shift to lower wavenumbers suggested the involvement of hydrogen-bond interactions.

Spectroscopic analysis also revealed that both NIMO and HC were encapsulated with remarkably high efficiencies, reaching $96.4 \pm 1.3\%$ and $96.9 \pm 1.2\%$, respectively. Furthermore, as shown in **Table A7**, the incorporation of PF didn't lead to any significant change in these values. For example, when PF concentration was 25 wt/wt% with respect to C₆-chit, the encapsulation efficiencies were $95.4 \pm 1.0\%$ for NIMO and $97.3 \pm 2.6\%$ for HC. These high encapsulation efficiencies could be ascribed to the abundance of nitrogen and oxygen atoms within the C₆-chit matrix, which can offer multiple hydrogen-bonding sites for interactions with the encapsulated drugs.

Table A7 also provides a summary of the DLS measurements. As can be seen, the drug-loaded NPs exhibited a noticeably larger hydrodynamic diameter compared with the unloaded counterparts (**Figure 27A**). For instance, the hydrodynamic diameter of the (25 wt/wt% PF)-C₆-chit NPs was about $124 \pm 10\text{ nm}$, which increased to $197 \pm 10\text{ nm}$ upon NIMO loading and to $203 \pm 25\text{ nm}$ upon HC loading, indicating successful drug incorporation [60]. One can also observe that all drug-loaded samples displayed relatively low PDI values (0.249–0.284), showing that the formulations maintained good size uniformity [207,208]. Regarding the ζ -potential measurements, no significant differences were detected between the drug-loaded NPs (**Table A7**) and their drug-free counterparts (**Figure 27B**), supporting the conclusion that both pharmacological agents are encapsulated within the NP core.

XRD spectra were also registered for the free drugs and the nanoparticle-encapsulated forms to examine crystallinity changes that may arise as a consequence of drug encapsulation [108,121]. As can be seen, in comparison with the diffraction pattern of free NIMO (**Figure 28**), the encapsulated forms presented noticeably diminished peak intensities, reflecting a reduction in the NIMO's crystallinity [121]. This decrease in crystallinity was anticipated because drug

loading leads to NIMO being incorporated into the NP matrix, reducing its ability to form the well-defined micron-sized needle-like crystals observed for the free drug [121]. A similar decrease in crystallinity was also detected for HC upon encapsulation [251]. Typically, highly crystalline drugs have lower solubility than their amorphous forms, which possess greater surface area, as drug solubility depends on the formation hydrogen bonds between the solvent and the drug molecules. Therefore, reducing drug crystallinity is advantageous, as it enhances drug solubility, promoting faster dissolution and improving bioavailability [121,274].

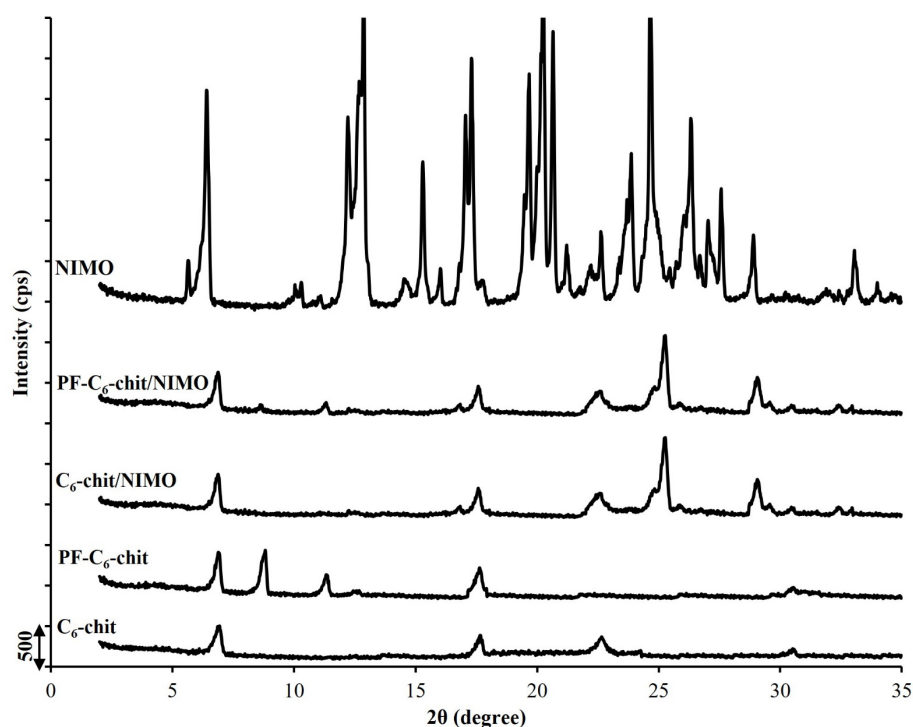


Figure 28. XRD spectra of the uncoated (free) NIMO, C₆-chit polymer, PF-C₆-chit NPs, NIMO-loaded NPs. The drug content is ~11 wt% relative to C₆-chit [251].

Moreover, to better understand the effect of encapsulating HC or NIMO within C₆-chit or PF-C₆-chit particles, the stability of both the free drugs and the encapsulated counterparts was evaluated in aqueous PBS (pH 7.4, 0.9% NaCl). As illustrated in **Figure 29**, the existence of the polymeric shells markedly improved the dispersibility of both drugs in aqueous medium. According to **Figure 29A and 29B**, the relative turbidity of the NIMO- and HC-loaded particles remained largely stable throughout the 8-hour period, showing reductions of approximately 3% and 5%, respectively. In contrast, the free drugs experienced a marked drop in turbidity, falling by 83% for NIMO and 81% for HC.

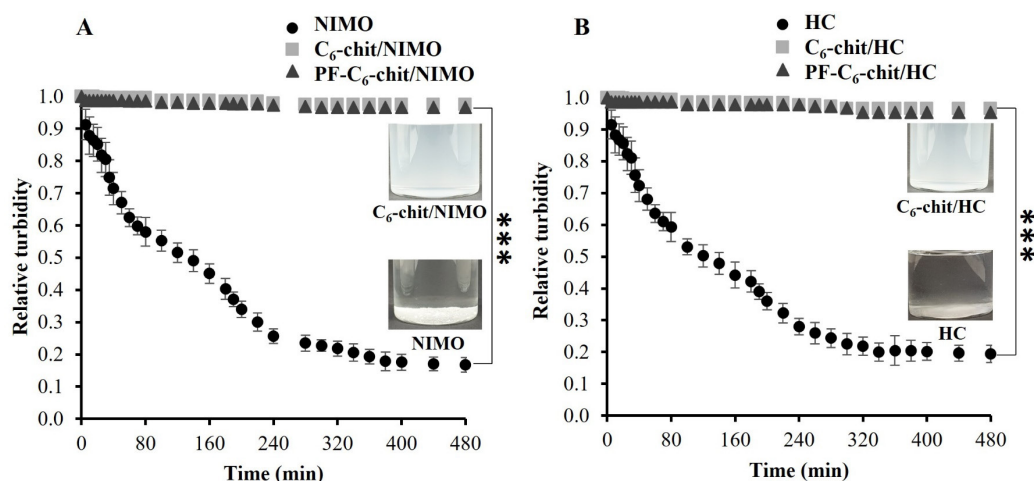


Figure 29. (A) Change in turbidity values over time for NIMO and NIMO-loaded NP dispersions in aqueous PBS (10 ml) at a NIMO concentration of 0.2 mg/ml. (B) Time-dependent turbidity for HC and HC-loaded NP dispersions in aqueous PBS (10 ml) at a HC concentration of 0.2 mg/ml. Statistical analysis: one-way ANOVA with Dunnett's post hoc test (control group: bare drugs). *** $p < 0.001$ [251].

The limited dispersibility of both NIMO and HC can be explained by their hydrophobic character [151,275] as well as their low ζ -potentials at pH 7.4 (NIMO: -1.0 ± 5.5 mV; HC: 0.7 ± 1.7 mV), which indicate insufficient surface charge to maintain a stable dispersion in aqueous medium, thereby promoting aggregation and rapid sedimentation [276]. In contrast, the improved dispersibility of the NIMO- and HC-loaded polymeric particles can be ascribed to their small particle size and their cationic surface charge (see **Table A7**). The relatively high ζ -potential values (approximately 24 mV) reflect a sufficient surface charge to generate electrostatic repulsion between particles, supporting the formation of stable dispersions [275,276]. The stability of the NIMO- and HC-loaded polymeric particles was further assessed over an approximately one-month storage period. As presented in **Figure A19**, the drug-loaded nanoparticle samples showed high stability, with minor changes in turbidity, DH, and ζ -potential over time.

These results support the use of C₆-Chit NPs to improve the aqueous solubility/dispersibility of NIMO and HC, thereby reducing the need for ethanol-based solubilization approaches, which are widely used for these poorly water-soluble drugs and are associated with local adverse effects, as noted in the introduction [155–157]. Moreover, because of the poor aqueous solubility of these drugs when diluted with saline solutions during intravenous administration, they can crystallize, posing risks to patient safety [121,156]. However, based on the stability assessment in phosphate-buffered saline, C₆-Chit NPs can maintain the drugs in a dispersed state, indicating a reduced tendency for crystallization and supporting safer, more reliable injection. Overall, these findings are promising for the prospective intravenous administration of HC and NIMO.

5.3.8 *In vitro* drug release study

Based on our observations, C₆-chit can form stable particles at physiological pH, and these particles can undergo dissolution when the pH drops below the polymer's cut-off pH (~6.74). This behaviour suggests that the polymeric system could effectively release both NIMO and HC under the acidic conditions typically associated with ischemic tissues (pH 6.5–6.0) [147]. To check the pH-triggered release behaviour of the polymeric particles, drug-release studies were conducted in PBS at 37 °C under two different pH conditions: pH 7.4 to mimic the pH of normal brain tissues and pH 6.0 to simulate the pH of ischemic regions.

Figure 30A shows the release curves of both the bare and the polymer-coated NIMO at pH 7.4, whereas **Figure 30B** presents the corresponding profiles for HC. As demonstrated, after 8 hours of release measurements, only 2.5% of NIMO and 2.4% of HC were released from the C₆-chit particles; while the bare drugs exhibited much higher release levels of about 40% and 85%, respectively. The negligible drug release from C₆-chit NPs can be ascribed to the closed, undissolved state of the particles at physiological pH (see **Figure 24C**), which restricts drug diffusion. Moreover, it may be ascribed to the greater tendency of the hydrophobic bioactive agents to remain within the hydrophobically modified chitosan particles rather than be released into the hydrophilic buffer solution. The release study at pH 7.4 also revealed a clear difference between drug release from the unmodified chitosan and from the C₆-chit particles. The unmodified Chit NPs released much higher percentages of NIMO (29%) and HC (71%), and this behaviour can be attributed to their partially opened structure at this pH (see **Figure 24C**).

The NIMO- and HC-release profiles at pH 6 are also displayed in **Figures 30C** and **30D**. Under this pH condition, the C₆-chit particles released the encapsulated compounds far more efficiently, yielding markedly higher release percentages for both NIMO (47%) and HC (98%). This increase can be explained by the improved dissolution behaviour of the C₆-chit coatings at pH 6.0 (**Figure 24C**). In addition, the C₆-chit particles showed greater drug release than the unformulated HC (86%) and NIMO (39%). This improvement is attributable to the reduced crystallinity and smaller particle size of the encapsulated compounds relative to the unformulated ones, as these attributes can contribute to enhanced dissolution [121]. Moreover, the release study demonstrated that PF effectively prolonged the release of both NIMO and HC. For instance, incorporating 25 wt/wt% PF resulted in final release levels of 22% for NIMO (**Figure 30C**) and 68% for HC (**Figure 30D**) after 8 hours. These findings are consistent with several reports that have used PF to design delivery systems capable of sustaining drug release [109,111]. As described in these reports, PF can inhibit some drug-diffusion paths and thus slow the overall release process. Moreover, PF is recognized for its capacity to form

semisolid gels at physiological temperature. Greater PF contents produce more firm gels, which consequently can extend the drug-release duration [109,111].

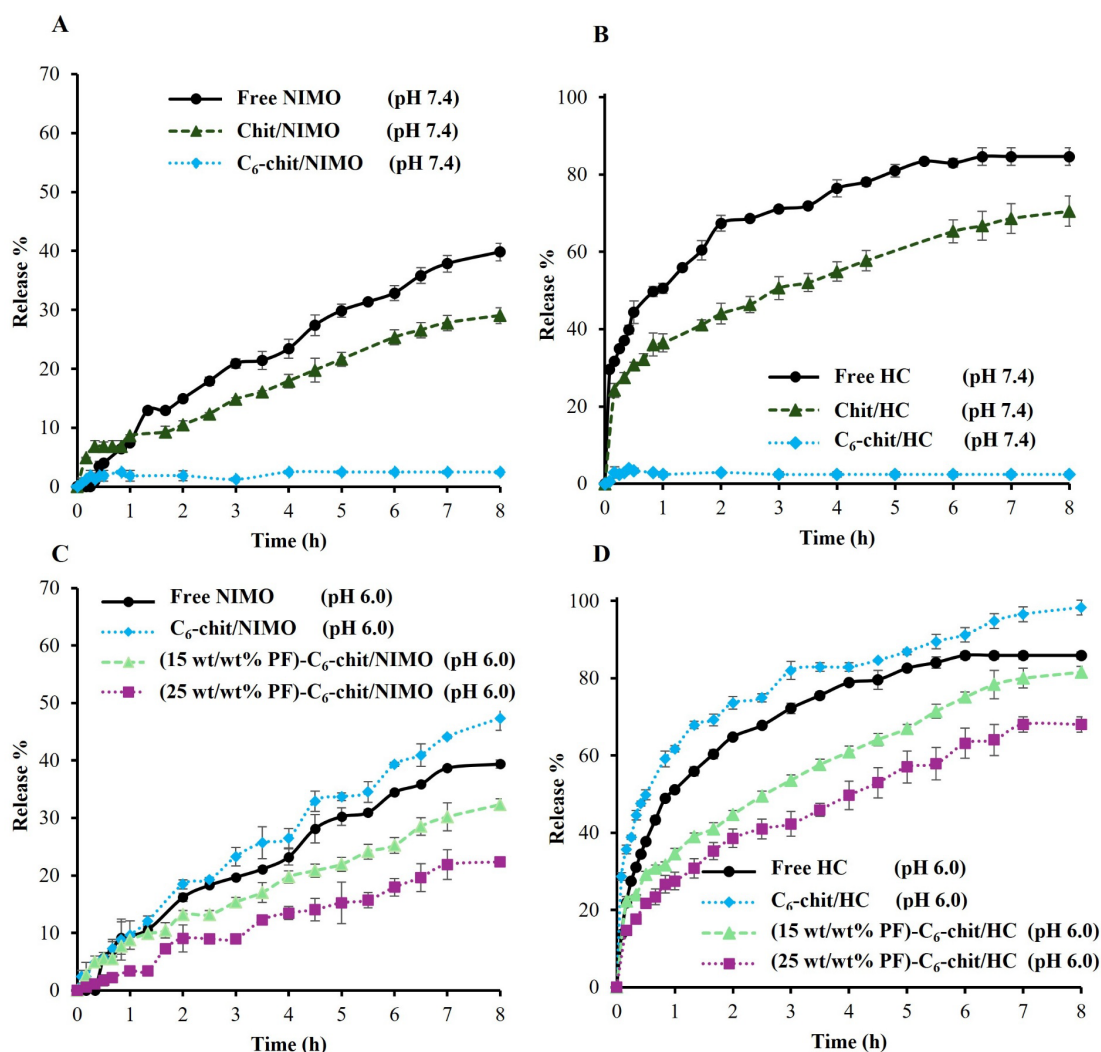


Figure 30. The *in vitro* drug release profiles for (A) NIMO and NIMO-loaded NPs at pH 7.4, (B) HC and HC-loaded NPs at pH 7.4, (C) NIMO and NIMO-loaded NPs at pH 6, and (D) HC and HC-loaded NPs at pH 6 [251].

The release profiles were further analyzed with several kinetic models to gain insight into the underlying release mechanisms. As shown in **Table A8**, the Higuchi model offered the best fitting for free NIMO, indicating diffusion-controlled release [121,185]. In contrast, the release of bare HC was better described by the Korsmeyer–Peppas model, as shown in **Table A9**; however, the low release exponent ($n = 0.269$) shows that its release is likewise governed by diffusion [88,226]. For both NIMO- and HC-loaded particles, the Korsmeyer–Peppas model showed the best fit, but since the resulting n values ranged from 0.435 to 0.843, one can say that the release is governed by both polymer-shell erosion and drug diffusion [88]. The release rate constants further demonstrated the sustained-release effect caused by PF. For example, the (25 wt/wt% PF)-C₆-chit/HC particles exhibited a much lower rate constant (27.7 h^{-n}) compared

to the PF-free particles (62.2 h^{-n}). Based on that, PF-C₆-chit NPs can be considered a promising platform for the extended release of drugs in ischemic tissues.

To conclude, in this part, we developed a polymeric drug-delivery system aimed at improving the aqueous dispersibility of NIMO and HC while enabling pH-responsive, sustained drug release in ischemic brain regions (pH 6.5–6.0). As a summary, we can state that hydrophobic modification of Chit with aliphatic aldehydes altered its hydration and progressively lowered the cut-off pH for NP formation—from 7.45 (native Chit) to 6.74 (C₆-chit) and 2.49 (C₁₂-chit)—thereby identifying C₆-chit as the most suitable polymer derivative for releasing drugs more efficiently in ischemic regions than in normal tissues. NIMO and HC were efficiently encapsulated (EE $\approx$ 95–97%, loading $\approx$ 8–10%) into $d \leq 280 \text{ nm}$ PF-C₆-chit particles (PF content: 0–25 wt/wt%) via desolvation method. The polymeric nanocapsules reduced drug crystallinity and improved the water solubility of the drugs. The NIMO- and HC-loaded particles showed high colloidal stability in aqueous medium (3–5% decrease in relative turbidity over 8 hours) and maintained stable size and ζ -potential over time, supporting potential intravenous administration without organic solvents. The *in vitro* drug-release studies further demonstrated the pH-responsive nature of C₆-chit particles, with minimal drug release at pH 7.4 (2.5% NIMO; 2.4% HC) and markedly higher release at pH 6.0 (47% NIMO; 98% HC), an advantageous feature that can minimize premature drug leakage in healthy tissues and enable pH-triggered drug release under ischemic conditions. Varying the PF content further enabled tuning of the release rate, supporting more sustained drug availability under ischemic conditions. Overall, these drug formulations, with their good aqueous dispersibility and stability, pH-sensitivity, and enhanced or sustained drug release under ischemic pH conditions, offer a promising platform for intravenous administration and for the controlled release of both NIMO and HC.

5.4 Development of catalyst-free mucoadhesive poly(ethylene thiomalate) NPs for improved oral delivery of MPA

The poor aqueous solubility of the anti-inflammatory corticosteroid MPA compromises suspension stability and lowers the dissolution rate, thereby lowering its oral bioavailability. The objective of this topic was to develop a mucoadhesive polymeric nanocarrier system for MPA to enhance its aqueous stability and dissolution, prolong its gastrointestinal residence time, and thus support its potential for enhanced oral bioavailability. Therefore, a biodegradable thiolated polyester (poly(ethylene thiomalate), PET) was planned to be synthesized with different molecular weights through a catalyst-free polycondensation reaction, and the chemical and physical properties of the resulting polymers were studied. Then, MPA-loaded polyester particles were prepared via nanoprecipitation approach. The drug-loaded NPs were

characterized to evaluate the impact of encapsulation on drug properties, and *in vitro* release studies were conducted to assess how polyester NPs of different molecular weights influence MPA dissolution. The mucoadhesive behaviour of the PET-based NPs was also investigated to determine their capacity for mucosal adhesion.

5.4.1 Structural characterization of the starting monomers and the developed polyesters

PET polymer was synthesized by a direct polycondensation approach, in which equivalent moles of the diol (ethylene glycol, EG) and the dicarboxylic acid (thiomalic acid, TA) [113] were reacted under catalyst-free conditions at 165 °C. As illustrated in **Figure 31**, the PET formation was signalled by the development of a highly viscous, yellow, wax-like product from the initially clear reactants [120,277]. PET M_w was controlled via increasing the polycondensation time of this stepwise polymerisation process [121].

