

Review

Recent Advances in Functionalization of Hyaluronic Acid for Biomedical Applications

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Abstract

Hyaluronic acid (HA) has emerged as a promising biopolymer for various biomedical applications due to its biocompatibility, biodegradability, and intrinsic ability to interact with cell surface receptors, making it an attractive candidate for numerous biological processes. Since HA cannot be gelled alone, it must be functionalized through different modification techniques, such as chemical, physical, bio-conjugative, and enzymatic, to activate the various sites present in it. This review analyzes these strategies and applications of HA in different biomedical fields. The first part of the review focuses on the different advanced functionalization strategies of HA, highlighting hydroxyl, amino, and carboxyl group-based reactions, as well as advanced techniques such as oxidation, sulfation, Cu-free click chemistry, thiol–ene coupling, hydrazone formation, bio-conjugation, nano-metal conjugation, and dynamic crosslinking methods. The second part of the review evaluates the use of functionalized HA in the development of advanced biomedical materials, including nano- and microparticles, hydrogels, and mucoadhesive materials with tailored drug release profiles, tissue engineering, skin wound healing, site-specific targeting, and stimuli-responsive behavior. Overall, this review critically consolidates the current advances and future perspectives in the functionalization of HA, underscoring its immense potential to drive the development of biomaterials with diverse biomedical applications.

Keywords: hyaluronic acid; functionalization; tissue engineering; drug delivery

1. Introduction

The creation of novel medical materials and drug delivery methods is enormously impacted by polymers derived from natural polysaccharides and their derivatives [1]. Among these, hyaluronic acid (HA) is the most prevalent glycosaminoglycan present in vertebrate tissues and is in charge of a number of essential functions [2]. It is a vital structural and functional component of the extracellular matrix (ECM) in connective, epithelial, and neural tissues. HA is a linear glycosaminoglycan made up of repeating units of N-acetyl-D-glucosamine and D-glucuronic acid with the monosaccharide units linked together via alternating β -1,3 and β -1,4 glycosidic bonds. Its chemical structure is substantially consistent, except for occasional deacetylated glucosamine residues [2,3]. In physiological conditions, HA takes the form of a sodium salt that is negatively charged and highly hydrophilic. The typical molecular weight (MW) of hyaluronic acid (HA), with the repeating unit $(C_{14}H_{21}NO_{11})_n$, has been investigated across a wide range in biomaterial



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applications, including 5 kDa, 60 kDa, 800 kDa, and 3 MDa. However, HA polymers having more than 1.8 MDa MW have been made commercially available [3]. HA possesses exceptional physicochemical properties such as biocompatibility, biodegradability, and non-inflammatory, non-toxicity, and non-immunogenic behavior [2]. Furthermore, understanding the dynamic metabolism and physiological behavior of HA is necessary for its biosynthesis. Hyaluronidases and oxidative species quickly break down about 30% of the body's HA, while liver and lymphatic channel endothelial cells catabolize the remaining 70%. The tissue half-lives of HA vary from minutes in the bloodstream to weeks in cartilage [4]. Under physiological settings, HA is a polyanion linked to extracellular cations, e.g., Na^+ , Ca^{2+} , Mg^{2+} , and K^+ [4]. In eukaryotic cells, integral membrane proteins catalyze the biosynthesis of HA at the inner surface of the plasma membrane [5]. Balazs used the extraction and purification procedure to extract the first pharmaceutically pure HA from rooster combs [5,6]. Microbial fermentation is the primary method used in the industrial manufacture of HA. Initially, *Streptococci* strains A and C were used, but more recently, commercially available strains such as *Streptococcus equi*, *Streptococcus zooepidemicus*, and even genetically engineered bacterial strains have been used [7,8].

The distinct reactive functional groups of HA enable diverse and controllable chemical modifications, which are crucial for tailoring its properties and performance in biomaterial applications. The HA disaccharide unit comprises three main reactive groups: hydroxyl (-OH), carboxyl (-COOH), and N-acetyl (-NHCOCH₃), as shown in Figure 1. These reactive groups serve as chemical handles for different functionalizations, allowing for the incorporation of new reactive sites or crosslinkers that define material performance [9]. The main reactive functional groups of hyaluronic acid and their characteristics are as follows: The hydroxyl (-OH) groups are located at the C6 position of N-acetyl-D-glucosamine and the C₂/C₃ positions of D-glucuronic acid. These groups are nucleophilic and relatively mild in reactivity, allowing for reactions such as esterification, etherification, and methacrylation [10,11]. The carboxyl (-COOH) group is found at the C6 position of D-glucuronic acid. It is acidic and reacts readily with amines, making it suitable for amidation (such as EDC/NHS coupling) and drug conjugation processes [12]. The amide (-NHCOCH₃) group occurs at the C₂ position of N-acetyl-D-glucosamine. It is weakly reactive but can be modified after deacetylation to allow for amine substitution or grafting reactions [13,14]. Although HA-based materials are widely applied in biomedical systems, native HA exhibits several inherent limitations, including rapid enzymatic degradation, limited drug-binding capacity, and short *in vivo* residence time [15]. Over the past decade, HA functionalization has evolved from classical reactions targeting its hydroxyl, carboxyl, and N-acetyl groups to highly sophisticated chemistries that offer unprecedented control over molecular structure and biomedical behavior [13]. Many reviews focus predominantly on specific categories of functionalization, such as chemical derivatization [16–20], hydrogel formation [21–23], or drug conjugation [23–25], without providing a holistic comparison across chemical, physical, enzymatic, and bio-orthogonal functionalization strategies. Moreover, numerous reviews adopt a thorough, catalog-style approach, summarizing reaction chemistries and applications without critically evaluating how different functionalization pathways influence material properties, biological exchange, and translational possibilities, or often do not focus on structure–property–function relationships and/or rational design methodologies [26–31]. However, several traditional methods like carbodiimide coupling [16], esterification/etherification [32], thiol modification [33], hydrazone/Schiff-base formation [34], sulfation [35], selective oxidation (periodate) at C₂–C₃ positions [36], and hydrogen bonding of intra- and intermolecular HA [37] provided precise molecular-weight tuning for viscosity, diffusion, and biodegradability control. These techniques revealed how structural modifications alter HA's persistence length, crosslinking efficiency,

hydration behavior, immune activation, and degradation kinetics. These chemical routes collectively expand the functionality of HA and establish it as a versatile biomaterial for designing advanced systems in drug delivery, tissue engineering, and regenerative medicine.

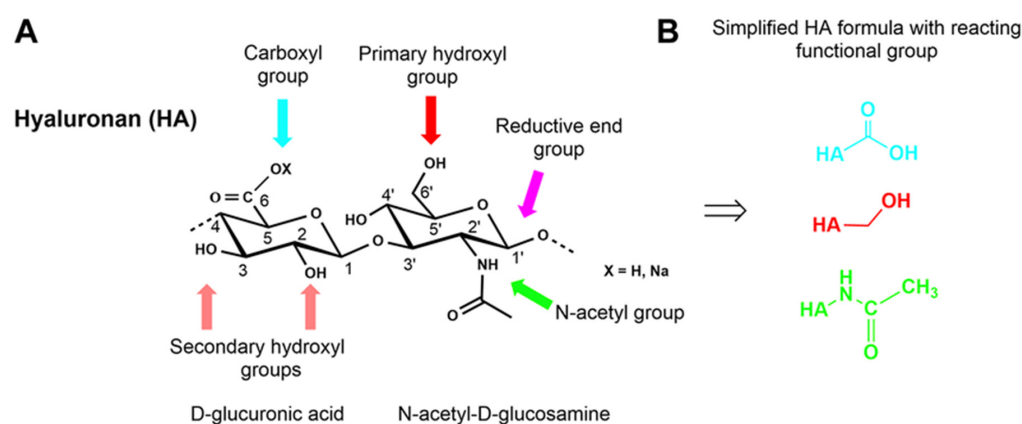


Figure 1. Structure (A) and reactive functional groups (B) of HA [20].

The field has since enlarged into advanced functionalization paradigms that emphasize precision, bio-orthogonality, and multifunctionality. Herein, this review particularly focuses on modern functionalization approaches, including strain-promoted copper-free click chemistry, thiol–ene crosslinking, dynamic hydrazone coupling, and oxidation methods (TEMPO/TCC (2,2,6,6-Tetramethylpiperidine-1-oxyl radical/Trichloroisocyanuric Acid) at C₆ position and H₂O₂-based non-specific oxidation). It also covers supramolecular physical crosslinking strategies such as coating/assembly formation through electrostatic/ionic interactions, guest–host chemistry, hydrophobic interactions, advanced hydrogen bonding, enzyme-mediated crosslinking (HRP/H₂O₂, transglutaminase, tyrosinase), and HA–drug/protein/lipid/antimicrobial conjugation. Together, these approaches enable programmable gelation, controlled drug release, mechanically tunable scaffolds, and stimuli-responsive behavior. In addition, these chemistries support the development of injectable hydrogels, self-healing biomaterials, immune-modulating matrices, and precision drug delivery carriers. Most importantly, this review demonstrates that functionalization parameters—including the degree of modification, reaction environment, ionic strength, linker selection, and crosslinker architecture—directly govern network formation, biomechanical strength, degradation behavior, bioactivity, and therapeutic performance, as well as the associated characterization techniques.

Simultaneously, the integration of HA with other biopolymers (gelatin, chitosan, alginate, collagen, etc.) has emerged as a compelling composite system that combines the biological cues of natural ECM components with the mechanical tunability of synthetic polymers and contributes to multifunctional therapeutics. HA–drug conjugates such as HA–paclitaxel, HA–camptothecin, HA–doxorubicin, and HA–curcumin exemplify how chemical conjugation can enhance solubility, prolong cellular retention, enable receptor-mediated targeting, and support sustained release. Furthermore, enzyme–HA hybrid systems, HA-modified nanoparticles (HA–Au, GOQD–HA (Graphene Oxide Quantum Dot–Hyaluronic Acid), HA–Fe etc.) and dynamic crosslinking illustrate new opportunities for immobilized catalysis, ROS (reactive oxygen species)-responsive materials, and bioactive multifunctional nano-therapeutics.

Despite impressive progress, some key challenges remain, such as reproducibility, scalability for translational use, potential cytotoxicity from chemical reagents, and incomplete understanding of structure–function relationships. Addressing these gaps is crucial for the development of truly next-level HA-based biomaterials. Taken together, these classical

and emerging methodologies signal a paradigm shift: functionalization is no longer an auxiliary modification but a central design philosophy for engineering next-level HA biomaterials. By connecting molecular chemistry to macroscopic function, critically evaluating mechanistic instincts regarding biomedical performance, and integrating these modules to clinical application, this review highlights HA functionalization as a powerful and modular toolkit for the rational design of intelligent biomaterials.

2. Functionalization Methods

HA functionalization strategies can be classified based on the mode of interaction and level of structural control, rather than solely on individual chemical reactions (Figure 2). At first, chemical crosslinking methods depend on the covalent modification of HA to generate static networks or tailored conjugates with improved mechanical strength and degradation resistance. Secondly, physical crosslinking strategies exploit non-covalent interactions to construct dynamic and reversible HA assemblies, offering advantages such as injectability, self-healing behavior, and stimulus responsiveness. Thirdly, bio-conjugation strategies focus on attaching biologically active entities such as drugs, proteins, peptides, or complementary polymers to HA to introduce specific therapeutic functions, targeting capability, or synergistic biological effects. Recently, enzymatic functionalization approaches have emerged as highly specific and biologically compatible substitutes, enabling controlled HA modification under mild conditions while minimizing structural damage and cytotoxicity. Finally, nanoconjugation techniques illustrate an advanced design paradigm, where HA is incorporated with a multifunctional hybrid system for molecular-level modification and nanoscale engineering. Together, these functionalization strategies form a hierarchical design framework for engineering next-generation HA-based biomaterials with tunable structure, function, and biomedical performance.

Functionalization Strategy of Hyaluronic Acid (HA)	Chemical Crosslinking & Modificaton	OH Group Modification
		COOH Group Modification
		N-acetyl group modification
		Oxidation of HA
		Sulfation of HA
	Click Chemistry	Cu-free Click Chemistry
		Thiol-ene Crosslinking
		Hydrazone Coupling
	Physical Crosslinking	Ionic Interaction
		Guest-Host Interaction
		Hydrogen Bonding
		Hydrophobic Interaction
	Biomolecule Conjugation	HA-Drug Conjugation
		HA-Protein Conjugation
		HA-Lipid Conjugation
		HA-Antimicrobial Conjugation
		Other Biopolymers
	Advanced and Specialized Strategies	Enzymatic Crosslinking
		Nano-based Metal Conjugation
		Dynamic Crosslinking (Reversible networks of HA)

Figure 2. HA functionalization strategies.

2.1. Functionalization by Chemical Crosslinking

Chemical crosslinking is a well-established technique for improving the physical and chemical properties of native HA, including its mechanical strength, swelling behavior,

and rheological characteristics. Specifically, covalent crosslinking makes it easier for hydrogels, cryogels, sponges, and other solid structures to form [38]. Typically, the most common crosslinkers are tiny compounds with at least two functional groups and a spacer designed to join HA chains, like 4-butanediol diglycidyl ether (BDDE) [39], divinylsulfone (DVS) [40,41], poly (ethylene glycol) diglycidyl ether (PEGDE) [42], octenylsuccinic anhydride [43,44], glutaraldehyde [34], methacrylic anhydride [45], maleic anhydride [46], diamines [47,48], biscarbodiimides [9], etc. The crosslinker's chemical composition can also affect the gel's properties; for example, a polyfunctional crosslinker might lead to more difficult degradation, while a long and hydrophilic crosslinker backbone could result in excessively large swelling properties [49].

2.1.1. OH Group Modification

The modification of hydroxyl group-containing biopolymers with mono- and dialkylating agents to form ethers is a classical reaction for many polysaccharides. Alkylation of the HA macromolecule brings about a strong change in the rheological properties of the biopolymer [20] (Figure 3). In 2006, Creuzet et al. performed an alkylation reaction in homogeneous aqueous conditions (NaCl) from an HA derivative having C₈, C₁₀, C₁₂, C₁₄, and C₁₆ chains selectively modified with adipic dihydrazide (HA-ADH) and followed by 1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide (EDC). C₁₀- and C₁₂-alkylated HA derivatives in aqueous solutions should find application in the field of viscosupplementation, where the physical crosslinkage enhances the rheological performance of the polymers [50]. Pavan et al. followed a similar trend and analyzed five amide derivatives of HA that were synthesized with C₈, C₁₂, C₁₅, C₁₆, and C₁₈ linear alkylamines and observed viscoelastic behavior. They observed that C₁₅, C₁₆, and C₁₈ showed a significant stabilization of viscoelastic properties during thermal treatment. Furthermore, the amide side chain exhibited similar behavior to chemically crosslinked HA and improved performances with respect to linear HA in terms of the preservation of viscoelasticity after chain depolymerization [51,52]. Mlčochová et al. adopted another approach for the activation of the hydroxyl groups of glycosaminoglycans, in which HA reacts with cyanogen bromide, followed by the nucleophilic addition of alkylamine [53]. In contrast to other alkyl derivatives of hyaluronan, these derivatives preserve unaltered carboxyl groups, which are utilized for the hydrophobization of hyaluronan. Due to the obtained hydrophobic properties, the derivatives might be potential candidates for targeted delivery systems.

Yang et al. showed that under harsh conditions, epoxides serve as electrophiles in the Williamson etherification process through ring opening. This reaction creates a bridge between the HA chains, resulting in an elastic crosslinked HA hydrogel. The degree of modification for HA fillers crosslinked with BDDE (1,4-Butanediol Diglycidyl Ether) ranges from 1 to 10% [39]. Oudshoorn et al. generated sodium-HA polymerized with methacrylate residues, exhibiting scrupulous control over the degree of substitution. The synthesis of methacrylated hyaluronic acid (HA-MA) was performed in dimethyl sulfoxide (DMSO), employing glycidyl methacrylate (GMA) and 4-(N,N-dimethylamino)pyridine as a catalyst. Rheological characterization showed that with increasing DS (degree of substitution), the strength of HA-MA hydrogels increased, but swelling properties decreased with the increase in DS [45]. Kristiffer and Corinne noted that the OH group of HA can also be esterified with octenyl succinic anhydride or methacrylic anhydride [43,54]. JY Lai explored for the first time that the crosslinking of HA with glycidyl methacrylate-HA takes place at a pH above 13 and room temperature. Conducting the reaction at normal temperature helps prevent acid-induced breakdown of HA, which can happen at higher temperatures [40]. Zhang et al. established a novel scheme under mild environments at C₆ hydroxyl groups via an ether bond to synthesize a cysteine-hyaluronic acid combination.

This conjugate is suitable to prepare injectable and in situ-formed hydrogels crosslinked by native chemical ligation and Michael addition [41]. This process forms sulfonyl-bis-ethyl ether bonds between the hydroxyl groups of HA. The cytocompatibility of HA hydrogels crosslinked with DVS (Divinyl Sulfone) at varying concentrations (0–100 mM) was studied. Investigations suggested that as the concentration of DVS increased, the amount of sulfur and sulfonyl-bis-ethyl crosslink increased, as well as its mechanical durability and resistance to enzymatic degradation. This implies that the crosslinker plays a critical role in changing the structure and properties of this polysaccharide biomaterial. In addition, Min Yoo et al. revealed the synthesis of an HA derivative by reacting -OH groups with maleic anhydride (MA) [46]. Because MA is a highly reactive anhydride, it easily undergoes a ring-opening reaction in an organic solvent to form non-toxic compounds. In this process, HA is dissolved in DMSO and heated to 60 °C, allowing the reaction between the hydroxyl groups of HA and maleic anhydride to occur [46].

