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Review

Chemical, enzymatic and biological synthesis of hyaluronic acids

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ABSTRACT

Hyaluronic acid (HA) is a major glycosaminoglycan, a family of structurally complex, linear, anionic hetero-*co*-polysaccharides. HA is important in various anatomical structures including the eyes, joints, heart and myriad intricate tissues, and is currently widely used in the therapeutics and cosmetics areas. The synthesis of HA of well-defined and uniform chain lengths is of major interest for the development of safer and more reliable drugs and to gain a better understanding of its structure-activity relationships. However, HA has received less attention from the synthetic carbohydrate community compared with other members of the glycosaminoglycan family. In this review, we examine the remarkable progress that has been made in the chemical and chemoenzymatic synthesis of HA, providing a broad spectrum of options to access HA of well controlled chain lengths.

Contents

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1. What is hyaluronic acid?

Hyaluronic acid (HA) is a major glycosaminoglycan (GAG), a family of structurally complex, linear, anionic, hetero-*co*-polysaccharides. HA is primarily found in the extracellular matrix (ECM) of animals and is polysaccharide composed of the repeating disaccharide unit →4) β-D-GlcA (1 → 3) β-D-GlcNAc (1 → (Fig. 1) [1,2] of molecular weight ~400 Da. Intact HA polysaccharide chains can be comprised of as many as 25,000 disaccharide units corresponding to a molecular weight 10^7 Da. HA is generally a sodium salt under physiological conditions and is, thus, referred to as sodium hyaluronate. The spatial architecture of HA is an amphiphilic two-fold helix with a hydrophobic patch consisting of 8 CH groups and the hydrophilic domains of carboxyl and hydroxyl groups [3]. In addition, the spatial structure is strongly dependent on pH, concentration and HA molar mass [3,4]. For example, HA is a highly hydrated random coil as determined by crystallography at physiological pH, but at pH 2.5–4, HA structure is in helical compacted forms such as rod-like (single strands) and coiled-coil (double helix strands) as determined from molar ellipticity spectra and molecular dynamics simulations [5].

Currently, commercially available HA is polydisperse and is either isolated from rooster combs or produced through microbial fermentation [2,6,7]. Due to the hydroxyl group that can bind water molecules tightly to the chain through hydrogen bonds, HA is highly hydrophilic and making it responsible in imparting flexibility to the tissues. For example, HA is important in various anatomical structures, including the eyes, joints, heart and myriad intricate tissues [8]. HA also interacts with cell surface receptors and subsequently regulates various intracellular signaling pathways [9]. Modified HA polysaccharides have been prepared, such as chemically sulfated HA, with display surprising biological properties as biomaterials, improving the healing of dermal wounds and the regeneration of bone material and these sulfated HA polymers are considered and promising candidates for the development of implant coatings and tissue-regeneration with artificial versions of the ECM [10,11]. Various clinical studies of oral HA treatments have shown its moderate efficacy in reducing pain and improving knee functionality, without observed side effects [12]. As a cosmetic component, HA has been used for skin-care and is an excellent moisturizer, inhibiting skin wrinkles and improving the condition of skin and acting as a filler for facial rejuvenation [13–17].

2. Why is HA of well-defined chain length needed?

HA biosynthesis in both prokaryotes and eukaryotes results in HA that is polydisperse containing a large number of molecules of different chain lengths. HA isolated from animal tissues, skin, rooster combs, vitreous, synovial fluid, and umbilical cord, shows seasonal variations, based on environmental factors, variations based on the organ tissue collected, animal health, and animal feed and these contribute to the wide variety of observed chain lengths, resulting in HA molecular weights ranging from 50,000 and 8,000,000 Da [7,18]. Moreover, animal tissues contain other GAGs, such as chondroitin and heparan sulfate that can be structural similar to HA limitating HA purification and the lack of simple and reliable analytical methods, cross-contamination of animal-derived GAGs can frequently occurs [19–21]. Animal-derived HA also possess additional safety concerns, such as the risk of infection from animal virus and prior impurities [19]. Microbial fermentation provides an alternative approach to produce HA but it is challenging to obtain HA with the same molecular weight properties as animal-sourced HA and these products can still contain impurities such as nucleic acids, proteins and microbial toxins [22]. The physiological and pharmacological functions of HA varies with its molecular weight. For example, inhaling high molecular weight (HMW)-HA has been applied to treat inflammation clinically while low molecular weight (LMW)-HA exhibit pro-inflammatory characteristics [23]. Additionally, absorption characteristics of intestinal epithelial cells are different for HA having different molecular weights [15,24]. Certain diseases require optimal absorption oral HA, thus, the preparation of low polydispersity or monodisperse HA of appropriate molecular weight is highly desirable.