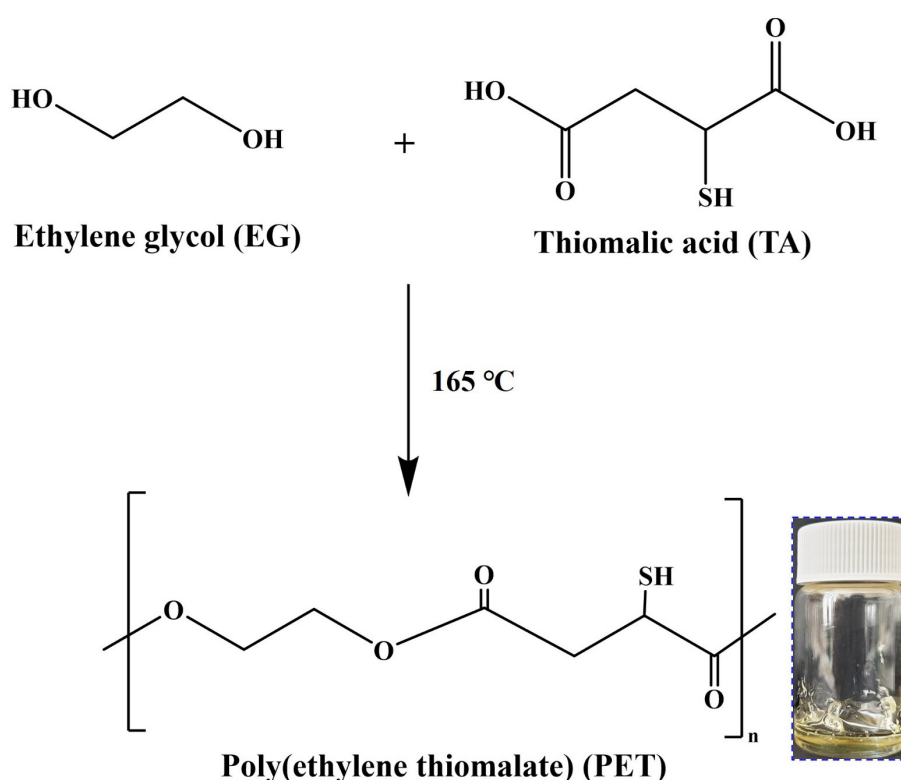


Figure 31. Synthesis of poly(ethylene thiomalate) (PET) polyester via the condensation reaction between ethylene glycol (EG) and thiomalic acid (TA). The inset image presents the PET polyester sample obtained after a 120 min polymerization time [278].

By examining the FTIR profiles (**Figure 32A**) of the precursor monomers EG and TA, along with the PET produced after 120 minutes of polymerization, the successful progression of the reaction was verified. As illustrated in **Figure 32A**, TA exhibited its key FTIR bands at 3012 cm^{-1} (O–H stretching), 2910 cm^{-1} (CH_2 stretching), 2551 cm^{-1} (S–H stretching), 1680 cm^{-1} ($\text{C}=\text{O}$ stretching), 1413–1171 cm^{-1} (COO^- stretching and C–O stretching), and 674 cm^{-1} (C–S

stretching) [252,279]. In addition, EG showed its main FTIR bands at 3325 cm^{-1} (O–H stretching), 2938 and 2865 cm^{-1} (CH stretching), 1464 cm^{-1} (CH_2 bending vibration), 1400 cm^{-1} (C–O–H bending vibration), 1080 cm^{-1} and 1040 cm^{-1} (C–O stretching vibration), and 877 cm^{-1} (CH_2 rocking vibration) [280]. The polyester formation was confirmed through the evaluation of the PET FTIR profile, as it exhibited a new IR band at 1722 cm^{-1} corresponding to the C=O stretching vibrations of the ester bonds. Additionally, the disappearance of the characteristic peak associated with the O–H stretching vibrations of EG, along with the peak attributed to the C=O stretching vibrations of TA, further confirmed the polyester formation [112,120]. The IR bands observed in the $1235\text{--}1150\text{ cm}^{-1}$ region are assigned to the C–O–C ester bond stretching vibrations, whereas the absorption at 1030 cm^{-1} is associated with the –O–C–C– stretching [120,252]. Moreover, the spectrum displayed peaks at 680 cm^{-1} and 2551 cm^{-1} associated with the C–S and S–H stretching vibrations, respectively, along with peaks at 2830 cm^{-1} and 2948 cm^{-1} corresponding to the CH stretching vibrations [252,279].

Further confirmation of the reaction's success was obtained from the ^1H NMR analysis of the synthesized polyester samples. As illustrated in **Figure 32B**, the PET-120 min spectrum exhibited distinct peaks aligned with the anticipated chemical structure. The CH_2 protons located within the repeat unit, originating from the EG monomer (**d** and **c**), were observed as triplet resonances at 4.02 and 3.96 ppm, respectively. Likewise, the methylene protons at the chain end (**g** and **h**) appeared as triplet signals at 3.84 and 3.45 ppm, respectively [120,281]. The observation of these triplets, instead of singlets, affirmed the successful polymerization, since EG in its unreacted state would have yielded singlet signals [282]. In addition, the triplet resonances at 3.58 and 3.63 ppm were attributed to the CH protons situated next to the SH functionality in the terminal moiety (**b**) and in the repeating segment (**f**), respectively [281]. Moreover, the doublet at 2.42 ppm, together with the doublet at 2.51 ppm, which partially overlapped with the DMSO resonance, was assigned to the CH_2COO protons in the end group (**a**) and in the repeating unit (**e**), respectively. These findings affirmed the polymer structure and offered additional confirmation of successful polymerization.

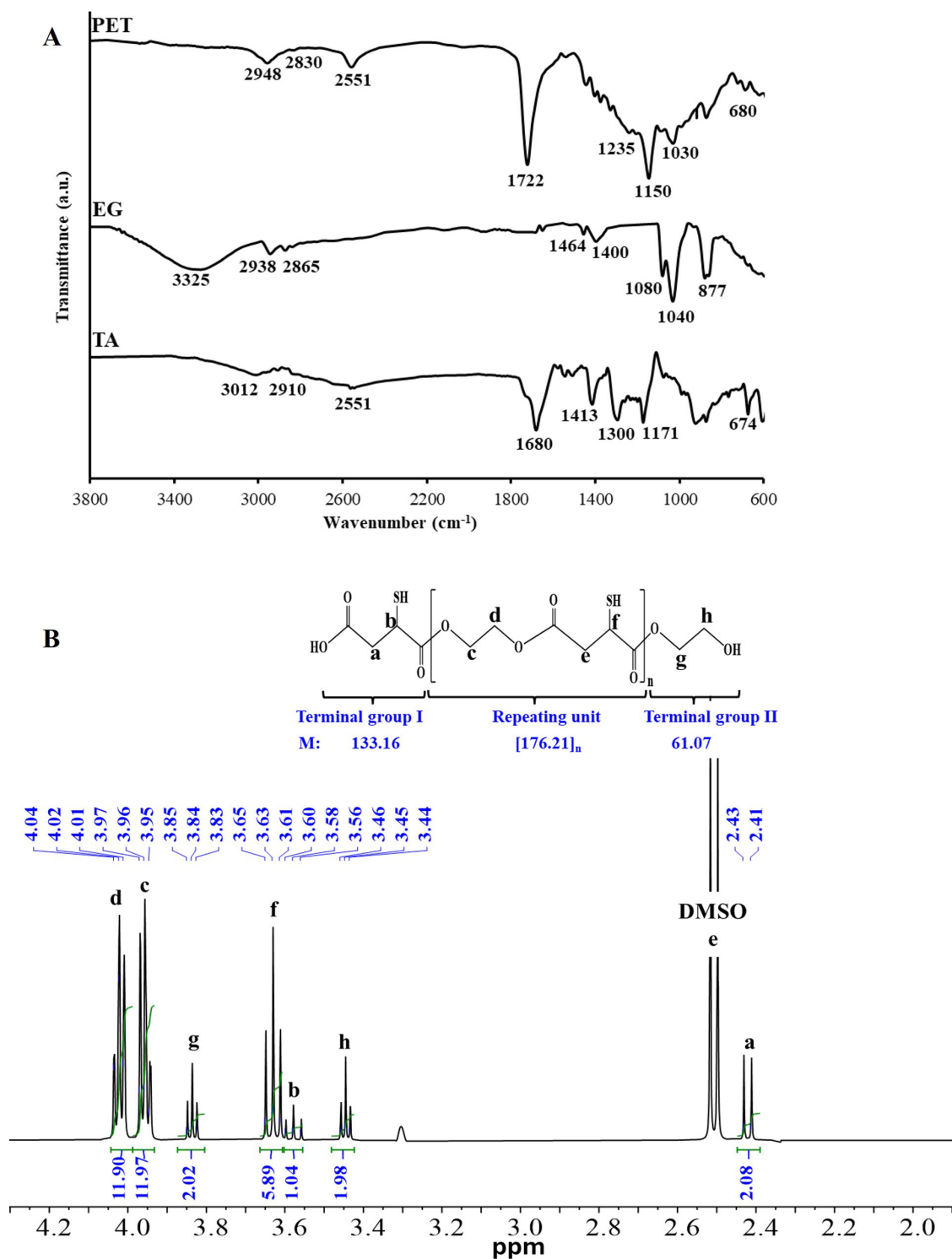


Figure 32. (A) FTIR patterns of the initial monomers (TA and EG) and the synthesized PET polyester obtained after a 120-minute polymerization time. (B) Expanded partial $^1\text{H-NMR}$ spectrum of PET-120 minutes with signal annotations and computed molecular masses of its building blocks [278].

5.4.2 Thermal characterization of the starting monomers and the developed polyesters

Through DSC and TG analyses, the thermal properties of both the precursor monomers and the resulting PET samples were examined to determine the temperatures most suitable for polymerization and to evaluate the thermal stability of the final polyester materials. As shown in **Figure 33A**, the DSC thermograms of the starting monomers revealed decomposition points of 227 °C for TA and 212 °C for EG, while TA also displayed a melting point at approximately 160 °C [283,284]. In the light of these thermal events, the polymerization temperature should be maintained between 160 °C and 190 °C, since this temperature window enables TA melting while preventing monomer decomposition.

The lack of monomer-related signals in the DSC thermograms of the PET samples produced at 165 °C for different reaction durations (**Figure A20**) confirmed successful purification. **Figure 33B** presents the DSC and the TG thermograms of the PET-120 min polyester as a representative example. As seen, the polyester crystallization temperature was about 55 °C while the melting temperature was around 86 °C. As shown in both curves, PET started to decompose above ~232 °C, exhibiting two decomposition steps. The first took place between 240 °C and 311 °C and accounted for approximately 46% weight loss, while the second observed between 311 °C and 420 °C, resulting in an additional 44% loss. These thermal characteristics were consistent with those observed in structurally similar polymers, such as poly(ethylene succinate) [120]. Considering these observations, for PET synthesis, polymerization temperatures above 200 °C should not be used, since elevated temperatures can heighten the likelihood of thermal degradation and, in turn, lead to molecular-weight reduction. This conclusion agreed with previous research [112,285], which used sub-200 °C temperatures for synthesizing PET or structurally analogous polyesters, but offered no reason for the effectiveness of this condition. It is further important to mention that the produced PETs showed a continuous increase in melting point, from about 62 to 86 °C, as polymerization time increased (**Figure A20**), and this can be linked to the increase in molecular weight (discussed later).

5.4.3 Determination of degree of polymerization (X_n) for the synthesized PET polyesters

The polymerization yield of the synthesized PETs was further evaluated at the optimal polycondensation temperature (~165 °C). Through an acid–base titration approach, the amount of TA that had reacted with EG was quantified and afterward normalized to the initial content to determine the polymerization percentage (P%) over time, as seen in **Figure 33C**. The data showed a gradual increase in P% as the polymerization duration was extended. This rise can be ascribed to the step-growth polymerization process, where the reaction proceeds by the

successive forming of dimers, trimers, oligomers, and ultimately long chains [120]. Increasing the reaction duration raised the likelihood of interactions between the carboxyl groups of the diacid (TA) and the hydroxyl groups of the diol (EG), which led to a gradual decrease in the available carboxyl moieties over time, resulting in a maximum P% of about 83% after 120-140 minutes of reaction. X_n values were subsequently computed via Carothers' equation and represented against the P%. The data (**Figure 33D**) affirmed that the polymerization of TA and EG followed a step-growth mechanism, as a progressive increase in X_n was observed with rising conversion, ultimately reaching a value of 5.83 at the 83% conversion.

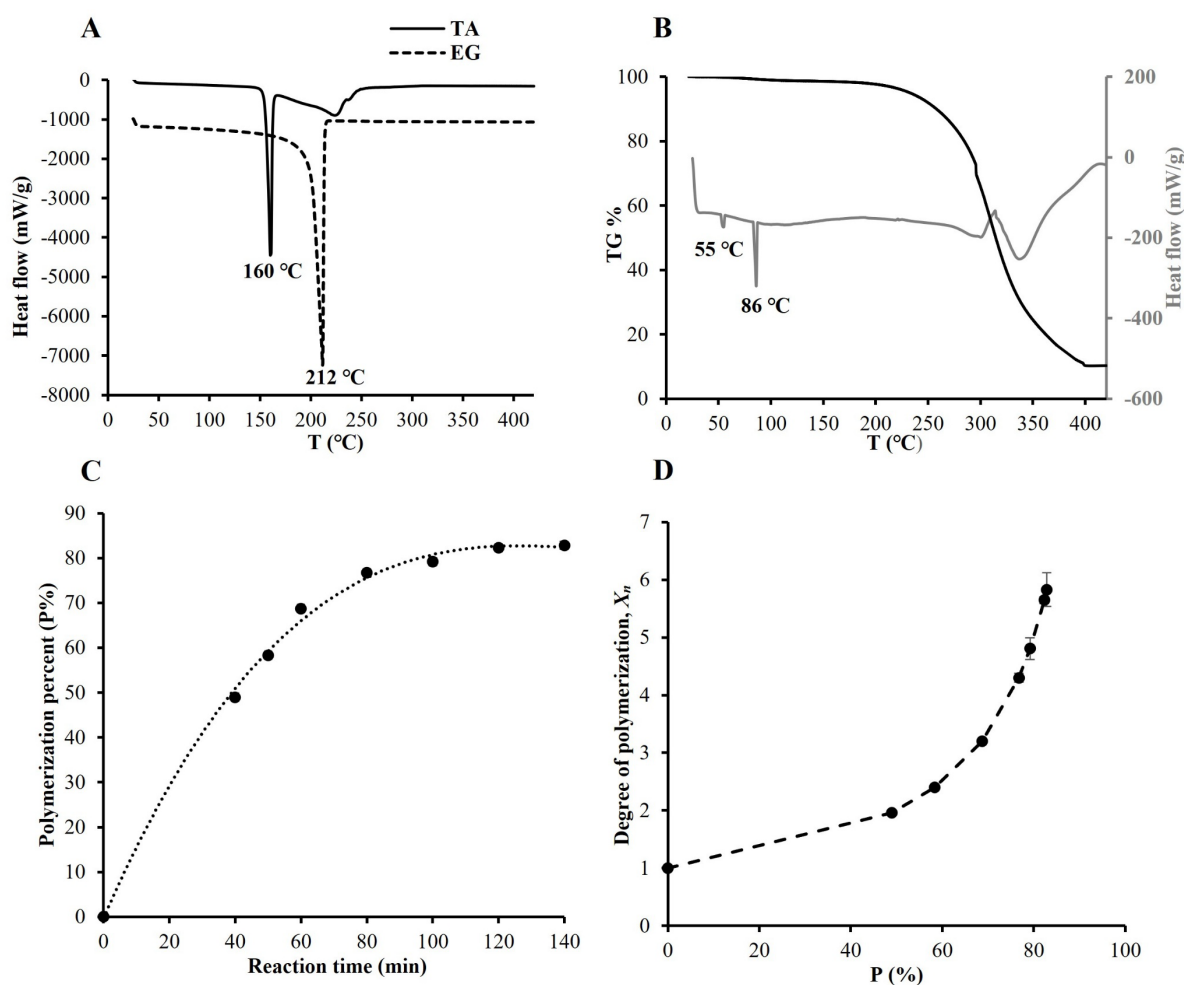


Figure 33. (A) DSC curves of TA and EG. (B) DSC and TG curves of the PET polyester obtained after a 120 min polymerization time. (C) Polymerization percentage (P%) of PET formation over time. (D) Degree of polymerization (X_n) with respect to polymerization percentage (P%) [278].

5.4.4 Determination of molecular weight of the prepared PET samples

¹H-NMR spectroscopy was used to determine the M_n values of the resulting polyester samples and to assess the effect of reaction time on molecular mass. Because PET was prepared from equimolar amounts of TA and EG, one can expect that the produced polymer chains will statistically end with a COOH group at one terminal (terminal group I) and an OH group at the other (terminal group II), as demonstrated in **Figure 32**. The number of repeating units for each polyester sample was determined from the integrated intensities of the characteristic proton peaks, and the M_n values were then calculated by applying equation 20 [120].

$$M_n = 133.16 + \left(\frac{I(c)}{I(g)} \times 176.21 \right) + 61.07 \quad (20)$$

where $I(c)$ and $I(g)$ represent the peak areas of the respective methylene protons and the numbers 176.21, 133.16 and 61.07 correspond to the molecular-weight values of the repeating unit, the terminal group I, and the terminal group II of the PET polymer, respectively (see **Figure 32**).

The M_n values displayed in **Table 2** showed that relatively low-molecular-weight polymers ($M_n = 552$ – 1238 Da) were synthesized without the use of catalysts. The results also showed that prolonging the polymerization period to approximately 2 h led to a continuous rise in molecular weight; but further prolongation beyond this point caused the M_n to decline, decreasing to 1071 Da. These findings indicate that the optimal period for the polycondensation reaction is around 120 minutes, as extended durations can lead to thermal degradation, even under the optimized temperature (165 °C).

Table 2. The number of repeating units (n), the number-average molecular weights (M_n), the weight average molecular weights (M_w), and the polydispersity index ($PDI = M_w/M_n$) for the synthesized PET samples [278].

Sample	I(c)*	I(g)*	Number of repeating units (n)	M_n^* (Da)	M_w^{**} (Da)	M_w/M_n
PET-40 min	4.10	2.02	2	552	908	1.644
PET-60 min	5.95	2.02	3	713	1002	1.405
PET-80 min	8.29	2.02	4	917	1250	1.363
PET-100 min	10.03	2.02	5	1069	1422	1.330
PET-120 min	11.97	2.02	6	1238	1652	1.334
PET-140 min	10.05	2.02	5	1071	1469	1.372

*Obtained by integrating the corresponding peaks in the related ¹HNMR spectra

** Obtained by light scattering measurements

The M_w values of the synthesized PETs were also determined from the Debye plots [278] constructed from the light scattering data by applying **equation 11**. As demonstrated in **Table 2**, lengthening the polycondensation period from 40 min to 120 min led to a continuous increase

in M_W from about 908 to 1652 Da. This trend was similar to that noticed for M_n , but the M_W values were always greater since M_W gives more weight to heavier chains. In addition, the PDI values obtained from the M_W/M_n ratio (**Table 2**) showed that the polymers formed were relatively monodisperse [120].

5.4.5 Determination of water contact angles (Θ_W) for the synthesized PETs

The surface hydrophilicity of the prepared PETs was evaluated through water contact angle analysis. As seen in **Figure 34A**, extending the polycondensation period resulted not only in a higher molecular weight but also in a higher Θ_W . Specifically, the PET-40 min sample (M_W 908 Da) showed the lowest Θ_W value ($38.0 \pm 2.8^\circ$), while the PET-120 min sample (M_W 1652 Da) exhibited the highest value ($75.1 \pm 3.4^\circ$). This Θ_W rise reflected a decrease in hydrophilicity, which was expected because the continuous increase in polyester chain length and molecular mass leads to a reduction in the relative proportion of hydrophilic chain ends [121,286]. Based on the resulting values, the obtained PETs can be considered to possess moderately hydrophilic surfaces [287], making them appropriate for encapsulating sparingly water-soluble compounds, like MPA, and improving their stability in aqueous systems (as shown later). Studies have showed that moderately hydrophilic polymers are beneficial for achieving compatibility with both the hydrophobic drugs and the aqueous environments. They can offer improved stability for hydrophobic drugs in aqueous media, thus increasing their bioavailability. In addition, moderate hydrophilicity can accelerate polymer biodegradability because, during hydrolysis, water can penetrate and attack moderately hydrophilic polymer chains more readily than the chains of highly hydrophobic polymers. Moreover, studies have demonstrated that cells attach and grow more rapidly and effectively on moderately hydrophilic surfaces than on hydrophobic ones, which makes these polymeric materials well-suited for biomedical applications [121,288].