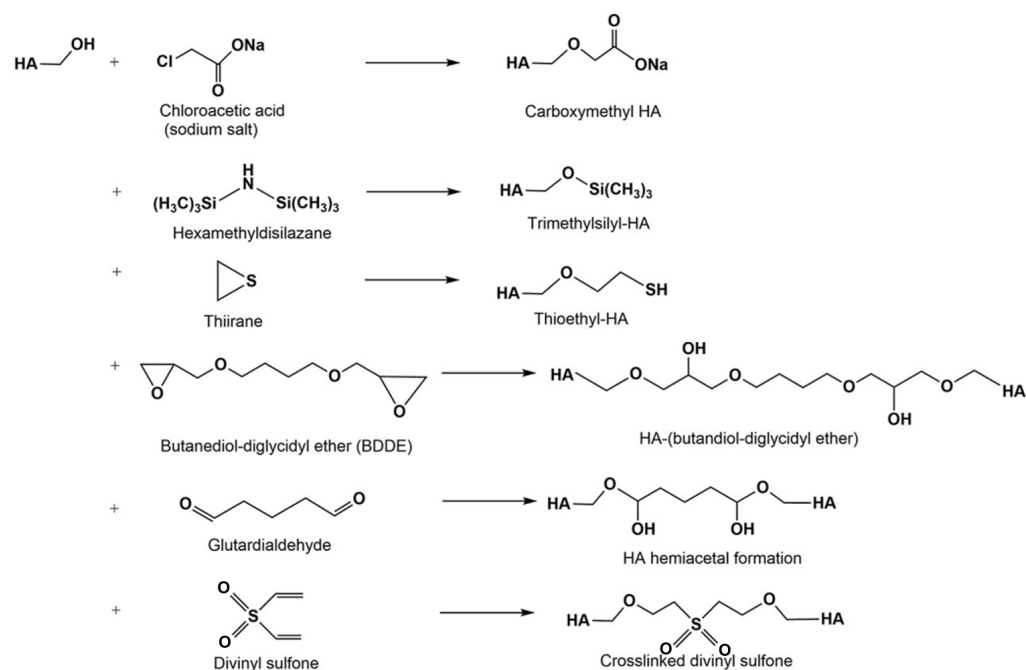


Figure 3. Crosslinking of hydroxyl groups of HA. Reproduced from Frontiers Review [20].

Tomihata and Ikada noted a hemiacetal crosslinking bond between the OH group of HA and glutaraldehyde in acetone–water mixtures in the presence of HCl. The hydroxyl group of HA can be converted to hemiacetal or full acetal in an acid-catalyzed reaction [34]. The optimal HCl concentration for HA crosslinking was found to be 0.01 N. Here, special care must be taken throughout the reaction due to the poisonous nature of glutaraldehyde. Baier Leach et al. and Moller et al. showed the reaction of epoxides with the hydroxyl group of HA and described the possible methacrylation mechanism using glycidyl methacrylate [55,56]. The obtained methacrylated HA products were subsequently crosslinked using UV radiation.

2.1.2. COOH Group Modification

Activation of the COOH group of HA often involves several crosslinking reactions like esterification and amidation (Figure 4). Bencherif et al. showed that two simultaneous reactions took place between HA and glycidyl methacrylate in the presence of triethylamine catalyst. A reversible transesterification occurs via the primary hydroxyl group of HA and an irreversible ring-opening conjugation via the HA-COOH group to the most modified carbon of the epoxide [57]. However, the concentration of ring-opening products

gradually increases with time, suggesting the necessity for longer times to promote epoxide ring-opening products.

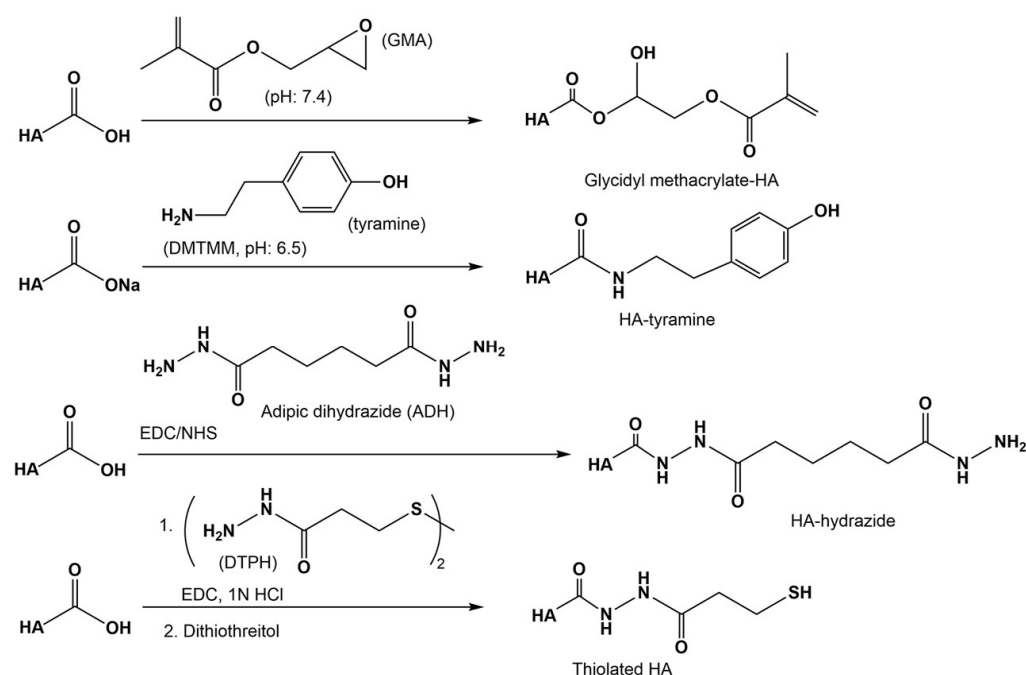


Figure 4. Reactions of the HA carboxyl group with epoxides, amines, and hydrazides. Reprinted from Frontiers Review [20].

Kuo et al., Ponedel'kina et al., and Liu et al. showed that the carboxyl groups of HA can be activated by carbodiimide (i.e., N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride, EDC) and using co-activators such as N-hydroxysuccinimide (NHS), 1-hydroxybenzotriazole, or triazine compounds [16,58,59]. Schanté et al. devised a synthesis of HA with N-alanyl-hyaluronamide derivatives using different amidation methods performed either in water, a water/acetonitrile mixture, or anhydrous dimethylformamide [17]. The resulting amidation of hyaluronic acid led to a DS of the carboxylic groups of up to 100% and demonstrated that the N-alanyl-hyaluronamides presented enhanced resistance towards enzymatic digestion. Kim et al. synthesized injectable tyramine-modified hyaluronic acid (HA-Tyr) hydrogels via amide coupling and enzymatic crosslinking using horseradish peroxidase/H₂O₂ [60]. The dexamethasone-encapsulated hydrogel was used as a model drug for the treatment of rheumatoid arthritis (RA). The hydrogel showed sustained release of dexamethasone up to a month in vitro and in vivo. In collagen-induced arthritis models, the HA-Tyr hydrogels impressively reduced interleukine-6, prostaglandin E2, and multiple cytokines, and this histological analysis confirmed the therapeutic efficacy of the hydrogels for the treatment of RA [60].

2.1.3. N-Acetyl Group Modification

HA's N-acetyl groups play a massive role in skin and age-related issues. It helps in maintaining skin hydration and elasticity. HA gradually loses its N-acetyl groups in the body as it grows older, which contributes to the formation of wrinkles and other signs of skin aging. Zhang et al. observed the generation of two highly N-deacetylated HAs, dHA-6 and dHA-10, via NH₂NH₂-HIO₃, and the N-deacetylation degrees (DD) were determined by ¹H NMR. The N-deacetylated HAs exhibit markedly lower moisture absorption (Ra) and moisture retention (Rh) capacities compared to their corresponding re-N-acetylated products, which may contribute to the manifestations of aged skin, such as laxity, sagging, and wrinkling [61]. The results showed that both HAs and dHAs could activate the nu-

clear factor-kappa B (NF- κ B) transcription factor in antigen-presenting cells (APCs) and stimulate cytokine production via the Toll-like receptor (TLR)/Myeloid Differentiation Primary Response 88 (MyD88) pathway. However, the cytokine-inducing ability of dHAs was lower than that of HAs. Moreover, the TLR-2 pathway was more active in response to the appearance of zwitterionic motifs in dHAs [62]. Sedláček et al. utilized nitrous acid and *Streptococcus pneumoniae* hyaluronan lyase for the selective deacetylation of HA to reveal the amino group distributions within, as reactive amino groups are crucial for further modification of HA [63]. The resulting oligomers were assessed using liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS), nuclear magnetic resonance (NMR), and high-performance liquid chromatography (HPLC). The findings show that deacetylation does not occur randomly, which might be attributed to the steric hindrance of N-acetyl-D-glucosamine (GlcNAc) resulting from the electrostatic interactions between D-glucosamine (GlcN) and D-glucuronic acid residues (GlcA) [63]. Babasola et al. produced low-molecular-mass (reducing Mw to 30–214 kDa) HA (LMM-HA) by hydrazinolysis (partial N-deacetylation) to reveal the role of LMM-HA in stimulating inflammation [64]. The resulting free NH₂ functional groups were acylated (re-acetylation) using acyl anhydride. The results revealed that the re-acetylated HA strongly stimulated pro-inflammatory cytokine production in THP-1 macrophages via TLR-4 (Toll-like receptors) receptor system, while partially deacetylated HA showed no activity. Notably, N-butyrylated LMM-HA suppressed cytokines most among all tested partially N-acylated hyaluronan derivatives indicating it as a promising anti-inflammatory candidate [64]. Saturnine et al. showed that acetylated HA (HA–Acet) holds as much antioxidant and anti-inflammatory activity comparable to native HA. However, in terms of bioavailability, HA–Acet showed a six-fold increase in bioavailability and slightly improved radical scavenging in lipopolysaccharide (LPS)-stimulated macrophages. Both HA and HA–Acet were non-cytotoxic (IC₅₀ > 100 μ M); however, HA–Acet showed enhanced stability and biological performance without the loss of native activity [65]. Together, this study highlights biological properties and therapeutic uses of acetylated HA derivatives.

2.1.4. Oxidation of HA

Oxidation is mainly used to introduce aldehyde groups. Three major oxidation strategies used in HA chemistry: periodate oxidation and hydrogen peroxide oxidation/degradation. Kristiansen et al. and França et al. showed that partial periodate oxidation of hyaluronan (1–20%) leads to ring opening at the C₂ and C₃ positions of the 4-linked D-glucuronate (GlcA) residues (Figure 5i) [36,66]. The electrostatic contribution to the total persistence length increased with increasing degree of oxidation (DO). Similarly, Pandit et al. analyzed the modification of HA with sodium metaperiodate (NaIO₄), which results in the formation of oxidized HA. This OHA (oxidized HA) contains multiple aldehyde groups, which easily react with amino functionality via the formation of Schiff base linkage, resulting in the formation of OHA-based hydrogel scaffolds [67]. In one study, K-carrageenan (KC) is exposed to oxidation by sodium periodate (NaIO₄) in acidic medium [68]. The optimal conditions are NaIO₄ to KC 0.5:1; temperature: 40 °C; time: 3 h. Then, OKC was mixed with HA and polyvinyl alcohol (PVA) to deliver an electrospun arrangement, and a ratio of HA, OKC, and PVA at 2.5:2.5:5 was accomplished for uniform, normal, and globule-free nano-filaments [68].

Shan et al. observed that the degree of oxidation by the N-acetylglucosamine unit with (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO)/trichloroisocyanuric acid (TCC) oxidation can be further increased by using anhydrous DMF as the solvent to avoid overoxidation of the primary alcohol group to carboxylic acid (Figure 5ii) [69]. Moreover, hydrogels were formed using ADH hydrazine as a crosslinker and applied to the chondrogenic differenti-

ation of human mesenchymal stromal cells (hMSCs). Jiang et al. showed that the rate of TEMPO-mediated oxidation decreased with an increase in the ionic strength of the reaction mixture [70]. Ponedel'kina et al. also performed the selective oxidation of hyaluronic acid (HA) by 2,2,6,6-tetramethylpiperidine-1-oxoammonium chloride (TEMPO⁺) in harsh conditions (pH > 8) [71]. The results indicated that high-efficiency HA oxidation occurred, and unusual behavior of the oxidation system was observed, such as dioxygen evolution in the decomposition system.

Hydrogen peroxide (H₂O₂) generates free radicals that cause non-specific chain scission of HA [72]. However, this is not a functionalization reaction but is widely used to tune molecular weight (MW), viscosity, and biodegradability [73]. For example, Abbasi et al. found that the treatment of reactive oxygen species (ROS) on cancer cells with H₂O₂ + HA was significantly more effective than H₂O₂ alone [74]. Lin et al. and Chen et al. show that HA can be effectively degraded by an admixture involving ultrasound, hydrogen peroxide, and copper ions. In acidic medium, the content of glucuronic acid was 36.56%, and the yield of LMW-HA was 81.71% [75,76]. In another study, Elvitigala et al. reported the degradation of phenolated HA by oxidative cleavage using H₂O₂-induced degradation [77]. The variation in H₂O₂ exposure helps to modulate the physicochemical properties of the prepared composite hydrogel systems. The higher the exposure time, the higher the Young's modulus and degradation time; however, there is a threshold value of exposure time above which (60 min) both decrease. Mouse myoblast C₂C₁₂ cells underwent myogenic differentiation onto the stiff composite hydrogels, as shown in Figure 5iii,iv [77].

2.1.5. Sulfation of HA

Sulfation of hyaluronic acid (HA) is a process that adds sulfate groups to the HA molecule, creating sulfated hyaluronic acid (SHA). This modification is achieved through numerous methods and makes SHA more stable, with a slower degradation rate and anti-inflammatory and anti-angiogenic properties [78]. Sulfation of HA occurs at different hydroxyl groups, i.e., at the C₂, C₃, C₄, or C₆ position of OH, depending on the concentration of the sulfating reagent, temperature, and reaction time, and increases the HA molecule's negative charge density [79]. By varying reaction times, D.S. Bhattacharya et al. generated selectively (ssHA), moderately (msHA), and completely (csHA) sulfated hyaluronic acid using a multistep synthetic approach to alter binding specificities for CD44 and P-selectin to tumor cell interaction [80]. While P-selectin binding relies on electrostatic interactions with sulfate groups, CD44 binding is less sensitive to sulfation (Figure 6(iA–iF)). Nanoparticles made from ssHA/msHA/csHA (labelled with Cy7.5/IRDye800) showed higher uptake by S2-013 tumor cells. Combination antibody blocking gave the greatest reduction for dual-binding SHA-NPs. (Figure 6(iiA–iiH)) [79]. According to Feng et al., SHA using hyaluronidase degraded more slowly than wild-type HA. They summarized that the sulfation reduces the available HA octasaccharide substrate needed for the effective catalytic action of hyaluronidase. HA hydrogel sulfation exerts no negative effect on the viability of encapsulated human mesenchymal stem cells (hMSCs). Moreover, the sulfated HA hydrogels significantly improve protein sequestration, thereby effectively extending the availability of the proteinaceous drugs in the hydrogels [80]. Behera et al. explored the impact on bone morphogenetic protein-2 (BMP-2) conformation, aggregation, and mechanistic pathways of aggregation using diverse optical and rheological methods in the presence of HA and SHA. They showed that SHA led to rapid amorphous aggregates, while HA promoted amyloid fibrils with a lag phase and sigmoidal kinetics. Aggregations were also influenced by viscosity and excluded volume. Higher viscosity, low diffusivity of protein, and higher excluded volume in the presence of HA promote fibrillation, with sizes in the micrometer range. Low viscosity, high diffusivity of protein, and lesser

excluded volume in the presence of SHA lead to an amorphous aggregate, with sizes in the nanometer range [81]. In the same way, Thönes et al. divulged hyaluronan/collagen hydrogels containing sulfated hyaluronan that improve wound healing by sustained release of heparin-binding EGF (HB-EGF)-like growth factor [82]. Molecular modeling (MM) and surface plasmon resonance (SPR) analyses demonstrated that sulfation of HA increases binding strength to HB-EGF, which supports the creation of SHA-infused hydrogels for use in promoting epithelial tip growth in wounds, as demonstrated in a porcine skin organ culture model, along with their effectiveness as wound dressings.

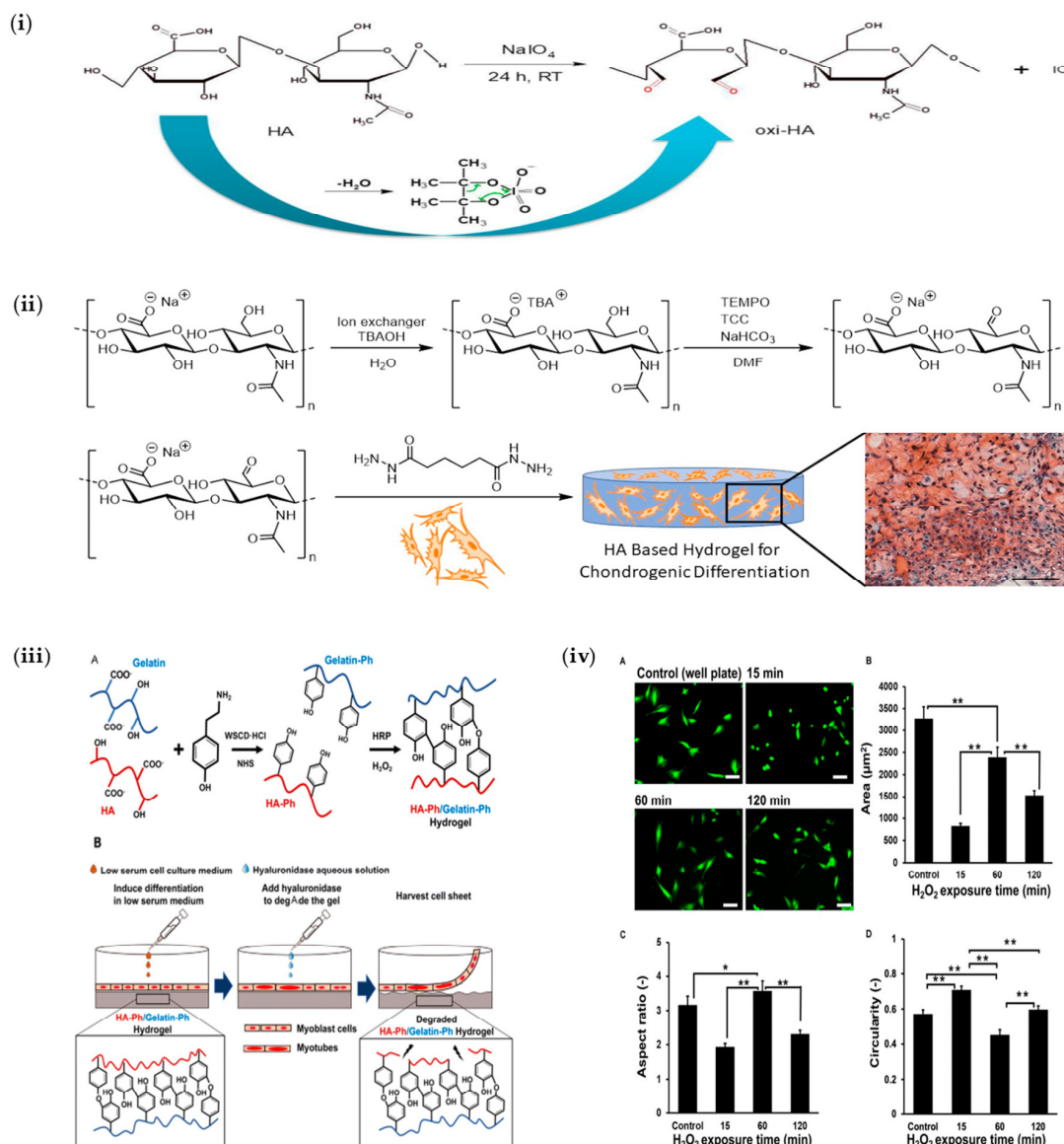


Figure 5. (i) Partial oxidation of hyaluronic acid (HA) with sodium periodate (NaIO_4) to form oxidized hyaluronic acid (oxi-HA). In red, aldehyde groups, and in green, the bond breaks during oxidative cleavage. Reprinted from MDPI [66]. (ii) Selective oxidation of the primary alcohol of HA by using TEMPO/TCC in anhydrous DMF, followed by hydrogel formation by AdH hydrazine crosslinkers. Reprinted from MDPI [69]. (iii) A. synthesis of HA-Ph and Gelatin-Ph using WSCD-HCl and NHS, followed by HRP-mediated crosslinking of these polymers, utilizing hydrogen peroxide (H_2O_2) to yield the hydrogel; B. After inducing myogenic differentiation on the hydrogel, the cell sheet is obtained by treating the HA-Ph/Gelatin-Ph hydrogel with hyaluronidase. (iv) A. Fluorescence micrographs of C2C12 cells on hydrogels stained with Calcein-AM. Scale bars: 100 μm . B. Area. C. Aspect ratio. D. Circularity of cells on hydrogels (* $p < 0.05$ and ** $p < 0.01$). Reprinted from Royal society of chemistry [77].

