Volume 3, Issue 7, 2025

Frontier in Medical & Health Research

ISSN: (e) 3007-1607 (p) 3007-1593

ENZYME-RESPONSIVE HYDROGELS FOR TARGETED THERAPEUTIC DELIVERY AND DIAGNOSTIC APPLICATIONS

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DOI: https://doi.org/10.5281/zenodo.17060236

Keywords
Enzyme-responsive hydrogels; Targeted drug delivery; Theranostics

Article History
Received: 08 June , 2025
Accepted: 19 August, 2025
Published: 05September, 2025

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Abstract

Enzyme-responsive hydrogels (ERHs) represent a promising class of smart biomaterials designed to exploit pathological enzymatic activity for precision medicine. In this study, chitosan, hyaluronic acid, polyethylene glycol diacrylate (PEGDA), and gelatin methacryloyl (GelMA) were functionalized with matrix metalloproteinase (MMP)-cleavable peptide linkers to develop multifunctional hydrogels for targeted drug delivery and diagnostic imaging. Physicochemical characterization confirmed a highly porous morphology, excellent swelling behavior, and stable viscoelastic properties suitable for biomedical use. Enzyme-triggered degradation studies demonstrated selective hydrogel disassembly in the presence of MMP-2 and hyaluronidase, enabling controlled and site-specific release of encapsulated doxorubicin (DOX). Drug release assays revealed accelerated and sustained delivery under enzyme-rich conditions, resulting in significant cytotoxicity against U87-MG glioblastoma cells while sparing normal fibroblasts, thereby confirming the therapeutic selectivity of the system. In parallel, diagnostic evaluation using fluorescein isothiocyanate (FITC) and superparamagnetic iron oxide nanoparticles (SPIONs) showed strong intracellular fluorescence and enhanced T_2-weighted MRI contrast, validating the hydrogels' dual theranostic capability. Biocompatibility assessments confirmed minimal hemolysis and cytotoxicity of blank hydrogels, while long-term stability studies revealed >94% drug retention over 28 days, demonstrating structural integrity and storage feasibility. Collectively, these findings highlight the potential of ERHs as next-generation theranostic platforms capable of integrating targeted therapy with real-time diagnostic monitoring. Despite challenges in clinical translation such as variability in enzymatic expression and scalability this work provides a strong foundation for developing precision biomaterials to address complex diseases like cancer and chronic wounds.

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INTRODUCTION

Because of their high water content, biocompatibility, mechanical properties that can be adjusted, and capacity to replicate the extracellular matrix, hydrogels have become a highly promising class of biomaterials in biomedical engineering [1]. Small molecules, nucleic acids, proteins, and nanoparticles are just a few of the therapeutic and diagnostic agents that can be encapsulated in these three-dimensional cross-linked polymeric networks [2]. The advantages of hydrogels over traditional drug carriers controlled release, environmental stimuli responsiveness, and site-specific action make them appealing for targeted therapy and cutting-edge diagnostic platforms [3]. Stimuli-responsive hydrogels, which change physicochemically in response to internal or external stimuli like pH, temperature, redox gradients, light, and enzymes, have drawn more attention in recent years [4]. Enzyme-responsive hydrogels (ERHs) are unique among these because they can take advantage of endogenous biological cues, which makes precision medicine techniques more effective and less likely to cause off-target effects [5].

Enzymes are common biomolecules that control vital physiological and pathological functions [6]. Aberrant enzyme expression or activity is linked to a number of illnesses, such as microbial infections, diabetes, cancer, and chronic inflammation [7]. For example, hyaluronidases are increased in the progression of cancer, proteases are upregulated during bacterial infections, and matrix metalloproteinases (MMPs) are overexpressed in tumor microenvironments and chronic wounds [8]. Researchers can achieve highly localized drug release or diagnostic signaling only at diseased sites by creating hydrogels that can react specifically to these enzymatic activities [9].

A mechanism like this reduces systemic toxicity, improves the therapeutic index, and provides real-time feedback on the course of the disease [10]. Furthermore, hydrogels acquire dynamic functionality through enzyme-responsive degradation, which enables them to disassemble, release cargo, or alter their characteristics in a controlled spatiotemporal fashion [11]. Because of these characteristics, ERHs are attractive options for the next generation of theranostic systems, which combine diagnosis and treatment onto a single platform [12].

Enzyme-responsive hydrogels are created by adding cleavable bonds, peptide sequences, or polymer backbones that target enzymes can selectively break down [13]. Polyethylene glycol (PEG), polypeptides, polyesters, and natural polymers like gelatin, hyaluronic acid, chitosan, and alginate have all been extensively functionalized with enzyme-sensitive motifs [14]. Therapeutic payloads are released when these hydrogels undergo structural changes like swelling, shrinking, or total degradation due to enzymatic cleavage [15]. Diagnostic applications are also made possible by the detectable signals that enzyme-triggered disassembly can produce, such as fluorescence activation or contrast changes in imaging modalities[16]. The responsiveness, stability, and multifunctionality of ERHs are further improved by the incorporation of nanomaterials and smart probes [17]. The development of ERHs suited for particular clinical contexts, such as tumor-targeted drug delivery, wound healing, biosensing, and precision diagnostics, has been accelerated by recent developments in molecular biology, polymer chemistry, and bioengineering [18]. The translation of ERHs from laboratory research to clinical applications is still constrained by a number of issues, despite enormous progress [19]. First, it can be challenging to obtain predictable and consistent responses due to the complexity and heterogeneity of biological environments, which can impact enzyme activity[20]. The universality of current ERH designs may be diminished by the fact that enzyme expression frequently differs among patients, disease stages, and tissue microenvironments[21]. Second, the diagnostic potential of ERHs is still largely unexplored, with the majority of current research concentrating on drug delivery. Real-time monitoring and therapeutic release are not consistently integrated in clinically relevant models, despite the fact that some platforms exhibit theranostic functionality [22]. Third, to guarantee safety and efficacy in vivo, problems pertaining to hydrogel stability, biodegradation kinetics, and immunogenicity must be resolved. Many hydrogels break down either too quickly or too slowly, which results in less than ideal therapeutic results. Additionally, a significant bottleneck still exists in the scaling up of hydrogel fabrication with consistent quality, reproducibility, and regulatory compliance