5.4.6 The solubility/precipitation behaviour of the prepared PETs

Nanoprecipitation is broadly applied for the encapsulation of drugs with varying solubilities; however, this method requires an awareness of the solution behaviour of both the polymer and the drug [120]. Therefore, the solubility/precipitation behaviour of the prepared PETs was analysed employing biocompatible DMSO as the good solvent and water as the precipitating agent (or poor solvent). The selection of this solvent pair was guided by its capacity to act as a good–poor solvent system for the produced polyester samples as well as for a wide range of water-insoluble drug compounds, such as MPA [121,166,189].

As a first step, the Θ -composition of the biocompatible solvent system was established for PET via turbidimetric titrations [187]. During this study, the PET-120 min DMSO-based solutions (0.2–1.0 w/v%) were subjected to dropwise water addition, and the turbidity changes were

tracked to determine the Φ_{water} corresponding to the onset of PET precipitation. As shown in **Figure 34B**, the log–log plot of Φ_{water} versus Φ_{PET} yielded an intercept of -0.6065 . This finding revealed that the Θ -solvent for PET is at a Φ_{water} of about 0.247 in water/DMSO binary solvent system, an outcome that aligned well with the results depicted in **Figure 34C**.

In **Figure 34C**, the study focused on elucidating the effect of polyester molecular weight on its precipitation behaviour. The results indicated a reverse correlation between the H₂O needed for precipitation and PET molecular weight. Specifically, the PET polymer with the smallest molecular weight (~ 552 Da) needed the largest volume of H₂O ($\Phi_{water} = 0.394$) to start precipitating. In contrast, the PET polymer with the largest molecular weight (~ 1238 Da) needed the least volume of H₂O ($\Phi_{water} = 0.259$) for the precipitate to appear. These findings were consistent with the widely known influence of polymer chain length on phase separation and solubility [286]. Differences in molecular weight likewise caused clear variations in the maximum turbidity values detected when 10 mL of distilled H₂O was introduced into 10 mL DMSO-based 0.2 w/v% PET solutions. As demonstrated, the turbidity value observed in the case of PET-40 min (~ 552 Da) was 183 NTU, while PET-120 min (~ 1238 Da) showed a much higher value of 716 NTU. This behaviour is useful for monitoring the polyester precipitation and determining the molecular weight [120].

The determined M_n values (**Table 2**) and their associated Φ_{water} data (**Figure 34C**) were used to construct a $1/M_n$ versus Φ_{water} plot (**Figure A21**), from which the A (15.58) and B (12987) coefficients of the Schulz equation (**Equation 17**) were obtained [120,188]. These constants (**Table A10**), which are specific to PET in the DMSO (solvent)–water (precipitant) system, were identified in this study for the first time. Based on Schulz theory, having these coefficients available enables molecular mass determination with a simple precipitation-based method [188]. Consequently, researchers will have the capacity to identify the molecular mass of PET prepared with comparable procedures with ease and reliability.

On the basis of the preceding results, one can say that the specified reaction conditions (reaction temperature of 165 °C, polymerization duration of 40-120 minutes, and an equimolar catalyst-free reaction) yielded moderately hydrophilic PETs with M_n values of 552–1238 Da and M_w values of 908–1652 Da. Even though the molecular weight range was relatively narrow, the PET solubility/precipitation behaviour within the DMSO/water mixture showed significant variation, with Φ_{water} ranging from 0.26 to 0.39. These changes in molecular mass and solubility characteristics can significantly affect fundamental attributes such as drug release kinetics [121], which in turn enables the formulation of tailored controlled-release systems. Moreover, the moderate hydrophilic nature of the prepared PETs can make them suitable for

improving the bioavailability and aqueous stability of MPA. Taken together, these findings highlight the potential of the developed PETs for biomedical use, especially for encapsulating sparingly water-soluble compounds, like MPA. Therefore, the work next examined MPA encapsulation.

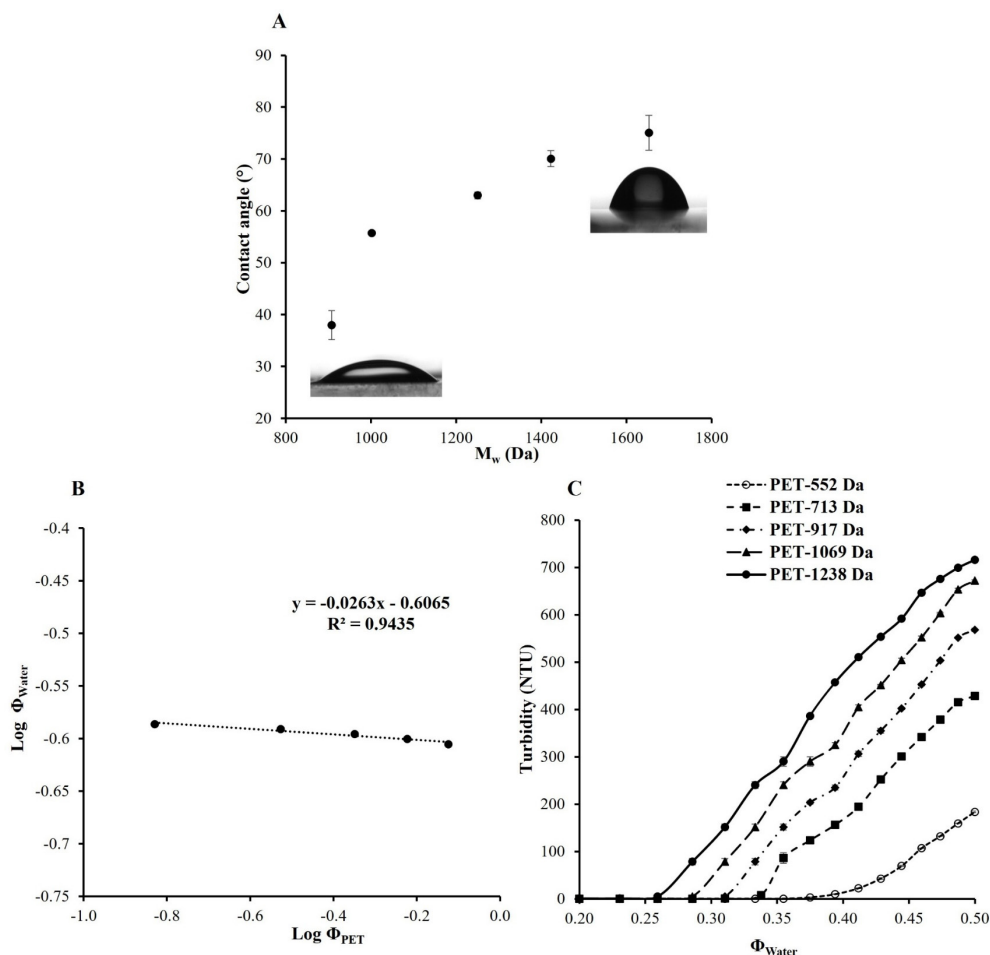


Figure 34. (A) Water contact angle (θ_w) characterization for PET films with various M_w . (B) Turbidimetric titration of the DMSO-based PET solution (polymerization period=120 min, $M_w = 1652$ Da) upon gradual water addition; the plot presents the logarithmic relationship between the volume fraction of water (Φ_{water}) required to induce precipitation and the associated volume fraction of polymer (Φ_{PET}). (C) The precipitation curves of the produced PET polyesters with varying molecular weights. In all cases, measurements were performed on 10 mL 0.2 w/v% DMSO-based PET solutions, precipitated via the dropwise addition of water [278].

5.4.7 Preparation and characterization of MPA-loaded polyester particles

To establish an efficient strategy for preparing drug-loaded PET nanoparticles, the solubility of the MPA drug in the DMSO/water solvent system was examined. First, the dropwise introduction of water (the precipitant) into the separate 0.2 w/v% MPA and 0.1 w/v% PET DMSO solutions was performed, during which the absorbance of each sample was continuously monitored (Figure 35A).

Compared with the MPA sample, which demanded a substantial amount of water for precipitation to begin ($\Phi_{water} = 0.468$), the PET sample exhibited much earlier precipitation, initiating at a Φ_{water} of about 0.265. Moreover, the polyester approached its plateau precipitation level at nearly $\Phi_{water} = 0.5$, a value similar to the water fraction needed to trigger MPA precipitation. According to these observations, the encapsulation of MPA into PET applying this precipitation strategy—where DMSO-based solutions is subjected to water addition—is not advisable, since MPA will begin to precipitate near the completion of PET precipitation.

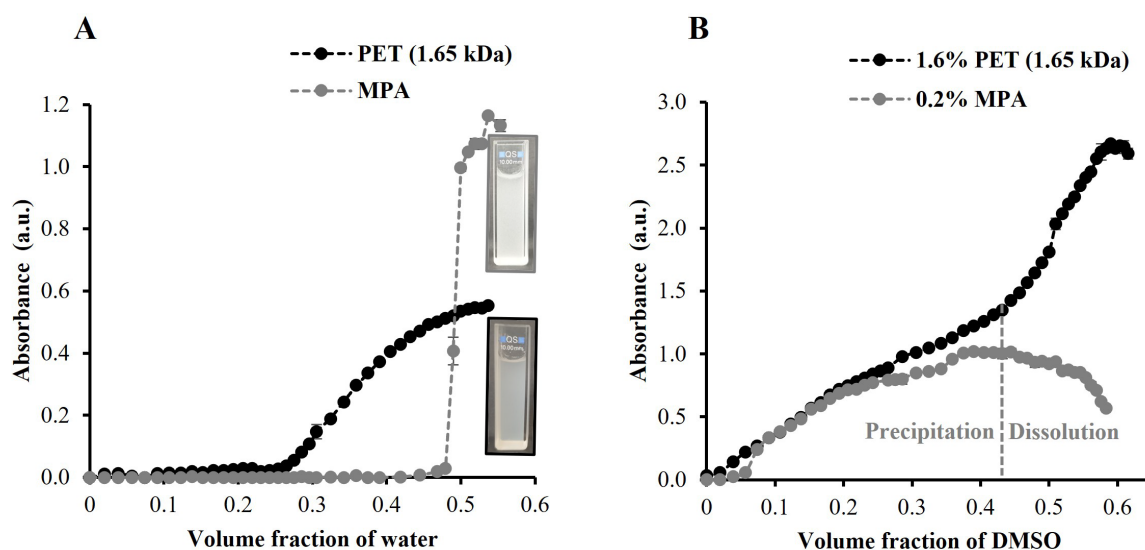


Figure 35. (A) Precipitation curves of the DMSO-based solutions of MPA (0.2 w/v%) and PET (0.1 w/v%) following the gradual introduction of distilled water to them. (B) Precipitation curves of 0.2 w/v% of MPA and 1.6 w/v% of PET-120 min following their gradual incorporation into distilled water [278].

As a result of these findings, a reversed precipitation strategy was implemented, whereby the DMSO-based drug and polymer solutions were introduced dropwise into the aqueous phase while continuous magnetic stirring was applied. In particular, a 1.6 w/v% DMSO-based PET-120 min ($M_w = 1.65$ kDa) solution was gradually added to 2.5 mL of distilled water, and particle formation was tracked through absorbance recording. The DMSO-based MPA solution (0.2 w/v%) was also subjected to the previously described procedure, and the findings were presented in **Figure 35B**. As shown, implementing the process in reverse enabled the simultaneous precipitation of MPA and PET, effectively overcoming the limitation of sequential precipitation. MPA achieved its absorbance plateau at a Φ_{DMSO} of about 0.444. Above this amount, turbidity began to diminish since higher DMSO volume fractions introduced dilution effects that caused the precipitated MPA to redissolve. This indicated that throughout the procedure, the introduction of the DMSO-based solution must be discontinued at

$\Phi_{DMSO} = 0.444$ (grey dashed line in **Figure 35B**) to prevent the redissolution of MPA. This ratio also allowed the synthesis of sufficiently small NPs (as it will be seen later). Taken together, the outcomes suggested that the established strategy was effective for producing MPA-loaded nanoparticles. The detailed procedures employed to prepare both MPA-loaded and unloaded NPs can be seen in the methods section.

The encapsulation of MPA into PET polymers was verified via FTIR analysis (**Figure A22**). The distinctive FTIR peaks for the anti-inflammatory drug, specifically those arising from the stretching vibrations of the alcoholic O–H functionalities (3392 cm^{-1}), CH_2 units (2910 and 2843 cm^{-1}), the C=C moieties ($1654\text{--}1609\text{ cm}^{-1}$), and the C–O linkages in esters and alcohols (1227 cm^{-1}), were all detected in the drug-loaded polyester particles [252,289,290]. Some merging was also observed between the drug and polymer signals; for example, the C=O ester and ketone peaks of MPA ($1748\text{--}1726\text{ cm}^{-1}$) showed overlap with the polymer's C=O ester band (1722 cm^{-1}), and this is most likely due to drug encapsulation within the polymeric matrix [289]. Overall, the analysis verified the loading of the drug into the polyester particles.

UV-Vis spectroscopic analysis also revealed that the drug was successfully encapsulated, with efficiencies in the range of 91–95%. In particular, the average encapsulation efficiencies for MPA in PET polyesters with M_w of 1652 Da, 1422 Da, 1250 Da, 1002 Da, and 908 Da were 95.0%, 95.2%, 94.3%, 94.2%, and 91.2%, respectively. According to these results, the drug content in the polymer particles was about 106.2, 106.3, 105.5, 105.4, and 102.3 mg/g respectively.

The particle size measurements in PBS (pH 7.4, 0.9% NaCl) further supported the encapsulation of MPA. As shown in **Figure 36A**, the hydrodynamic diameters of the drug-loaded particles were higher than those of the corresponding unloaded particles. As an example, the PET-1652 Da polyester yielded NPs with an average DH of about $260 \pm 15\text{ nm}$. With MPA encapsulated, the DH shifted upward to $275 \pm 8\text{ nm}$, indicating successful drug incorporation [60]. Regarding the ζ -potential analysis (**Figure 36B**), no significant difference was detected between the MPA-loaded and the MPA-free particles. For instance, particles produced from the PET-1652 Da polymer showed an average ζ -potential of $-39.7 \pm 3.7\text{ mV}$ in the unloaded state, which was similar to that of the MPA-loaded formulation ($-42.4 \pm 1.6\text{ mV}$). These outcomes suggest that the drug is mostly present in the nanoparticle core. TEM imaging also revealed the spherical morphology of both the polyester particles and the drug-loaded ones, as shown in **Figure 36C** and **36D**, respectively.

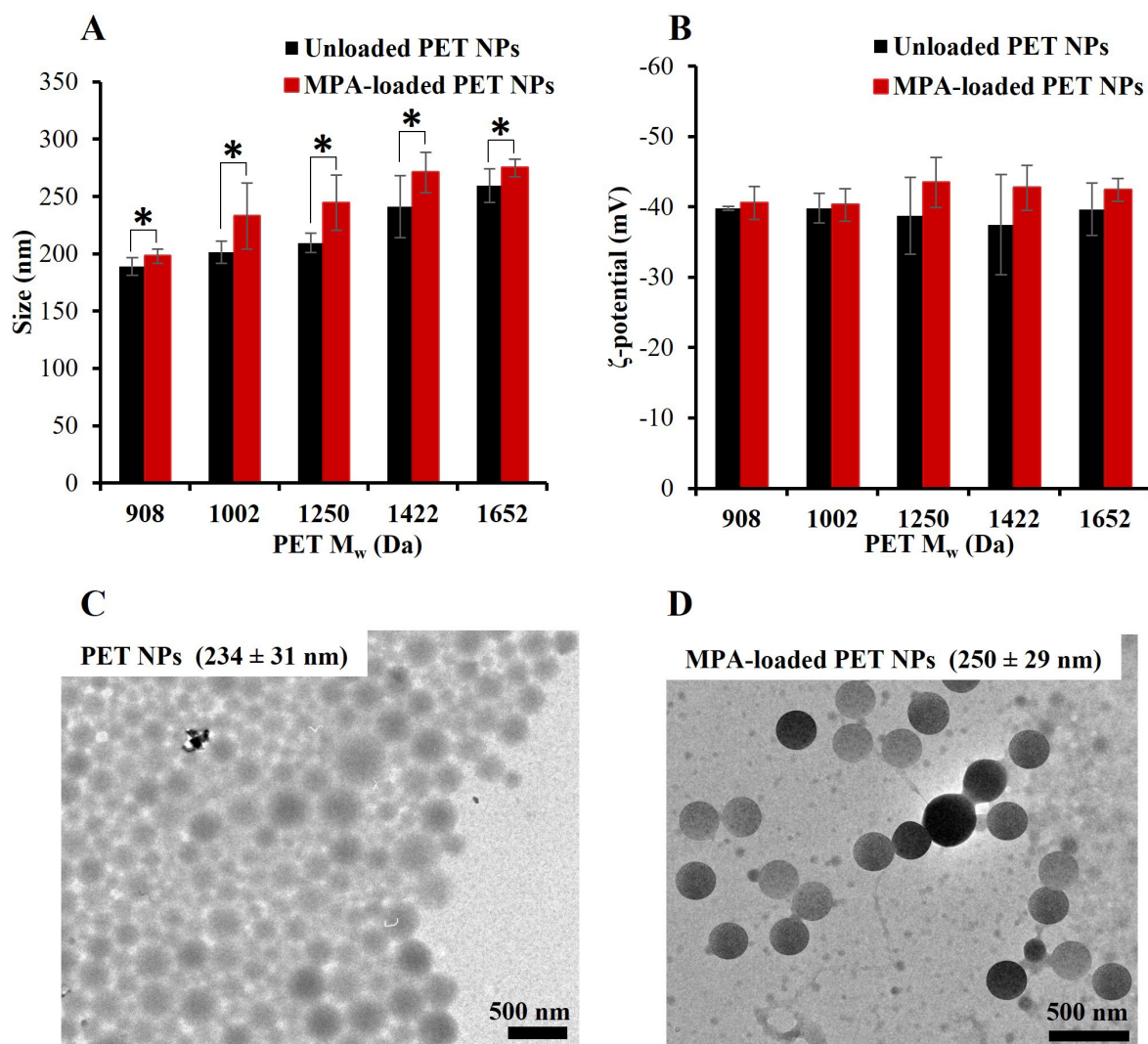


Figure 36. (A) Particle size and (B) ζ -potential values of PET NPs and MPA-loaded PET NPs synthesized from PET polyesters with molecular weights ranging from 908 Da to 1652 Da. Particles were dispersed in PBS (0.9% NaCl, pH 7.4). TEM images of (C) PET NPs and (D) MPA-loaded PET NPs, both synthesized from PET polyester with M_w of 1652 Da. Statistical analysis of the results was performed using a two-sample t-test. * $p < 0.05$ [278].

To examine crystallinity changes that may arise as a consequence of drug encapsulation, XRD spectra were registered for the free MPA and the nanoparticle-incorporated MPA [121,278]. As can be seen, in comparison with the diffraction pattern of free MPA (**Figure 37**), the encapsulated form presented noticeably diminished peak intensities, reflecting a reduction in the MPA's crystallinity [121]. These observations suggested that the drug encapsulation process promoted a shift toward a more amorphous form. This can be especially beneficial for poorly water-soluble drug compounds such as MPA, because—as mentioned earlier—the transition from a crystalline to a more amorphous form can enhance solubility and promote faster dissolution, which can in turn increase aqueous stability, optimize release characteristics, improve bioavailability, and strengthen overall therapeutic efficacy [108,121].