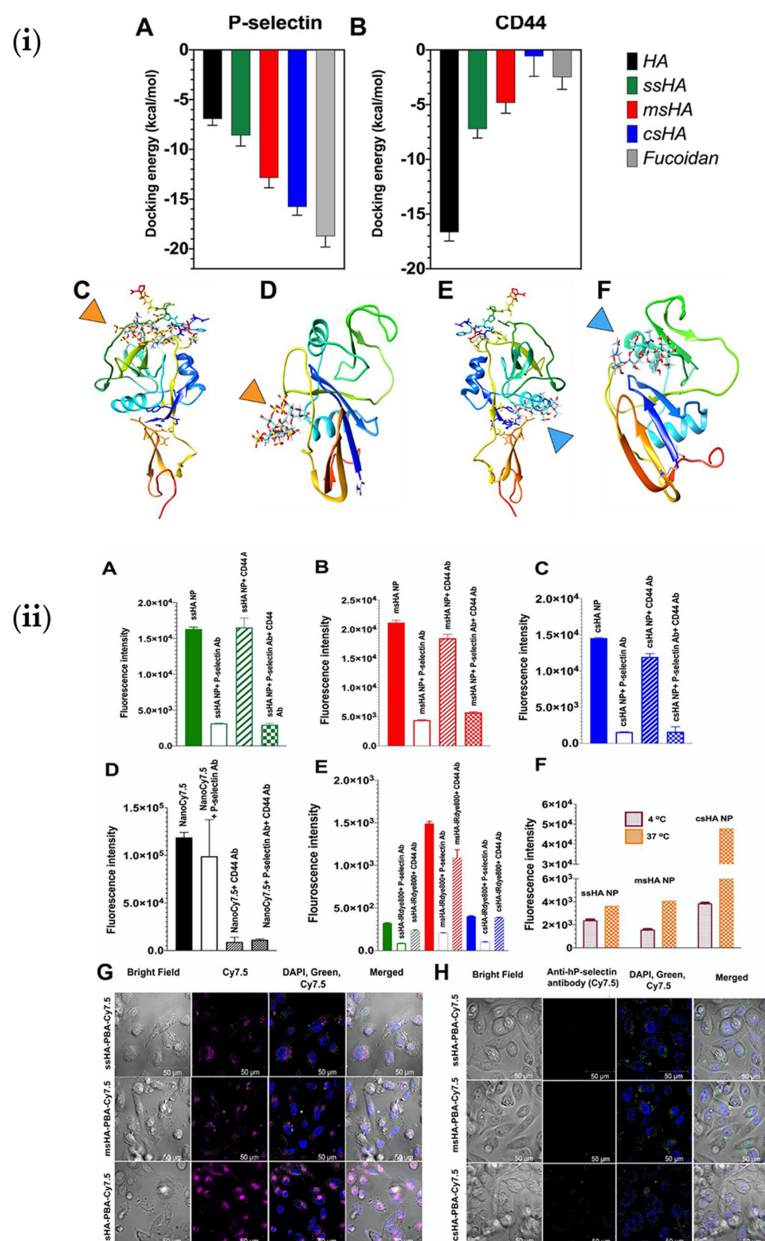


Figure 6. (i) (A,B) Computational docking energies for sulfated derivatives of HA against P-selectin and CD44 (n = 10 runs). (C–F) Images obtained from the docking program; arrows indicate the binding pose of sulfated HA (orange) and HA (blue) in the protein structure. (ii) (A) Uptake analysis of ssHA–NP with anti–hCD44, anti–hP–selectin, and combination of both antibodies in S2–013 cells (green) (n = 5). (B) Uptake analysis of msHA–NP under anti–hP–CD44, anti–hP–selectin, and combination of both antibodies in S2–013 cells (red) (n = 5). (C) Uptake analysis of csHA–NP under anti–hP–CD44, anti–hP–selectin, and combination of both antibodies in S2–013 cells (blue) (n = 5). (D) Uptake analysis of NanoCy7.5 nanoparticle formulation under antih-CD44, anti–hP–selectin, and combination of both antibodies in S2–013 cells (black) (n = 5). (E) Evaluation of uptake of ssHA, msHA, csHAIRDye800 conjugates in the presence of anti–hP–selectin antibody in S2–013 cells (n = 5). (F) Temperature-dependent effect on the uptake of three derivatives of the HA-NP system at 4 °C vs. 37 °C by FACS. (G,H) CSLM images acquired of S2–013 cells with DAPI (blue; nucleus), NIR (Cy7.5), and green (endosomal marker Rab5 protein) staining to visualize uptake of sNanoCy7.5 in the presence and absence of anti–hP–antibody. The scale bar represents 50 μ m on all images. Reprinted from *ACS Biomater. Sci. Eng.* **2020**, *6*, 3585–3598 [79].

A comparative study of chemical modifications of the reactive groups of HA is reported in Table 1.

Table 1. Comparative study of chemical modifications of reactive groups of HA.

Functional Groups	Types	Reagents/Crosslinkers	Reaction Conditions	Characterization Techniques	Advantages	Disadvantages	Refs.
OH group	Alkylation	HA-ADH, EDC/NHS, R-NH ₂ , CNBr	Aqueous (NaCl), nucleophilic substitution, pH ~4.5–8, RT to 50 °C.	¹ H/ ¹³ C NMR, FTIR, SEC/GPC, rheology (viscosity), DLS	Good control of graft length; improved viscoelasticity and thermal stabilization; retains carboxyl groups;	Multistep; requires controlling substitution degree; possible chain degradation.	[25–29]
	Esterification	GMA, DMAP, OSA, MAA, DMSO R-COOH,	Alkaline conditions, RT–50 °C, inert atmosphere	NMR (DS), FTIR, rheology, photo-rheology (UV-crosslinked), swelling, and mechanical tests	Introduces amphiphilicity; polymerizable groups; useful for drug delivery and surfactant properties.	Requires aprotic solvent and ion-exchange salts for solubility; residual reagents need removal.	[31–33],
	Etherification	BDDE, DVS, Cysteine–HA (C ₆ ether)	High pH (often >13), fast reaction, mild heating (25–60 °C),	NMR (DS), FTIR, swelling ratio, SEC, SEM, elemental analysis	Covalent ether linkage forms a stable hydrogel; enzymatic resistance; tunable by DVS concentration.	Harsh alkaline conditions can cause HA depolymerization; residual crosslinker toxicity must be controlled.	[34,35,39],
	Schiff-base crosslinking	Glutaraldehyde hemiacetal	Aqueous, pH: 4.5–7.5, RT; limited crosslinking reaction	FTIR (C=O), swelling, cytotoxicity assays	Simple, fast crosslinking reaction forms insoluble films/gels; strong mechanical properties.	Glutaraldehyde is cytotoxic and requires thorough washing or quenching; potential uncontrolled.	[34]
COOH group	Amidation	Biscarbodiimide, CMPI, DMTMM	Anhydrous DMF or polar aprotic solvent, short reaction times, optimized pH	¹ H NMR, FTIR, Ellman's or colorimetric assays, gelation tests with HRP/H ₂ O ₂	Efficient amidation under neutral pH; better for sensitive biomolecules than EDC/NHS; good for hydrophobic amide substitution.	Solvent compatibility and purification challenges; potential HA backbone depolymerization	[37–39]
	Esterification	GMA, HA-ADH coupling + succinate ester	Aqueous or mixed solvents. pH dependent, temp varied,	FTIR, solid-state NMR, rheology, swelling, mechanical testing, cytocompatibility assays.	Introduces vinyl groups for photo-crosslinking; Direct covalent crosslinking of HA via stable bis(N-acylurea) linkages.	Possible O-acylisourea side-products need to quench/remove EDC urea byproducts; limited reaction efficiency without NHS.	[16,17,40]

Table 1. Cont.

Functional Groups	Types	Reagents/Crosslinkers	Reaction Conditions	Characterization Techniques	Advantages	Disadvantages	Refs.
NHCOCH ₃ group	N-deacetylation	NH ₂ NH ₂ , HIO ₃ , HNO ₂ , Streptococcus pneumoniae hyaluronan lyase	Strong nucleophile, cleaves the amide bond.	1H NMR, LC-ESI-MS, HPLC.	Removal of N-acetyl groups using NH ₂ NH ₂ –HIO ₃ produces dHA with varying DD; Results in loss of moisture-retention due to exposed amino groups.	Hydrazine (NH ₂ NH ₂) is toxic, harsh, and not biologically safe; limited biomedical scalability.	[61]
	Re-acetylation	Acetic anhydride (Ac ₂ O), TAE/NaHCO ₃	Mild organic solvent, pH 7–8.	1H NMR, FTIR.	Chemically restores N-acetyl groups on dHA, increasing water-binding ability, and recovering HA's native hydration properties.	Deacetylation is not random, making precise control difficult at high DD.	[47]
	Biological response	NF-κB activation, TLR/MyD88 signaling	Reduced cytokine output by dHA	Rheology, swelling, cytocompatibility assays.	dHAs show reduced cytokine induction and enhanced TLR-2 activity due to zwitterionic motifs.	Enzymatic digestion (SpHyl) requires strict conditions and yields variable oligomer lengths.	[62]

Comparative study of oxidation of HA is reported in Table 2.

Table 2. Comparative study of oxidation of HA.

Types of Oxidation	Target of Oxidation	Reagents/Crosslinkers	DO	Advantages	Disadvantages	Refs.
Periodate	C ₂ and C ₃ position	NaIO ₄ , Adipic acid dihydrazide (ADH)	1–20%	Enables covalent crosslinking for stable hydrogels/scaffolds; Tunable mechanical properties/stiffness by varying DO and crosslinker content.	Overoxidation or excessive DO may negatively affect biocompatibility/cytotoxicity; it changes chain flexibility/persistence length.	[9,21,49,50]
TEMPO	C ₆ position	TEMPO/TCC, ethylene glycol, NaHCO ₃ , NaCl	≥50%	Much higher DO achievable; The backbone ring structure remains intact, preserving HA's core polysaccharide scaffold; Hydrogels formed are more stable over time	Oxidation leads to significant MW reduction; Use of anhydrous DMF and HA salt-exchange.	[51–53]
H ₂ O ₂	does not oxidize selectively	H ₂ O ₂ , CuCl ₂	N/A	Under optimal conditions, degraded HA gives ~81.7% yield of LMW-HA; glucuronic acid content of degraded HA is ~36.56%.	Cannot be controlled where glycosidic bonds break; may risk further unwanted side reactions or damage to the HA chain if not carefully controlled.	[54–57]

2.2. Functionalization by Click Chemistry

Direct crosslinking of HA can be considered unsuitable for hydrogel design since they go through harsh reaction conditions, toxic by-products can be formed, and the crosslinking agents used are cytotoxic [23]. Nowadays, one of the most promising strategies for hydrogel preparation turns out to be click chemistry due to its high specificity, high yield, bio-orthogonality, and mild reaction conditions [83].

2.2.1. Cu-Free Click Chemistry (SPAAC)

The Huisgen azide–alkyne cyclo-addition is a chemical reaction between an azide and an alkyne that forms triazoles, typically in the presence of a copper (Cu^+) catalyst, as first described by Rostovtsev et al. [84] and Tornøe et al. [85]. This reaction has become very popular for making hydrogels because it offers high yield, efficiency, fast reaction rates, and excellent biocompatibility. Manzi et al. created nanohydrogels (range: 80–150 nm) using HA and riboflavin through this copper-catalyzed reaction [86]. However, Cu is toxic to the cell. To solve this problem, it is observed that cyclooctyne-functionalized molecules can react with azides without copper, a promising process known as strain-promoted azide–alkyne cycloaddition (SPAAC) [87]. This copper-free hydrogel design maintains the same advantages while being safer for biological use. Sang-Soo Han et al. developed copper-free click hydrogels by reacting dibenzocyclooctyne (DBCO)-modified HA with 4-arm PEG-azide for cartilage tissue engineering. DBCO-modified HA was synthesized via 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (EDC)/N-hydroxysuccinimide (NHS) coupling. Hydrogels were formed by mixing HA–PEG4–DBCO with PEG-azide at concentrations of 0, 0.25, 0.5, 1, and 2.5 mM. The fastest gelation occurred at 1 mM PEG-azide, while 2.5 mM showed cytotoxicity [88]. Chondrocytes encapsulated in the hydrogels were cultured for 1 and 5 days; confocal microscopy confirmed cell survival and cartilage tissue regeneration. Optimal PEG-azide concentration (1 mM) balances gelation rate and cytocompatibility, providing a supportive 3D environment for cartilage regeneration [88]. In the same way, Fu et al. developed an injectable HA–PEG hydrogel by first modifying HA with 2-(aminoethoxy) cyclooctyne and then reacting it with azide-functionalized PEG [89]. The final hydrogel showed quick gel formation, strong mechanical strength, and high stability. In contrast, Kasana et al. reviewed the limitations of azide–alkyne cycloaddition, highlighting issues related to catalyst instability and recyclability. To address these challenges, they emphasized the development of heterogeneous catalysts, such as Cu nanoparticles (Cu-NPs) supported on carbonaceous materials, Cu-supported zeolitic imidazolate frameworks (ZIFs), and Cu catalysts immobilized on magnetic supports. These heterogeneous catalytic systems have attracted considerable attention owing to their broad industrial applicability, offering advantages such as cost-effectiveness, enhanced stability, superior thermal resistance, facile catalyst recovery, and improved recyclability compared with their homogeneous counterparts [90].

2.2.2. Thiol–Ene Crosslinking

In 2002, Shu et al. made a reversible HA hydrogel from thiol-modified HA (HA–dithiobis(propanoic dihydrazide) (DTPH), HA–dithiobis(propanoic dihydrazide) (DTBH)) with a controllable DS of 20–70% and pKa values of 8.87 (DTPH) and 9.01 (DTBH), enabling air-oxidizing gelation followed by dilute H_2O_2 . The reversible sol–gel behavior (dithiothreitol (DTT) as reducible) might be applicable for a controlled drug release model and fibroblast encapsulation [91]. Later, they created HA–gelatin hydrogels (DS: $\leq 50\%$) by blending thiol-modified HA and gelatin [92]. Gramlich et al. devised a photopatterned HA-based hydrogel by reacting norbornene-modified HA (NorHA) with DTT. Several crosslinkers produce different hydrogels with elastic moduli ranging from 1000 Pa to

70,000 Pa. At first, they used a primary crosslinker with a lower grade, and subsequently, the thiol–norbornene reaction could be performed to further modify the hydrogel [93]. A self-crosslinked HA–SH hydrogel was developed by Bian et al. via reagent-free air oxidation, enabling tunable thiol substitution (10–60%) and molecular weights (0.1–1.0 MDa) to achieve storage moduli up to 44.6 kPa. This non-toxic, tunable 3D environment supports long-term fibroblast and chondrocyte culture, demonstrating high cell viability and robust extracellular matrix deposition [94]. Bi et al. analyzed a series of alkene-functionalized polyamidoamine dendrimers that have been synthesized for the preparation of in situ-forming hydrogels with HS–HA through thiol–ene crosslinking. The gelation time and mechanical strength of hydrogels increase with the decrease in the electron density of alkenyl end groups on the dendrimer [95]. In another study, Ramanathan Yegappan et al. developed thiol-functionalized HA using cysteamine and HA (HA–Cys) via EDC–NHS crosslinking chemistry [96]. The developed HA–Cys conjugate was crosslinked using dimethylolallylglycine (DMOG) loaded polydopamine (PDA) nanoparticles via the Michael-type addition reaction. DMOG is a proangiogenic agent that acts as a competitive analog of 2-oxoglutarate, inhibiting the hypoxia-inducible factor prolyl hydroxylase (HIF–PH) pathway. Through this mechanism, DMOG enhances endothelial cell migration, adhesion, and in vitro tube formation in human umbilical vein endothelial cells (HUVECs). These findings suggested that PDA-crosslinked thiol-functionalized hydrogels could be used as tissue engineering scaffolds as well as drug delivery vehicles. Recently, a novel pH-stimulative HA–SH hydrogel was developed by Noreen et al., showing 2.4-fold higher viscosity, sustained 5-FU release in colonic fluid, 3-fold higher cellular uptake in HT29 cells, reduced viability, strong mucoadhesion, and site-specific in vivo colon accumulation, demonstrating effective targeted delivery for colorectal cancer therapy [97].