3. How to prepare monodisperse HA

3.1. Degradation of HA polymer

3.1.1. Chemical methods

Degradation of the HA polysaccharide is a step-wise process that can occur using chemical or enzymatic reactions. Acidic/alkaline hydrolysis is a common chemical approach for HA degradation, as acidic condition cleaves the glucuronic acid moiety while alkaline hydrolysis occurs on *N*-acetylglucosamine units [25]. This type of degradation occurs in a random fashion, is difficult to control, and often results in disaccharide fragment production [26]. Traditional hydrolysis is usually conducted in a homogeneous solution, however, heterogeneous hydrolysis in ethanolic hydrochloric acid was recently developed to hydrolyze HA into HA oligosaccharides of low molecular weight material [27], and this approach provided several advantages compared to homogenous hydrolysis such as lower ethanol and water consumption and the possibility to hydrolyze a large amount of HA in a small solvent volume. HA can also be depolymerized using reactive oxygen species (ROS) [28], generated by chemical reagents or radiation. Combined physical and chemical methods are becoming more popular approaches for preparing HA of reduced molecular weight and reduced polydispersity. For example, a combination of ultrasound, hydrogen peroxide, and copper ion has been used for the controlled degradation of HA resulting in a low molecular weight (LMW)-HA of a pristine primary structure not affected by this degradation method [29]. Nevertheless, physical and chemical degradation, while reducing HA molecular weight, still afford polydisperse mixtures. These mixtures must then be extensively fractionated to obtain homogenous HA ligosaccharides.

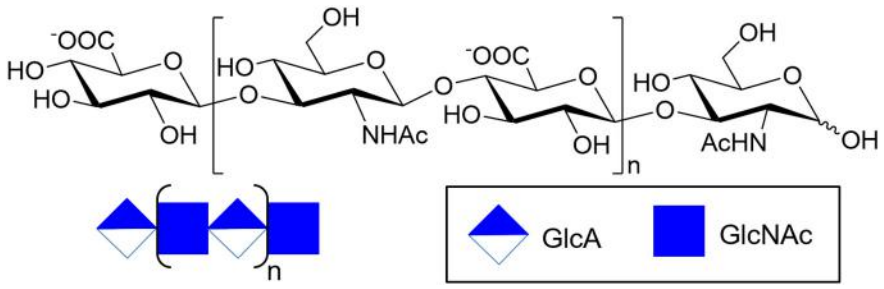

Fig. 1. Structure of hyaluronic acid. The chemical structures of the saccharide residues as well as their symbolic representation are shown. The number of disaccharide repeating units (n) controls the biological activity of HA. When n is a single value HA is considered monodisperse (Mw/Mn = 1). When n covers a range of values, then HA is polydisperse with a polydispersity index (Mw/Mn) > 1.

3.1.2. Enzymatic methods

HA can be enzymatically degraded by cleaving it with hyaluronidase (HAase) [30]. HAase, an endoglycosidase, is a hydrolase obtained from animal tissues, such as mammalian testes, or prepared as a recombinant enzyme in a microbial host, catalyzes the hydrolysis of HA into HA oligosaccharides [31,32]. Treatment of HAse can be followed by anion exchange chromatography to prepare a more uniform HA oligosaccharides. The length of HA oligosaccharides obtained can be controlled by adjusting the enzymatic hydrolysis time as well as the concentration of the enzyme [33]. Immobilized enzyme technology can also be applied for HA depolymerization. Microbial enzymes that cleave HA are HA lyases (or chondroitin lyases) [34]. These enzymes act either as endo (or exo) eliminases breaking the linkage between GlcNAc and GlcA to form an unsaturated uronic acid residue at the non-reducing end of the HA oligosaccharide product [34]. Lee et al. used the recombinant HA lyase, extracted and immobilized by submicron-sized immobilized metal affinity magnetite (IMAM), to cleave HMW-HA into HA oligosaccharides with a molecular weight of 1400 to 1660 Da [35]. This approach broadened the pH range of the reaction and increased the recyclability of the enzyme. While enzymatic degradation provides an HA oligosaccharide mixture with a relatively narrow molecular weight range, the chain size of the resulting product cannot be precisely controlled. Thus, this method results in a relatively low yield and requires chromatographic separation.