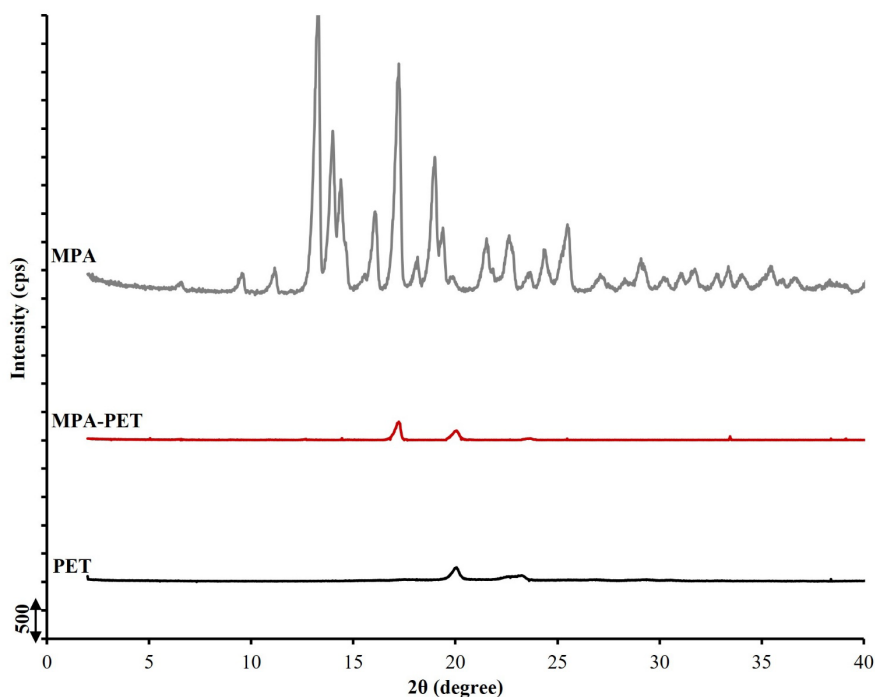


Figure 37. XRD spectra of bare MPA, PET (M_w of 1422 Da), and MPA-loaded PET NPs (11 w/w% MPA content).

Considering these outcomes, it became necessary to assess the stability of both free MPA and its nanoparticle-loaded counterparts in aqueous PBS (pH 7.4, 0.9% NaCl) to determine how encapsulation and the accompanying reduction in crystallinity affect the drug's behaviour in aqueous media. As illustrated in **Figure 38A** and **38B**, the presence of PET polymer shells markedly boosted the MPA's aqueous dispersibility. According to turbidity analyses, the MPA-loaded PET particles remained highly stable over time, showing only modest reductions in relative turbidity (~10% reduction for the formulation synthesized using PET-908 Da and 8% reduction for the formulation synthesized using PET-1652 Da after the 8 h measurement period). On the other hand, the uncoated MPA showed a sharp decline in turbidity of almost 91%, underscoring its poor stability in aqueous media, which is consistent with its low water solubility and hydrophobic character [164]. In addition, the low ζ -potential value measured for the uncoated drug at pH 7.4 (-5.6 ± 8.1 mV) may also be a contributing factor to the observed rapid sedimentation, as such weak surface charge provides insufficient electrostatic repulsion to maintain a stable dispersion [276]. In contrast, the good dispersibility of the MPA-loaded particles can be related to their small particle size (**Figure 36A**), high ζ -potential values (**Figure 36B**), and reduced crystallinity (**Figure 37**).

The stability of the drug-loaded polymeric particles was further assessed after 24 h of dispersion in PBS (pH 7.4, 0.9% NaCl), and both the particle size and ζ -potential showed no significant changes, confirming their colloidal stability. Based on these results, encapsulating MPA within

the PET particles represents a promising strategy for overcoming the drug's poor aqueous solubility and instability, thereby supporting the development of stable aqueous oral formulation of MPA. Because of the improved solubility and dispersibility of the drug, the developed formulations hold considerable potential for improving bioavailability and therapeutic efficacy, addressing long-standing challenges [153,278].

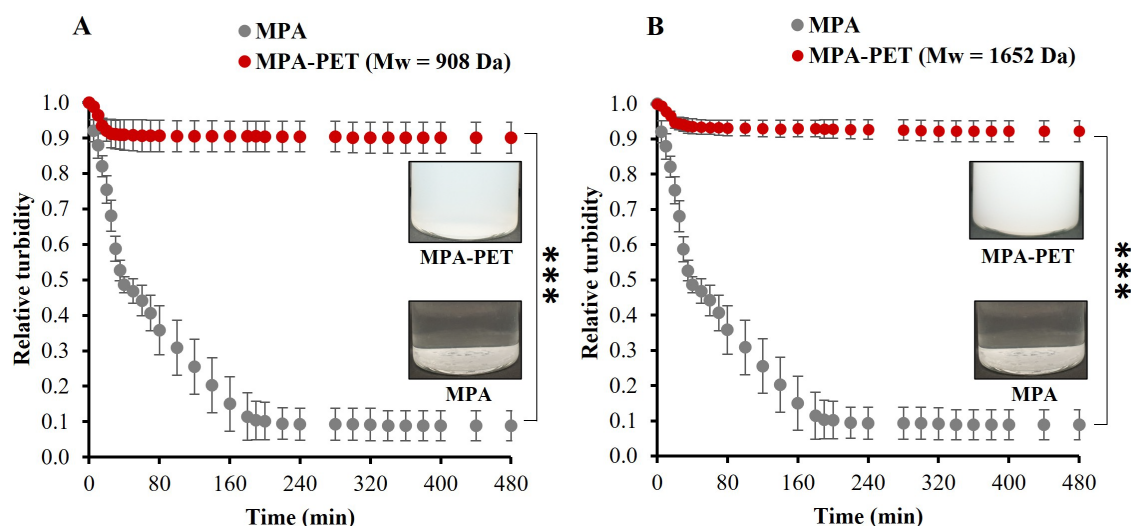


Figure 38. (A) Change in turbidity values over time for bare MPA and MPA-loaded PET NPs synthesized from PET polyester with M_w of 908 Da. (B) Turbidity variation over time for MPA-loaded PET NPs synthesized from PET polyester with M_w of 1652 Da. All samples were dispersed in 10 mL aqueous PBS (pH 7.4, 0.9% NaCl). MPA and PET concentrations in the nanoparticle dispersions were 0.01 w/v% and 0.09 w/v%, respectively. The bare MPA dispersion contained MPA at 0.01 w/v%. Statistical analysis of the results was performed using a two-sample t-test. *** $p < 0.001$ [278].

5.4.8 In vitro drug release study

The release of MPA from the bare drug sample and from the PET formulations with various molecular weights was evaluated at pH 7.4, and the resulting release profiles are depicted in **Figure 39**. As shown, in comparison with the bare drug, which achieved only 30.0% release after 8 hours at 37 °C, the drug-loaded PET-40 min ($M_w = 908$ Da) and PET-60 min ($M_w = 1002$ Da) formulations demonstrated markedly greater release percentages of 53.0% and 41.9%, respectively. This enhanced drug release is attributable to the lower crystallinity and the reduced particle size of the drug incorporated into the low molecular mass PETs [121,251]. These features considerably enhanced the drug's solubility and diffusion, resulting in better release behaviour and higher bioavailability compared with the crystalline bare drug.

The study also revealed that variations in PET molecular weight strongly influenced the release behaviour of MPA (**Figure 39B**). For instance, compared with PET-908 Da and PET-1002 Da, the higher molecular weight PETs (PET-1652 Da and PET-1422 Da) showed more prolonged

drug release behaviour, reaching 16.5% and 23.0% release after 8 h, respectively. This observation is consistent with earlier studies that showed how increases in polymer M_W and chain length can extend drug-release kinetics [121].

The release profiles were further analyzed with several kinetic models to gain insight into the underlying release mechanisms. **Table A11** showed that the release profile of free MPA was best described by the Higuchi model, indicating diffusion-controlled release [121,185]. The MPA release data obtained from both MPA-PET (40 min, $M_W = 908$ Da) and MPA-PET (60 min, $M_W = 1002$ Da) were best described by the first-order model. This indicated that the drug-release rate is concentration-dependent, meaning that the greater the drug concentration in the system, the quicker the release process will be [226]. The effect of the polyester M_W was also evident, as MPA-loaded PET-60 min (1002 Da) exhibited a lower release rate constant than MPA-loaded PET-40 min (908 Da). The MPA release data obtained from both MPA-PET (100 min, $M_W = 1422$ Da) and MPA-PET (120 min, $M_W = 1652$ Da) were best described by the Korsmeyer-Peppas model. Moreover, the obtained diffusional exponent values ($n = 0.61$ – 0.76) indicated that the release mechanism follows anomalous (non-Fickian) diffusion [88]. This suggested that both diffusion and erosion contribute to governing the drug-release process, ultimately leading to a reduced amount of drug released and a prolonged release duration.

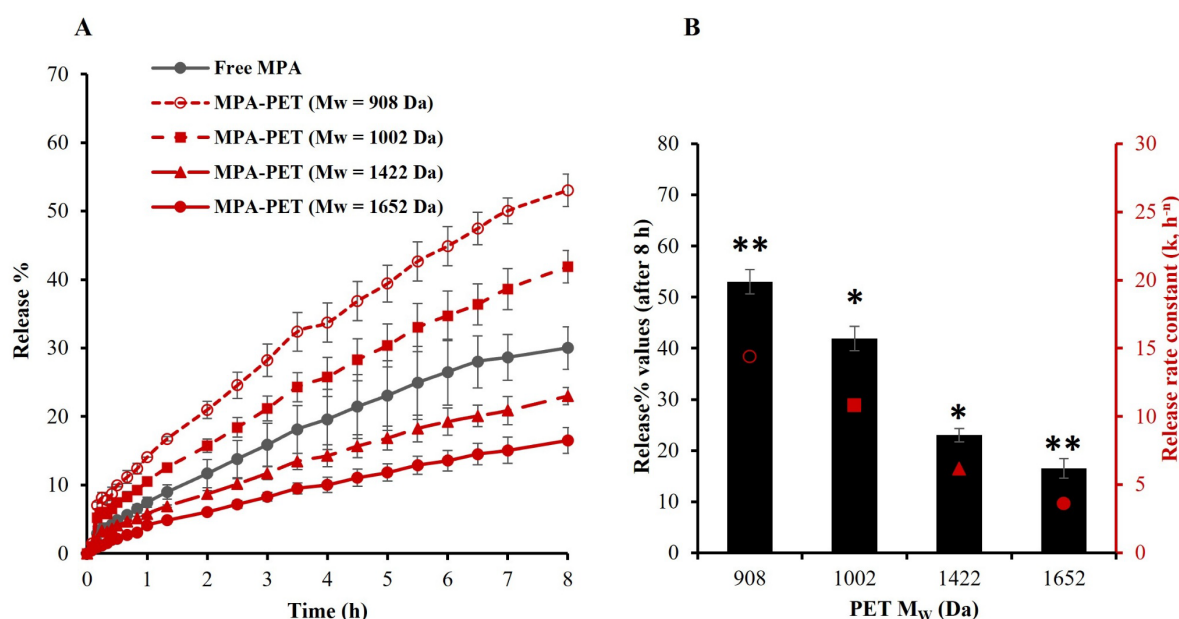


Figure 39. (A) In vitro release profiles of free MPA and MPA encapsulated within PET with varying molecular weights. (B) Release% values (after 8h) and k values as a function of M_W to prove that the PET can accelerate and prolong the drug release, depending on its molecular weight. Statistical analysis was performed using a two-sample t-test, with free MPA serving as the control. * $p < 0.05$; ** $p < 0.01$ [278].

Overall, the outcomes demonstrated that while higher M_W PETs were effective in prolonging the release, the relatively lower M_W PETs were able to enhance the release. These conclusions were further supported by the dissolution efficiency (DE) and mean dissolution time (MDT) values listed in **Table A12**.

5.4.9 Mucoadhesive characteristics of the developed PET NPs

An important goal of the work was to design a mucoadhesive carrier that could potentially improve the systemic and local bioavailability of MPA. Therefore, examining the mucoadhesivity of the resulting PET particles was an important step. The pendant thiol functionalities ensured that the prepared NPs possessed the requisite mucoadhesive properties. Using Volhard's analytical procedure [182], the thiol contents were determined as 3.21, 3.20, 3.10, 2.95, and 2.38 mmol/g for PET samples with M_W s of 1652, 1422, 1250, 1002, and 908 Da, respectively.

Rheological analysis was employed to verify the mucoadhesive features of the produced polymer particles, as combining the aqueous dispersion of mucin with that of PET particles (synthesized using PET-1652 Da or PET-908 Da) gave rise to a cross-linked, viscous hydrogel (**Figure 40**).

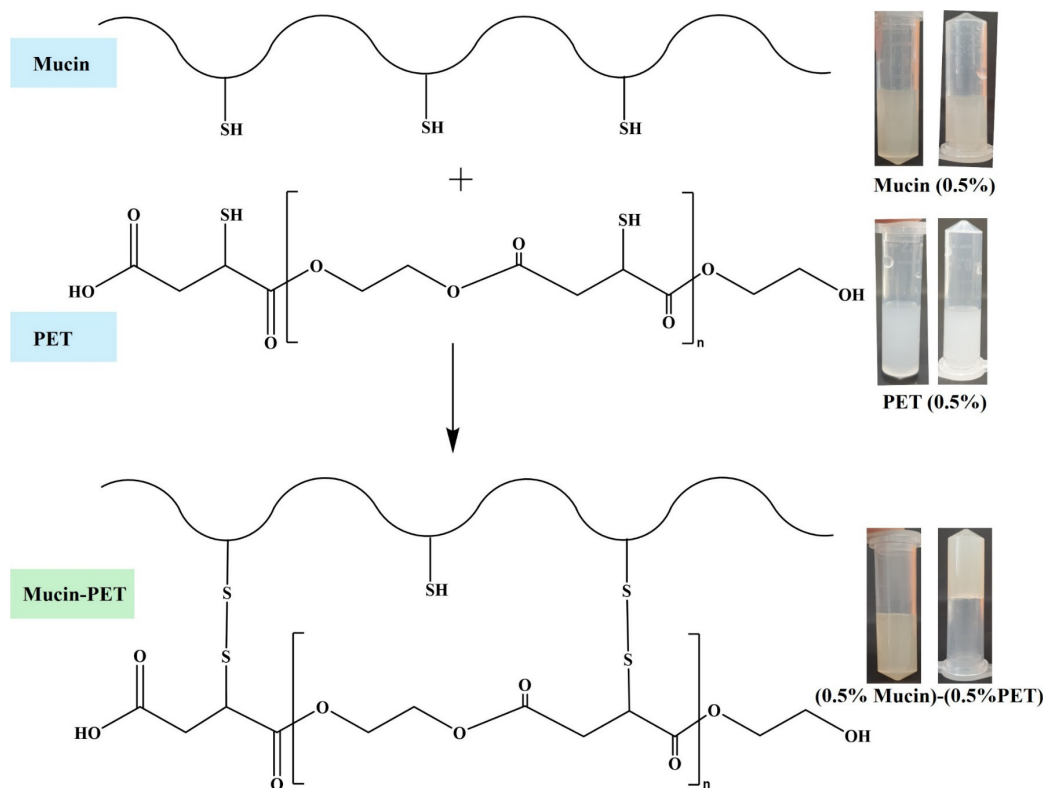


Figure 40. Schematic representation of Mucin crosslinking with PET via disulfide bond formation. The inserted images illustrate the hydrogel formation from the aqueous dispersions of the mucin and PET (1652 Da) [278].

The outcomes shown in **Table 3** and **Figure A23** provide valuable information about the storage modulus (G'), which reflects the material's elastic behaviour; the loss modulus (G''), which reflects its viscous characteristics; and the complex modulus (G^*), which summarizes the overall mechanical performance, accounting for both viscosity and elasticity [88]. As demonstrated, both Mucin-PET 1652 Da and Mucin-PET 908 Da showed a clear predominance of the G' over the G'' , and this validated the crosslinked hydrogel formation, as gels generally show solid-like characteristics when G' is higher than G'' [291]. Moreover, the presented G'/G'' values further reinforce the mechanical robustness of the resulting hydrogels, with the higher ratio observed for Mucin-PET 1652 Da reflecting enhanced gel integrity and mechanical strength [88,291].

The formation of these hydrogels, together with their ability to preserve their viscous appearance for more than 6 months, offered clear evidence for the strong associative interactions between the mucin and the polymer particles, thereby validating their mucoadhesive functionality [88,278]. These hydrogel networks were stabilized through hydrogen bonding and disulfide linkages (**Figure 40**). As indicated in the literature, thiomers can form disulfide bonds with mucin primarily through thiol–disulfide exchange mechanism. Thiol–disulfide exchange reactions between thiomers and mucosal surfaces are widely discussed in the literature and are commonly supported, as in our work, by rheological measurements, since these interactions induce strong gelation [3,88,292,293].

Table 3. Storage modulus (G'), loss modulus (G'') and complex modulus (G^*) data for mucin, PET (908 Da and 1652 Da), and their mixtures [278].

Sample	G' (Pa)	G'' (Pa)	G^* (Pa)	G'/G''
0.5% Mucin	0.00005	0.96306	0.96311	0.00005
0.5% PET 908 Da	0.00002	0.48988	0.48990	0.00005
0.5% Mucin-PET 908 Da	61.8130***	6.18134	67.9944	9.9999
0.5% Mucin	0.00005	0.96306	0.96311	0.00005
0.5% PET 1652 Da	0.00002	0.48680	0.48682	0.00005
0.5% Mucin-PET 1652 Da	288.066***	22.1413	310.207	13.0103

*Statistical analysis was performed using t-test to assess significant increases in G' with respect to G'' across sample groups. *** $p < 0.001$.*

Surface adsorption experiments also confirmed the Mucin-PET interaction. In these experiments, pig intestinal membranes were employed as model mucosal surfaces and immersed in slowly agitated buffered aqueous dispersions of the polyester particles (0.025 wt/v%, pH 7.4). The reduction in turbidity, attributable to particle adsorption, was followed over time. To isolate the adsorption effect, the measurement was performed without submerging the mucosal membrane (**Figure 41**). As seen, surface adsorption of PET-908 Da

particles (**Figure 41A**) and PET-1652 Da particles (**Figure 41B**) progressed continuously, leading to turbidity decreases of 64.7% and 73.4% within 7 hours, respectively. Meanwhile, the control experiments exhibited only 4.6% and 4.0% turbidity loss, respectively, demonstrating that the pronounced turbidity reductions in the membrane-containing samples were driven by mucoadhesion of the PET particles rather than passive effects. Furthermore, the turbidity reductions noted in the membrane-immersed dispersions after 7 hours remained almost unchanged after 24 hours, demonstrating the permanence of the formed surface-bonding state. Taken together, these findings confirmed that the developed polyester NPs possess strong mucoadhesive properties. Such mucoadhesive behaviour can potentially prolong NP residence within the intestinal mucus layer, thereby supporting improved local and systemic MPA bioavailability [115,167].

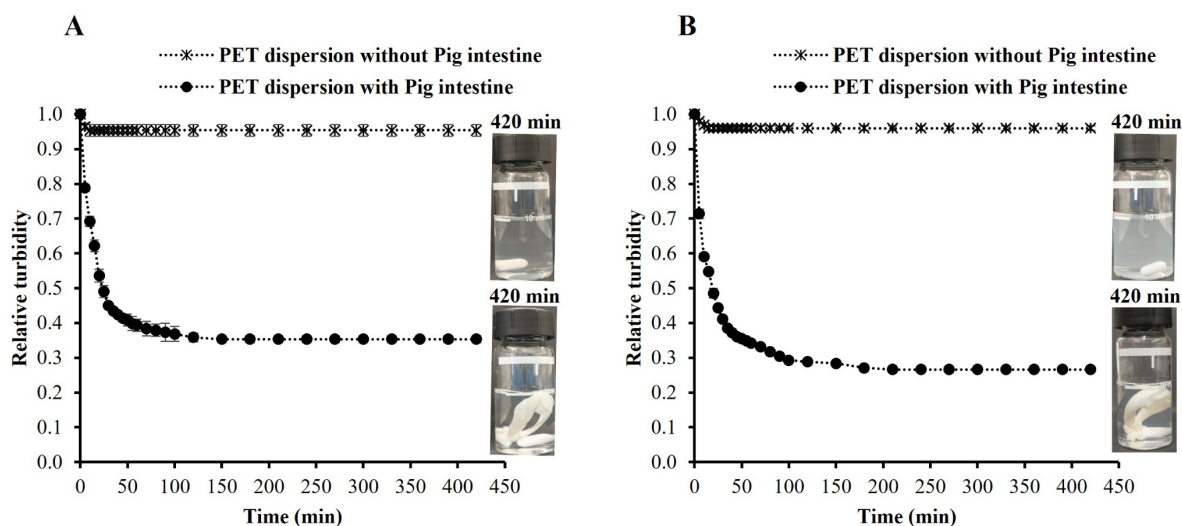


Figure 41. Surface adhesion measurements of PET particles on pig intestinal membranes: Variations in relative turbidity of 0.025 w/v% aqueous buffered dispersions of (A) PET-908 Da NPs and (B) PET-1652 Da NPs, attributed to particle adhesion on the surface of an 8 cm pig intestinal membrane immersed in the dispersions. The inset images on the right illustrate the aqueous buffered particle dispersion following measurement completion [278].