2.2.3. Hydrazone Coupling

Hydrazine is covalently crosslinked with aldehyde (hydrogels) to form hydrazones (Figure 7A), which have demonstrated great potential for tissue engineering applications. Su et al. first developed a temperature-responsive in situ hydrogel based on an oxidized hyaluronic acid/adipic acid dihydrazide (oxi-HA/ADH) hydrogel, which showed a liquid-to-gel transformation within 3–8 min. This degradable hydrogel retained 60% of its initial weight after a 5-week-long degradation test and showed good biocompatibility. This hydrogel assists in nucleus pulposus cell synthesis of type-II collagen and gene delivery [98]. Similarly, Chen et al. created an injectable HA–pectin hydrogel by reacting HA/ADH with biofunctionalized pectin–dialdehyde followed by coupling with hydrazine-modified elastin-like protein. This hydrazone crosslinking was simple, and the hydrogel's stability and mechanical integrity were adjustable by varying the weight ratio of hydrazide and aldehyde derivatives [99]. Importantly, HA/pectin-based hydrogels supported chondrocyte phenotype and facilitated chondrogenesis. Wang et al. proposed another injectable dynamic hydrogel based on dynamic hydrazone chemistry that used hydrazine-modified elastin-like protein (ELP) and aldehyde-modified hyaluronic acid [100]. This hydrogel provides substantial mechanical protection to encapsulated human mesenchymal stem cells during syringe injection and rapidly recovers its structural integrity after injection. Similarly, Esther A. Mozipo et al. used statistical modeling design of experiments (DoE) to efficiently optimize the physicochemical properties of hydrazone-crosslinked HA hydrogels for protein delivery. The DoE identified the optimal formulation, which exhibited a gelation time of 3.7 min, a compressive modulus of 62.1 Pa, and minimal mass loss over a 28-day period [101].

Acylhydrazone linkages are closely related to hydrazones (Figure 7B) and are commonly employed in the fabrication of biomedical hydrogels through reactions between

aldehyde-functionalized polymers and dihydrazide compounds or polymers modified with acylhydrazide groups [102]. Recently, H An et al. prepared injectable hydrogels by reacting pectin aldehyde (pectin-CHO) with the acylhydrazide-functionalized polymer poly(N-isopropylacrylamide-stat-acylhydrazide) P(NIPAM-stat-AH). The dynamic acylhydrazone bonds impart self-healing capability to the hydrogel, while its mechanical properties can be precisely tuned by adjusting the weight ratio of P(NIPAM-stat-AH) to pectin-CHO [103]. Moreover, this hydrogel can load anticancer drugs like doxorubicin hydrochloride (DOX-HCl) and combretastatin A4 disodium phosphate (CA4) [103]. The same group previously prepared self-healable hydrogels from TPE-[P(DMA-stat-DAA)]₂ and acylhydrazide-functionalized pectin. Here, tetraphenylethylene (TPE) units contribute to the opening of the hydrophobic pectin ring and trigger AIE properties [104]. Similar findings were reported by L. Chang et al., who developed a limonin-loaded pectin/cellulose hydrogel that significantly inhibited the growth of lung adenocarcinoma in a xenograft mouse model [105].

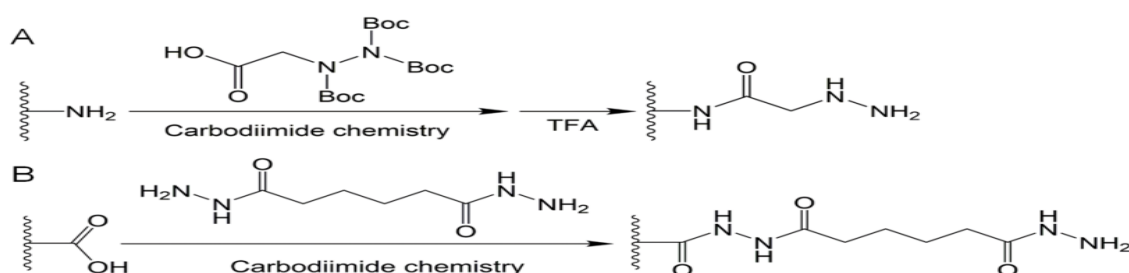


Figure 7. (A) Synthesis of hydrazide-functionalized polymer via carbodiimide chemistry coupling with tri-Boc-hydrazinoacetic acid for amide formation and subsequent trifluoroacetic acid (TFA) deprotection of tert-butoxycarbonyl (BOC) groups. (B) Synthesis of acylhydrazide-functionalized polymer using carbodiimide chemistry, coupled with dihydrazide-containing compounds, for example, adipoyldihydrazide, and coupled with a carboxyl group by amide formation. Reprinted from MDPI by Xu et al. [102].

2.3. Functionalization by Physical Crosslinking

The physical crosslinking process mainly depends on the natural properties of HA and the functional groups added to it without forming covalent bonds [13]. They generally form gels under mild conditions without crosslinkers or catalysts, which helps reduce cytotoxicity [106]. These interactions are reversible and allow them to create injectable hydrogels with shear-thinning and self-healing properties, which are suitable for extrusion bioprinting and injection.

2.3.1. Ionic Interaction

G. Prakash et al. developed a hyaluronic acid hydrogel crosslinked with divalent metal ions (e.g., Mg^{2+} , Mn^{2+} , and Fe^{2+}) without requiring chemical modification. The hydrogel exhibited tunable rheological properties, which could be readily adjusted by varying the molecular weight of hyaluronic acid (HA) and the concentrations of metal ions/NaOH. Mg^{2+} -HA hydrogels evolved self-healing and stimulus-responsive behaviors, highlighting their potential for biomedical applications [107]. Damouny et al. developed a hybrid injectable hydrogel composed of hyaluronic acid (HA), gelatin, and cationic cellulose nanocrystals (cCNCs), which formed a stable three-dimensional (3D) network through electrostatic interactions and hydrogen bonding. The hydrogel exhibited excellent shear-thinning injectability, maintained structural stability under stresses below 15 Pa, demonstrated rapid self-healing, and showed reduced swelling and degradation at cCNC concentrations exceeding 3%. Hydrogel elutions manifested biocompatibility with 5% cCNC formulations, enhancing fibroblast enhancement to 200% of the control after 11 days,

usable in dermal fillers, bioprinting inks, and soft-tissue scaffolds [108] (Figure 8). Recently, M. Riopedre-Fernandez et al. developed a novel strategy demonstrating that hyaluronic acid (HA) binds preferentially to positively charged peptides. Using ^1H NMR spectroscopy and molecular dynamics simulations (MDS), they showed that arginine-rich peptides (R4) exhibit significantly higher binding affinity toward HA than lysine-rich (K4) or glycine-rich (G4) peptides [109]. This binding is electrostatically driven via COOH and NHCOCH_3 groups of HA, enabling additional nonpolar and π interactions. However, HA–peptide interactions are characterized by dynamic, transient, and multivalent binding, leading to the formation of bridging complexes instead of stable, rigid assemblies, with these interactions being largely independent of the molecular weight of HA.

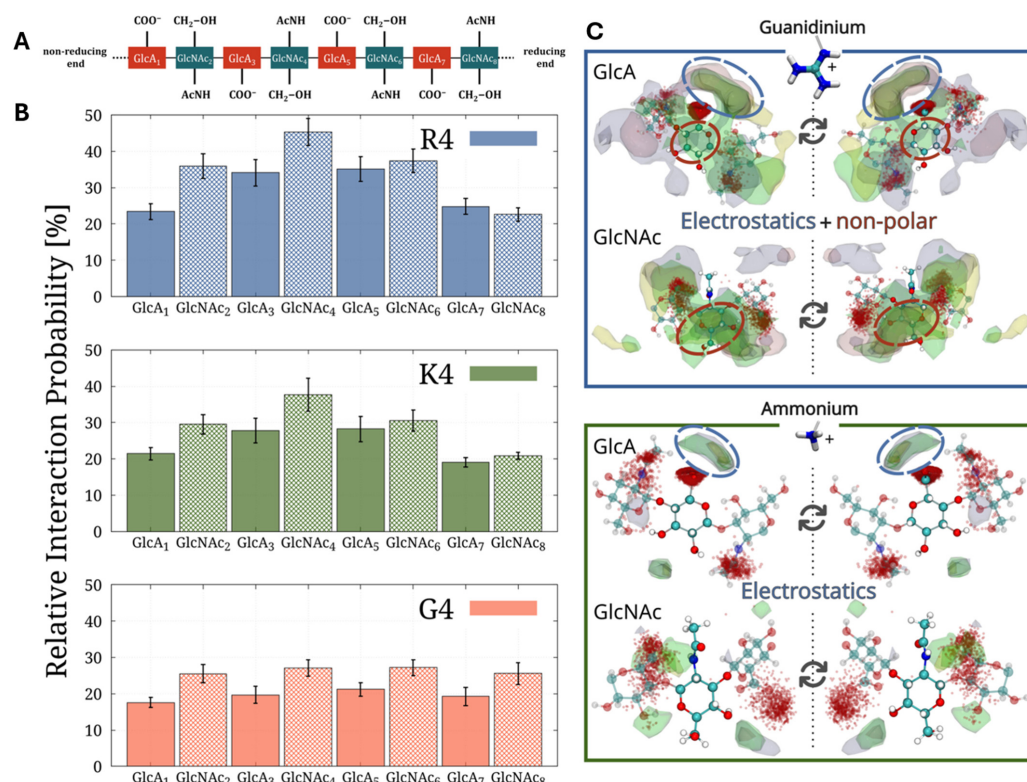


Figure 8. (A) Hyaluronan octamer scheme showing its key functional groups. (B) Relative percentage of frames in which the tetrapeptide binds to each hyaluronan monosaccharide, as determined from MD simulations using the proECCo75 model. (C) Volumetric densities of peptide side chains (guanidinium carbons or ammonium nitrogens) in the vicinity of HA, with trajectories centered on all ring atoms of either GlcA or GlcNAc monosaccharides. Overlaid density maps, with different colors representing distinct monosaccharides: yellow for GlcA₁ and GlcNAc₂, green for GlcA₃ and GlcNAc₄, gray for GlcA₅ and GlcNAc₆, and pink for GlcA₇ and GlcNAc₈. Reprinted from Elsevier [109].

2.3.2. Guest–Host Interaction

Guest–host interaction is an advanced method involving the specific recognition between guest and host monomers, where crosslinking occurs through the self-assembly of polymers modified with these monomers. Typically, the host monomer has a site that hits the guest monomer. The strength of this bond depends on its structure and interactions. Rodell et al. and Chen et al. noted that cyclodextrin is the most widely used host monomer, while adamantane serves as a common guest monomer [110,111]. Miller et al. reported that HA can be functionalized with adamantane through controlled esterification and with β -cyclodextrin via amidation. These modified polymers can form HA hydrogels or hybrid hydrogels through noncovalent guest–host interactions. Such systems produce self-healing hydrogels due to their dynamic and reversible nature under physi-

ological conditions [112]. Similarly, Rosales et al. performed azobenzene-functionalized and β -cyclodextrin-modified HA, which can create supramolecular hydrogels with shear-thinning behavior under physiological conditions, and their properties can be finely adjusted. Yu developed HA hydrogels between β -cyclodextrin-modified HA and adamantane-modified 4-arm PEG, empowering efficient supramolecular crosslinking with tunable mechanics and swelling and controlled release of hydrophobic dexamethasone [113]. More recently, Soni et al. demonstrated that cyclodextrin nanoparticles ranging in size from 25 nm to >100 nm are efficiently internalized by macrophages in vitro. The uptake relies on the particle size: increasing particle size was correlated with greater uptake by macrophages. Both scavenger receptor A1 and mannose receptor contribute to this internalization, which indicates that additional pathways like pinocytosis are involved [114]. Undoubtedly, host-guest complexes in HA-based supramolecular assemblies offer significant opportunities for the development of drug delivery systems, wound healing platforms, and biomedical soft materials, although several challenges remain to be addressed.

2.3.3. Hydrogen Bonding

Hydrogen bonding plays a crucial role in hyaluronic acid-based systems by enabling physical crosslinking, stabilizing polymer–lipid and polymer–protein interactions, and promoting the formation of resilient, mucoadhesive, and tunable networks essential for biomedical applications such as hydrogels, scaffolds, and ocular formulations. Barretta et al. reported that tamarind seed polysaccharide (TSP) and hyaluronic acid (HA) work together in water to create improved eye drop formulations with better mucoadhesive (eye surface-sticking) properties. TSP is a neutral, branched polysaccharide with a cellulose-like backbone and side chains of xylose and galactoxylose [115]. Bełdowski et al. analyzed hyaluronic acid in the presence of phospholipids in an aqueous nano-environment that was responsible for its physical crosslinking. This physical distance is understood to be the H-bond length and defined in a coarse-grained picture of the molecule [116]. However, longer chains and the addition of lipids promote appreciably resilient networks that are important in biological systems. Tavsanlı et al. developed silk/HA-based hydrogels that were produced by reacting methacrylated hyaluronic acid (MeHA) with silk fibroin (SF) in water, using tetramethylethylenediamine (TEMED), ammonium persulfate (APS), and N, N-dimethylacrylamide (DMMA) as initiators and linking agents. The vinyl groups of MeHA were connected through DMMA. The presence of silk fibroin played a key role in these properties, as its β -sheet domains acted as physical crosslinking points that reinforced the hydrogel structure [117]. In the same way, Guan et al. reported another novel water-insoluble silk fibroin/hyaluronic acid (SF/HA) scaffold with tunable performances in aqueous processes. By employing freezing-induced assembly, silk I crystallization evolved in the SF/HA blends, trapping HA content with silk I crystals, forming a water-insoluble SF/HA scaffold. However, when HA content is too high, the content of silk I in the silk/HA blends may be insufficient to entrap the high amount of HA [118]. This type of non-chemically crosslinked protein/HA scaffold shows great promise for soft-tissue engineering owing to its excellent biocompatibility and tunable mechanical and biological properties.

2.3.4. Hydrophobic Interaction

HA is investigated as a model semi-flexible amphiphilic polyelectrolyte with remarkable biomedical potential. Mayol et al. modified HA with octenyl succinic anhydride (OSA–HA) to improve solubility and as a platform for the sustained release of hydrophobic drugs in the form of self-assembled micelles. OSA–HA solutions appear to be an appropriate tool to be used in viscosupplementation therapy owing to their suitable

viscoelastic features. The dissolution contribution of the drug is more relevant as the OSA–HA concentration increases [119]. Payne et al. developed computational models of self-assembly to examine the effects of hydrophobic modification on supramolecular behavior in three systematically designed HA derivatives substituted with various alkyl chain lengths. Among them, HA–octadecylamine forms well-defined hydrophobic domains due to stronger hydrophobic interactions. Their experimental hydrodynamic radius and polydispersity aligned with theoretical prognosis [120]. At first, they reacted hexylamine, dodecylamine, and octadecylamine with the HA solution and formed hexHA, dodHA, and ocdHA, respectively. The hydrodynamic radius (radius of gyration, R_g) and polydispersity were analyzed compared to native HA. The R_g for both hexHA and ocdHA reached a stable radius of gyration of around 2 nm compared to unmodified HA (4 nm); these radii demonstrated a compact conformation because of hydrophobically driven intramolecular interactions. These hydrophobic moieties are used to entrap dyes and in therapeutics. Similarly, Ondreas et al. demonstrated that oleyl-modified hyaluronic acid (HA) self-assembles into solvent-dependent nanostructures in water (H_2O) and phosphate-buffered saline (PBS). Pyrene fluorescence analysis confirmed the self-assembly behavior, while polymer chain stiffness was identified as the key factor governing the size and internal structure of the resulting aggregates. At 10 mg/mL, oleyl–HA—especially in PBS—produced markedly higher epidermal curcumin accumulation, proving that supramolecular structure dictates drug uptake and skin penetration [121]. Lin et al. investigated hyaluronic acid–cholesterol (HACH) conjugates as emulsifiers for stabilizing oil/water interfaces and their application in vaccine delivery. The results show that HACH20 can form stable squalene nanoemulsions (SQ@CH) with a uniform size of around 190 ± 2 nm and a polydispersity of 0.136 ± 0.027 , and that SQ@HACH can induce immune responses in mice immunized with OVA and SQ@HACH [122]. Li-Jung Kang et al. developed self-assembled hyaluronic acid nanoparticles (HA-NPs) that exhibit enhanced resistance to hyaluronidase-mediated degradation and prolonged retention within the knee joint. These HA-NPs also inhibit CD44-mediated NF- κ B activation and the expression of catabolic genes in chondrocytes. In vitro, HA-NPs reduce collagenase activity and PGE2 production, reversing IL-1 β - and CD44-induced osteoarthritis-like phenotypes. In DMM mouse models, intra-articular HA-NPs protected cartilage more effectively than FDA-approved HMW HA, identifying HA-NPs as a promising CD44-targeted therapeutic for osteoarthritis [123]. More recently, Zamora et al. demonstrated that methacrylated hyaluronic acid (HA–MA) functions as an effective bio-stabilizer for oil-in-water (O/W) emulsions, producing stable emulsions for more than one month at high degrees of methacrylation (DS: 50–100%) and HA–MA concentrations of 10–15 wt%. This emulsion can be used particularly with polar solvents (octyl palmitate, MCT) and at moderate surface tensions (to 63 mN/m). HA–MA emulsions were mild and inoffensive, which showed bioactivity and enhanced epidermal delivery of hydrophobic compounds by eight-fold, highlighting dual cosmetic and drug delivery functionality [124].

2.4. Functionalization by Biomolecule Conjugation

HA bio-conjugation involves covalently linking drugs, peptides, or biomolecules to its carboxyl, hydroxyl, or modified amino groups to enhance targeting, solubility, and controlled release [48]. Typical strategies include EDC/NHS-mediated amide formation for amine-containing drugs (e.g., DOX) [125], esterification via linker molecules for hydrophobic agents (e.g., PTX) [126], and thiol–maleimide chemistry for peptides such as Exendin-4 and others [127]. The incorporation of cleavable linkers further enables stimulus-responsive delivery and collectively broadens the therapeutic versatility of HA in modern drug delivery platforms [128].

2.4.1. HA–Drug Conjugate

HA-based nanocarriers have been designed to make drug delivery more efficient and to better target cancer cells while minimizing side effects and drug buildup in healthy tissues [25]. One major area of progress has been in developing HA-based prodrugs for anticancer treatments, including doxorubicin (DOX), paclitaxel (PTX), and camptothecin (CPT) [25].