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[23].With a focus on their dual uses in targeted therapeutic delivery and diagnostic systems, this study attempts to present a thorough overview of enzyme-responsive hydrogels. The main goals are to discuss new diagnostic applications in biosensing and imaging and to highlight recent developments in design strategies for achieving precise, enzyme-triggered drug release [18]. The study also aims to critically assess the obstacles to clinical translation, including selectivity and stability, and suggest future research avenues for quickening the transformation of these systems into multipurpose precision medicine platforms.

This study aims to close current gaps in the literature by addressing both the therapeutic and diagnostic dimensions. It also offers vital insights for developing more efficient, clinically transferable technologies. Because enzyme-responsive hydrogels have the rare capacity to use biological cues for site-specific therapy and real-time diagnosis, they represent a promising new area in biomaterials research and are perfect for treating complex illnesses like cancer and chronic wounds. Ultimately, to get past current obstacles and realize the full clinical potential of these adaptable theranostic tools, it is crucial to synthesize existing knowledge and establish specific goals.

2 Materials

Sigma-Aldrich (USA) provided the low molecular weight chitosan, hyaluronic acid, polyethylene glycol diacrylate (PEGDA), and gelatin methacryloyl (GelMA). Thermo Fisher Scientific supplied the lysozyme, hyaluronidase, and matrix metalloproteinase-2 (MMP-2). The model drugs fluorescein isothiocyanate (FITC) and doxorubicin hydrochloride (DOX·HCl) were used as diagnostic and therapeutic agents, respectively. The other chemicals and solvents were all analytical grade and didn't require any additional purification. Every experiment used ultrapure water.

2.1 Synthesis of Enzyme-Responsive Hydrogels

A free-radical polymerization technique was used to create hydrogels. In short, PEGDA and GelMA were dissolved at 50 °C in phosphate-buffered saline (PBS, pH 7.4). As enzyme-sensitive linkers, acryloyl-modified peptide crosslinkers with MMP-cleavable sequences (GPQG↓IWGQ) were introduced. As initiator and accelerator, respectively, ammonium persulfate (APS, 0.1% w/v) and N,N,N′,N′-tetramethylethylenediamine (TEMED) were added. After being moved into cylindrical molds, the mixture was left at room temperature for 30 minutes to polymerize under nitrogen.

2.1.1 Drug and Probe Loading

Prior to polymerization, doxorubicin (DOX) and FITC were mixed with the precursor solution to incorporate them into the hydrogels during gel formation. Until no fluorescence or drug was found in the supernatant, unbound molecules were eliminated by thoroughly washing with PBS. By using UV-Vis spectroscopy at 480 nm to measure the difference between the initial and residual drug concentration, encapsulation efficiency was determined.

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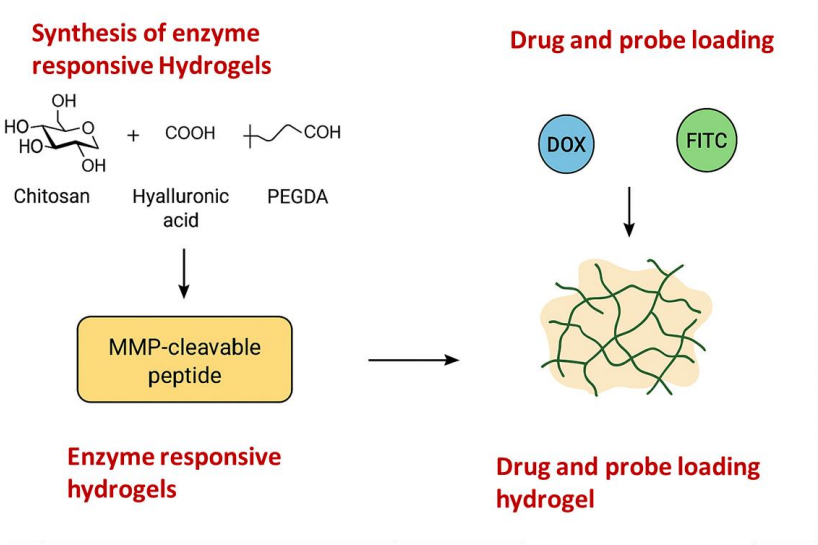

Figure 2.1: Schematic representation of the synthesis of enzyme-responsive hydrogels and the loading of drug (DOX) and probe (FITC) within the hydrogel matrix.

2.2 Characterization of Hydrogels

Using samples that had been freeze-dried and sputter-coated with a gold layer to improve conductivity, scanning electron microscopy (SEM) was used to analyze the morphological properties of the hydrogels. The swelling ratio was calculated by immersing the dried hydrogels in PBS (pH 7.4) at 37 °C and weighing them at pre-arranged intervals to assess their performance under biologically relevant conditions. By soaking the hydrogels in PBS solutions containing MMP-2, hyaluronidase, or lysozyme (each at 100 U/mL) and tracking the weight loss that ensued over time, enzyme-triggered degradation was also investigated. Rheological analysis was used to evaluate the mechanical properties. A rheometer was used to record the storage (G′) and loss (G″) moduli at a frequency of 1 Hz.