5.4.10 Cytotoxicity of PET particles

To assess carrier biocompatibility, the cytotoxicity of the prepared catalyst-free polymer particles was examined in MRC-5 cells over a final concentration range of 1–150 $\mu\text{g/mL}$. According to **Figure A24**, cell viability stayed consistently high (>90%) across the entire concentration range, with no significant dose-dependent decrease detected. These results demonstrate that the catalyst-free PET NPs didn't produce high cytotoxic responses, indicating good cytocompatibility within the examined concentration interval.

In this part, we aimed to develop a mucoadhesive polymeric nanocarrier system for MPA to enhance its aqueous stability and dissolution, prolong its gastrointestinal residence time, and thus support its potential for enhanced oral bioavailability. As a summary, we can state that the produced PET (M_w 908–1652 Da) is appropriate for encapsulating MPA (encapsulation efficiency = 91–95%) via nanoprecipitation approach. Compared with bare MPA, the MPA-loaded particles showed significantly improved dispersibility and stability in aqueous medium (~8–10% reduction in relative turbidity), attributable to their high ζ -potential (~40 mV) and relatively small sizes (198–275 nm). Moreover, the encapsulated MPA's release behaviour could be tuned based on the polymer M_w , allowing for either sustained release or enhanced dissolution in comparison to the bare drug. Furthermore, the presence of the thiol groups (2.38–3.21 mmol/g) provided mucoadhesive characteristics for the NPs as confirmed by adsorption (~65–73%) and rheological (G'/G'' ratio $\approx$ 10–13) measurements, and these characteristics are expected to prolong NP residence at the absorption site and in turn support enhanced local and systemic MPA bioavailability. Overall, the newly developed MPA-loaded PET particles exhibit several promising features—such as nanoscale dimensions, good aqueous stability, enhanced or sustained drug dissolution, and strong mucoadhesive properties—that support the development of stable aqueous oral MPA formulations and hold potential for improved oral delivery and bioavailability of MPA through enhanced dissolution and mucoadhesion.

6. Summary

Compared to conventional drug-delivery methods, polymer-based controlled delivery platforms offer multiple advantages, including reduced toxicity, increased stability, improved bioavailability, controlled and sustained drug release, and more precise site-specific therapeutic targeting. Throughout my doctoral research, novel polymer-based drug delivery systems were developed to potentially overcome critical barriers associated with different pharmacological agents, including the hydrophilic drug Mitomycin C (MMC) and the hydrophobic compounds Nimodipine (NIMO), HC-067047 (HC), and Methylprednisolone Acetate (MPA). Specifically, four distinct polymeric platforms—each exhibiting unique structural and functional features—were constructed by synthesizing or modifying biocompatible polymers to enhance therapeutic applicability and overcome limitations inherent to conventional drug-delivery approaches. The overall summary of these works is outlined below:

During the first part of the work, to address the harsh side effects and rapid clearance associated with MMC, I developed multifunctional, pH-responsive PVA-based NPs capable of encapsulating MMC, prolonging its release, enabling preferential drug release under acidic tumor-like conditions, while also providing fluorescence-based imaging. Through a straightforward reaction, a partially succinylated PVA (PVA-SA) containing both OH (13.3 mmol/g) and COOH (2.5 mmol/g) functional groups was synthesized to enable pH-sensitive amide bonding with MMC, support interactions with the fluorescent RB, and promote spontaneous nanoparticle formation. The coexistence of the abovementioned functional groups in the resulting polymer enabled ester bond formation upon EDC activation, driving spontaneous self-assembly into ~12 nm particles within 15 minutes, as validated by turbidimetric assessment and TEM analysis. Prior to MMC encapsulation, RB-loaded PVA-SA particles were synthesized using various nominal RB concentrations (0.5–6.0 wt% relative to PVA-SA) via a one-pot approach in order to evaluate dye-loading feasibility and to identify formulations with favourable physicochemical properties relevant to cancer-oriented applications. Increasing the initially added dye amount led to a marked rise in binding efficiency (27.35% → 40.37%) and loading percentage (0.13% → 2.42%), confirming the capacity of the polymer particles to incorporate different amounts of RB. Nevertheless, because particles <200 nm are generally preferred for effective cancer treatment [213–215], the (0.5% RB)-PVA-SA NPs and (1.5% RB)-PVA-SA NPs were selected for subsequent investigations. These NPs enhanced the photophysical performance of RB by increasing its quantum yield from 29.8 % to ≥ 42.7 %, maintaining emission stability under continuous excitation at 554 nm for 2 h, and increasing its fluorescence lifetime with minimum increases of 2.4-fold in τ_1 and 1.7-fold in τ_2 . Two amounts of MMC (4 mg and 12 mg) were then applied

to synthesize the MMC-loaded fluorescent NPs (DH: ~118–194 nm; ζ -potential: ~–24 to –58 mV) in a single-pot process. Conjugating MMC through amide bond formation markedly prolonged its *in vitro* release; whereas bare MMC reached ~90% cumulative release within ≤ 30 h, the conjugated formulations (~10% drug loading) required 7–9 days to reach their highest measured release (~20–79%), owing to the slow hydrolysis of the amide bonds. The linkages further enabled clear pH-responsive behaviour, yielding higher MMC release under acidic conditions relative to neutral ones. After 9 days, the ((0.5% RB)-PVA-SA)-MMC and ((1.5% RB)-PVA-SA)-MMC NPs released approximately 79% and 62% MMC at pH 5.8, compared to ~50% and ~20% at pH 7.4. The MMC-loaded NPs also exhibited potent toxicity under tumor-like acidic conditions (IC_{50} = 0.6–0.7 μ g/mL MMC at pH 5.8) but markedly reduced activity at physiological pH (IC_{50} = 6.5–18.4 μ g/mL MMC at pH 7.4), unlike free MMC, which displayed nearly identical IC_{50} values at both pH levels (0.5 μ g/mL at pH 5.8 and 0.6 μ g/mL at pH 7.4). *In vitro* cellular uptake experiments further demonstrated that the NPs were internalized by cancer cells and accumulated in the perinuclear and cytoplasmic areas, while even at the lower nominal RB loading (0.5 wt%), the NPs emitted intense red fluorescence, enabling high-contrast imaging and highlighting their potential as dual-function, imaging-guided therapeutics. Overall, these MMC-loaded fluorescent NPs, with their ability to self-assemble, their favourable nanoscale size for prolonged circulation, their sustained pH-responsive drug-release behaviour, their potent cytotoxicity under tumour-like acidic conditions compared to physiological conditions, and their clear fluorescence emission with efficient cellular uptake, offer a versatile platform for controlled MMC release with integrated bioimaging capability, which could be helpful in supporting safer and more efficient cancer-related therapy.

In the second part, to further reduce off-target exposure and side effects associated with MMC, I developed magnetically responsive, MMC-loaded polymeric NPs to support field-guided accumulation of drug-loaded carriers at tumour sites. The previously developed PVA-SA polymer (containing OH (13.3 mmol/g) and COOH (2.5 mmol/g) functional groups) was used as the matrix for conjugating MMC and MNPs. MNPs-PVA-SA NPs were first prepared at varying MNP loading (5–20 wt%) to determine the maximum magnetic content that could be incorporated while maintaining suitably small particle size and colloidal stability. The results indicated that 10 wt% is the maximum amount of MNPs that can be used to synthesize MNPs-PVA-SA NPs while still maintaining uniform particle size distribution (DH: ~100–110 nm, PDI: ~0.124–0.126) and acceptable colloidal stability (>90%) at pH 7.4 over time. MMC-loaded magnetic composite NPs were then synthesized using this MNP composition via a one-pot synthesis approach, achieving ~98% MMC binding efficiency and exhibiting a relatively small

average particle size (DH: ~128 nm at pH 7.4 and ~134 nm at pH 5.8) with stable size characteristics over 28 days. The *in vitro* release studies showed that the NPs possessed the capability to provide sustained and pH-responsive MMC release. Bare MMC reached plateau levels of ~90% at both pH 7.4 and pH 5.8 within ≤ 30 h, whereas the MMC-loaded formulation (MNPs–PVA–SA–MMC; ~10 wt% MNPs, ~3 wt% MMC) released the drug more gradually, reaching ~50% at pH 7.4 and 80% at pH 5.8 after nearly 7 days. More importantly, the synthesized magnetic particles demonstrated strong potential for site-specific, magnet-assisted drug delivery. This was supported by the NPs' rapid migration in aqueous medium toward an external magnetic field (0.35 T) with visible accumulation within seconds, their magnetic collection efficiencies after 60 s of magnet exposure (49% for MNPs–PVA–SA and 46% for MNPs–PVA–SA–MMC), the preservation of magnetic performance after 28 days of storage, and the measured saturation magnetization values (8.649 emu/g for MNPs–PVA–SA and 8.541 emu/g for MNPs–PVA–SA–MMC), which are adequate for magnetic-targeting applications [246–248]. Overall, these findings highlight the potential of the developed composite NPs as a magnetically guidable platform for spatially controlled MMC delivery in cancer therapy.

In the third part, I developed a hydrophobically modified chitosan–Pluronic nanocarrier to improve the aqueous dispersibility of NIMO and HC and to enable pH-responsive, sustained drug release in ischemic brain regions (pH 6.5–6.0). The hydrophobic modification of Chit with aliphatic aldehydes altered its hydration and progressively lowered the cut-off pH for NP formation—from 7.45 (native Chit) to 6.74 (C₆-chit) and 2.49 (C₁₂-chit)—thereby identifying C₆-chit as the most suitable polymer derivative for releasing drugs more efficiently in ischemic regions than in normal tissues. The optimal concentration range for C₆-chit NP formation via the desolvation method was determined to be 0.2–0.6 wt/v%, as it produced relatively small NPs (DH = 161–163 nm) with narrow size distributions (PDI $\approx$ 0.3) and sufficient surface charge (ζ -potential = 24.1–25.1 mV). Moreover, using ethanol as the precipitant at a volume fraction of 0.29 v/v and TPP ionic crosslinker at 10 wt/wt% relative to C₆-chit was most effective for producing uniform and colloiddally stable particles. NIMO and HC were each efficiently encapsulated (encapsulation efficiency $\approx$ 95–97%, loading $\approx$ 8–10%) into DH $\leq$ 280 nm PF–C₆-chit particles (PF content: 0–25 wt/wt% relative to C₆-chit) via a simple encapsulation process. XRD investigations showed that the polymeric nanocapsules had decreased drug crystallinity, which contributed to the improved aqueous solubility and stability of drugs. The NIMO- and HC-loaded particles (ζ -potential = ~23–25 mV at pH 7.4) demonstrated high colloidal stability in aqueous medium, with turbidity decreasing by a maximum of 3% for NIMO and 5% for HC over 8 hours, compared to 83% for free NIMO

(ζ -potential = -1.0 ± 5.5 mV at pH 7.4) and 81% for free HC (ζ -potential = 0.7 ± 1.7 mV at pH 7.4). The drug-loaded particles also maintained stable size and ζ -potential over time, potentially enabling their use intravenously without the need for organic solvents. The *in vitro* drug-release studies further demonstrated the pH-responsive nature of C₆-chit particles. At pH 7.4, the C₆-chit NPs released only 2.5% of NIMO and 2.4% of HC within 8 hours, which is mostly due to the closed, undissolved state of the NPs at this pH. In contrast, at pH 6.0, drug release increased markedly (47% for NIMO and 98% for HC), and this increase can be explained by the enhanced dissolution of the C₆-chit coatings at pH 6.0. This feature can help minimize premature drug leakage in healthy tissues and enable pH-triggered release under ischemic conditions. The studies also showed that increasing the PF content from 0 to 25 wt/wt% relative to C₆-chit slowed the release process, supporting more sustained drug release under ischemic conditions. Overall, these drug formulations, with their good aqueous dispersibility and stability, pH-sensitivity, and enhanced or sustained drug release under ischemic pH conditions, offer a promising platform for intravenous administration and for the controlled release of both NIMO and HC.

In the fourth part, I developed a mucoadhesive polymeric nanocarrier system for MPA to overcome its low water solubility, improve its aqueous stability and dissolution, prolong its gastrointestinal residence time, and thereby support enhanced oral bioavailability and the development of stable, safe, and effective oral MPA formulations. To achieve this, a biodegradable thiolated polyester, poly(ethylene thiomalate) (PET), was synthesized and its solubility/precipitation characteristics were examined. PET was prepared via a direct polycondensation approach in which equivalent moles of the diol (EG) and the dicarboxylic acid (TA) were reacted under catalyst-free conditions at an optimal polycondensation temperature of 165 °C. These conditions yielded relatively low molecular weight PETs (M_n = 552–1238 Da; M_w = 908–1652 Da), and the molecular weight was tailored by systematically varying the polycondensation time (40–120 min). Our solubility studies revealed an inverse correlation between PET molecular weight and its solubility in the DMSO/water system. From the obtained data, the Schulz equation coefficients ($A = 15.58$ and $B = 12987$) were calculated, enabling direct determination of PET molecular weight through a straightforward precipitation-based method. Increasing molecular weight also enhanced thermal stability ($T_m = 62$ – 86 °C) and hydrophobicity ($\theta_w = 38$ – 75°) of the samples, yielding materials with moderately hydrophilic character suitable for encapsulating sparingly water-soluble drugs. MPA was efficiently encapsulated into these PET polyesters via nanoprecipitation approach (encapsulation efficiency ≈ 91 – 95% , loading ≈ 10 – 11%). XRD investigations showed that the encapsulation process had decreased the crystallinity of MPA, which supported its improved

aqueous solubility and stability. The MPA-loaded particles (DH $\approx$ 198-275 nm; ζ -potential around -40 mV at pH 7.4) demonstrated high colloidal stability in aqueous medium, with turbidity decreasing by approximately 8–10%, compared to a 91% decrease for free MPA (ζ -potential = -5.6 ± 8.1 mV at pH 7.4). Based on these results, encapsulating MPA within the PET particles represents a promising strategy for overcoming the drug's poor aqueous solubility and instability, thereby supporting the development of stable oral preparations. The *in vitro* drug release studies confirmed that the dissolution of MPA can be either enhanced or prolonged depending on the M_W of the PET carrier. Lower- M_W PET polyesters (908 Da and 1002 Da) facilitated a faster drug-release process ($k_1 = 0.090$ h $^{-1}$ and 0.064 h $^{-1}$; cumulative release of 53.0% and 41.9% after 8 h) compared to bare MPA ($k_1 = 0.046$ h $^{-1}$; 30.0% after 8 h), due to reduced crystallinity and smaller particle dimensions. In contrast, higher- M_W PET variants (1422 Da and 1652 Da) significantly slowed the drug-release process ($k_1 = 0.030$ h $^{-1}$ and 0.023 h $^{-1}$), yielding lower cumulative release values (23.0% and 16.5% after 8 h). Together, these results support extended therapeutic action and improved oral bioavailability. The presence of the thiol groups (2.38–3.21 mmol/g) also provided mucoadhesive characteristics for the NPs as confirmed by adsorption ($\sim$ 65-73%) and rheological (G'/G'' ratio $\approx$ 10-13) measurements, and these characteristics are expected to prolong NP residence at the absorption site and in turn support enhanced MPA bioavailability. Overall, the newly developed MPA-loaded PET particles exhibit several promising features—such as nanoscale dimensions, good aqueous stability, enhanced or sustained drug dissolution, and strong mucoadhesive properties—that support improved oral delivery and bioavailability of MPA through enhanced dissolution, controlled release, and mucoadhesion.

7. References

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8. Appendices

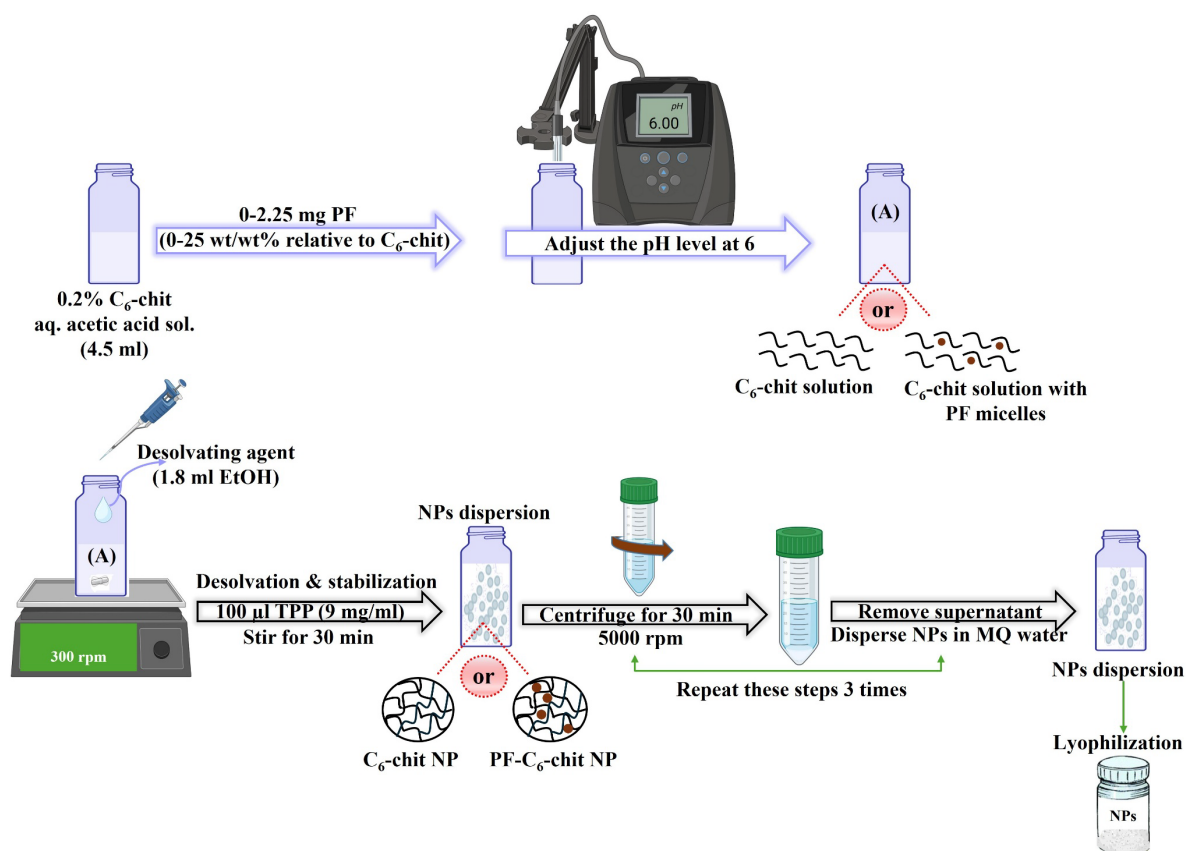


Figure A1. Schematic illustration of the synthesis process for the C₆-chit and PF-C₆-chit NPs [251].