In summary, though HA oligomers could be prepared by depolymerization of HA polymers using either enzymatic or chemical approaches, this type of process cannot be controlled to provide pure HA oligosaccharides of distinct molecular weights. Thus, the alternative step-wise synthesis of pure HA oligomers from simple building blocks is often required to access structurally defined oligosaccharides.

3.2. HA assembly strategy

3.2.1. Chemical synthesis

Chemical approaches of HA oligosaccharide synthesis are generally classified into two types, pre-glycosylation oxidation strategy and post-glycosylation oxidation approach, and differ based on the way that glucuronic acid (GlcA) is installed [36]. In the pre-glycosylation oxidation strategy, the GlcA residues are introduced prior to glycosylation, affording low to moderate yields in glycosylation due to the low reactivity of GlcA. For this reason, the post-glycosylation oxidation approach, by which glucose units are oxidized to GlcA after glycosylation have been developed. However, the oxidation of these intermediates is a complex and challenging synthetic step. A typical example for the chemical synthesis of an HA oligosaccharide, based on repetitive steps of protection, activation, coupling and de-protection, is presented in Fig. 2A.

Although several studies on the synthesis of HA oligosaccharides have been undertaken, there are still several challenges remaining in this field. Due to the stereo-selectivity and regio-selectivity of sugars, the chemical synthesis of HA remains complicated and costly [37]. Achieving fast access to inexpensive monosaccharide building blocks or the disaccharidic repeating units and the installation of the sensitive GlcA residue remains challenging. Liberation of the target compounds and the purification of the polyanionic target molecules is another roadblock to the large scale chemical synthesis of HA oligosaccharides [36].

3.2.2. Chemoenzymatic synthesis

Over the past decade, a chemoenzymatic synthesis approach, integrating the flexibility of chemical derivatization with the specificity of enzyme-catalyzed reactions, mimicking the biosynthetic pathway of HA, has been developed [7]. This represents a promising strategy to solve these synthetic challenges mentioned above, and offering a potential alternative for the targeted preparation of homogeneous HA oligosaccharides [38–40].

The glycosyltransferase used for chemoenzymatic synthesis comes from a variety of different sources and allows synthetic chemists to mimick the HA biosynthesis *in vitro* [41–43]. The enzyme PmHAS are prepared from the pathogenic bacteria *P. multocida* that biosynthesizes a capsular polysaccharide, hyaluronan [44]. PmHAS catalyzes the formation of HA chains by adding GlcNAc and GlcA monosaccharides from the corresponding sugar nucleotide donors. Moreover, PmHAS can also elongate HA chains using HA oligosaccharides as acceptors in a synchronized non-processive manner [42,45,46].

3.2.2.1. Semi-synthesis. The semi-synthesis of HA oligosaccharides employs readily available HA oligosaccharide acceptor, obtained from depolymerization of HA polysaccharide, followed by backbone modification through enzymatic or chemical reactions. DeAngelis et al. used a mutated version of PmHAS to synthesize HA 20-mer using an HA tetrasaccharide acceptor and UDP-sugar substrates [47]. Using QuikChange system, PmHAS was mutated to two single-action enzymes: $pmHAS^{1-703}$ (D527N, D529N and $pmHAS^{1-703}$(D247N,D249N). The former can transfer GlcNAc from UDP-GlcNAc and the latter has the ability to transfer GlcA from UDP-GlcA. After purifying and immobilizing the two enzymes above, HA tetrasaccharide in the reaction buffer can be extended to a target length of HA by cyclic reaction using UDP-GlcNAc and UDP- GlcA as raw materials, respectively (Fig. 2B).

3.2.2.2. Total-synthesis. Total synthesis refers to the synthesis of monosaccharide into HA polysaccharide targets using enzymes. Imitating the pathway of intracellular PmHAS catalyzed HA synthesis, DeAngelis et al. synthesized HA *in vitro* using recombinant PmHAS, UDP-GlcNAc and UDP-GlcA [48]. At the same time, they found the synthesis rate of HA polymers were controlled by acceptor/UDP-sugar ratio and the selected acceptors (Fig. 2C) [49].

4. Novel synthetic approaches

4.1. Bond-selective degradation of HA polymers

HA oligosaccharides can have complete different biological activities than low molecular weight HA [50,51]. Zhao et al. successfully prepared oligosaccharides having an odd and even number of saccharide units by using 0.1 mol/L and 1 mol/L hydrochloric acid to cleave HA, followed by gel permeation chromatography (GPC) fractionation (Fig. 3A) [50]. The results indicate that the HA oligosaccharides with an odd number saccharide units had a GlcA reducing end, while those with an even number of saccharide units had a GlcNAc at the reducing end. The authors speculated that low concentration hydrochloric acid could cleave $\beta(1 \rightarrow 3)$ glycosidic bonds, but at higher concentrations of hydrochloric acid, $\beta(1 \rightarrow 4)$ glycosidic bonds were more likely to break. The homogeneous HA oligosaccharides obtained by this approach could be used for the exploration of HA oligosaccharides in the physiological functioning of certain cancer cells and for the preparation of anticancer drugs.