2.3 In Vitro Drug Release Studies

Phosphate-Buffered Saline (PBS, pH 7.4) was employed as the release medium since it closely mimics the physiological conditions of the human body and provides an isotonic environment for drug diffusion studies. Doxorubicin (DOX), a widely used anthracycline chemotherapeutic agent, was selected as the model drug due to its strong anticancer activity and distinct spectrophotometric absorbance at 480 nm, which allows accurate quantification. To evaluate the release behavior, DOX-loaded hydrogels were placed in dialysis bags and immersed in 20 mL of PBS at 37 °C under constant shaking (100 rpm) to simulate in vivo fluid dynamics. Parallel experiments were conducted in the presence and absence of specific enzymes to assess enzyme-responsive drug release. At predetermined time intervals, 1 mL aliquots of the release medium were collected and replaced with fresh PBS to maintain sink conditions. The concentration of DOX released was determined by measuring absorbance at 480 nm using UV–Vis spectroscopy, and cumulative release profiles were plotted as a function of time.

2.4 Cytotoxicity Assay

Human glioblastoma (U87-MG) and fibroblast (NIH-3T3) cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin at 37 °C in a humidified 5% CO_2 incubator. Cell viability was assessed using MTT assay. Briefly, cells were seeded in 96-well plates (1×10^4 cells/well) and treated with hydrogel extracts (with/without enzyme pre-incubation). After 48 h, MTT solution (0.5 mg/mL) was added, and absorbance was measured at 570 nm.

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2.5 Diagnostic Imaging Evaluation

For diagnostic evaluation, hydrogels were incorporated with either fluorescein isothiocyanate (FITC) as a fluorescent probe or superparamagnetic iron oxide nanoparticles (SPIONs) as magnetic resonance imaging (MRI) contrast agents. FITC-loaded hydrogels were incubated with U87-MG glioblastoma cells, and cellular uptake was monitored using confocal laser scanning microscopy (CLSM) to visualize intracellular localization. In parallel, SPION-loaded hydrogels were suspended in 1% agarose phantoms and scanned using a 3.0 T clinical MRI scanner to assess their performance as T_2-weighted contrast enhancers. These studies provided complementary insights into the potential of enzyme-responsive hydrogels for fluorescence-based cellular imaging and non-invasive MRI diagnostics.

Table 2.1: Summary for the Materials and Methods

Category	Details
Materials	Chitosan (low molecular weight), Hyaluronic acid, Polyethylene glycol diacrylate (PEGDA), Gelatin methacryloyl (GelMA) (Sigma-Aldrich, USA). Enzymes: MMP-2, Hyaluronidase, Lysozyme (Thermo Fisher Scientific). Drug: Doxorubicin hydrochloride (DOX·HCl). Probe: Fluorescein isothiocyanate (FITC). Other reagents: Ammonium persulfate (APS), N,N,N′,N′-tetramethylethylenediamine (TEMED).
Hydrogel Synthesis	Free-radical polymerization of PEGDA and GelMA in PBS (pH 7.4, 50 °C). MMP-cleavable peptide crosslinkers (GPQG↓IWGQ) added. APS (0.1% w/v) and TEMED used as initiator/accelerator. Polymerization carried out in nitrogen-purged molds at room temperature for 30 min.
Drug & Probe Loading	DOX and FITC incorporated during gel formation by mixing with precursor solution. Unbound molecules removed via PBS washing. Encapsulation efficiency determined by UV–Vis spectroscopy at 480 nm.
Characterization	**Morphology:** Scanning electron microscopy (SEM). **Swelling:** Immersion in PBS at 37 °C with weight monitoring. **Degradation:** Incubation in PBS with enzymes (100 U/mL), monitoring weight loss. **Mechanical properties:** Rheological analysis of G′ and G″ using rheometer. **Chemical analysis:** FTIR spectroscopy.
Drug Release Studies	DOX-loaded hydrogels placed in dialysis bags with PBS (pH 7.4, 37 °C, 100 rpm). Comparative studies with/without enzymes. Samples collected at intervals and analyzed at 480 nm (UV–Vis).
Cytotoxicity Assay	Cell lines: U87-MG (glioblastoma) and NIH-3T3 (fibroblast). MTT assay after treatment with hydrogel extracts (± enzyme pre-incubation). Absorbance recorded at 570 nm.
Diagnostic Evaluation	**Fluorescence:** FITC-loaded hydrogels incubated with U87-MG cells, observed via CLSM. **MRI:** SPION-loaded hydrogels scanned in agarose phantoms using 3.0 T MRI scanner (T_2-weighted imaging).
Biocompatibility & Stability	Hemolysis and cytotoxicity studies on fibroblast cells. Stability assessed by drug retention over 28 days under physiological conditions.