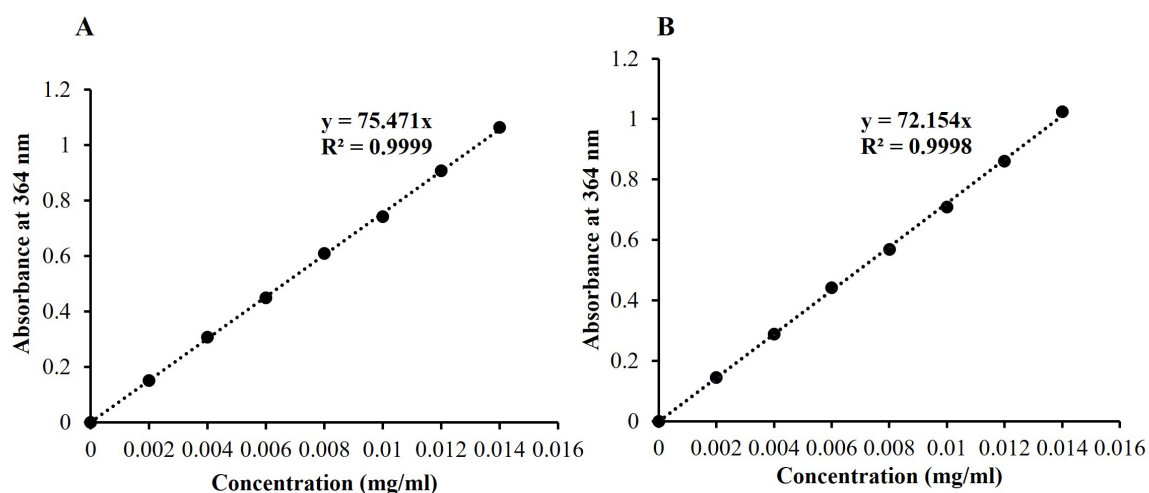


Figure A2. Calibration plot for MMC in PBS medium containing 0.9% NaCl at pH (A) 7.4 and (B) 5.8 [195].

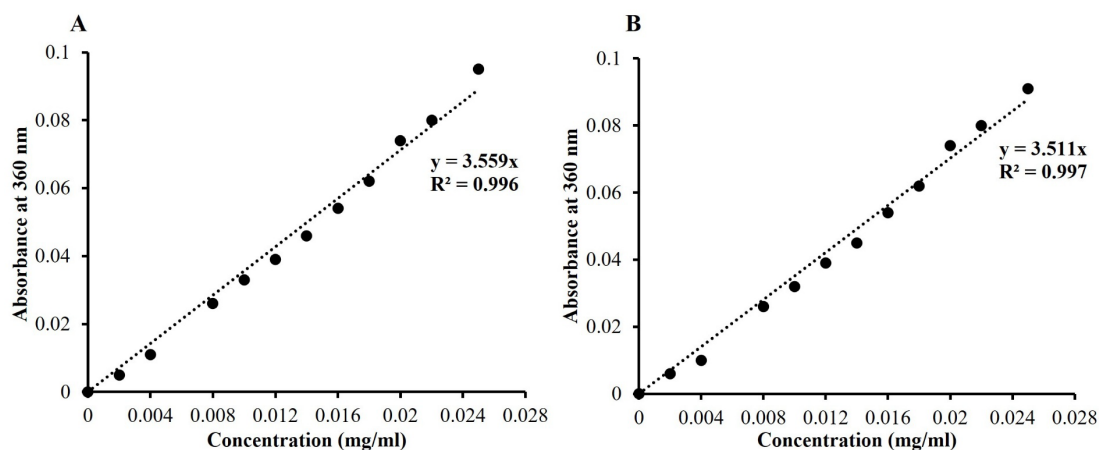


Figure A3. Calibration plot for NIMO in PBS medium containing 0.5% SDS and 0.9% NaCl at pH (A) 7.4 and (B) 6.0 [251].

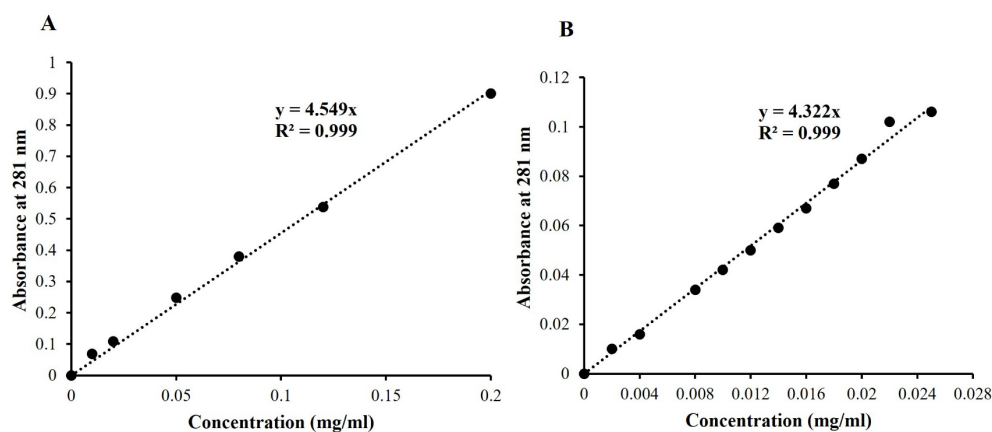


Figure A4. Calibration plot for HC in PBS medium containing 0.5% SDS and 0.9% NaCl at pH (A) 7.4 and (B) 6.0 [251].

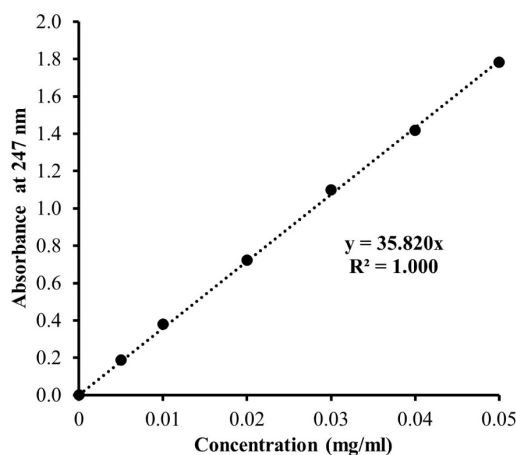


Figure A5. Calibration curve for MPA in PBS medium containing 0.5% SDS and 0.9% NaCl at pH 7.4 [278].

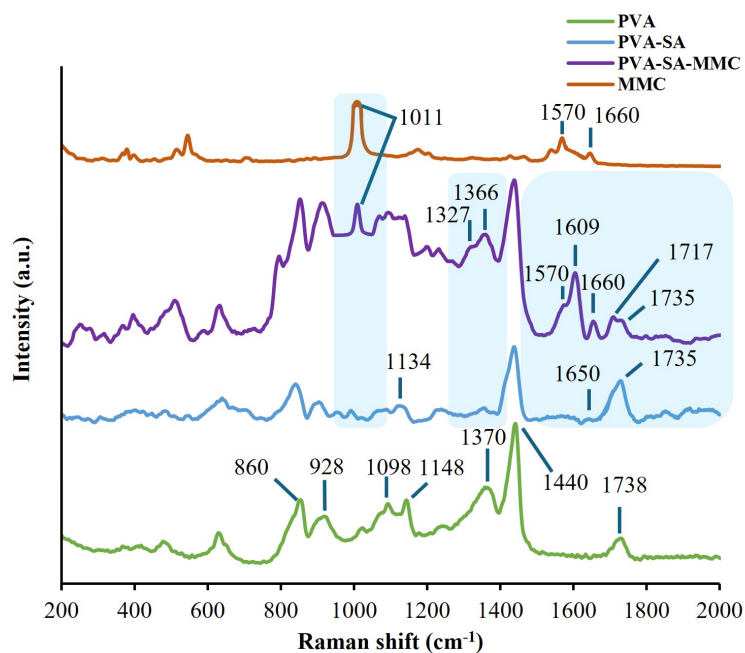


Figure A6. Raman spectra of PVA, PVA-SA, PVA-SA-MMC and MMC [195].

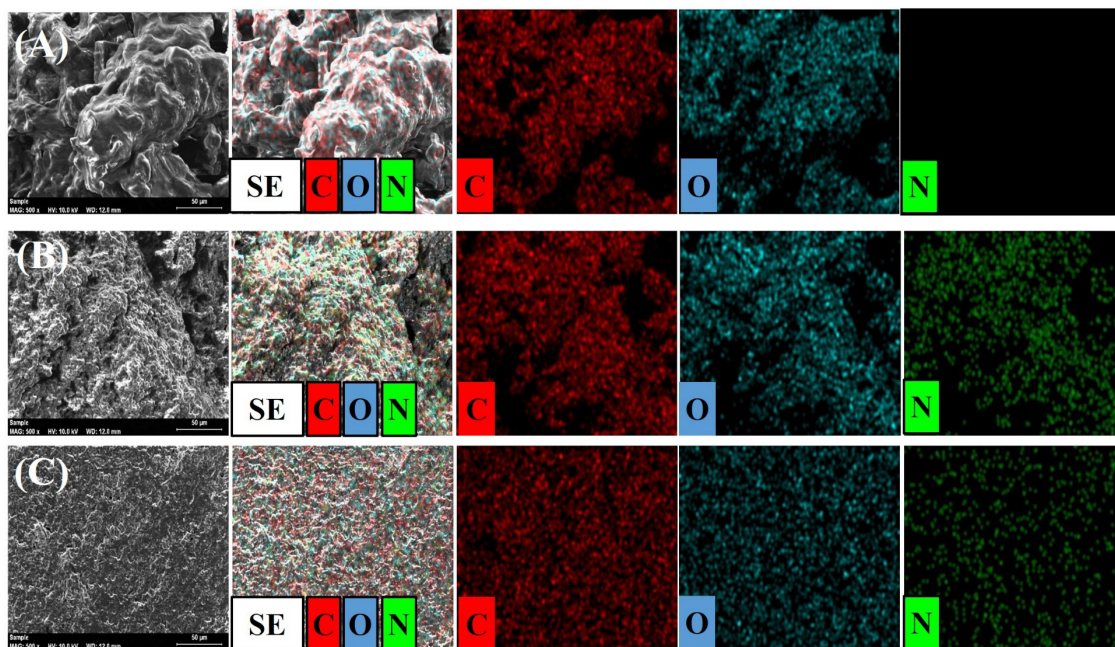


Figure A7. EDX of (A) PVA-SA and (B) RB-PVA-SA as well as (C) RB-PVA-SA-MMC particles, where C,O, and N are carbon, oxygen, and nitrogen elements, respectively [195].

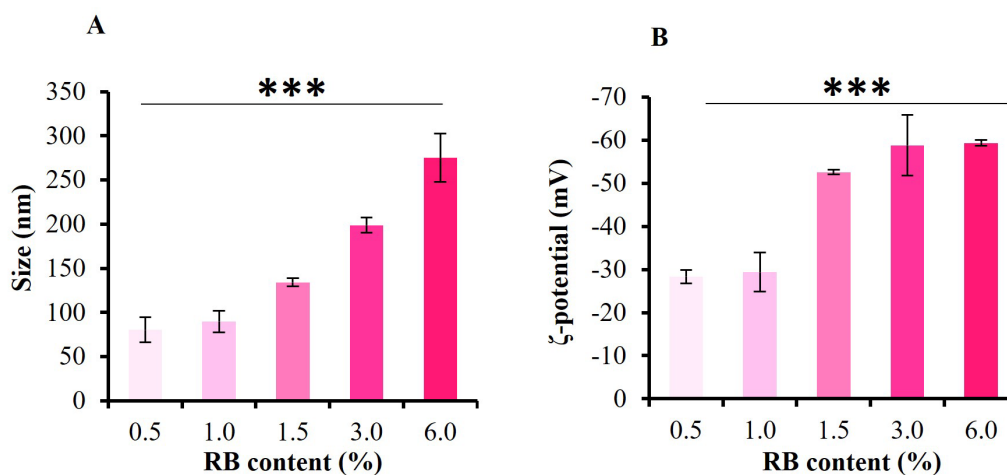


Figure A8. (A) The average particle sizes and (B) the ζ potential values of RB-loaded NPs dispersed in PBS (pH 5.8, 0.9% NaCl). Statistical analysis of the results: one-way ANOVA with Tukey's HSD post-hoc analysis. The added asterisks in the figure represent the summarized p value obtained from the analysis. *** $p < 0.001$ [195].

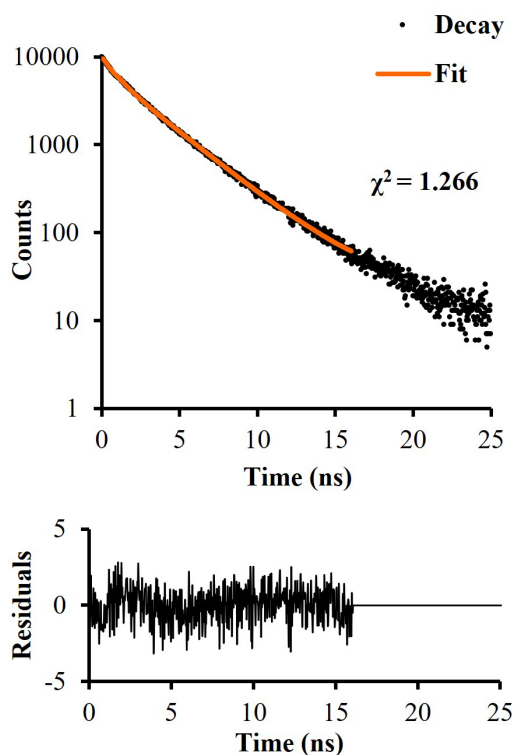


Figure A9. The typical fluorescence decay profile for (1.5% RB)-PVA-SA NPs with the fitting [195].

Table A1. The DLS data (at pH 5.8) for the two samples obtained from applying the first MMC amount (4 mg) either with the 0.5% or 1.5% RB [195].

Sample	DH (nm)	PDI	ζ -potential (mV)
((0.5% RB)-PVA-SA)-MMC	127 ± 3	0.276 ± 0.02	-27.9 ± 0.7
((1.5% RB)-PVA-SA)-MMC	156 ± 23	0.214 ± 0.07	-54.8 ± 2.0

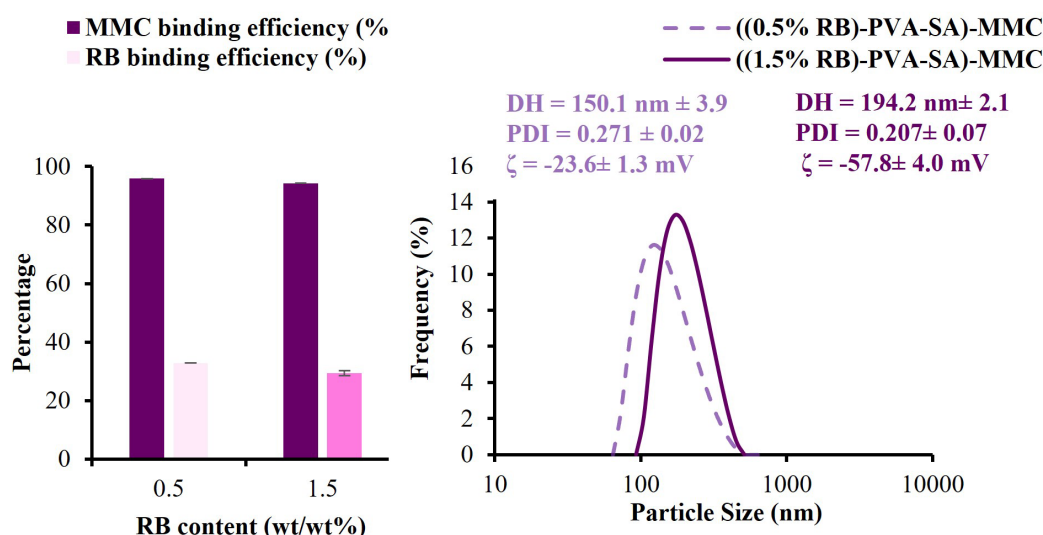


Figure A10. (A) The MMC and RB binding efficiency results as well as (B) the DLS data at pH 7.4 for the two samples obtained from applying the second MMC amount (12 mg) either with a 0.5% or 1.5% RB [195].

Table A2. Outcomes of fitting various kinetic models to MMC release data from fluorescent NPs [195].

	<i>Zero-Order model</i>		<i>First-Order model</i>		<i>Higuchi model</i>		<i>Hixson-Crowell model</i>		<i>Korsmeyer-Peppas model</i>		
	R^2	$k(h^{-1})$	R^2	$k(h^{-1})$	R^2	$k(h^{-1/2})$	R^2	$k(h^{-1/3})$	R^2	n	$k(h^{-n})$
Free MMC (pH 7.4)	0.312	0.164	0.426	0.007	0.499	2.996	0.390	0.006	0.702	0.148	48.31
Free MMC (pH 5.8)	0.106	0.140	0.149	0.005	0.224	2.891	0.134	0.005	0.537	0.200	49.43
((0.5% RB)-PVA-SA)-MMC (pH 7.4)	0.966	0.221	0.987	0.002	0.986	3.637	0.981	0.004	0.988	0.771	0.818
((0.5% RB)-PVA-SA)-MMC (pH 5.8)	0.960	0.409	0.983	0.009	0.965	6.084	0.985	0.010	0.988	0.771	1.403
((1.5% RB)-PVA-SA)-MMC (pH 7.4)	0.970	0.101	0.978	0.001	0.971	1.418	0.976	0.002	0.979	0.625	0.701
((1.5% RB)-PVA-SA)-MMC (pH 5.8)	0.950	0.325	0.988	0.005	0.975	4.763	0.980	0.007	0.991	0.831	0.861

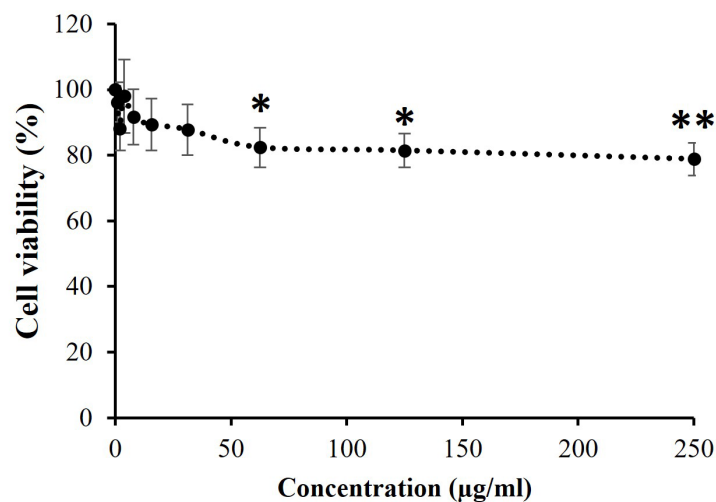


Figure A11. Cell viability (%) of CCD cells incubated for 72 h with (1.5% RB)-PVA-SA NPs at various NP concentrations. Statistical analysis of the difference in cell viability results was performed using one-way ANOVA with Dunnett's post hoc test (control group: untreated cells). * $p < 0.05$; ** $p < 0.01$ [195].

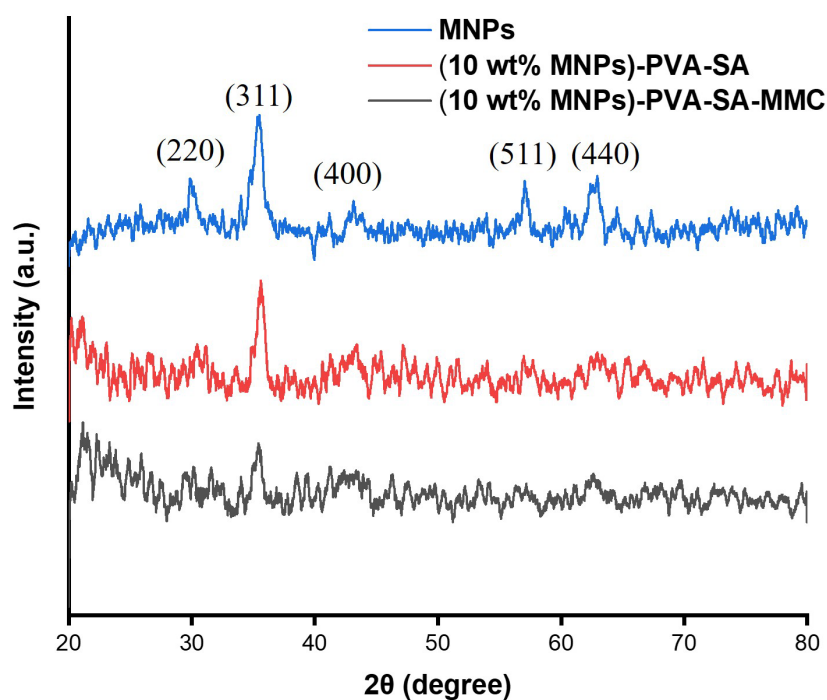


Figure A12. XRD for MNPs, (10 wt% MNPs)-PVA-SA and (10 wt% MNPs)-PVA-SA-MMC [193].