Doxorubicin (DOX) is a highly effective chemotherapy drug, but it exerts some toxicity to the heart, liver, and kidneys; its chemical structure is reported in Figure 9a. To analyze and reduce these side effects, Cai et al. attached DOX to HA (35 kDa) through a pH-sensitive acylhydrazone bond with drug loadings of 5–15% (*w/w*) [129]. Oommen et al. created a novel HA–DOX prodrug by forming an amide bond between the amino group of DOX and the carboxyl group of HA (150 kDa) using carbodiimide coupling. In vitro, this type was much less toxic to normal mouse fibroblast (NIH3T3) and low-CD44 human breast cancer (MCF-7) cells but remained highly toxic to high-CD44 human colon cancer (HCT116) cells [130]. Later, fluorescein-based supramolecular HA-NPs and CS-NPs (chondroitin sulfate) enabled CD44-mediated uptake and indicated zero-order DOX release [131]. However, CS–DOX showed higher cytotoxicity in HCT116/MCF-7 cells and activated at 0.08–0.8 mg/mL, and both had blood clotting risks of 0.8 mg/mL. Another HA–DOX prodrug was developed by Yin et al. to respond to both acidic and reducing tumor environments by linking DOX to HA through disulfide bonds and hydrazine bonds [132]. This dual-responsive system enabled targeted drug release in tumor tissues and showed the strongest cancer-killing and apoptosis-inducing effects among all the tested formulations. Similarly, Lee et al. developed pH-sensitive hyaluronic acid–glycol chitosan (HA–GC) nanoparticles (~150 nm) co-loaded with doxorubicin (DOX) and celecoxib. The HA-conjugated nanoparticles exhibited CD44-targeted, pH-responsive drug release under acidic conditions (pH 6.0–4.0), effectively suppressing tumor proliferation in non-small cell lung cancer (NSCLC) cells and xenograft mouse models while reducing the expression of inflammatory and metastasis-associated markers [133]. Lately, Malfanti et al. reported a hyaluronic acid–doxorubicin hydrazone conjugate (HA–Hz–DOX), which demonstrated superior efficacy compared with the amide-linked conjugate (HA–NH–DOX) and the adipic hydrazone conjugate (HA–AdpHz–DOX) for local glioblastoma treatment. The HA–Hz–DOX formulation exhibited potent antitumor activity, enhanced diffusion within brain tissue, and reduced systemic toxicity [134].

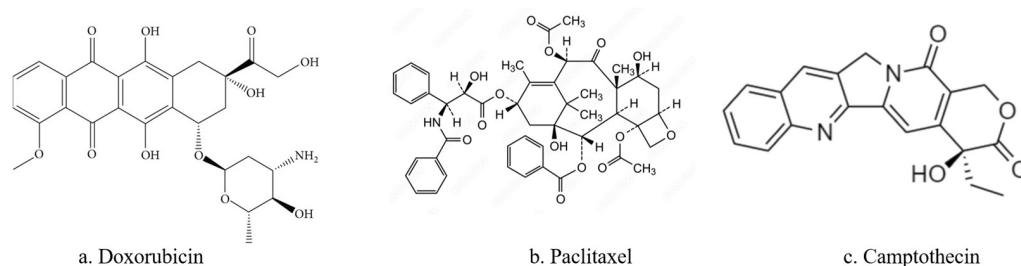


Figure 9. Chemical structures of (a) doxorubicin (DOX), (b) paclitaxel (PTX), and (c) camptothecin (CPT).

Paclitaxel (PTX), composed of hydrophilic β -cyclodextrin units and hyaluronic acid (HA) units, is widely used to treat many types of cancer, including ovarian, breast, lung, prostate, esophageal, and cervical cancers, melanoma Kaposi's sarcoma, and other solid tumors. Its chemical structure is reported in Figure 9b. For example, Xu et al. developed hyaluronic acid–paclitaxel (HA–PTX) nanosystems (~200 nm) that facilitated the cytosolic delivery of paclitaxel (PTX) through multiple endocytic pathways while bypassing the lysosomal–endosomal trafficking system. This delivery strategy resulted in enhanced tumor

accumulation and a several-fold reduction in tumor volume compared with free PTX after 14 days of treatment [135]. In another study, Y Chen et al. designed an advanced HA-6-PTX by chemically grafting PTX to the C-6 position of N-acetyl-D-glucosamine (GlcNAc) of HA using hexanediamine as the linker. The drug conjugate, as high as 21.8%, showed water solubility of 168 mg/mL and delivered the drug without affecting the carboxylated-HA chain. However, HA-6-PTX showed increased cytotoxicity and apoptosis-inducing effects against HepG2 and A549 cells when compared to free PTX [136]. Luo et al. devised self-assembled mPEG-PLA/HA-PTX composite nanoparticles (~116 nm), which showed antitumor efficacy with a tumor inhibition rate of 75.9% in vivo and outperformed conventional PTX formulations [137]. However, PTX is associated with some problems like solubility issues, strong multidrug resistance (MDR), lower intrinsic potency, dose-limiting side effects, etc. [138]. To tackle this problem, a stimuli-responsive dendronized HA-docetaxel (HA-DTX-Dendron, HADD) conjugate was synthesized by Wang et al. for the specific delivery of DTX with high biocompatibility into tumor tissues via the CD44+ receptor mechanism [139]. Ravari et al. fabricated water-soluble, chitosan-coated HA-DTX nanoparticles via the polyelectrolyte complex (PEC) method and systematically optimized the formulation using a Box-Behnken design [140]. Biological evaluation of nanoparticles was conducted in CD44+ cancer cells, and the results showed that chitosan-coated HA-DTX nanoparticles were more effective against CD44+ cells than free DTX. Again, Fang et al. developed redox-responsive, crosslinked hyaluronic acid-based nanoparticles (DTX-CMHN; 69–96 nm) synthesized from HA-g-polytyrosine lipoic acid. These nanoparticles exhibited redox-triggered docetaxel (DTX) release and a prolonged circulation half-life of 5.75 h, highlighting their potential for enhanced drug delivery [141]. Gaio et al. introduced a therapeutic approach using HA-NPs that simultaneously delivers DTX and the photosensitizer meso-tetraphenyl chlorine disulfonate (TPCS2a) for the targeting and killing of CD44-overexpressing breast cancer (BC) cells. This co-loaded system (HA@DTX/TPCS2a-NPs) showed markedly better performances over monotherapies (HA@DTX-NPs or HA@TPCS2a-NPs) in reducing the self-renewal capacity [142].

In one study, Song et al. developed hyaluronic acid-modified liposomes co-loaded with doxorubicin (DOX) and paclitaxel (PTX) (HA-D/P-Lip) to exploit the synergistic therapeutic effects of the two drugs. The HA-functionalized liposomes specifically targeted MCF-7 cells with high CD44 receptor expression, thereby enhancing antitumor efficacy. This formulation had a suitable particle size of 125.5 ± 0.79 nm with a negative surface charge of -9.56 ± 0.62 mV and acceptable encapsulation efficacy of $93.6 \pm 0.51\%$ (DOX) and $70.4 \pm 1.46\%$ (PTX). In acidic conditions, this dual-edge drug showed a superior response compared to neutral conditions, thereby facilitating selective drug release in the tumor microenvironment. This dual drug-loaded, HA-functionalized system ultimately resulted in improved therapeutic efficacy due to the combined effects of active targeting, controlled release, and drug synergism [143].

Camptothecin (CPT) is a natural compound that allows DNA to be cut but prevents it from being rejoined, leading to DNA strand breaks and cell death; its chemical structure is reported in Figure 9c. However, CPT has very low water solubility and an unstable ring, which easily breaks down in aqueous solutions [25]. To overcome these problems, Xu et al. created two HA-CPT prodrugs using HA with different molecular weights (8 kDa and 100 kDa). At first, they modified CPT with ADH/NHS ester to improve CPT's solubility in water from only 2.08 µg/mL to 420 µg/mL (HA-CPT-8k) and 620 µg/mL (HA-CPT-100k), which prolonged CPT's retention time and improved its potential as a cancer therapy [144]. Yang et al. synthesized an HA-CPT prodrug in which about 5% of HA's carboxyl groups were replaced with aldehyde groups. These modified sites are linked to L-tartaric acid bishydrazide and then to the cholesterol group. The final product

was a cholesterol–HA–CPT that enabled self-assembly into nanoparticles in solution [145]. Recently, Z. Chen et al. developed a camptothecin–hyaluronic acid (CPT–HA) conjugate linked through 3,3'-dithiodipropionic acid that self-assembled into reduction-responsive micelles with degrees of substitution (DS) ranging from 4% to 20%. The optimized formulation achieved a balance between enhanced CD44-mediated cellular uptake and potent cytotoxicity against 4T1 cells [146]. More recently, the same group site-specifically conjugated camptothecin (CPT) to an ultra-low-molecular-weight hyaluronic acid tetrasaccharide (HA4), generating three well-defined amphiphilic conjugates: HA-S-S-CPT (disulfide-linked), HA-CH₂-CH₂-CPT (alkyl-linked), and 2HA-S-S-CPT (dual HA chain disulfide-linked). These conjugates subsequently self-assembled into stable nanoparticles. Among them, disulfide-linked HSC showed superior in vitro antitumor and targeting activity [147]. In another study, HA-conjugated DOX and CPT (DOCTOR) were released by nucleophilic acyl substitution with a fixed synergistic drug ratio (DOX: CPT 15:1). Their study demonstrated that the formulation released camptothecin (CPT) more rapidly than doxorubicin (DOX), achieving approximately 70% CPT release compared with 11% DOX release over 5 days. The conjugates also self-assembled into salt-induced hyaluronic acid (HA) nanoparticles and achieved ex vivo skin deposition of approximately 20% for DOX and 10% for CPT. Topical DOCTOR has seen no IL-1 α induction and is safe across primary human cells, 3D skin, and mice [148].

2.4.2. HA–Protein Conjugation

Some of the clinical trials of therapeutic proteins indicate a lack of site-specific targeting. Here, HA–protein conjugation has gained renewed attention as a modern strategy to overcome these challenges. For example, Oh et al. reviewed target-specific long-acting delivery of protein, peptide, and nucleotide therapeutics using HA derivatives [149]. Ferguson et al. explored HA conjugates, using the Polymer-masked Unmasked Protein Therapy (PUMPT) concept, to enhance protein stability for restoring activity after enzymatic degradation [150]. HA–trypsin conjugates showed increased activity and greater stability, indicating potential for tissue repair applications, whereas HA–EGF (EGF: epidermal growth factor) conjugates showed limited bioactivity. In another study, Lim et al. revealed that hyaluronic acid–polydopamine (HA–PDA) complexes self-assemble into stable nanocarriers with diameters of 100–300 nm, enabling the efficient encapsulation of lysozyme without compromising its structural integrity or thermal stability. These complexes effectively protect the protein and act as a promising delivery system [151]. In line with these findings, Huang et al. reported that dopamine polymerization (PDA) under alkaline conditions, achieved by the addition of NaOH, facilitates the co-deposition of hyaluronic acid (HA) onto substrate surfaces, resulting in the formation of a hybrid PDA/HA film. It is assumed that an electrostatic repulsion between PDA and HA probably prevents the aggregation of PDA, resulting in protein adsorption [152]. Shendi et al. developed tunable hyaluronic acid (HA) hydrogels via rapid Michael addition, demonstrating their ability to support cell survival, migration, and axonal outgrowth, thereby highlighting their potential as scaffolds for neural tissue regeneration [153]. Functionalization (e.g., anti-Fas) can further modulate immune responses while keeping neural stem cells viable. In line with this hypothesis, Sun et al. developed a dual-function anti-angiogenic hyaluronic acid–peptide conjugate (HA–ES2–AF) by combining the VEGFR1-binding peptide AF with ES2. The conjugate exhibited enhanced aqueous solubility, efficient CD44-mediated tumor targeting, potent anti-proliferative, anti-migratory, and anti-angiogenic activities, as well as a prolonged in vivo circulation half-life [154]. Recently, Tsuji et al. demonstrated the interaction between the recombinant CD44 (rCD44) molecule and the carboxyl groups of hyaluronic acid (HA), followed by the covalent conjugation of bovine serum albumin (BSA) through

a dehydration reaction. These conjugates, which explicated high affinity for rCD44, were highly internalized by CT26 and could be used as potent tools for protein delivery to cancer tissues [155].

Exendin-4 is a naturally derived peptide composed of 39 amino acids originally separated from the saliva of the Gila monster (*Heloderma suspectum*) [156]. It is an exogenous analogue of human glucagon-like peptide-1 (GLP-1), sharing approximately 53% sequence homology with native GLP-1 but possessing markedly higher resistance to degradation by dipeptidyl peptidase-4 (DPP-4) [157]. Chen et al. reviewed that Exendin-4–HA conjugates have emerged as an effective strategy to overcome the pharmacokinetic limitations [158]. Kong et al. developed a long-acting hyaluronic acid–exendin-4 (HA–Ex-4) conjugate via Michael addition, achieving high bioconjugation efficiency with 5–30 exendin-4 molecules conjugated per HA chain. The resulting conjugates exhibited nearly a 20-fold improvement in in vitro serum stability while preserving the hypoglycemic and glucose-regulatory bioactivities of exendin-4 [159].

2.4.3. HA–Lipid Conjugation

HA–lipid conjugation provides a versatile and biocompatible platform that enhances targeted delivery, cellular uptake, and therapeutic efficacy. In one study, Ruhela et al. introduced an efficient method to produce HA–lipid conjugates by ozonolysis, then linked them to dipalmitoyl phosphatidylethanolamine (DPPE) via reductive amination. This method offers biocompatible material suitable for tissue repair and engineering [160]. Wojcicki et al. developed hyaluronic acid–dioleoyl phosphatidylethanolamine (HA–DOPE) lipoplexes that efficiently targeted CD44-positive A549 lung cancer cells. Among the formulations evaluated, a lipid-to-DNA ratio of 2:1 with 10% HA–DOPE achieved the highest transfection efficiency through CD44- and caveolae-mediated cellular uptake [161]. In another study, Hayward et al. employed HA-conjugated liposomes (HALNPs) and Glioblastoma Multiforme (GBM) cells, which led to brain malignancy. The drawbacks of this conjugation were that astrocytes and microglia showed high HALNP–lysosome co-localization, which led to reduced anticancer effectiveness [162]. Recently, Lee et al. developed hyaluronic acid–polyethylene glycol (HA–PEG)–lipid conjugates to coat natural killer (NK) cells, thereby enhancing their ability to recognize and eliminate cancer cells (Figure 10i). They tested three conjugated lipids as 1,2-dimyristoyl-sn-glycero-3-phosphatidylethanolamine (HADM-NK cell), 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine (HADS-NK cell), and cholesterol (HACH-NK cell). Among them, HADS showed improved NK cell anticancer activity and strong potential for use in cancer immunotherapy, as shown in Figure 10(iiA–iiE) [163]. These findings are consistent with those of Park et al., highlighting the potential of this approach as a promising strategy for triple-negative breast cancer (TNBC) immunotherapy [164]. However, BI Koo et al. developed “conjugation-free” multilamellar protein–lipid nanovesicles (MPLVs) that self-assemble from unilamellar lipid vesicles and co-deliver protein antigens and adjuvants [165]. These MPLVs showed strong immune activation and significantly improved antigen-specific responses in cells and mice, suggesting great potential as an effective nano-vaccine platform and gene delivery.

2.4.4. HA–Antimicrobial Conjugation

Antimicrobial conjugation of HA involves chemically linking HA with antibiotics or antimicrobial peptides (AMPs) to create smart drug delivery systems, offering slow release, reduced toxicity, and targeted delivery for treating bacterial infections, especially intracellular ones, by leveraging HA’s biocompatibility and tumor/macrophage targeting [166]. In 2018, Wang et al. addressed the limited efficacy of aminoglycoside antibiotics, such as amikacin, against intracellular bacterial infections caused by their poor cellular penetra-

tion resulting from high hydrophilicity. To overcome this limitation, they synthesized a hyaluronic acid–amikacin (HA–AM) conjugate using an amidation strategy. HA–propargyl amide was formed, and amikacin was converted to an azide derivative, followed by linking them together. The conjugate exhibited antibacterial activity against planktonic bacteria and showed extended intracellular bactericidal activity compared to free amikacin, as well as requiring lower antibiotic dosages [167]. Later, Zhang et al. reported HA particles loaded with vancomycin (355 mg/g) and ciprofloxacin (25 mg/g) releasing antibiotics over 100 h, inhibiting bacteria like *P. aeruginosa* and *S. aureus* for up to a week, with no cytotoxicity to fibroblasts or corneal cells [168]. Dubashynskaya et al. developed hyaluronic acid–colistin (HA–CT) conjugates using carbodiimide-mediated coupling, achieving degrees of substitution (DS) of 3–10% (129–377 µg CT/mg). The conjugates self-assembled into nanoparticles ranging from 30 to 600 nm with a ζ -potential of approximately -20 mV and exhibited sustained drug release, with only 1–5% colistin released over 24 h. Notably, the conjugates retained their antimicrobial activity at a DS of 8%. They also reduced colistin nephrotoxicity by 20–50%, indicating safer, effective nanodrug potential. Petit et al. showed acylated-HA (AcHA) hydrogels functionalized with gelatin and antimicrobial peptide (AMP) using thiol–acrylate crosslinking. AcHA-G showed fast gelation, good cell adhesion (optimal at 0.5% G), but AcHA-AMP showed strong antibacterial activity against *S. aureus* and *E. coli* [169]. M Atrash et al. designed a new approach in which Rose Bengal conjugated to hyaluronic acid (RB–HA, ~ 3 RB per 10 HA units) showed high singlet oxygen yield ($\Delta\Phi = 0.90$) and slow photobleaching, providing effective antibacterial activity against *S. aureus* and *E. coli* for at least 80 days under light and ultrasound, making it a promising long-lasting photodynamic therapy for dental applications [170].

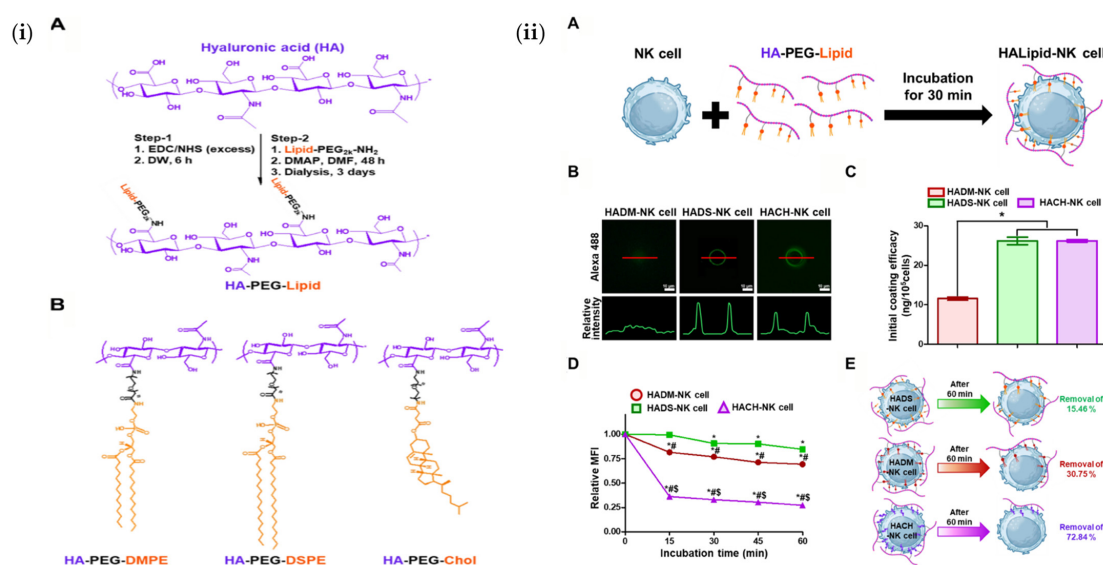


Figure 10. (i) (A) Conjugation of lipid-PEG-NH₂ to the carboxylic acid in the HA backbone via EDC/NHS coupling chemistry. (B) Chemical structures of HA-PEG-lipid with three different lipid types (i.e., HA-PEG-DMPE, HA-PEG-DSPE, and HA-PEG-Chol). (ii) Characterization of HA-lipid-NK cells. (A) Schematic representation of NK cell coating with HA-PEG-lipid and fabricated HA-lipid-NK cells. (B) Fluorescence images of HADM-NK cell, HADS-NK cell, and HACH-NK cell. (C) Quantitative assessment of NK cell coating performance of HA-lipid-NK cells. (D) Retention of cell membrane anchoring of Fluor-HA-PEG-lipid detected by flow cytometry at 0, 15, 30, 45, and 60 min. (E) The average amount of HA-PEG-lipid removal from NK cells after 60 min. Reprinted from Springer Nature [163]. (*) indicates a significant difference compared to 0 h for each group ($p < 0.05$). (#) indicates a significant difference compared to HADS-NK cells at the same time point ($p < 0.05$). (\$) indicates a significant difference compared to HADM-NK cells at the same time point ($p < 0.05$).