4.2. Branched HA as potential anticancer reagents

HA polysaccharides have advantages in cell recognition, while HA oligosaccharides have higher biological activity. Buffa et al. designed a copolymer HA-spacer-oliHA that connecting HA polymer and HA oligomer (oliHA) to incorporate both advantages (Fig. 3B) [52]. A stable HA aldehyde derivative was first prepared by simply oxidizing the 6-hydroxyl group of GlcNAc in HA. The resulting product was further conjugated with oliHA-spacer, which was obtained by the conjugation between oliHA and hydrazides, hydrazines or oxyamines, followed by reduction with $NaBH_3CN$ to get the stable conjugate—HA-spacer-oliHA. Subsequent biological evaluation showed that HA-spacer-oliHA had selective cytotoxicity against some cancer cells. A variety of other

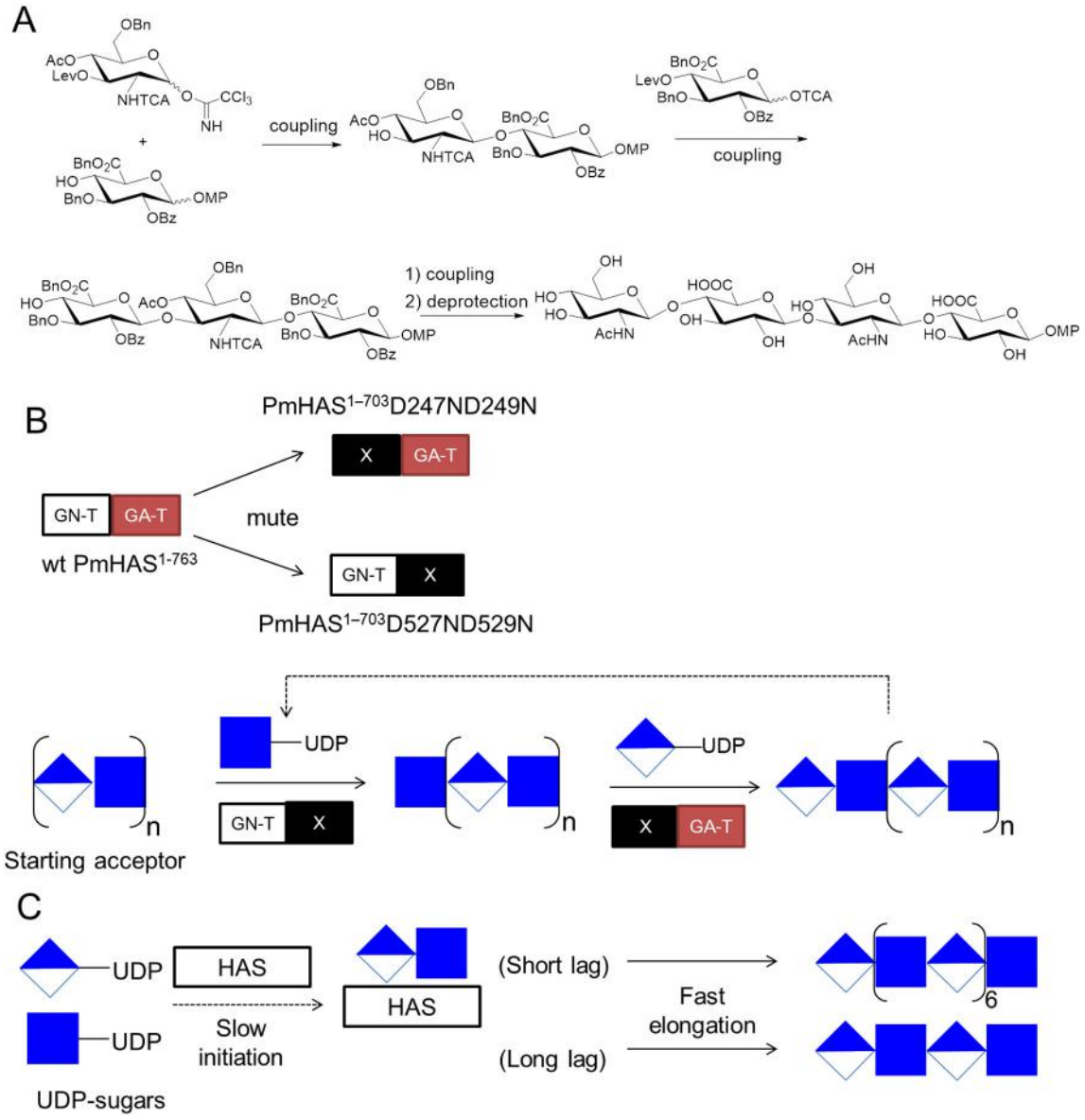

Fig. 2. Synthesis of HA oligosaccharides. (A) Chemical synthesis of pure HA tetrasaccharide. (B) The semi-synthesis starting from hyaluronic oligosaccharides using a chemoenzymatic approach using UDP-activated donors. (C) The total synthesis of hyaluronic acid through a chemoenzymatic approach.

similar polysaccharides having branched chemical structures might also be prepared using this method.

4.3. Fluorous-tagging techniques