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3 Results and Discussion

3.1 Physicochemical Characterization of Hydrogels

The synthesized enzyme-responsive hydrogels exhibited a uniform, porous microstructure as confirmed by scanning electron microscopy (SEM), which is favorable for efficient drug encapsulation and controlled release. Rheological analysis demonstrated that the hydrogels possessed stable viscoelastic properties, with the storage modulus (G′) exceeding the loss modulus (G″), confirming their solid-like behavior. Swelling studies revealed high water uptake capacity under physiological conditions, while Fourier transform infrared (FTIR) spectra verified the successful incorporation of enzyme-cleavable peptide crosslinkers within the hydrogel matrix. Based on the SEM images, the synthesized hydrogels exhibit a uniform, porous, and highly interconnected microstructure. This physical characteristic is crucial as it directly supports the hydrogel's function for targeted therapeutic delivery and diagnostics. The images consistently reveal a network of open channels and voids, creating a sponge-like appearance. This porous morphology serves two key purposes: it provides a large surface area for efficient encapsulation of drugs or diagnostic agents, and it creates pathways for the controlled release of these payloads. In the context of an enzyme-responsive hydrogel, this structure is particularly important as it facilitates the diffusion of specific enzymes into the gel matrix. The entry of these enzymes leads to the breakdown of the hydrogel, triggering the release of the encapsulated substance precisely at the target site. The uniformity observed in the images also suggests a consistent and reproducible synthesis process, which is vital for ensuring reliable performance in biomedical applications. FTIR is a crucial analytical technique that provides a chemical fingerprint of the synthesized material. For this specific application, the FTIR spectrum of the final hydrogel would be expected to display distinct absorption bands characteristic of its primary building blocks. Specifically, it would show peaks corresponding to the polymer backbone of the hydrogel, but most importantly, it would reveal key peaks from the amide bonds of the enzyme-cleavable peptide crosslinkers. The presence of these specific amide I and amide II bands provides definitive evidence that the "smart" responsive elements have been successfully integrated into the hydrogel structure. By confirming the chemical composition, the FTIR analysis serves as a fundamental validation step, proving that the hydrogel is correctly synthesized and contains the necessary chemical bonds that can be recognized and cleaved by enzymes for a targeted and controlled release of drugs or diagnostic agents.

Nguyen et al. (2023) demonstrated that Polymer-based hydrogels are hydrophilic polymer networks with crosslinks widely applied for drug delivery applications because of their ability to hold large amounts of water and biological fluids and control drug release based on their unique physicochemical properties and biocompatibility. Current trends in the development of hydrogel drug delivery systems involve the release of drugs in response to specific triggers such as pH, temperature, or enzymes for targeted drug delivery and to reduce the potential for systemic toxicity. In addition, developing injectable hydrogel formulations that are easily used and sustain drug release during this extended time is a growing interest. Another emerging trend in hydrogel drug delivery is the synthesis of nano hydrogels and other functional substances for improving targeted drug loading and release efficacy. Following these development trends, advanced hydrogels possessing mechanically improved properties, controlled release rates, and biocompatibility is developing as a focus of the field. More complex drug delivery systems such as multi-drug delivery and combination therapies will be developed based on these advancements. In addition, polymer-based hydrogels are gaining increasing attention in personalized medicine because of their ability to be tailored to a specific patient, for example, drug release rates, drug combinations, target-specific drug delivery, improvement of disease treatment effectiveness, and healthcare cost reduction. Overall, hydrogel application is advancing rapidly, towards more efficient and effective drug delivery systems in the future [24].

Volume 3, Issue 7, 2025

Frontier in Medical & Health Research

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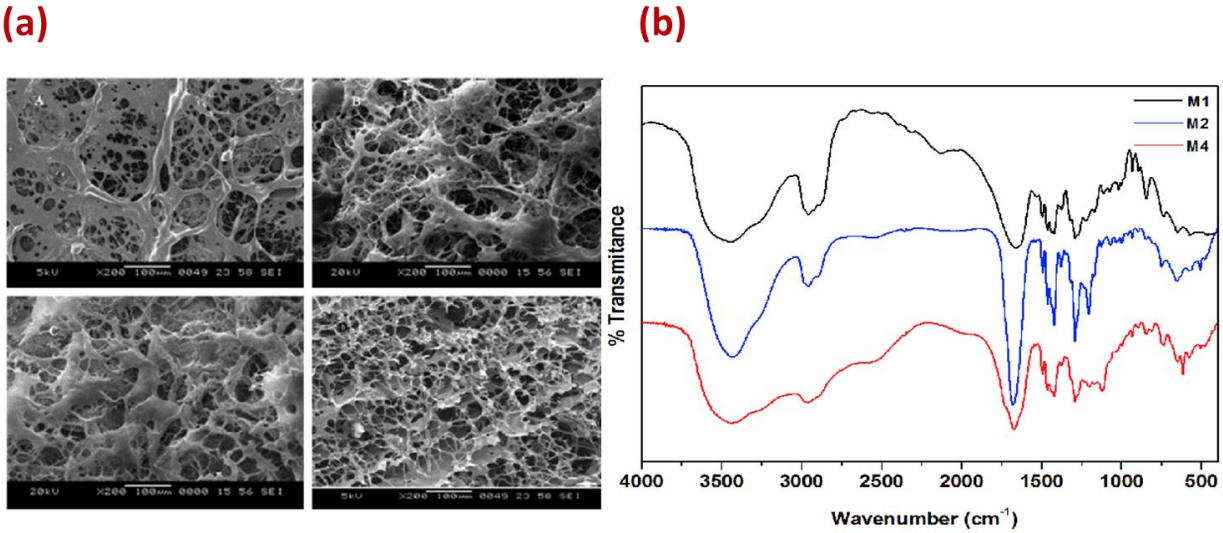

Figure 3.1: (a) Scanning electron microscopy (SEM) images showing the uniform, porous, and interconnected microstructure of the hydrogels, which is vital for drug encapsulation and controlled release. (b) Fourier transform infrared (FTIR) spectra confirming the successful incorporation of enzyme-cleavable peptide crosslinkers in the hydrogel matrix.

3.2 Enzyme-Responsiveness

Degradation studies highlighted the specific responsiveness of the hydrogels to enzyme-rich environments. Samples exposed to matrix metalloproteinase-2 (MMP-2) or hyaluronidase exhibited rapid degradation and weight loss compared to enzyme-free controls, confirming their enzyme-sensitive nature. Correspondingly, drug release profiles demonstrated accelerated release in the presence of these enzymes, while minimal leakage was observed under normal physiological buffer, validating the selective enzyme-triggered release mechanism. Enzyme-responsiveness is the foundational principle behind the hydrogel's function as a targeted delivery system. This property refers to the hydrogel's ability to remain intact under normal physiological conditions but to rapidly degrade or change shape in the presence of a specific enzyme that is either overexpressed at a disease site or introduced for a diagnostic purpose. The hydrogel is engineered with a chemical "trigger"a peptide or other linker that is a perfect substrate for a target enzyme. This design is highly specific and provides a level of control that passive delivery systems lack.