2.4.5. Bioconjugations with Other Biopolymers

In recent years, biopolymer-based bioconjugation has been extensively employed to develop biomaterials such as nanogels, hydrogels, and cryogels (Table 3). This approach overcomes the limitations of individual biomaterials while exploiting the synergistic advantages of the constituent biopolymers [132]. Recently, Kotla et al. reviewed the key opportunities and challenges in HA-based materials, highlighting chemically modified diverse HA–bioconjugates and crosslinked systems [48]. In a study, Manivong et al. synthesized chitosan–hyaluronic acid (CS–HA) polyionic nanocomplexes (~214 nm) loaded with Gd-DTPA via carbodiimide-mediated conjugation. The nanocomplexes exhibited a positive ζ -potential of 19.9 mV and provided enhanced and prolonged magnetic resonance imaging (MRI) contrast in articular joints [171]. Shad et al. reported that folate-conjugated HA-coated alginate nanogels encapsulating oxaliplatin were executed (200.3 nm, 22.0 mV). F-HA-AL-OXA nanogels exhibited superior antitumor activity in HT29 colorectal cancer cells [172]. Similarly, Thanh et al. developed a printable hyaluronic acid–alginate (HA–Alg) bioink via Schiff base crosslinking between HA–NH₂ and aldehyde-functionalized alginate (Alg–CHO). At the optimal equivalent volume ratio, the bioink exhibited excellent shear-thinning behavior and printability while supporting mesenchymal stem cell encapsulation and chondrogenic differentiation over a 4-week culture period. Then, HA–Alg was linked to silk–fibroin-reinforced networks without compromising mechanical properties for extrusion-based 3D bioprinting [173]. Gheran et al. divulged the biocompatibility of Gd-loaded HA–chitosan nanogels in SVEC4-10 endothelial cells, with no Nrf-2 activation after 1 day. They experimented with lower chitosan content (1.5 mg·mL^{−1}), which induces slight genotoxicity at high doses, while superior quality was achieved for nanogels containing 2.5 mg·mL^{−1} chitosan [174]. Lai consolidated HA-functionalized gelatin micro-carriers via periodate oxidation and aldimine linkage with a grafting yield of 26.9 ± 0.1%, significantly increasing collagen and GAG production [175]. Shinji Sakai et al. developed ultrafast blue light-induced gelatin–Ph/HA–Ph hydrogels that exhibited rapid gelation within 5–20 s and tunable mechanical softness, with a Young’s modulus ranging from 0.2 to 1.2 kPa. However, the resulting hydrogels remained relatively soft (Young’s modulus < 2 kPa) and lacked long-term degradation assessment [176]. In a more recent study, Lee et al. reported a novel ternary bioink composed of collagen, hyaluronic acid, and alginate (Ternary-CHA), which was crosslinked with Ca²⁺ (0–2.5 wt%). The Ca²⁺ ions induced the formation of “egg-box” networks through ionic coordination with alginate, together with hydrogen bonding among the matrix components, thereby enabling ion-responsive gelation and high water retention. Pumping Ca²⁺ content raised physiochemical networks with tunable rheology [177]. Lee et al. investigated the skin-moisturizing potential of a blend comprising 2,2,6,6-tetramethylpiperidine-1-oxyl radical-oxidized cellulose nanofibrils (TOCN), hyaluronic acid (HA), and poly- γ -glutamic acid (γ -PGA). The formulation exhibited enhanced water retention capacity and upregulated the expression of skin hydration-related genes; however, its thickening effect remained moderate [178]. In another study, Afzali et al. developed and compared two hydrogel scaffolds: a Ca²⁺-crosslinked scaffold composed of sodium alginate, fish collagen, and hyaluronic acid (CC scaffold), and a chemically crosslinked scaffold consisting of hyaluronic acid, polyethylene glycol diglycidyl ether (PEGDE), and itaconic acid (IPC scaffold). The CC scaffold exhibited superior yield strength, porosity, and cell proliferation, whereas both scaffolds effectively served as carriers for proteins such as basic fibroblast growth factor (bFGF), demonstrating their potential for chronic wound healing [179].

Table 3. Comprehensive Data Matrix of Modified Hyaluronic Acid-Based Gels for Biomedical Applications.

Gel Type	Structure	Physical & Structural Properties	Drug Loading	Advantages	Limitations	Refs.
Hydrogel	Polysaccharides + H ₂ O ₂ (Hydrogen Peroxide) + Fe ²⁺ (Ferrous Iron)	Viscosity reduced by 65%; molecular weight down to 10–50 kDa	N/A (low-MW fraction tracking)	High yield (~81.7%) functional fractions	High risk of arbitrary chain scission	[74]
	HA (Hyaluronic Acid) + EDC (1-Ethyl-3-(3-(dimethylamino)propyl)carbodiimide)/NHS (N-Hydroxysuccinimide)	Storage modulus (G') 1.2–2.5 kPa; swelling ratio > 25	Macromolecular oncology therapeutics	Exceptional ECM-mimicking profile	Complex multistage synthetic modification	[13]
	HA–HYD (Hydrazide Hyaluronic Acid) + GelMA (Gelatin Methacrylate)	Shear-thinning index < 0.4; yield stress ~ 45 Pa	Live bone marrow stem cells	High structural print fidelity	UV crosslinking cell-viability risk	[180]
	AHA (Aldehyde Hyaluronic Acid) + DTPH (3,3'-Dithiobis(propionyl hydrazide)) + QCS (Quaternized Chitosan)	100% self-healing recovery; bacterial inhibition > 75%	Anti-infective agents	Intrinsic antimicrobial activity (~100%)	Rapid erosion under continuous high shear	[181]
	AcHyA (Acrylated Hyaluronic Acid) + G (Gelatin) + AMP (Antimicrobial Peptide)	Gelation < 5 min; Swelling 150%; G' 1210 Pa; Mesh 80–130 nm	0.01% AMP	Kills bacteria; Cell adhesion; Secretes	No passive release; Slow degradation	[182]
Nanogel	HA backbone crosslinked with an acid-cleavable 2,2'-(propane-2,2-diylbis(oxy)) bis(ethane-2,1-diyl) bis(2-methylacrylate) spacer)	Monodisperse spherical structures (~70 nm); Zeta potential: −22 mV; Shrinks to ~50 nm upon drug encapsulation.	16% Content <i>w/v</i> Doxorubicin (DOX); 62% encapsulation efficiency.	Targeted CD44 cellular internalization	Risk of premature systemic cargo leakage	[183]
	HA–TA (Hyaluronic Acid-Tyramine) + Ge-TA (Gelatin-Tyramine)	Core size drops to 68 µm; High needle injectability	MG-63 Osteogenic cells	Seconds-scale gelation; local calcium production.	Precision environmental sensor triggers	[184]
	HA–g-poly (AM-co-IA) (Hyaluronic Acid-graft-poly (acrylamide-co-itaconic acid))	High crosslinking swelling index (~450%); Excellent mechanical skin adhesion	Drug-free matrix; 0% payload	~95% diabetic wound closure; downregulates inflammation.	Low loading capacity for heavy drugs	[185]
	HA + LPEI (Linear Polyethyleneimine)	Monodisperse size: ~220 nm; Low polydispersity index: PDI < 0.15; Highly stable negative surface charge.	High loading density tailored for DOX (~14.5% loading capacity).	Sustained active cargo release lasting up to 15 days; CD44 receptor internal cell uptake.	water-immiscible solvent selection.	[186]
Cryogel	HA + HNTs (Halloysite Nanotubes) + DVS (Divinyl Sulfone)	Crosslinking at −18 °C increases Young's modulus (38 ± 1 to 99 ± 4 kPa), with low hemolysis (1.37 ± 0.11%).	Rat Mesenchymal Stem Cells (MSC), HeLa, and HCT116 lines.	2.5-fold increase in mechanical Young's modulus; Highly blood compatible	High thrombogenicity; clotting index 17.3 ± 4.8.	[187]
	HA + GMA (Glycidyl Methacrylate)	Pore sizes 80–120 µm; interconnectivity >95%	High cell-density uniform seeding	Promotes rapid cellular migration	Low elastic modulus under high strain	[117,188]

Table 3. Cont.

Gel Type	Structure	Physical & Structural Properties	Drug Loading	Advantages	Limitations	Refs.
Microgel	MeHA (Methacrylated Hyaluronic Acid) + PDMAA (Poly(N,N-dimethylacrylamide))	Freezing at -18°C with variable spacer; porosity 90–99%; pore size 90–150 μm	Compressive strength up to 3.6 MPa.	Storage modulus up to 0.37 MPa; reversible strain dependent gel–sol transition.	High spacer loading; pore diameter reduced to 90 μm .	
	HAGM (Hyaluronic Acid Glycidyl Methacrylate) + CD44 (Cluster of Differentiation 44)	High-throughput spheroid alignment; $\sim 400\text{ }\mu\text{m}$ spheroids; interconnected pores.	MCTS; 4T1 breast cancer lines.	30-day cell migration mapping; high oncology array precision.	Aggressive drug-resistant chemotherapy phenotype.	[189]
	PVA (Poly(vinyl alcohol)) + HA (Hyaluronic Acid) + Ag_2O (Silver Oxide Nanoparticles)	Multi-cycle cryo-exposure with nanoparticles $\geq 0.05\text{ wt}\%$; tensile strength peak at 0.5 wt%; thermal stability up to 200°C .	Non-toxic to MDCK and L-929 cell lines at concentrations $\geq 0.1\text{ wt}\%$.	Complete microbial killing (<i>S. aureus</i> , <i>E. coli</i>); Zero mutagenic or genotoxic traits.	High concentration profiles decrease the overall water absorption coefficient.	[190]
	MeHA (Methacrylated Hyaluronic Acid) + Cationic Payloads	Polymer presolution concentration 50 mg mL^{-1} ; uniform size range 114–250 μm ; negative surface potential	Dual payload tracking; Doxorubicin, Methylene Blue, Besifloxacin + fluorescent biomolecules.	Sequential cargo delivery; enzyme-responsive release kinetics	Physiological environment triggers an initial rapid burst release within a few hours.	[191]
	HA–TA (Hyaluronic Acid-Tyramine Conjugate) + Cationic Proteins	Seconds-scale HRP/ H_2O_2 crosslinking; high water content; polyanionic surface charge	Cationic protein encapsulation; Lysozyme + TGF- $\beta 1$.	4-week sustained molecular release; non-toxic to hMSCs	Initial burst release; stable diffusion control.	[192]
	HA (Hyaluronic Acid) + Granular Tyramine Nodes	Extrusion 3D bioprinting; secondary crosslinking; tunable porosity 9–21%; stiffness up to 200 kPa.	Extrusion carrier hosting mature chondrocytic lines.	63-day cartilage tissue development; high shape fidelity.	Larger microgels increase foreign body reaction and inflammation in vivo.	[193]
Smart Gel	GelMA + responsive backbones; dynamic functional network;	pH 4.5–6.5 optimization; reversible pore shifts; dynamic swelling.	Targeted small molecules; bioactive growth factors	90% radical scavenging; high antioxidant activity;	High synthesis cost; monomer purification.	[194]
	Macrocycle + Fe/ Fe^+ redox recognition; supramolecular meshwork	Chemical/electrochemical triggers; HRP/ H_2O_2 coupling; reversible volume/color changes.	Dual drug configuration; cargo control.	Real-time color sensing; switchable optical barriers	Fe precipitation risk; long-term tissue data.	[195]

2.5. Functionalization by Advanced & Specialized Strategies

2.5.1. Functionalization by Enzymatic Crosslinking

Enzymatic crosslinking is a useful method for making HA-based hydrogels because it takes place under mild reaction conditions, forms gels quickly, and produces materials with good mechanical strength [196]. Xu et al. developed HA–tyramine hydrogels by crosslinking the tyramine groups of HA using horseradish peroxidase (HRP) and hydrogen peroxide. By adjusting the amounts of enzyme and hydrogen peroxide, they were able to create hydrogels with different levels of stiffness and tested them as scaffolds for stem cell growth. They found that the hydrogel with an elastic modulus of 350 Pa best supported stem cell proliferation [197]. Similarly, Raia et al. used an enzymatic crosslinking method to combine tyramine-functionalized HA with silk fibroin, which further improved the mechanical strength and stability of the HA–tyramine hydrogels [198]. In contrast, previously, C.E. Schanté et al. noted that amino acids containing carboxyl groups, like aspartic acid, or hydroxyl groups, such as threonine, serine, or tyrosine, provided an especially strong shield against enzymatic breakdown. Then, HA–amino acid derivatives were crosslinked with butanediol diglycidyl ether (BDDE), and the resulting hydrogels had swelling properties similar to native HA but maintained higher resistance to hyaluronidase degradation. These findings indicate that amino acid-modified HA derivatives could be promising materials for viscosupplementation and drug delivery applications [199]. Another study by Atiroğlu immobilized lipase on HA-modified Fe₃O₄ magnetic nanoparticles coated with graphene oxide (GO) using crosslinked enzyme aggregates (CLEAs), achieving 96.5% activity recovery with 55% ammonium sulfate and 125 mM glutaraldehyde at 4 °C for 10 h. This immobilization greatly enhanced the enzyme's stability and reusability compared to free lipase [200]. Similarly, Farhan et al. achieved the modification of magnetic nanoparticles (MNPs) with HA, followed by crosslinked enzyme fine aggregates of isocitrate dehydrogenases (IDH/HA/MNP–CLEAs), and later bovine serum albumin (BSA) incorporated as a co-feeder on the IDH/BSA/HA/MNP–CLEAs. The IDH was effectively immobilized on the HA/MNP surface. This technique can be used as a biocatalyst and biosensor [201]. Recently, Arnold et al. hypothesized that enzymes can be immobilized on hyaluronic acid (HA)-based complexes to enhance stability, control, and efficiency for biomedical applications [202].

2.5.2. Functionalization by Nano-Metal Conjugation

Nano-metal conjugation (MNP) has emerged as a promising prospect in biosensor engineering, signal amplifiers, and the ultrasensitive detection of biomolecules through electrochemical, optical, and magnetic transducer mechanisms in the body. More recently, Li and Chen reviewed the applications of hyaluronic acid nanoparticles (HA-NPs) as biosensors and in three-dimensional (3D) bioprinting for biomedical applications [203]. Jingchao Li et al. developed polyethyleneimine (PEI)-modified Fe₃O₄ nanoparticles conjugated with fluorescein isothiocyanate (FITC) and hyaluronic acid (HA) of two molecular weights (6 and 31 kDa), yielding Fe₃O₄–PEI–FITC–HA nanoparticles with diameters of 15–16 nm. The nanoparticles exhibited excellent dispersibility and colloidal stability, enabling targeted magnetic resonance (MR) imaging in both cellular models and HeLa xenograft tumors [204]. Similarly, Meysam Soleymani et al. developed a one-pot hydrothermal method to directly coat Fe₃O₄ nanoparticles with hyaluronic acid (HA), yielding Fe₃O₄@HA nanoparticles. The HA-coated nanoparticles exhibited a four-fold higher cellular uptake in CD44-positive MDA-MB-231 breast cancer cells than in normal L929 cells and compared with dextran-coated Fe₃O₄ nanoparticles, demonstrating their enhanced CD44-mediated targeting capability. The resultant NPs showed superior magnetic hyperthermia performance under safe AMF conditions [205]. More recently, Chen et al. reported a similar strategy by de-

veloping Fe_3O_4 -hyaluronic acid nanogels (Fe_3O_4 -HA NGs; ~ 125 nm) (Figure 11i,ii). The nanogels exhibited superparamagnetic behavior with a saturation magnetization (M_s) of 62.8 emu/g, excellent biocompatibility (96.7% astrocyte viability at 100 $\mu\text{g}/\text{mL}$), and minimal plasma protein adsorption [206]. However, there is some adverse therapeutic effect on $\text{A}\beta$, and blood–brain barrier penetration efficacy remained unaddressed. In another study, Bayoumi et al. developed hyaluronic acid (HA)-conjugated, chitosan-coated iron oxide nanocrystals and demonstrated that their therapeutic efficacy was significantly enhanced when combined with ^{131}I -labeled cetuximab, resulting in a synergistic radio-photothermal therapeutic effect [207].

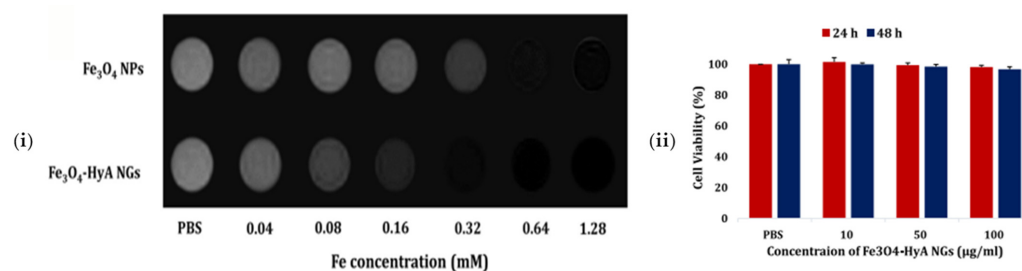


Figure 11. (i) T2-weighted MRI images of colloidal dispersions of Fe_3O_4 -NPs and Fe_3O_4 -HyA NGs at diverse Fe concentrations, showing a dose-dependent dark contrast generation in vitro. (ii) Cell viability of a normal astrocyte cell line (C8-D1A) treated with Fe_3O_4 -HyA NGs for 24 h and 48 h. The data were reported as the mean value $\pm$ SD of three independent assays. Reprinted from *Arabian Journal of Chemistry*, 2022 [206].