Fluorous separation techniques have found increasing synthetic uses in facilitating the isolation and purification of intermediates and products, based on the high affinity of perfluoroalkyl chains toward fluorous surfaces and solvent. A perfluorooctyl (C_8F_{17}) or a perfluorohexyl (C_6F_{13}) group is the most popular fluorous group for tagging on a target compound and this is followed by product purification using fluorous solid-phase extraction (FSPE). Nieto et al. reported a HA trisaccharide and tetrasaccharide synthesis using a pre-glycosylation oxidation strategy, where GlcA units were directly coupled with suitably protected GlcNAc derivatives (Fig. 3C) [53]. A fluorous tag C_8F_{17} was attached to the carboxylic acid of the reducing-end GlcA moiety by an esterification reaction, simplifying the purification of synthetic intermediates. However, the overall yield of the fluorous-assisted synthesis of the hyaluronic acid trisaccharide was quite low.

4.4. Chemical synthesis of HA disaccharides functionalized at C-6 of the glucuronic acid moiety

Bräse et al. reported the synthesis of a library of protected HA disaccharides bearing new functional groups at C-6 of the GlcA moiety by applying their previous established orthogonal protecting-group patterns (Fig. 4A) [54]. The resulting disaccharides could be under further oligomerized to access chemically modified higher hyaluronic acid oligomers by using only commercially available D-glucose and D-glucosamine hydrochloride.

4.5. Ph_2SO/Tf_2O-mediated glycosylations

Since the oxidation of glucuronic acid residues is a challenging synthetic step in a post-glycosylation strategy, several efforts for optimizing