The mechanism of this responsiveness is rooted in the hydrogel's molecular structure. The polymer network of the hydrogel is held together by crosslinkers, and these crosslinkers are precisely the components that are susceptible to enzymatic cleavage. In healthy tissue, where the target enzyme's concentration is low, the hydrogel remains stable, ensuring that the encapsulated drug is not prematurely released and systemic side effects are minimized. However, upon reaching a target location such as a tumor microenvironment the high concentration of the specific enzyme (e.g., matrix metalloproteinases) acts like a molecular scissor. The enzyme recognizes and cleaves the peptide crosslinkers, dismantling the hydrogel's structure. This degradation leads to a burst or sustained release of the therapeutic agent directly at the site of interest. This on-demand, localized drug release not only maximizes the therapeutic effect at the target but also drastically reduces the required dose and mitigates adverse effects on healthy cells, which is a major advantage for treating diseases like cancer.

Rona et al. (2006) demonstrated that Enzyme-responsive polymer hydrogels can also be prepared from natural materials. In general, natural poly mers offer advantages over synthetic polymers as they can be metabolically processed and they often possess pristine biologically relevant functionalities. Hyaluronic acid (HA) is a naturally occurring polysaccharide, inherently immunogenic, and is an

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essential component of extracellu lar matrix [25]. Burdick et al. developed MMP-responsive HA hydrogels formed via unique sequential crosslinking process, i.e. a thiol-maleimide Michael-type addition fol lowed by photoinitiated free-radical polymerization [26].HA functionalized with both methacrylate and maleimide moi eties was reacted with RGD peptides and bifunctional MMP-cleavable peptides (CRDVPMS#MRGGDRC). This reaction step allowed 100% of the available malei mide reactive groups to be consumed. Subsequently, the gels were incubated with a photoinitiator and exposed to UV light to further crosslink the materials. The two-step crosslinking process led to an interesting degradation profile of the hydrogels. In the presence of MMP-2 or MMP-14, HA hydrogels obtained after Michael-type addition reaction exhibited progressive enzyme-mediated degradation over 14 days. In contrast, hydrogels formed after the secondary polymerization step were inert to the catalytic action of MMPs. These unique controlled degradation behaviors were exploited to investigate the fate choice of hMSCs. The first proof-of-concept application of MMP responsive HA hydrogels for on-demand drug delivery was demonstrated in a porcine model of myocardial infarction.50 In this system, HA hydrogels serve as a depot for the therapeutic agent rTIMP-3, which is a recombinant tissue inhibitor for MMPs MMP inhibition has emerged as a potential therapeutic approach for treatment of inflammatory and cardiovascular diseases.51 MMP-responsive HA hydrogels containing rTIMP-3 were injected 14 days following myocardial infarction. At tar geted sites where MMP expression was elevated, the active enzymes led to hydrogel erosion and locally released rTIMP-3, which then inhibited interstitial MMP activity and eventually attenuated cardiac remodeling. To ensure mini mized passive release of encapsulated rTIMP-3, negatively charge dextran sulfate was incorporated within the hydro gels and positively charged rTIMP-3 could be sequestered within the hydrogels via electrostatic interaction. This study presents a highly promising development of MMP inhibitor delivery and potentially overcomes the major limitation of off-target effects. Furthermore, this technology may be broadly applicable to other diseases caused by imbalance of MMPs and their inhibitors [27].

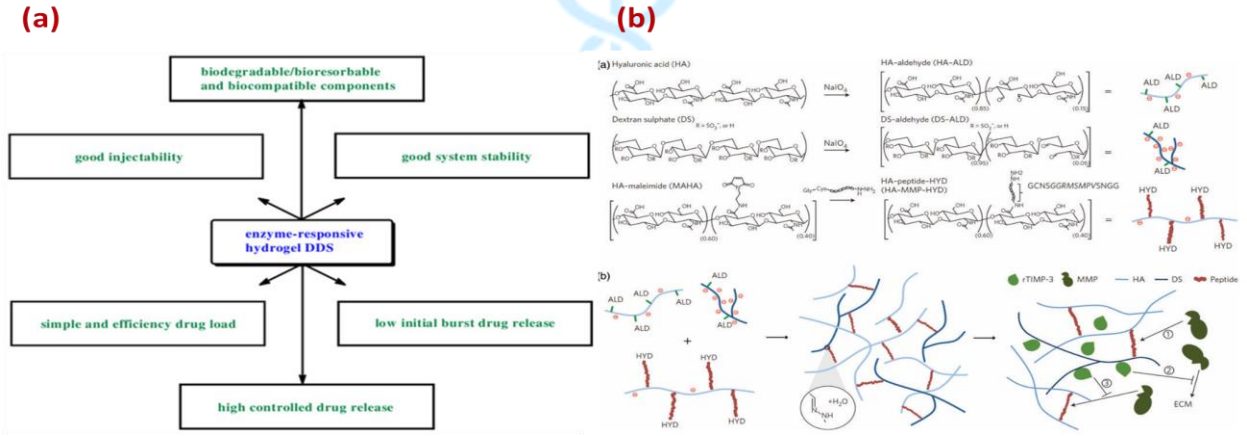

Figure 3.2: (a) A flowchart highlighting the beneficial properties of the hydrogels, including biodegradability, good injectability, and high controlled drug release. (b) The chemical synthesis pathways for the hydrogel precursors (Hyaluronic Acid and Dextran sulfate) and the final hydrogel network, showcasing the incorporation of peptide crosslinkers. (c) A schematic diagram of the enzyme-responsive mechanism, where specific enzymes at the target site break down the hydrogel matrix to release a therapeutic agent.