Zhang et al. developed hyaluronic acid-coated silver nanoparticles (HA-AgNPs) using sodium borohydride as the reducing agent and demonstrated their potential as dual-modality contrast agents for computed tomography (CT) and single-photon emission computed tomography (SPECT) imaging [208]. Recently, Hussein & Abdullah reviewed AgNP-based drug delivery systems for cancer treatment, which were functionalized with HA, liposomes, and lipid NPs [209]. R.H. Al-Serwi et al. developed hydrophilic hyaluronic acid–quercetin (HA–Qt) conjugates decorated with silver nanoparticles (AgNPs) and further functionalized with polyethylene glycol–folic acid (PEG–FA), yielding PF/HA–Qt–AgNPs (Figure 12i,ii). The multifunctional nanodrug exhibited significantly higher cytotoxicity against HeLa and Caco-2 cancer cells than free quercetin [210]. More recently, P. Thi et al. hypothesized the exploitation of Ag-NPs@HA as a smart nanocarrier to control DOX delivery through apoptosis-dependent programmed, i.e., increasing the therapeutic efficiency against cancer [211].

In one study, Lee et al. developed hyaluronic acid (HA)–end-functionalized gold nanoparticles (AuNPs) and demonstrated that reactive oxygen species (ROS)-responsive nanoparticle surface energy transfer (NSET) enabled sensitive optical imaging [212]. Again, Lee et al. demonstrated that the hyaluronic acid–gold nanoparticle/interferon- α (HA–AuNP/IFN α) complex induced significantly higher expression of 2',5'-oligoadenylate synthetase 1 (OAS1) in liver tissue than free IFN α , PEG-Intron, or the AuNP/IFN α complex, indicating enhanced antiviral activity [213]. More recently, Apaolaza et al. investigated the interaction between hyaluronic acid-coated gold nanoparticles (HA-GNPs) and the CD44 receptor, demonstrating their specific CD44-mediated binding [214]. Again, Kim et al. developed hyaluronic acid-coated gold nanoparticles (AuNPs–HA) via a one-pot electron beam (e-beam) irradiation method, which simultaneously reduced the molecular weight of hyaluronic acid (HA) and enabled the formation of drug-loaded nanoparticles. The resulting AuNP–HAs encapsulated sulfasalazine (SSZ) with an encapsulation efficiency of 94% and a drug-loading capacity of 70%, demonstrating their potential as an orally administered, intestine-targeted drug delivery system (Figure 13i,ii) [215]. In another study,

Hejazi et al. developed hyaluronic acid (HA)-functionalized AuNPs/PEI/RhB nanoparticles and demonstrated their selective targeting of CD44-overexpressing MCF-7 breast cancer cells [216]. In a hybrid application study, A. Hovhannisyan et al. investigated the synthesis of silver nanoparticles (AgNPs, 5–25 nm) and gold nanoparticles (AuNPs, 10–35 nm) within a hyaluronic acid (HA) matrix using plasma-treated water generated under both air and argon atmospheres. The resulting nanocomposites exhibited antimicrobial activity, although its extent varied depending on the synthesis conditions. However, HA–Ag surpassed HA–Au in antimicrobial activity but carried some toxicity risk, whereas HA–Au offers lower biological activity but better stability and safety in case of long-term medical use [217].

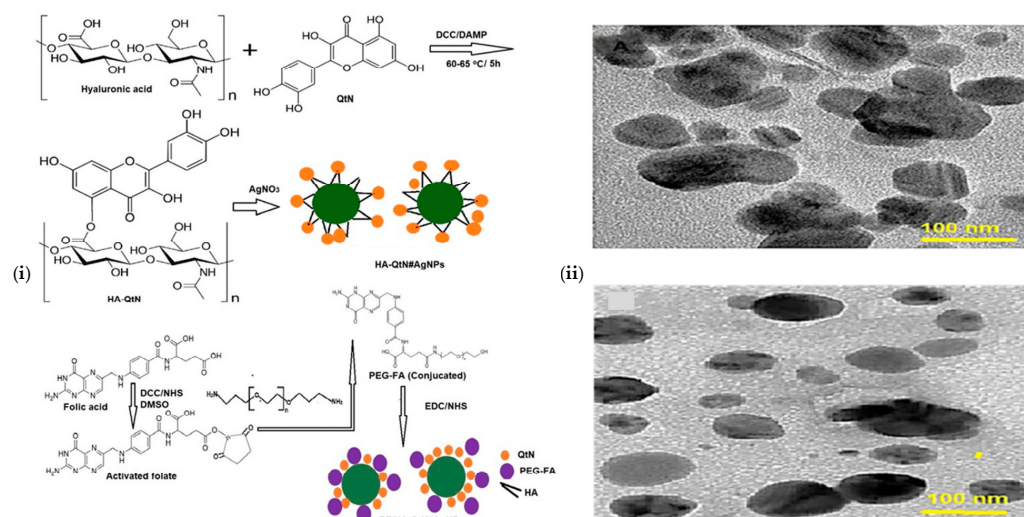


Figure 12. (i) Schematic representation of PF/HA-QtN-AgNP generation. QtN: quercetin; HA: hyaluronic acid; AgNPs: silver nanoparticles; AgNO₃: silver nitrate; FA: folic acid; PEG; polyethylene glycol; DMSO: dimethylsulfoxide; DCC: 1,3-dicyclohexylcarbodiimide; DAMP: 4-dimethylaminopyridine; EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, NHS: N-hydroxysuccinimide. (ii) TEM images of HA-QtN-AgNPs (upper) and PF/HA-QtN-AgNPs (lower). The scale bars indicate 100 nm. Reprinted from MDPI [210].

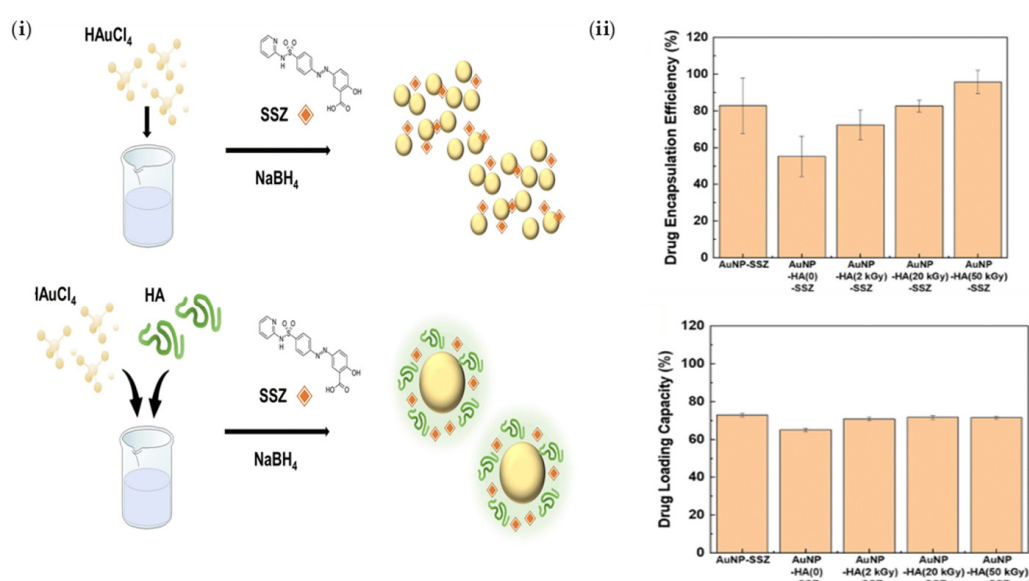


Figure 13. (i) Schematic illustration for one-pot synthesis of the gold nanoparticle–hyaluronic acid–sulfasalazine (AuNP–HA–SSZ). (ii) Drug encapsulation efficiency and drug loading capacity of the AuNP–SSZ and AuNP–HA–SSZ. Reprinted from Royal Society of Chemistry, 2023 [215].

Another promising advancement in biomedical imaging is the conjugation of hyaluronic acid (HA) with nanocarbon materials and their derivatives. For example, Goh et al. synthesized carbon dots (CDs) via the pyrolysis of citric acid in the presence of PEG diamine, followed by the covalent conjugation of hyaluronic acid (HA) through amide bond formation. This conjugate executed sensation-dependent multicolor fluorescence with HA receptor +B16F1 cells and liver-specific delivery via HaRe/CD44 [218]. A roughly advanced gemcitabine (GEM)–HA–PEG–CNTs approach to entrapping drug efficiency *in vivo* achieved approximately 90%, as shown by SK Prajapati et al. [219]. Silvia Arpicco et al. synthesized hyaluronic acid–dimyristoylphosphatidylethanolamine (HA–DMPE) conjugates using HA with molecular weights ranging from 6.4 to 1500 kDa. The conjugates were subsequently coated onto carbon nanotubes (CNTs) and loaded with doxorubicin to produce pH-responsive nanocarriers. Their findings identified 200 kDa HA as the optimal molecular weight for tumor-targeted CNT-based drug delivery, whereas higher-molecular-weight HA caused steric hindrance that reduced targeting efficiency [220]. Wu et al. discovered porous carbon-coated magnetite nanoparticles (PCCMNs) lined HA to obtain a nanohybrid with a good photothermal therapy/chemotherapy [221]. In the same way, H. Wu et al. developed hyaluronic acid (HA)-functionalized graphene oxide (GO) by first modifying nanosized GO with adipic acid dihydrazide (ADH), followed by the covalent conjugation of HA through amide bond formation. The resulting GO–HA nanoconjugates exhibited no significant *in vivo* toxicity, even at a high exposure dose of 10 mg kg^{−1} over 10 days. This conjugation acts as a good carrier of DOX [222]. More recently, Kunal Sarkar et al. developed hyaluronic acid-functionalized graphene oxide quantum dots (GOQD–HA) as a targeted drug delivery platform for the selective delivery of metformin to macrophages implicated in type 2 diabetes mellitus. Metformin–GOQD–HA effectively suppressed pro-inflammatory cytokines and restored antioxidant balance at lower doses than free metformin in palmitic acid-stimulated RAW264.7 cells and diet-induced obese mice (Figure 14i,ii). The nanocarrier contributes to therapeutic anti-inflammatory and antioxidant effects [223].

2.5.3. Functionalization by Dynamic Crosslinking

Recently, dynamic crosslinking strategies have come to the fore as a modern and versatile approach for engineered HA-based hydrogels to surpass the inherent limitations of conventional permanent covalent networks [224]. For example, Oliveira et al. formed a pH-sensitive dynamic boronate–ester crosslink between saccharide–HA (GLU–HA) and 3-aminophenylboronic acid–HA (APBA–HA), which enabled the smooth delivery of the anti-photoaging agent dihydrocaffeic acid (DHCA) [225]. However, the hydrogel showed uncontrolled release and limited injectable application [225]. Similarly, Han et al. developed an injectable hydrogel by combining methacrylated chitosan (CHMA) and oxidized hyaluronic acid (OHA) through electrostatic interactions and Schiff base crosslinking, followed by photopolymerization. This dual-crosslinking strategy produced hydrogels with enhanced mechanical strength, high modulus, and improved structural stability. Notably, the CHMA–OHA hydrogel possessed high stem cell viability (to 92%) [226]. Ma et al. developed an interpenetrated HA/γ-PGA adaptable hydrogel polymer network approach (>85% compressive strain), making it suitable for load-bearing tissue engineering [227]. Wang et al. reviewed some of the promising methods of dynamic covalent crosslinking to obtain a catalyst-free crosslinking strategy with high specificity and without any toxic by-products [228]. Y Wang et al. designed double-network hydrogel combining photocrosslinked gelatin methacrylate (5–20% sol.) and hydrazone–HA (mixing equal mass ratios of OHA and HA–ADH at 1.5–3% in PBS), exhibiting increased mechanical strength, making this a promising material for 3D-printed tissue engineering scaffolds [180]. In one study, Zhang et al. developed balanced reversible acylhydrazone bonds between aldehyde-

modified maleic sodium hyaluronate and 3,3'-dithiobis(propionylhydrazide), followed by photo-polymerization of maleic groups [229]. This obtained dynamic characteristic by dual crosslinking between acylhydrazone and disulfide linkages exerts fast gelation and self-healing properties [229]. More recently, Tavakoli et al. reported a novel ECM-mimetic HA hydrogel with a dual dynamic crosslinking nature (disulfide + thiazolidine), optimized for injectable stem cell delivery and 3D bioprinting. At first, they created a disulfide-crosslinked hydrogel via linking cysteine derivatives with HA carboxylate groups, followed by reacting excess cysteine residues with aldehyde-modified HA to develop a thiazolidine crosslinked product. The resulting bioink supported the bioprinting of human mesenchymal stem cells (hMSCs) and maintained stemness by inducing more than a two-fold increase in the mRNA and protein expression levels of the key stemness markers OCT3/4 and NANOG compared with the control group (Figure 15i–v) [230]. Previously, a similar approach was devised by Li et al.; however, they did not discuss stemness gene expression [231]. In another study, Yang et al. developed antibacterial, self-healing hyaluronic acid (HA) hydrogels via hybrid dynamic crosslinking of aldehyde-modified HA (AHA), 3,3'-dithiobis(propionyl hydrazide) (DTP), and fungal-derived quaternized chitosan (QAC) using a dual-syringe injection system. The hydrogel network was stabilized through hydrazone, disulfide, and Schiff base linkages, together with electrostatic interactions, resulting in rapid gelation and complete self-healing within 1 h. The hydrogels showed superior antibacterial resistance (up to 100% against *E. coli* and 75% against *S. aureus*) and good cytocompatibility for wound dressing, tissue engineering, and cell culture applications [181].

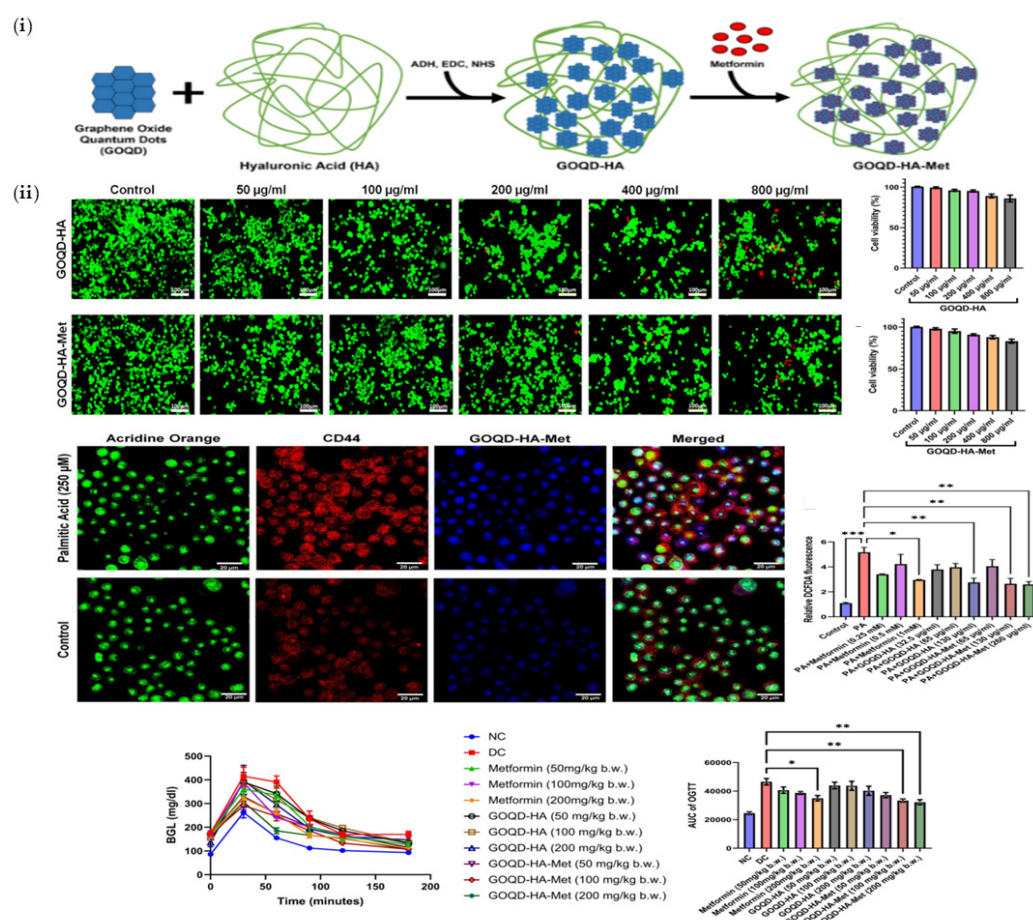


Figure 14. (i) Schematic synthesis scheme of hyaluronic acid functionalized graphene oxide quantum dots (GOQD-HA) and metformin-loaded GOQD-HA (GOQD-HA-Met). (ii) Cytocompatibility using Live/Dead assay by FDA/PI staining of RAW264.7 cells, cellular uptake, and dose optimization for GOQD-HA-Met (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). Reprinted from Springer Nature Link [223].