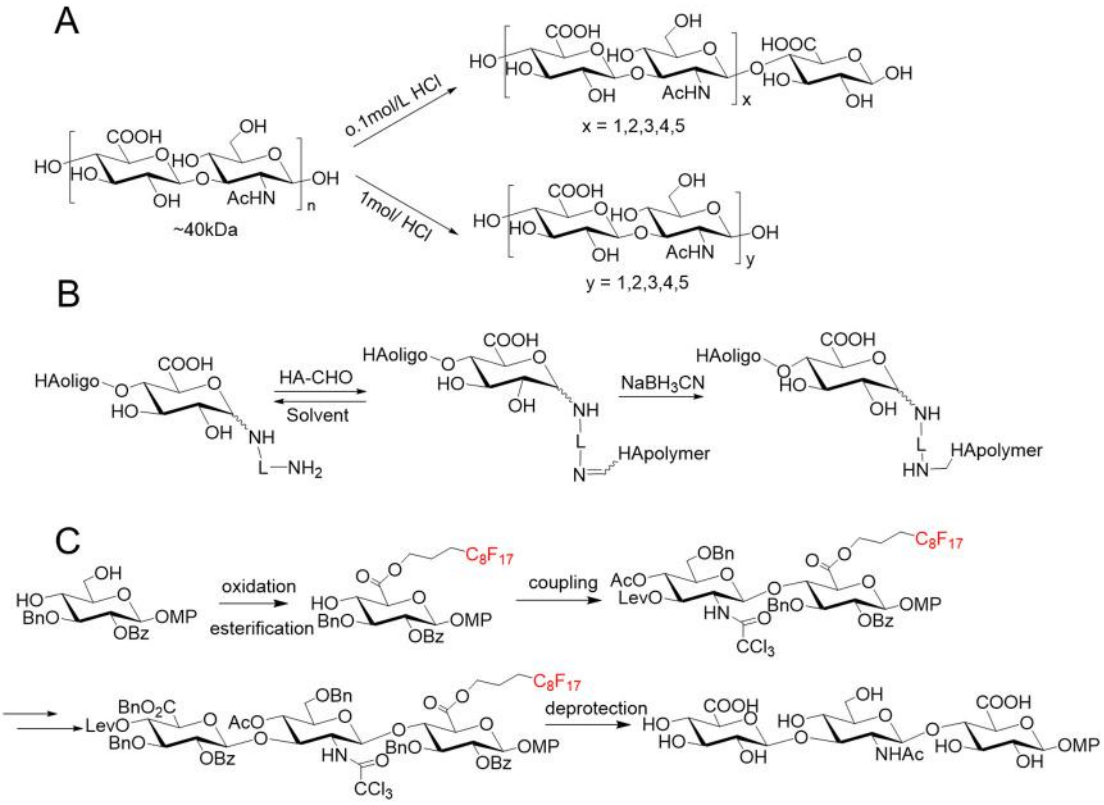

Fig. 3. (A) Preparation of HA oligosaccharides from HA polymer (~40 kDa) through hydrochloric acid cleavage. (B) The reaction of conjugates oliHA-spacer with polymeric HA, where L represents bis-hydrazido or bis-oxyamino spacers. (C) Synthesis of hyaluronic acid oligosaccharides involved with a fluorous-assisted approach.

the direct coupling for GlcA donor were developed. van der Marel et al. reported a method to couple either protected 1-hydroxyuronic acid or 1-phenylthio glucosamine donors, by making use of thioglycosides and 1-hydroxyglycosides, in combination with the Ph_2SO/Tf_2O/TTBP activating system, to form an azido modified HA oligosaccharide (Fig. 4B) [55]. The yields in the glycosidic bond formations are moderate and the azido functionalized compounds were suitable for future biological studies.

4.6. Automatic solid phase synthesis

Carbohydrate preparation would greatly benefit from developments of solid-phase synthesis approaches, by which the coupling reactions could be driven to completion by the use of excess reagents and repetitive coupling cycles [56,57]. However, solid-phase synthesis of carbohydrates has not yet become a routine operation due to

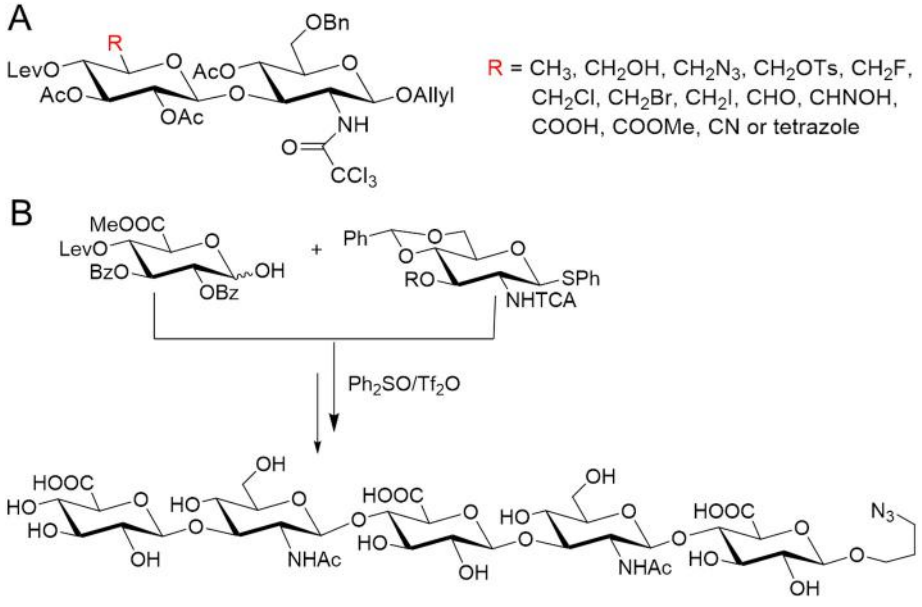

Fig. 4. (A) Structure of HA disaccharides functionalized at C-6 of the glucuronic acid moiety. (B) Ph_2SO/Tf_2O-mediated glycosylations.