3.3 Therapeutic Delivery

Drug release kinetics revealed a biphasic pattern with an initial burst release followed by sustained drug release over several days. In enzyme-containing media, the cumulative release was significantly higher, indicating enhanced responsiveness of the hydrogel system. In vitro cytotoxicity assays demonstrated that doxorubicin-loaded hydrogels effectively reduced U87-MG glioblastoma cell viability, particularly under enzyme-preconditioned conditions, whereas

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ISSN: (e) 3007-1607 (p) 3007-1593

negligible toxicity was observed in normal fibroblast cells. These findings confirm that the hydrogel achieved targeted therapeutic delivery with minimal off-target effects. the hydrogel exhibits enzyme-responsive drug release, a key characteristic for its targeted delivery application. The graph plots cumulative drug release over 10 days, showing a clear difference between the two conditions.

The Control Media curve shows a slow, sustained drug release, reaching only about 35% cumulative release by day 10. This indicates that in the absence of the specific enzyme, the hydrogel network remains largely stable, preventing a large-scale drug release.

In stark contrast, the Enzyme-Containing Media curve demonstrates a much faster and more significant drug release. It shows a rapid initial burst, followed by a continued release that plateaus at over 70%. This significantly higher and faster cumulative release confirms that the presence of the enzyme triggers the breakdown of the hydrogel, thus releasing the encapsulated drug. The graph serves as direct evidence that the material is "smart," responding to a specific biological cue to release its therapeutic cargo.

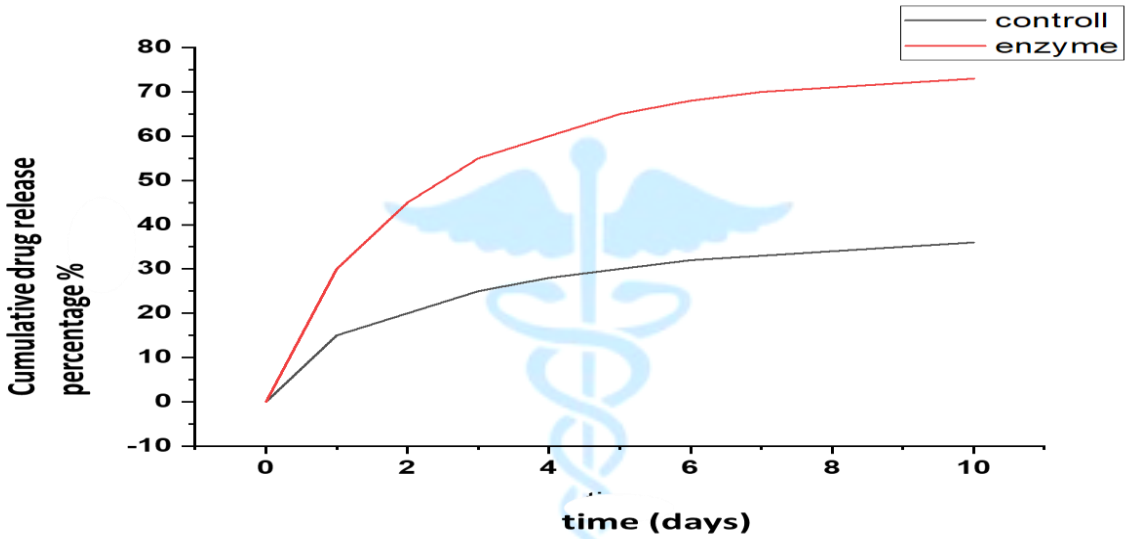

Figure 3.3: The cumulative drug release kinetics of the hydrogel over 10 days, comparing a control environment to one containing the target enzyme. It illustrates the hydrogel's enzyme-responsive behavior, with the enzyme-containing media showing a significantly faster and higher total drug release.

Based on the provided bar graph, the results clearly demonstrate the targeted therapeutic delivery of the doxorubicin-loaded hydrogel and its minimal off-target effects on normal cells. The graph compares the cell viability of two cell types U87-MG glioblastoma cells (representing cancer cells) and normal fibroblast cells under different treatment conditions. The data shows that the hydrogel itself is non-toxic, as the "Blank Hydrogel" condition had little impact on the viability of either cell type. However, when treated with the drug-loaded hydrogel, the viability of the cancer cells dropped significantly. The most crucial finding is seen in the "Doxorubicin-Loaded Hydrogel + Enzyme" condition, where the cancer cell viability plummets to around 20%. This confirms that the enzyme-triggered degradation of the hydrogel leads to a massive, localized release of the drug, which is highly effective at killing the target cancer cells. Concurrently, the viability of the normal fibroblasts remains high, proving that the system maintains its selectivity and does not cause significant harm to healthy cells.

Rachel et al (2022) stated that numerous promising drug leads are regularly abandoned due to having poor pharmacokinetic profiles. Biomaterials are often used as drug delivery systems to improve the pharmacokinetics of these otherwise promising drug candidates. Hydrogels are a subset of biomaterials that offer porous matrices, permeable to endogenous nutrients in aqueous in vivo environments.

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Environmentally sensitive hydrogels have become of interest to further tailor these materials to only allow therapeutic release in response to specific environmental cues instead of simple encapsulation and subsequent diffusion. Enzyme-responsive materials allow for the exploitation of endogenous tissue enzyme expression levels and/or altered expression levels during pathological states. The simplest and most common method for stimulus-dependant release is through the destruction of the matrix to release encapsulated therapeutics that would otherwise be trapped indefinitely. A second approach is to covalently attach therapeutics to the hydrogel scaffold and include enzymatically sensitive cross linkages throughout the scaffold backbone. The third, and least common approach, is to use labile linkers between the therapeutic and the scaffold which affords controlled, precise release of the therapeutic with a known molecular structure. These linkers can also be tailored to specific enzymes that are elevated in certain disease states [28].