Table A3. The DLS data, drug binding efficiency, and drug loading % for the prepared MMC-loaded magnetic NPs. Particles were dispersed in PBS (0.9% NaCl, pH 7.4 or 5.8) for DLS measurements [193].

pH	MNP content	DH (nm)	PDI $\pm$ SD	Zeta potential (mV)	Binding efficiency (%)	Drug loading (%)
7.4	10%	128.1 $\pm$ 9.5	0.284 $\pm$ 0.03	-18.5 $\pm$ 1.0	97.90 $\pm$ 0.83	2.6 $\pm$ 0.04
5.8	10%	134.1 $\pm$ 10.0	0.307 $\pm$ 0.04	-19.0 $\pm$ 1.4		

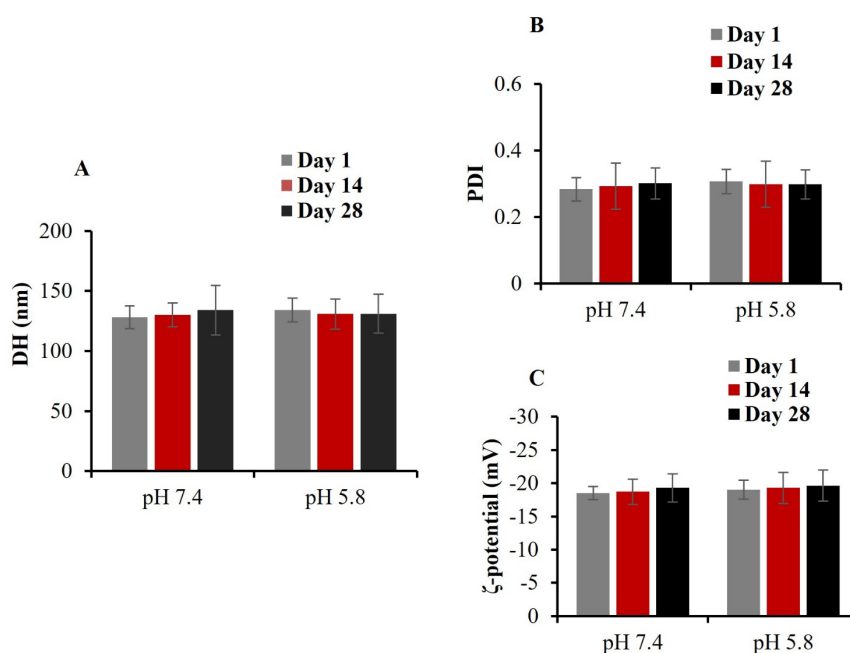


Figure A13. The change in (A) size, (B) PDI, and (C) ζ -potential values of MMC-loaded NPs ((10 wt% MNPs)-PVA-SA-MMC) over time [193].

Table A4. Outcomes of fitting various kinetic models to MMC release data from magnetic NPs [193].

	<i>Zero-Order model</i>		<i>First-Order model</i>		<i>Higuchi model</i>		<i>Hixson-Crowell model</i>		<i>Korsmeyer-Peppas model</i>		
	R^2	$k(h^{-1})$	R^2	$k(h^{-1})$	R^2	$k(h^{-1/2})$	R^2	$k(h^{-1/3})$	R^2	n	$k(h^{-n})$
Free MMC (pH 7.4)	0.327	0.212	0.452	0.009	0.520	3.496	0.412	0.008	0.713	0.157	47.97
Free MMC (pH 5.8)	0.100	0.188	0.142	0.007	0.237	3.659	0.127	0.006	0.570	0.224	49.20
PVA-MNPs-MMC (pH 7.4)	0.949	0.309	0.974	0.005	0.982	4.043	0.967	0.006	0.993	0.623	2.009
PVA-MNPs-MMC (pH 5.8)	0.936	0.523	0.970	0.009	0.979	6.853	0.965	0.013	0.994	0.617	3.565

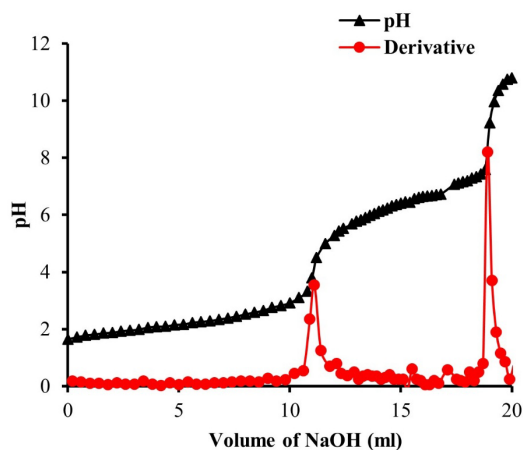


Figure A14. The potentiometric titration curve of Chit used to determine the deacetylation degree (DD%) [251].

Table A5. Substitution degree (*SD*) of Chit derivatives determined using equation 14 [251].

Samples	Molar ratio Aldehyde: D-glucosamine	Theoretical SD (%)	Obtained SD (%) ^a
C ₆ -chit	0.6:1	60	30.0 ± 0.5
C ₁₂ -chit	0.6:1	60	18.8 ± 0.8

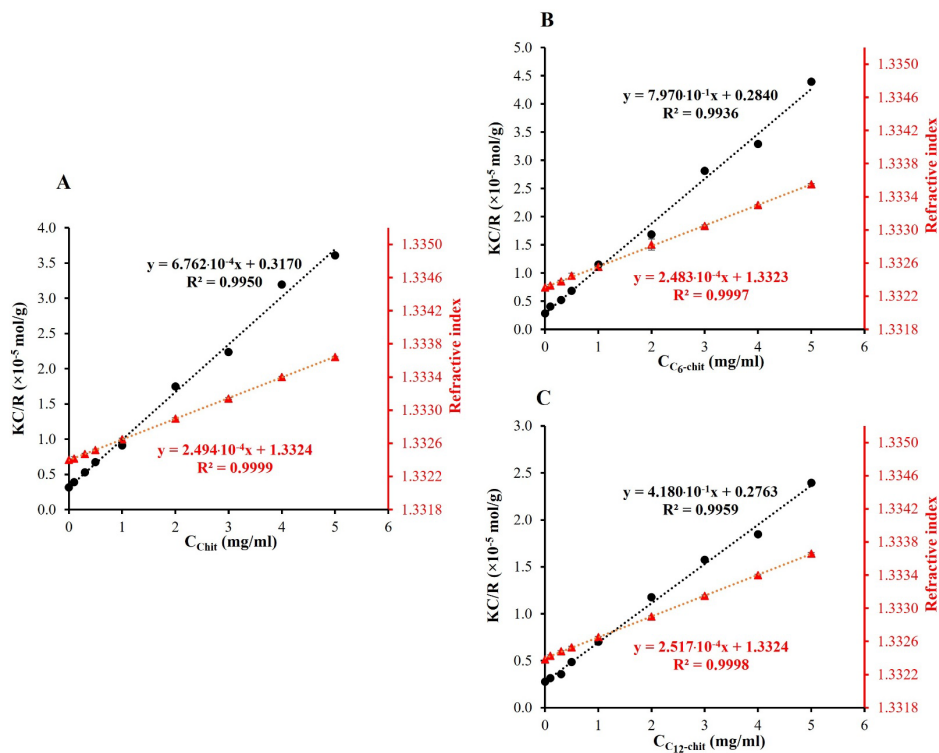


Figure A15. Determination of the M_w of polymers by light scattering measurements using Debye representations (●) and refractive indexes measurements (▲) for various concentrations of (A) Chit, (B) C₆-chit and (C) C₁₂-chit [251].

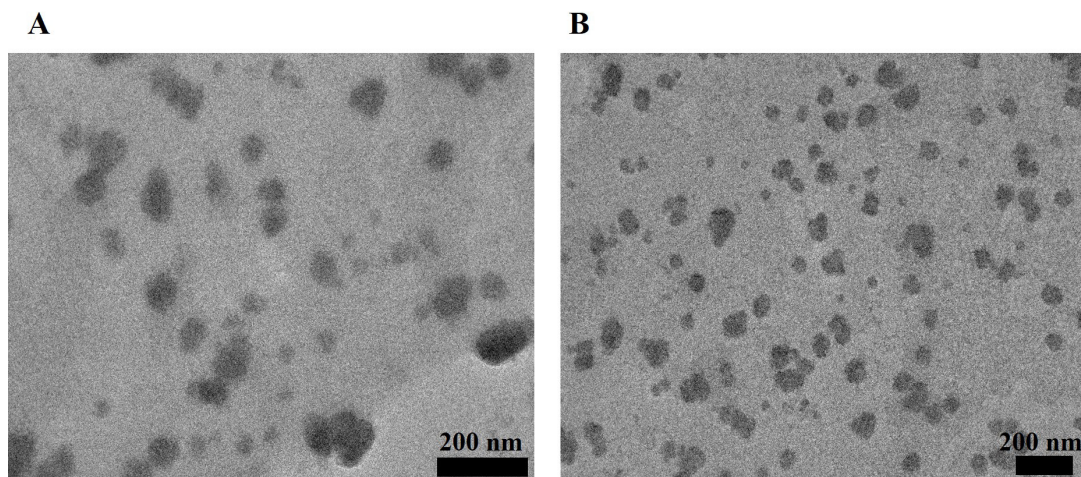


Figure A16. TEM images of C₆-chit NPs prepared using (A) methanol, and (B) acetone [251].

Table A6. DLS data of the NPs produced using different initial C₆-chit concentrations (0.2-1.2 wt/v%). Ethanol was used at a volume fraction of 0.29 and TPP was used at a concentration of 10 wt/wt% relative to C₆-chit [251].

C ₆ -Chit (w/v%)	DH (nm)			PDI		ζ-Potential (mV)	
0.2	161	±	13	0.302	± 0.036	24.1	± 1.2
0.4	163	±	10	0.309	± 0.059	24.3	± 0.4
0.6	163	±	09	0.304	± 0.061	25.1	± 1.8
0.8	227***	±	16	0.259	± 0.105	25.4	± 1.2
1.0	228***	±	10	0.274	± 0.103	26.7*	± 0.9
1.2	266***	±	39	0.294	± 0.078	27.2**	± 1.0

*Statistical analysis of the results: one-way ANOVA with Dunnett's post hoc test (control group: sample containing 0.2 wt/v% C₆-chit). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.*

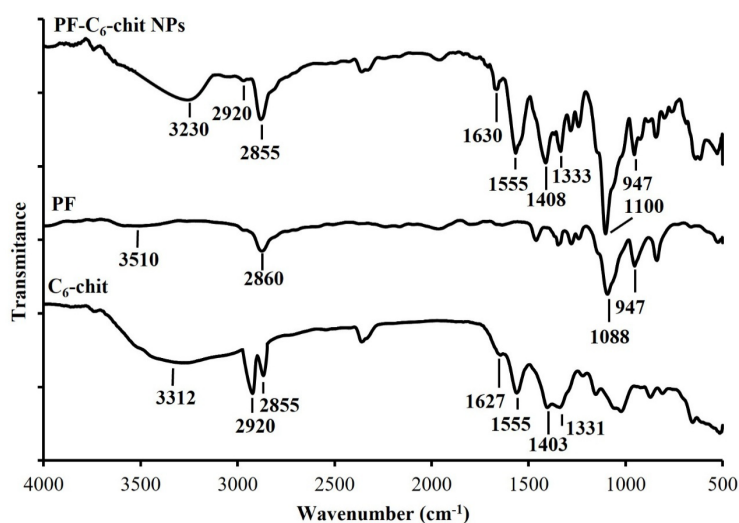


Figure A17. FTIR analysis of C₆-chit, PF and (25 wt/wt% PF)-C₆-chit NPs [251].

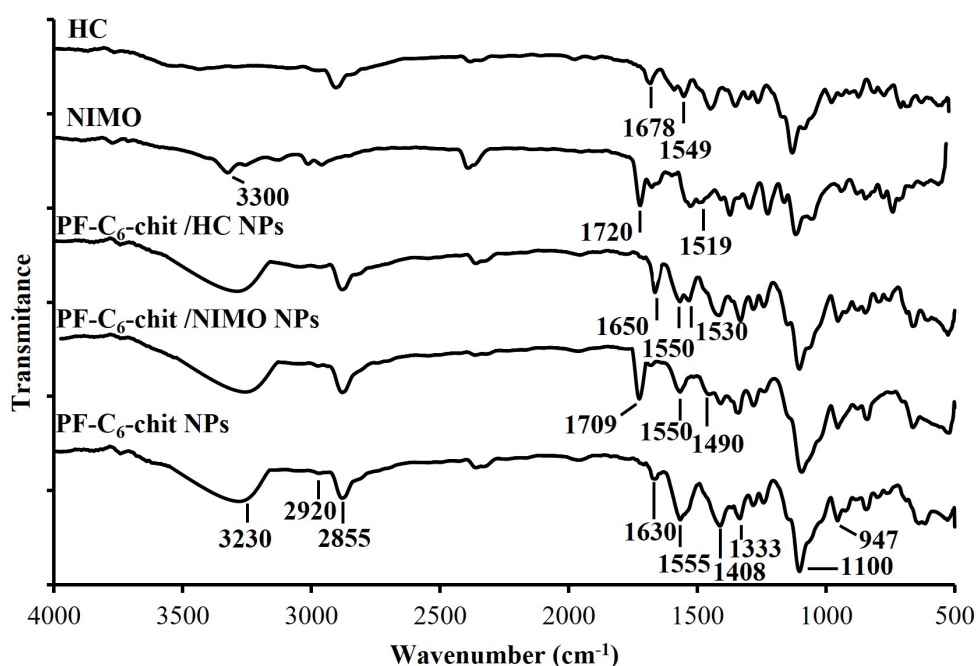


Figure A18. FTIR spectra of PF-C₆-chit NPs, NIMO-loaded PF-C₆-chit NPs, and HC-loaded PF-C₆-chit NPs, and bare drugs. PF content is 25 wt% relative to C₆-chit [251].

Table A7. Encapsulation efficiency results, as well as the DH, PDI, and ζ potential values of NIMO-loaded PF-C₆-chit NPs and HC-loaded PF-C₆-chit NPs at pH 7.4, as a function of increasing nominal PF content (0-25 wt% relative to C₆-chit) [251].

Drug	C _{PF} (wt/wt%)	EE (%)	DL (%)	DH (nm)	PDI	ζ -potential (mV)
NIMO	0.00	96.4 ± 1.3	9.73 ± 0.01	272 ± 10	0.275 ± 0.048	24.1 ± 0.5
	15.0	96.4 ± 0.4	8.53 ± 0.07	245 ± 48	0.256 ± 0.100	23.5 ± 1.1
	20.0	96.7 ± 0.7	8.30 ± 0.01	213** ± 19	0.255 ± 0.067	23.4 ± 0.8
	25.0	95.4 ± 1.0	7.84 ± 0.06	197** ± 10	0.265 ± 0.025	23.7 ± 0.8
HC	0.00	96.9 ± 1.2	9.74 ± 0.05	280 ± 25	0.284 ± 0.119	24.2 ± 1.2
	15.0	96.1 ± 2.3	8.47 ± 0.15	267 ± 12	0.249 ± 0.097	23.7 ± 0.8
	20.0	97.4 ± 0.9	8.29 ± 0.03	236** ± 30	0.260 ± 0.073	24.3 ± 1.5
	25.0	97.3 ± 2.6	7.99 ± 0.19	203*** ± 25	0.267 ± 0.073	24.8 ± 0.8

EE% stands for the encapsulation efficiency percentage, and DL% stands for the drug loading percentage. The drug content in all samples was approximately 11 wt% relative to C₆-chit. Statistical analysis of the results: one-way ANOVA with Dunnett's post hoc test (control group: PF-free sample). ** $p < 0.01$; *** $p < 0.001$.

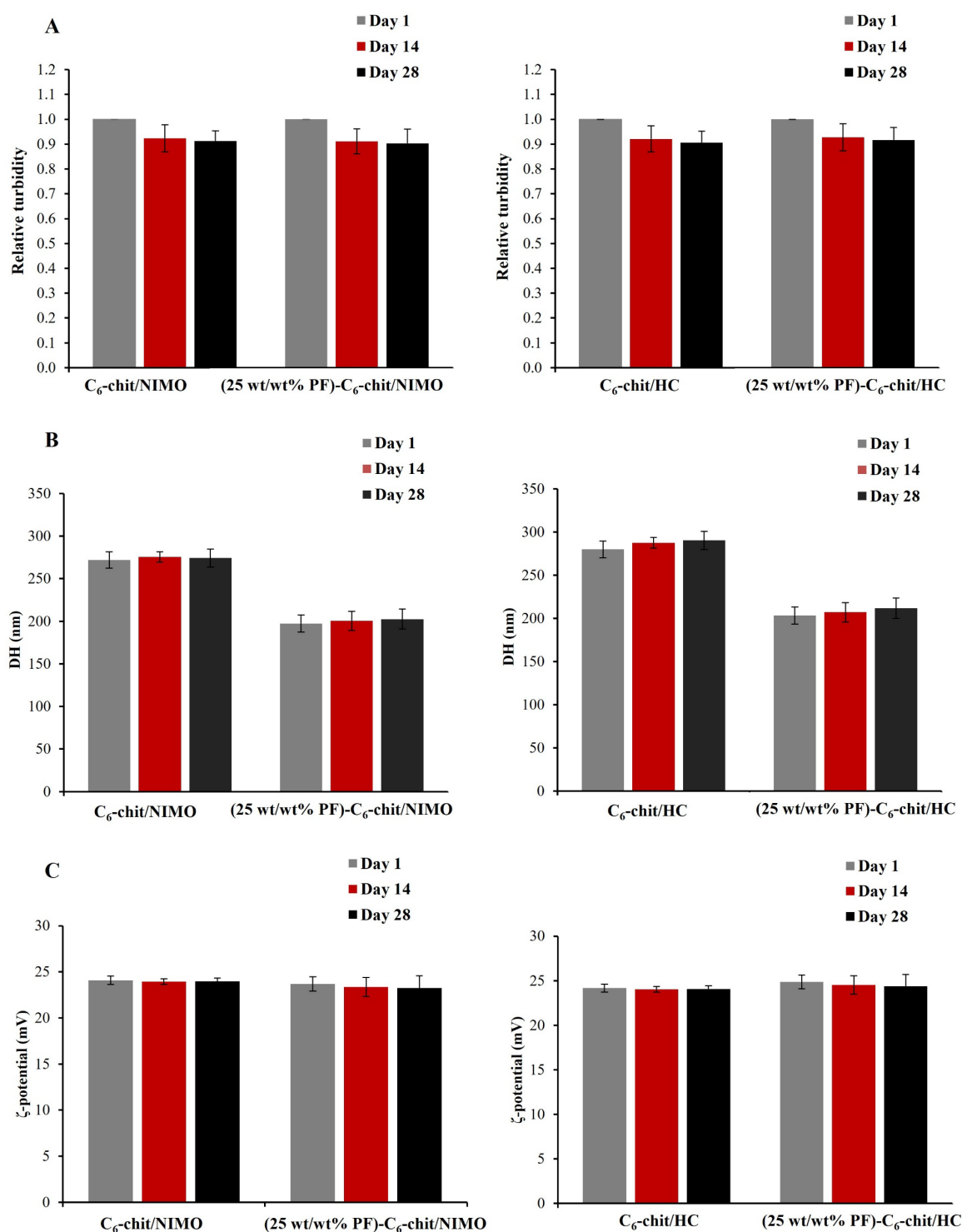


Figure A19. Variations in (A) turbidity, (B) particle size, and (C) ζ -potential for NIMO- and HC-loaded NP dispersed in 10 ml of PBS (0.9% NaCl, pH 7.4) over a 28-day storage period. Each sample contained a drug concentration of 0.2 mg/ml [251].

Table A8. Outcomes of fitting the NIMO release data (obtained at pH 6) to various kinetic models [251].