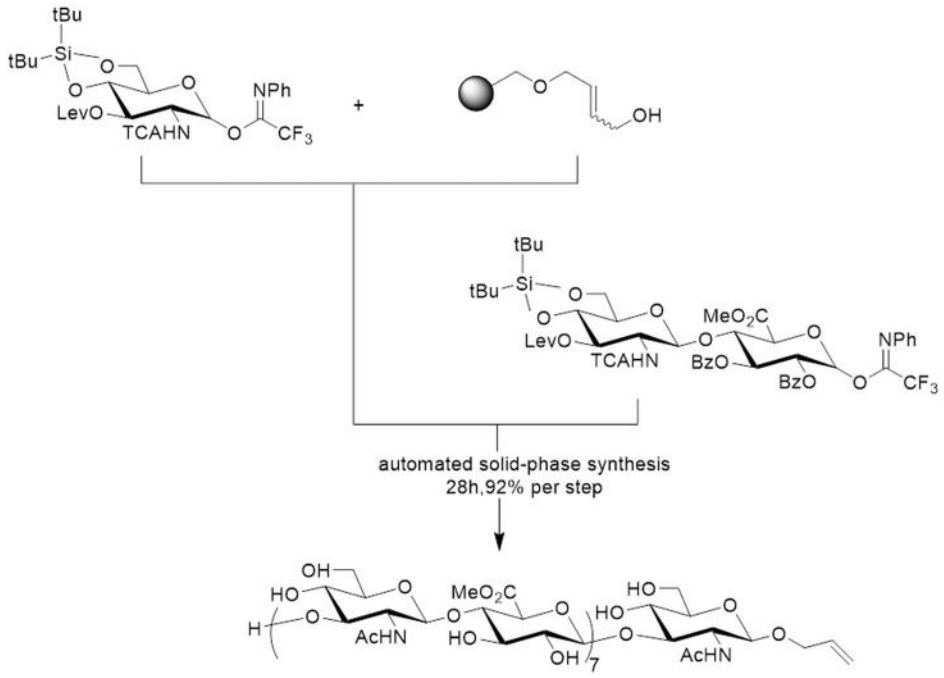

Fig. 5. Automated solid-phase HA-assembly.

the lack of a standard set of carbohydrate building blocks and coupling chemistry. The repetitive nature of the HA polysaccharide invites the assembly of well-defined oligomers by means of an automated solid-phase approach. Codée et al. reported a well-defined fragments of HA through a fully automated solid-phase oligosaccharide synthesis (Fig. 5) [58]. Disaccharide building blocks, featuring a disarmed GlcA donor moiety and a di-tert-butylsilylidene-protected glucosamine, were used in the rapid and efficient assembly of HA fragments up to the pentadecamer level, equipped with a conjugation-ready anomeric allyl function. This is the first automated solid-phase synthesis of a set of HA oligosaccharides, and also indicates that the automated solid-phase synthesis of other members of the glycosaminoglycan family is within reach.

4.7. One-pot reactions

In some instances, it might be more efficient to conduct multiple reaction sequences into one-pot reactions, where oligosaccharides or polysaccharides are prepared without the required isolation or purification of intermediates [59]. Fu et al. have described a one-pot synchronzied strategy to efficiently access homogeneous HA from GlcA-*p*NP acceptor catalyzed by PmHAS by controlling acceptor/donor molar ratio involved in the reaction system (Fig. 6A) [37] which was the key factor influencing final HA chain length. A series of HA-biotin and HA-drug conjugates were prepared using this general synthetic strategy, not only demonstrating the flexible substrate specificity of PmHAS, but also showing that this strategy could be applied to the