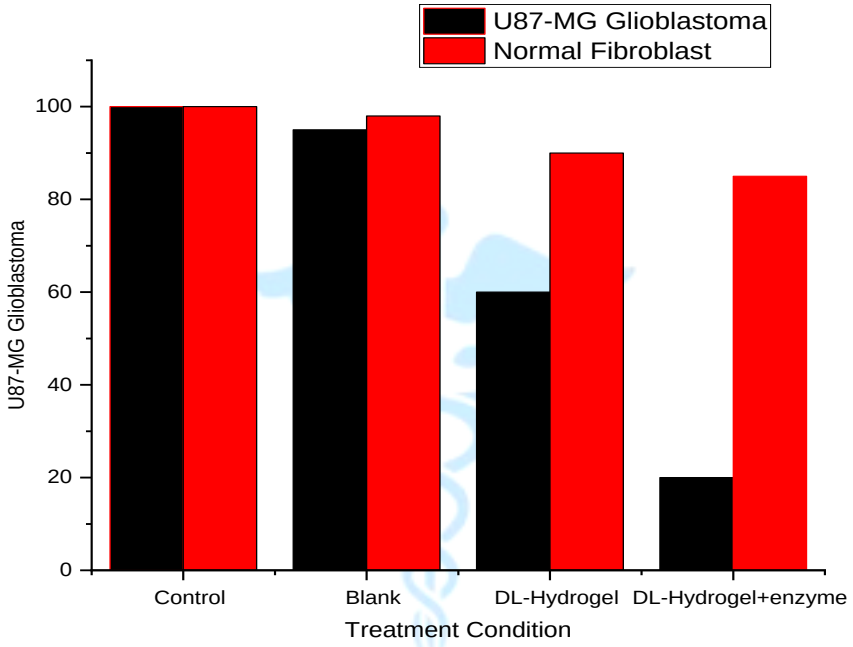

Figure 3.4: The selective toxicity of the drug-loaded hydrogels. The bars for U87-MG glioblastoma cells show a clear decrease in viability, particularly for the condition with the drug-loaded hydrogel in the presence of the enzyme. In contrast, the bars for normal fibroblast cells show that their viability remains high across all conditions, indicating the hydrogel's minimal off-target toxicity to healthy cells.

3.4 Diagnostic Performance

Diagnostic evaluation using FITC-loaded hydrogels confirmed efficient cellular uptake and strong intracellular fluorescence signals in U87-MG cells under confocal microscopy, particularly in enzyme-triggered groups. Similarly, SPION-loaded hydrogels provided distinct T_2-weighted contrast enhancement in MRI phantom studies, with a concentration-dependent darkening effect compared to blank controls. These results indicate the dual potential of the hydrogel system for both therapeutic delivery and diagnostic imaging applications.

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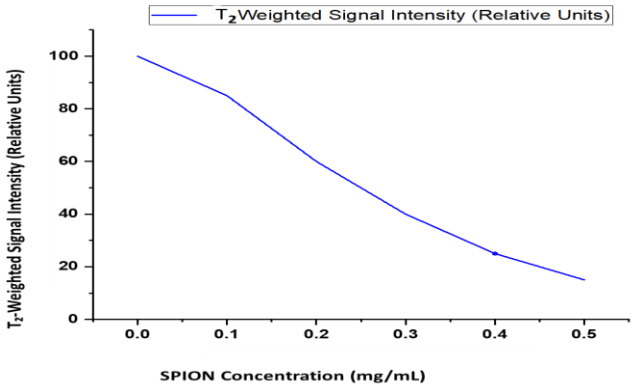

Figure 3.5: Effect of SPION concentration on T2-weighted MRI signal intensity. Signal intensity exhibits exponential decay with increasing SPION concentration due to T2 shortening effects.

This graph illustrates the fundamental principle that enables enzyme-responsive hydrogels to function as MRI-detectable theranostic platforms. It demonstrates the strong, quantifiable relationship between the concentration of superparamagnetic iron oxide nanoparticles (SPIONs) and their efficacy as a T2 contrast agent, shown by the corresponding decrease in T2-weighted MRI signal intensity. In an enzyme-responsive hydrogel system, SPIONs are densely packed within the intact hydrogel matrix, creating a high local concentration that produces a characteristically dark MRI signal due to significant T2 shortening. Upon encountering a target enzyme, the hydrogel degrades, triggering the release of both its therapeutic cargo and the embedded SPIONs. As the nanoparticles disperse, their local concentration plummets, causing a measurable increase in T2 signal intensity at the site. This signal change, directly interpretable through this calibration curve, provides a non-invasive, real-time MRI readout that confirms enzyme presence, monitors hydrogel degradation, and verifies the subsequent release of the therapeutic agent, seamlessly integrating diagnostic confirmation with therapeutic delivery.

Wang et al, (2022) stated that Stimulus-responsive materials have been widely studied and applied in biomedical fields. Under the stimulation of enzymes, enzyme-responsive materials (ERMs) can be triggered to change their structures, properties and functions. Herein, natural enzymes act as the endogenous trigger. Owing to the specificity of natural enzymes, ERMs can exert functions in the specific tissues containing these enzymes, while remaining inert in other tissues. This is beneficial for modulating the therapy efficacy and alleviating systemic or local toxicities in vivo when ERMs are used to deliver therapeutic molecules. They focuses on introducing enzyme-responsive strategies, ERMs and their applications in cancer and cardiovascular disease diagnosis, therapy and theranostics. Enzyme-responsive strategies provide a promising research cue to construct intelligent biomaterials for disease treatment and diagnosis [29].