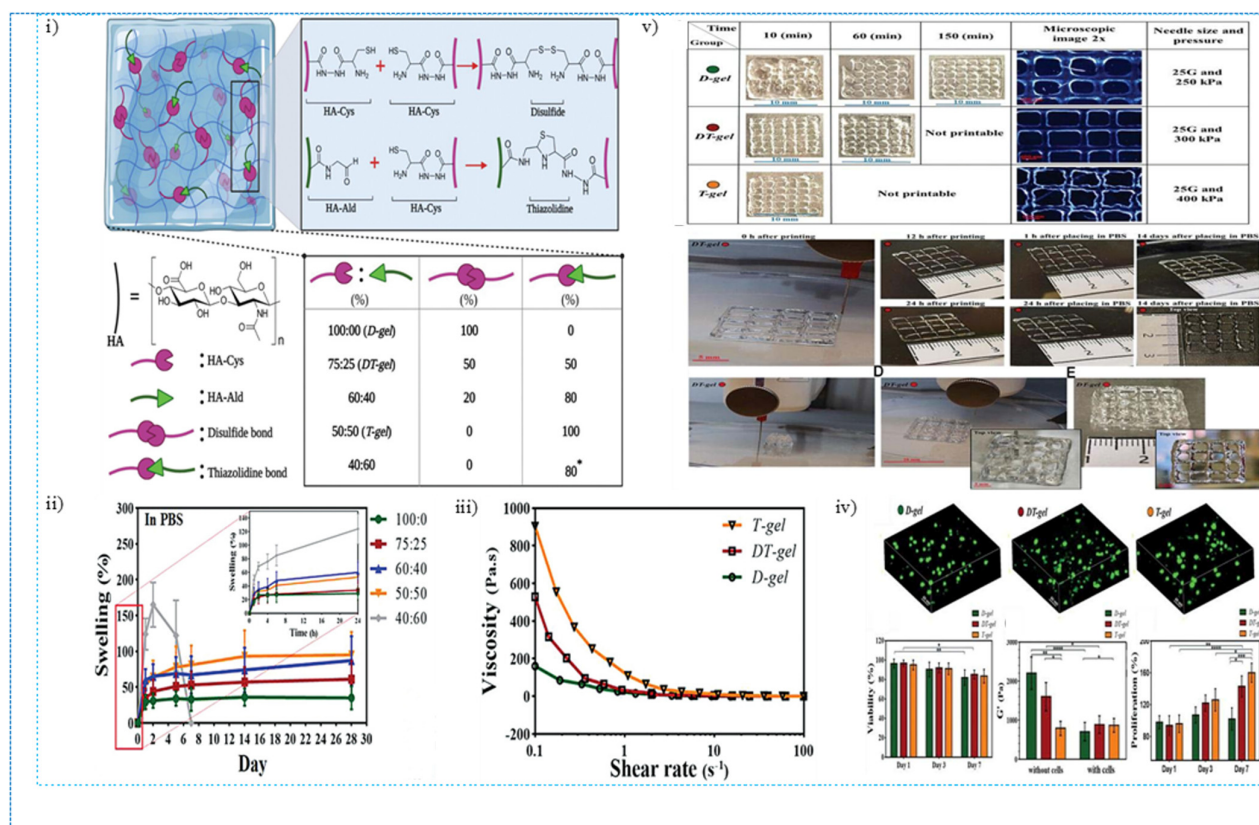


Figure 15. (i) Schematic representation of hyaluronic acid – cysteine (HA–Cys) and hyaluronic acid – aldehyde (HA–Ald) as well as their reactions to form disulfide and thiazolidine linkages upon mixing at different ratios of HA–Cys and HA–Ald. (ii) Swelling (%) of hydrogels in PBS over time at 37 °C over 28 days. (iii) Shear–thinning behavior of hydrogels obtained from viscosity measurement. (iv) Biocompatibility and support of hydrogels for cell proliferation and associated storage modulus of hydrogels. (v) 3D printing of hydrogels with different layers. Reprinted from Wiley Advanced Functional Material [230]. $p < 0.05$ was the statistical significance for all tests (**** = $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, and * = $p < 0.05$).

3. Characterization Techniques

Comprehensive characterization is indispensable to confirm successful functionalization, crosslinking, and bioactivity of HA-based systems, as well as to correlate chemical functionalization with physicochemical, mechanical, and biological performance. Correspondingly, a combination of spectroscopic, chromatographic, rheological, morphological, and biological techniques was analyzed to systematically verify the chemical structure, network formation, and functional properties of the modified HA materials. The techniques are summarized in Table 4.

Table 4. Characterization techniques of functionalization.

Functionalization Category	Primary Purpose	Key Techniques Used	Each Technique Proves/Measures	References
Covalent Chemical Functionalization	Chemically attach new groups to the HA backbone (–OH, –COOH, N-acetyl).	FTIR, $^1\text{H}/^{13}\text{C}$ NMR, XPS, GPC/SEC, DSC/TGA.	FTIR: C=O, amide, ester; NMR: degree of substitution (DS) & chemical shifts; XPS: surface elemental changes; GPC: molecular weight change; DSC/TGA: thermal transitions & organic content.	[20–65]

Table 4. Cont.

Functionalization Category	Primary Purpose	Key Techniques Used	Each Technique Proves/Measures	References
Covalent Crosslinking (Permanent Networks)	Oxidation and Sulfation of HA moderate the functional reactivity to a varying degree.	Rheology, Swelling Ratio, FTIR, NMR, CHNS, Zeta Potential, XRD.	Rheology: network stiffness; Swelling: crosslink density; FTIR: crosslink formation (C-O-C, amide), S=O stretching bands (1220–1260 cm ⁻¹), C-O-S vibration (800–850 cm ⁻¹); NMR: DS, DD, DO and confirmation of crosslinks vs. unreacted groups; Zeta Potential: surface charge; CHNS: presence of S and secondary confirmation.	[66–82]
Click chemistry	Bio-orthogonal, fast conjugation without toxicity, Light-controlled crosslinking & patterning, pH-responsive, self-healing networks.	FTIR, ¹ H NMR, Fluorescence labeling, Rheology, Cell viability, Ellman's assay, Photo-rheology, AFM, Microscopy, Swelling/Degradation.	FTIR: disappearance of azide peak (2100 cm ⁻¹), loss of C=C peak, C=N (imine) peak ~1620 cm ⁻¹ ; NMR: triazole formation, imine proton confirmation; Fluorescence: spatial conjugation; Photo-rheology: UV-triggered gelation kinetics; Rheology: rapid network formation; Swelling: pH-dependent behavior.	[83–105]
Physical Crosslinking	Form gels via hydrogen bonding, ionic interactions, and hydrophobic assembly, guest-host interactions.	Rheology, DLS/SEC, TEM/SEM, Zeta potential, FTIR.	Rheology: viscoelastic network formation; DLS: particle size/assembly distribution; TEM/SEM: morphology; Zeta: charge interactions; FTIR: hydrogen bond changes; Rheology: shear-thinning & recovery	[106–124]
Bio-conjugation (drug/peptide/protein/anti-microbial/other biopolymers)	Attach bioactive molecules to HA; impart antibacterial/antifungal activity to HA.	FTIR, NMR, UV-Vis/Fluorescence, HPLC/MS, XPS, Zeta potential, MIC/MBC assays, Live-Dead staining.	FTIR/NMR: linkage confirmation, covalent or ionic attachment of antimicrobial moiety; UV-Vis/Fluorescence: probe/drug presence; HPLC/LC-MS: purity & amount of conjugated bioactive; XPS: surface N ⁺ /metal ions (Ag, Zn); Zeta: charge reversal (often negative → positive).	[100–125]
Enzymatic Crosslinking (HRP, TGase, tyrosinase)	Mild, cell-friendly hydrogel formation.	FTIR, UV-Vis, Rheology (time & frequency sweep), Swelling, Cell viability.	FTIR: enzymatic coupling signatures; UV-Vis: reaction kinetics (enzyme activity); Rheology: gelation time; swelling: crosslink density.	[197–203]
Nano-metal conjugation	Drug delivery & targeting, self-wound healing, multifunctionality.	UV-Vis, ICP-MS, Rheology, FTIR, DLS, Zeta potential, TEM, and Release kinetics.	UV-Vis: coordination complex formation; ICP: metal content; DLS: size & PDI; Zeta: colloidal stability; TEM: morphology; Release: diffusion vs. degradation.	[204–224]
Dynamic Crosslinking	Reversible networks responsive to stimuli; 3D bioprinting applications	FTIR, Rheology, Swelling/Degradation, UV-Vis, Printability tests, Live-Dead assay.	FTIR: imine/hydrazone peaks; Rheology: self-healing recovery; Swelling: network dynamics; UV-Vis: reaction kinetics; Print tests: shape fidelity; Live-Dead: post-printing cell survival.	[181,225–230]

4. Application of HA

4.1. Application of HA in Tissue Engineering and Drug Delivery Systems

HA has been extensively engineered into advanced biomaterial systems for applications in tissue regeneration and targeted drug delivery. Owing to its diverse biological

and physicochemical attributes, such as biodegradability, biocompatibility, and inherent viscoelasticity, HA has attracted significant attention for functional modification. These modifications enable the development of tailored HA-based platforms with enhanced performance across a broad spectrum of biomedical applications. A comprehensive summary of these applications is presented in Table 5.

Table 5. Application of HA in tissue engineering and regenerative medicine, drug delivery & targeted therapy, wound healing & skin regeneration, gene therapy, cancer therapy and 3D printing and ophthalmology.

Application Types	Functionalization Strategy	Biomedical Application	References
Tissue engineering and Regenerative medicine	Chemical crosslinking (methacrylation, esterification, and thiolation); Enzymatic crosslinking (tyramin–HA); Physical modification (hydrogel, cryogel, and 3D-Scaffold); Bioconjugation with ECM protein and growth factor; HA–gelatin/silk/chitosan systems; Oxidation (periodate, TEMPO, N-acetyl-d-glucosamine).	Cartilage and bone regeneration; Skin and wound healing scaffolds; Cardiac patches and vascular grafts; Neural tissue engineering; Injectable cell-delivery hydrogels.	[38,46,55,60,69,70,76,126,176,182].
Drug Delivery & Targeted Therapy	Carboxyl and hydroxyl modification (EDC/NHS coupling); Schiff-base reaction for injectable depots; Nanoparticle conjugation (liposomes, polymeric carriers); pH- or enzyme-responsive functionalization; Self-assembled HA micelles.	Controlled drug release systems; Cancer-targeted drug delivery (CD44 receptor targeting); Protein and peptide carriers; Ocular drug delivery; Intranasal and pulmonary formulations.	[13,25,53,69,102,165,168,198,203].
Wound Healing & Skin Repair	Enzymatic HA modification for self-healing hydrogels; Photo-crosslinkable scaffolds for sprayable dressings; Bio-conjugated HA with antimicrobial peptides; Oxidized HA (aldehyde–HA) for rapid gelation; Guest–host (β -CD/adamantane, azobenzene–CD); (ionic-Mg ²⁺ /Fe ²⁺ , H-bonding).	Diabetic wound healing; Burn treatment; Scar reduction; Antibacterial dressings.	[100,109,111,112,125,134,160,177,196,199].
3D Bioprinting & Bioinks	Methacrylated HA (HA–MA); Thiolated HA (HA–SH); HA–alginate, gelatin, chitosan, collagen, hybrid formulations; Physical rheology tailoring (shear-thinning, viscoelasticity). HA–Fe, Fe ₂ O ₃ , C, Au, Ag, GO, GOQD, etc.	3D printed organs and tissues; Bioinks for cell-laden scaffolds. High-resolution vascular structures; Personalized implants.	[25,45,46,55,97,117,125,140,172,174,175,179,208,217,222,223].
Gene Delivery	Bioconjugates (iRNA/miRNA Delivery, Plasmid DNA (pDNA) Delivery, CRISPR/Cas9 Delivery, HA–peptide, HA–lipid); Hydrophobic modification (HA–cholesterol conjugates); Enzymatic degradation control (Tyramine–HA).	Used for gene silencing in cancer therapy; Enables sustained gene expression for tissue regeneration and immunotherapy.	[60,74,122,163,231–234]
Cancer Therapy & Targeted Immunotherapy	HA–drug conjugates (HA–DOX, PTX, DTX, CPT); HA–antibody conjugation; HA-coated nanoparticles; IDH/HA/MNPs–CLEAs; dual-drug HA systems.	Targeted tumor delivery (CD44 targeting); Immunotherapy carrier; Gene therapy vector; Theranostic imaging + therapy.	[79,125,126,134,140,142,170,217,220,223].
Anti-inflammatory & Antioxidant therapy	Enzyme-responsive HA conjugates; HA–curcumin, quercetin, resveratrol conjugates; HA-coated nanoparticles; HA–SH for ROS scavenging; N-deacetylation/re-acetylation.	Arthritis treatment; Inflammatory bowel disease; Neuro-inflammation control; Osteoarthritis therapy.	[11,15,61,65,74,88,90,111,165,235–237]
Ophthalmology	Crosslinked HA for viscoelastic eye drops; HA–chitosan nanoparticles for corneal delivery; Enzymatically degradable HA gels; Bioadhesive HA derivatives; Silk fibroin–HA conjugates.	Treatment of dry eye syndrome; Retinal drug delivery; Corneal wound treatment; Artificial vitreous substitutes.	[7,52,118,174,198,202,214]

4.2. Application of HA in Bio-Sensing Platform

Beyond structural tissue scaffolds, modified HA plays a critical role in advanced bio-sensing platforms. The table below (Table 6) details how various functionalization strategies and chemical modifications enable intelligent ion regulation, improve bio-interface architecture, and optimize bio-sensing transduction mechanisms for real-time diagnostic monitoring.

Table 6. Application of HA in bio-sensing platform.

Application Types	Functionalization Strategy	Target Reactive Site & Mechanism	Biomedical Applications	Technical Limitations	Refs.
Intelligent Ion Regulation Strategy	Polyelectrolyte Sulfation (HA-SO ₃ ⁻); Coupling β -Cyclodextrin (β CD) to Adamantane/Azobenzene; Aligning micro-porous spaces via AI modeling networks; Extending saccharide branches with synthetic side lines.	Carboxyl/Hydroxyl conversion altering surface charge profiles; hydrophobic cavity inclusion matching light/stress inputs; Reversible ester formation reacting directly to local carbohydrates; Carboxyl side chains crosslinked into high-density polyelectrolytes.	Dynamic iontronic membranes; bio-skin patches, and synaptic ASIC emulators; Smart therapeutic dressing platforms and automated active drug-release skins; Continuous wearable glucose profiling and metabolic test kits; Intracellular bioelectronic interfaces and local tissue-flux regulators.	Changes in surrounding moisture can cause baseline signal tracking errors; Overlapping sensor signal lines require complex algorithmic data separation; Chemical crosstalk risks if other competing sugar molecules enter the solution; Natural enzymatic material breakdown shifts the internal base signal metrics.	[238–242]
Bio-Interface Architecture	Inorganic Oxide Compositing (HA-SiO ₂ /HA-HAp); Organometallic Coordination (Ferrocene-HA); Conductive Plastic Blending (HA-PEDOT: PSS).	Covalent functionalization at -OH backbone (etherification; ratio ≤ 400); reversible Fc coordination; electrostatic interfacial loops (~5% polymer tuning); multivalent chelation at -COOH; matrix capping + H-bonding.	Orthopedic coatings, load-bearing joint repair; and structural bio-implant; Responsive skin matrices; tissue growth scaffolds; and wound heal patches; Flexible electronic nerve links and low-impedance brain interfaces.	Compressive modulus drops below 143 kPa if GPTMS/HA molar ratio stays under 400; Localized Fc phase precipitation risks under shifting cell media parameters; Soft organic layers peel from metal wires over extended strain periods	[243–245]
Bio-sensing Transduction Mechanism	HA-Based Soft-Sensor Arrays; Electronic-Driven Multimodal Gels (e-DMoH); Conductive PPy/PDA Nanohybrid Meshes	Soft-sensor state parameter predictions; Physical strain/pressure gating; Multifunctional target coordination matrices	Continuous software monitoring for commercial industrial fermentation factories; Point-of-care disease test kits and environmental contamination arrays.	Mean squared prediction error limits are fixed at 0.018–0.049 (g/L) ² ; Signal interference loops trigger under sweat fluctuations; Conductivity is confined to strict 10 ⁻⁴ to 10 ⁻³ S/cm ranges	[239,246,247]

5. Conclusions and Future Prospects

Although hyaluronic acid (HA)-based biomaterials have advanced significantly in drug delivery, tissue engineering, and regenerative medicine, conventional crosslinking strategies still face challenges in reproducibility, cytocompatibility, and scalability. Enzymatic systems such as horseradish peroxidase (HRP)/hydrogen peroxide (H₂O₂) offer mild conditions but may suffer from structural heterogeneity and residual oxidative stress. Future efforts should focus on precision fabrication, particularly automated microfluidic platforms with real-time monitoring, to produce uniform HA microgels with controlled architecture and minimized cytotoxic residues, thereby improving manufacturing consistency and scalability.

Uncontrolled initial burst release remains a key limitation in smart hydrogels, reducing therapeutic efficiency and causing local drug overload. Future systems should employ multifunctional interpenetrating and supramolecular networks integrating pH-, redox-, enzyme-, and host–guest responsiveness. The incorporation of dynamic motifs, such as ferrocene–cyclodextrin interactions and adaptable linkages, may enable more precise, sustained, and disease-specific release. Designing hydrogels that respond selectively to pathological environments, including acidic or oxidative conditions, can further reduce premature cargo leakage and improve therapeutic precision.

Future research on the functionalization of HA should focus on achieving precise spatiotemporal regulation of its biochemical and biophysical properties to better replicate the dynamic nature of the native extracellular matrix (ECM). Such advanced HA-based

biomaterials should be capable of providing controlled spatial distribution and temporal presentation of bioactive cues, mechanical properties, and degradation profiles in response to cellular activities or external stimuli. This level of dynamic regulation would more closely mimic the continuously remodeling ECM found in living tissues, thereby enhancing cell adhesion, migration, proliferation, differentiation, and tissue regeneration. Developing HA-based scaffolds with programmable and responsive spatiotemporal functionalities is expected to improve their performance in tissue engineering and regenerative medicine by creating a more physiologically relevant microenvironment.

Antimicrobial hydrogel platforms also require advancement beyond passive nanoparticle diffusion, which may lead to uncontrolled ion release and tissue toxicity. Future designs should emphasize self-healing, shear-thinning matrices incorporating covalently bound antimicrobial peptides or controlled inorganic components. Such systems can enhance antibacterial efficacy while maintaining cell compatibility, mechanical stability, and appropriate biodegradation behavior under dynamic biological conditions.

A major challenge remains the lack of non-invasive tools to monitor hydrogel degradation and drug release in vivo. The integration of imaging functionalities, including near-infrared fluorophores, MRI-active agents, and bio-orthogonal probes, into HA backbones can enable real-time tracking of degradation, distribution, and therapeutic performance in deep tissues.

Beyond therapy and imaging, an emerging frontier is the integration of bio-sensing within HA-based systems through organic–inorganic hybrid frameworks. Combining HA networks with inorganic nanostructures or metal–organic framework (MOF)-like architectures can enable intelligent ion regulation and selective interaction with physiological species. Embedding electrochemical, optical, or colorimetric transduction elements within these matrices allows real-time monitoring of pH, redox states, and ion flux. This hybrid approach enhances signal amplification, structural stability, and responsiveness, enabling advanced theranostic platforms that couple drug delivery with continuous bio-sensing.

Overall, future progress in HA-based hydrogels will depend on integrating precise fabrication, multifunctional responsiveness, bio-sensing, imaging, and thorough biological evaluation. Achieving reproducibility, scalability, immune compatibility, long-term safety, and regulatory feasibility will be essential for translating these systems into clinically viable and intelligent biomedical platforms.

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