	<i>Zero-Order model</i>		<i>First-Order model</i>		<i>Higuchi model</i>		<i>Hixson-Crowell model</i>		<i>Korsmeyer-Peppas model</i>		
	R^2	$k(h^{-1})$	R^2	$k(h^{-1})$	R^2	$k(h^{-1/2})$	R^2	$k(h^{-1/3})$	R^2	n	$k(h^{-n})$
Free NIMO	0.970	5.172	0.985	0.064	0.987	16.429	0.981	0.094	0.940	0.898	9.161
C ₆ -chit./NIMO	0.986	5.864	0.995	0.078	0.976	17.910	0.994	0.110	0.997	0.759	9.931
(15 wt/wt% PF)-C ₆ -chit./NIMO	0.984	3.761	0.990	0.046	0.976	11.560	0.989	0.066	0.990	0.622	8.299
(25 wt/wt% PF)-C ₆ -chit./NIMO	0.980	2.897	0.984	0.032	0.955	8.790	0.983	0.049	0.985	0.843	3.388

Table A9. Outcomes of fitting the HC release data (obtained at pH 6) to various kinetic models [251].

	<i>Zero-Order model</i>		<i>First-Order model</i>		<i>Higuchi model</i>		<i>Hixson-Crowell model</i>		<i>Korsmeyer-Peppas model</i>		
	R^2	$k(h^{-1})$	R^2	$k(h^{-1})$	R^2	$k(h^{-1/2})$	R^2	$k(h^{-1/3})$	R^2	n	$k(h^{-n})$
Free HC	0.877	5.389	0.946	0.203	0.951	20.928	0.928	0.200	0.981	0.269	62.18
C ₆ -chit./HC	0.942	5.186	0.925	0.378	0.981	19.729	0.974	0.286	0.988	0.435	62.23
(15 wt/wt% PF)-C ₆ -chit./HC	0.980	7.291	0.992	0.191	0.996	27.437	0.994	0.212	0.996	0.437	33.81
(25 wt/wt% PF)-C ₆ -chit./HC	0.978	6.151	0.988	0.124	0.990	23.076	0.988	0.151	0.991	0.438	27.73

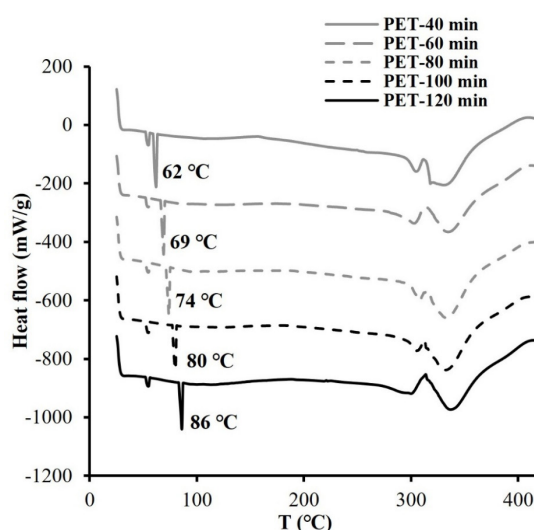


Figure A20. DSC curves of PET polyester samples synthesized at 165 °C under varying polycondensation times (40, 60, 80, 100, and 120 min) [278].

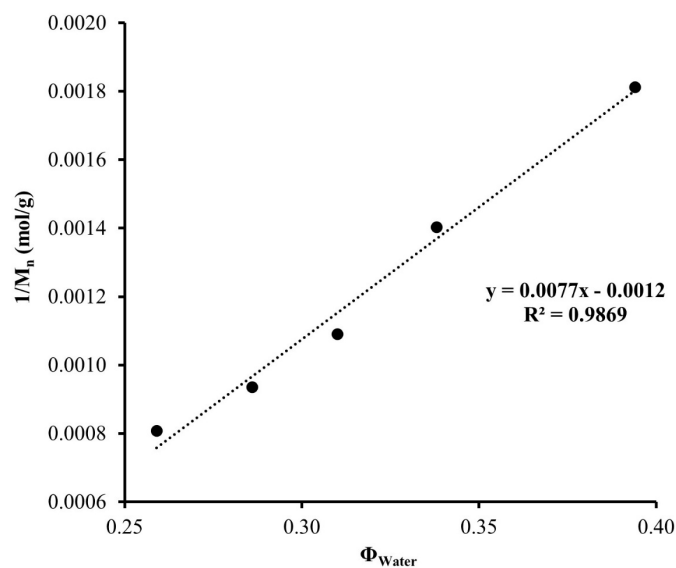


Figure A21. Reciprocal molecular weight values ($1/M_n$), obtained from ^1H -NMR, versus the water volume fraction (Φ_{water}) required to trigger precipitation of PET samples (40, 60, 80, 100, and 120 minutes) in DMSO/water system [278].

Table A10. The PET molecular weight and water volume fraction data employed for the calculation of A and B coefficients of Schultz-equation [278].

Polymerization Time (min)	M_n (Da)	$1/M_n$ (mol/g)	Volume fraction of H_2O	Data from Figure A21	Schulz constants
40 min	552	0.00181	0.394	Slope:0.0077	A=15.58
60 min	713	0.00140	0.338		
80 min	917	0.00109	0.310		
100 min	1069	0.00094	0.286	Intercept:-0.0012	B=12987
120 min	1238	0.00081	0.259		

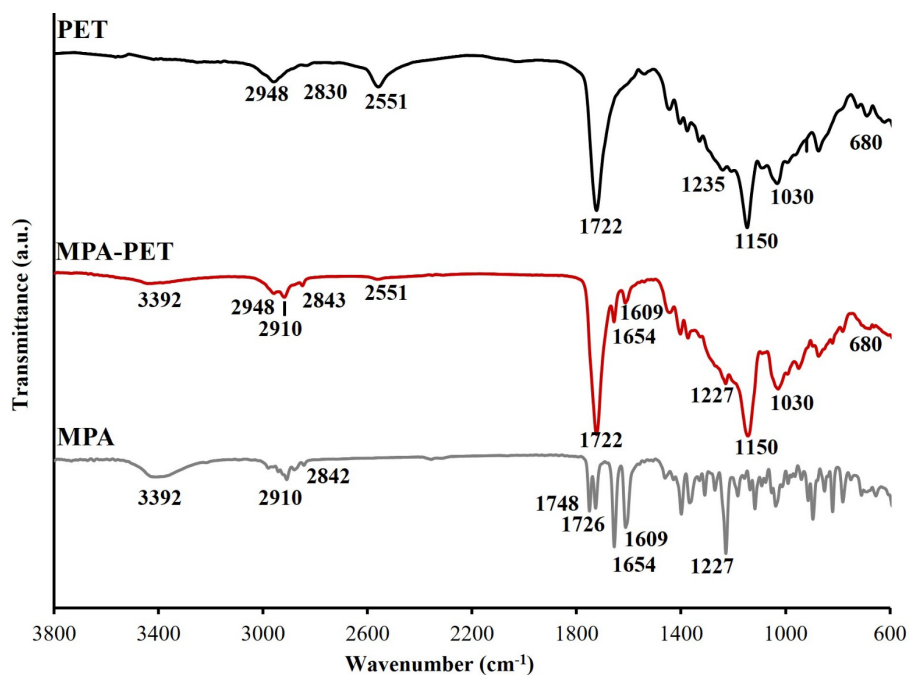


Figure A22. FTIR spectra of PET ($M_W = 1652$ Da), MPA-loaded PET NPs (11 w/w% MPA content), and bare MPA drug [278].

Table A11. Outcomes of fitting the MPA release data to various kinetic models [278].

	<i>Zero-Order model</i>		<i>First-Order model</i>		<i>Higuchi model</i>		<i>Hixson-Crowell model</i>		<i>Korsmeyer-Peppas model</i>		
	R^2	$k(h^{-1})$	R^2	$k(h^{-1})$	R^2	$k(h^{-1/2})$	R^2	$k(h^{-1/3})$	R^2	n	$k(h^{-n})$
Free MPA	0.982	3.799	0.990	0.046	0.991	11.886	0.988	0.066	0.950	0.737	7.21
MPA-PET ($M_W = 908$ Da)	0.984	6.329	0.997	0.090	0.991	19.776	0.994	0.124	0.951	0.641	14.36
MPA-PET ($M_W = 1002$ Da)	0.988	4.953	0.997	0.064	0.988	15.419	0.995	0.092	0.952	0.652	10.79
MPA-PET ($M_W = 1422$ Da)	0.988	2.724	0.993	0.030	0.990	8.489	0.992	0.046	0.994	0.613	6.18
MPA-PET ($M_W = 1652$ Da)	0.985	2.045	0.990	0.023	0.993	6.393	0.988	0.034	0.997	0.758	3.58

Table A12. Dissolution parameters derived from MPA and MPA-loaded NPs release data [278].

Formulation	$DE_{8\ h}$ (%)	$DE_{30\ h}$ (%)	MDT (h)	T10% (h)	T80% (h)
Free MPA	16.9	56.9	11.65	1.6	20.8
MPA-PET ($M_w = 908$ Da)	32.6	77.4	6.79	0.5	11.0
MPA-PET ($M_w = 1002$ Da)	25.0	67.8	9.65	0.9	13.2
MPA-PET ($M_w = 1422$ Da)	13.8	52.8	12.96	2.4	23.2
MPA-PET ($M_w = 1652$ Da)	9.7	46.1	14.97	3.8	25.6

$DE_{8\ h}$: dissolution efficiency calculated up to 8 h; $DE_{30\ h}$: dissolution efficiency calculated up to 30 h; MDT: mean dissolution time; T10%: time to release 10% drug release; T80%: time to release 80% drug release.

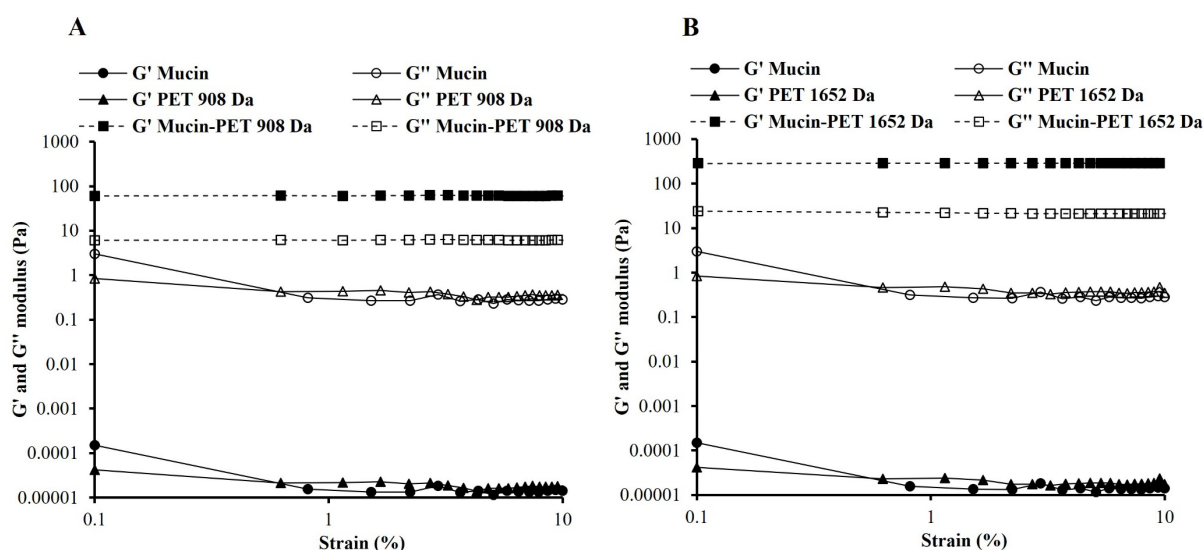


Figure A23. The Storage (G') and loss modulus (G'') as a function of strain % for (A) 0.5% mucin, 0.5% PET 908 Da, and 0.5% mucin-PET 908 Da, as well as (B) 0.5% mucin, 0.5% PET 1652 Da, and 0.5% mucin-PET 1652 Da [278].

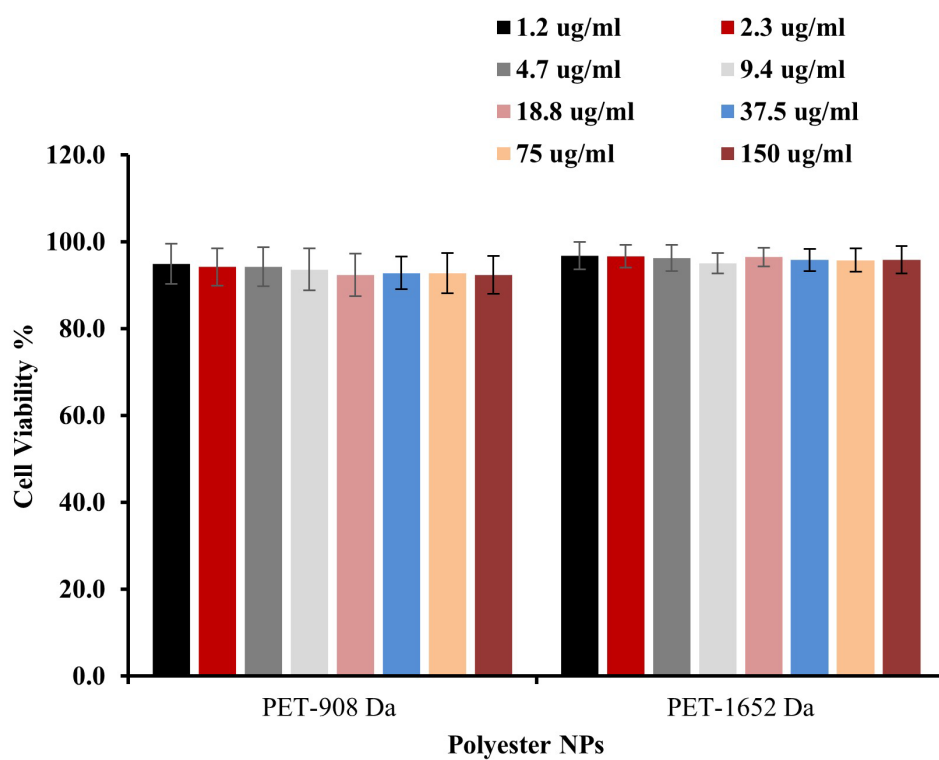


Figure A24. Cell viability (%) of MRC-5 cells measured by the MTT assay after 24 h treatment with PET NPs (synthesized from PET with M_w of 908 or 1652 Da) at concentrations of ~1–150 $\mu\text{g/mL}$ [278].

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List of publications

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Publications related to the doctoral work:

1. **Keristina Wagdi K. Amin**, Mohamed M. Abdelghafour, Viktória Hornok, Tamás Kiss, Diána Szabó, László Rovó, and László Janovák. Mitomycin loaded self-assembled colloidal prodrug nanoparticles for magnetic drug targeting. *Journal of Drug Delivery Science and Technology*, **2023**, 88, 104948.
<https://doi.org/10.1016/j.jddst.2023.104948> **IF = 5.2, Q1**
2. **Keristina Wagdi K. Amin**, Ágota Deák, Miklós Csanády, Nikoletta Szemerédi, Diána Szabó, Árpád Turcsányi, Ditta Ungor, Gabriella Spengler, László Rovó, and László Janovák. pH-Triggered Hydrogel Nanoparticles for Efficient Anticancer Drug Delivery and Bioimaging Applications. *Pharmaceutics*, **2024**, 16 (7), 931.
<https://doi.org/10.3390/pharmaceutics16070931> **IF = 6.9, D1**
3. **Keristina Wagdi K. Amin**, Ágota Deák, Eszter Farkas, Christine R. Rose, Ferenc Bari, and László Janovák. Hydrophobically modified Chitosan-Pluronic nanocarriers for controlled release of Nimodipine and HC-067047 drugs in ischemic tissues. *Materials Today Chemistry*, **2025**, 46, 102743.
<https://doi.org/10.1016/j.mtchem.2025.102743> **IF = 7.4, Q1**
4. **Keristina Wagdi K. Amin**, Ágota Deák, Médi Sándor, Diána Szabó, László Rovó, and László Janovák. Novel catalyst-free mucoadhesive polyester nanoparticles enabling enhanced dissolution and controlled release of methylprednisolone acetate. *Materials Today Nano*, **2026**, 33, 100750.
<https://doi.org/10.1016/j.mtnano.2025.100750> **IF = 7.6, D1**

Σ IF = 27.1

Other publications:

5. Viktória Hornok, **Keristina Wagdi K. Amin**, Alexandra N. Kovács, Adám Juhász, Gábor Katona, György T. Balogh, Edit Csapó. Increased blood-brain barrier permeability of neuroprotective drug by colloidal serum albumin carriers. *Colloids Surf. B Biointerfaces*, **2022**, 220, 112935.

<https://doi.org/10.1016/j.colsurfb.2022.112935>

IF = 5.9, Q1

6. Mohamed M. Abdelghafour, Ágota Deák, **Keristina Wagdi K. Amin**, Zsófia Czimer, Czike Flóra Veronika, Viktória Péter, Róbert Berkecz, Ferenc Bari, László Janovák. Near-Infrared Light-Responsive Molybdenum Disulfide Nanosheets for Controlling the Release of Nimodipine as NIR-Drug Delivery System. *Molecules*, **2025**, 30 (3), 497.

<https://doi.org/10.3390/molecules30030497>.

IF = 5.1, Q1

Σ IF = 11.0

$\Sigma\Sigma$ IF = 38.1

Lectures and posters related to the doctoral work

1. **Keristina Wagdi K. Amin**, Mohamed M. Abdelghafour, Viktória Hornok, Tamás Kiss, Diána Szabó, László Rovó, László Janovák “*Magnetic polymer nanoparticles for mitomycin delivery: synthesis, characterization, and in vitro drug release*” XLVI. Chemistry Lectures – an international conference for young professionals, October 17–19, **2023**, Szeged, Hungary. (Lecture)
2. **Keristina Wagdi K. Amin**, László Janovák “*Development of a targeted drug delivery system using modified polyvinyl alcohol and magnetic nanoparticles*” Autumn meeting of the MTA Colloid Chemistry Working Committee, October 30, **2023**, Szeged, Hungary. (Lecture)
3. **Keristina Wagdi K. Amin**, Ágota Deák, Diána Szabó, Árpád Turcsányi, Ditta Ungor, Spengler Gabriella, László Rovó, László Janovák “*pH-responsive nanoparticles for enhanced cancer therapy and bioimaging applications*” XLVII. Chemistry Lectures – an international conference for young professionals, October 29–31, **2024**, Szeged, Hungary. (Lecture)

4. **Keristina Wagdi K. Amin**, Ágota Deák, Eszter Farkas, Christine R. Rose, Ferenc Bari, László Janovák “*Development of hydrophobically modified chitosan-pluronic nanoparticles for sustained and targeted drug release in ischemic brain tissues*” Materials Today Conference, June 23–26, **2025**, Sitges, Spain. (Lecture)
5. **Keristina Wagdi K. Amin**, Ágota Deák, Eszter Farkas, Christine R. Rose, Ferenc Bari, László Janovák “*Development of hydrophobically modified chitosan-pluronic nanoparticles for sustained and targeted drug release in ischemic brain tissues*” Chemistry Physics and Biology of Colloids and Interfaces conference, June 15–19, **2025**, Eger, Hungary. (Poster)
6. **Keristina Wagdi K. Amin**, Ágota Deák, Médi Sándor, Diána Szabó, László Rovó, and László Janovák. Catalyst-free polyester particles for improved drug delivery. XLVIII. Chemistry Lectures – an international conference for young professionals, October 27–29, 2025, Szeged, Hungary. (Lecture)

Other presentations:

7. Viktória Hornok, **Keristina Wagdi K. Amin**, Alexandra N. Kovács, Edit Csapó “*Encapsulation of neuroactive drugs in serum album nanoparticles*” Biointerfaces International Zurich 2021 Conference, August 18–19, **2021**, Online. (Poster)
8. Viktória Hornok, Alexandra N. Kovács, **Keristina Wagdi K. Amin**, Edit Csapó “*Encapsulation of neuroactive drugs in desolvated serum album nanoparticle*” ECIS Conference, September 4–9, **2022**, Chania, Crete, Greece. (Lecture)