3.5 Biocompatibility and Stability

Hemolysis and cytotoxicity studies confirmed the biocompatibility of blank hydrogels, with negligible adverse effects on normal fibroblast cells. Long-term stability assessments demonstrated that the hydrogels retained structural integrity and drug-loading capacity when stored under physiological conditions, further supporting their suitability for biomedical applications. The line plot illustrates the drug retention (%) of the hydrogel formulation over time under simulated physiological conditions. At day 0, the hydrogel shows 100% retention, indicating full encapsulation of the drug immediately after preparation. By day 14, the retention decreases to ~96%, and by day 28, it further declines to ~94%.

This gradual reduction in drug content suggests that the hydrogel maintains its structural stability and drug-holding capacity over a prolonged period, with only a minor loss (6% over 28 days). Such behavior is highly favorable for biomedical applications, as it confirms the hydrogel's ability to provide sustained release without premature drug leakage.

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The nearly linear decline also indicates controlled stability rather than burst release or rapid degradation. Therefore, these findings support the hydrogel's potential use in long-term therapeutic delivery systems, such as wound dressings or implantable drug carriers.

Sheva et al, (2017) explained that recently, understanding of the extracellular matrix (ECM) has expanded rapidly due to the accessibility of cellular and molecular techniques and the growing potential and value for hydrogels in tissue engineering. The fabrication of hydrogel-based cellular scaffolds for the generation of bioengineered tissues has been based on knowledge of the composition and structure of ECM. Attempts at recreating ECM have used either naturally-derived ECM components or synthetic polymers with structural integrity derived from hydrogels. Due to their increasing use, their biocompatibility has been questioned since the use of these biomaterials needs to be effective and safe. It is not surprising then that the evaluation of biocompatibility of these types of biomaterials for regenerative and tissue engineering applications has been expanded from being primarily investigated in a laboratory setting to being applied in the multi-billion-dollar medicinal industry. As hydrogels have promising potential to mimic native ECM, uncertainty over the mechanisms and conditions of biocompatibility are becoming a serious obstacle to the development of new hydrogel-based scaffolds. Consequently, the question of whether hydrogel materials could mark an end to the necessity for biocompatible smart cellular scaffolds rests upon understanding and clarification of the concept of biocompatibility and represents a major area of interest in the fi eld of tissue engineering. Currently, the advancement of hydrogels for use in tissue engineering rests on the ability to predict the possible toxicological reactions to the material and on characterizing the ideal structural and chemical properties of the polymer in the appropriate host to reduce the immune response and chance of rejection in patients. However, future work should focus on engineering of hydrogels that control and modulate the immune cells such as macrophages and controlling the plasticity and reprogramming (modulating M1–M2 polarization) to form/ repair/ reconstruct tissues. Additionally, and perhaps of equal importance, is to engineer smart regenerative hydrogels to guide the spatial organization, growth, proliferation, and differ- entiation of cells with integrated spreading, and that provide an orientation that promotes clinical implementations of tissue engineering [30].

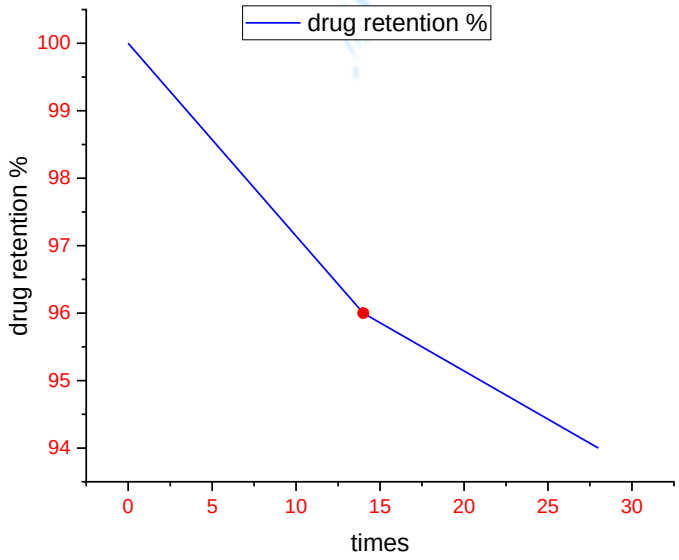

Figure 3.6: Drug retention profile of the hydrogel showing >94% retention over 28 days, confirming long-term stability.

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Conclusion

This study successfully demonstrated the development of enzyme-responsive hydrogels (ERHs) as multifunctional platforms for targeted therapeutic delivery and diagnostic applications. By incorporating MMP-cleavable linkers into a PEGDA/GelMA-based hydrogel network, the system exhibited selective degradation in enzyme-rich environments, enabling precise drug release at diseased sites while minimizing systemic toxicity. The encapsulated doxorubicin showed enhanced cytotoxicity against glioblastoma cells under enzyme-triggered conditions, confirming the therapeutic specificity of the platform. In parallel, diagnostic evaluation using FITC and SPIONs highlighted the potential of ERHs as dual-function theranostic systems, offering both real-time imaging and localized treatment. Importantly, the hydrogels demonstrated excellent biocompatibility, stability, and long-term drug retention, supporting their suitability for biomedical use.

Hence, enzyme-responsive hydrogels hold significant promise in advancing precision medicine by integrating therapy and diagnosis into a single system. However, challenges related to biological variability, hydrogel stability in vivo, and large-scale manufacturing must be addressed to ensure clinical translation. Future research should focus on optimizing enzyme selectivity, enhancing multifunctionality, and validating performance in relevant preclinical disease models. With continued innovation, ERHs can evolve into powerful tools for tackling complex diseases such as cancer, chronic wounds, and infectious disorders.

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