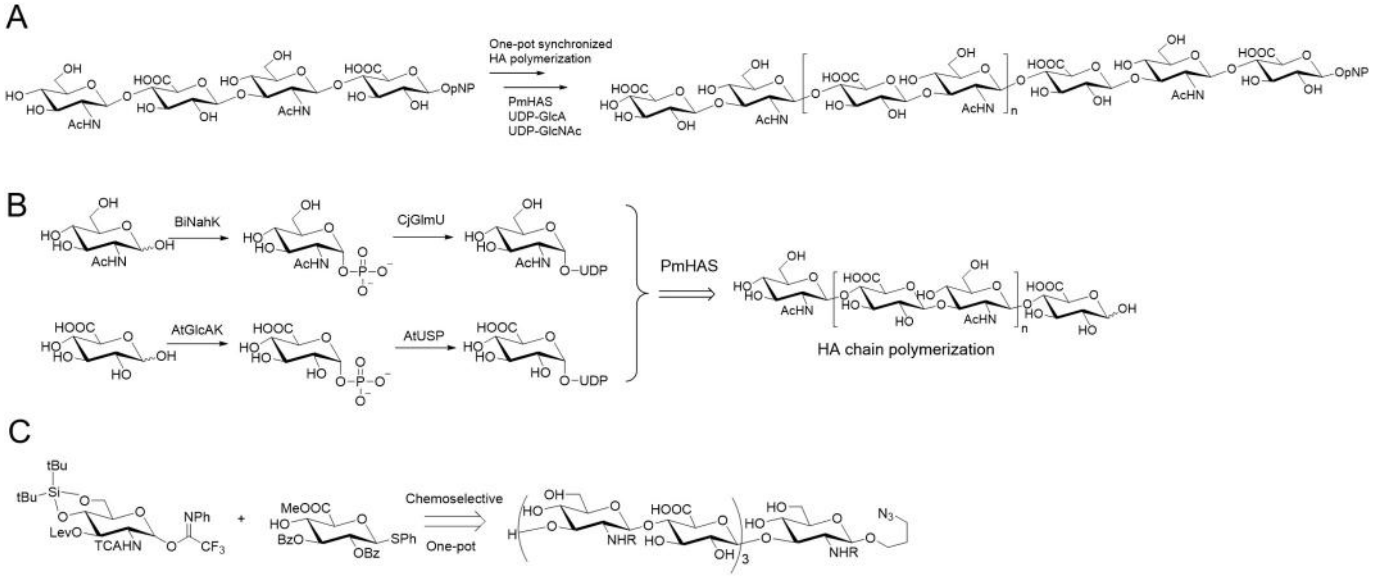

Fig. 6. One-pot approach to synthesize HA. (A) The general strategy used to chemoenzymatically synthesize homogeneous HA conjugates. (B) One-pot multienzyme reaction for the synthesis of HA polymers. (C) Pure chemical approaches.

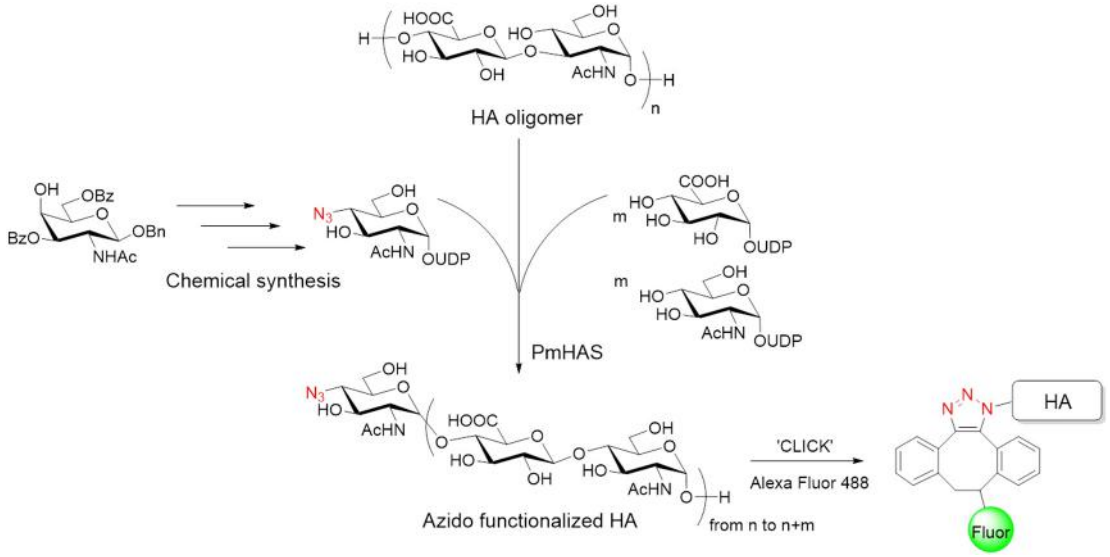

Fig. 7. UDP-4-N_3-GlcNAc preparation and incorporation into HA synthesis.

synthesis of any homogeneous GAG conjugates for potential biological applications and therapeutics.

However, the high cost of preparing UDP-GlcNAc and UDP-GlcA limits the large-scale application of this method [60,61]. Therefore, Fang et al. constructed a one-pot multi-enzyme (OPME) system which just needs cheaper monosaccharides (Fig. 6B) [62]. In the presence of ATP and UTP, GlcNAc can be converted to UDP-GlcNAc under the catalysis of both *N*-acetylhexosamine 1-kinase from *Bifidobacterium longum* (BlNahK) and GlcNAc-1-P uridyltransferase from *Campylobacter jejuni* (CjGlmU), and GlcA converted into UDP-GlcA under the action of GlcA 1-kinase from *Arabidopsis thaliana* (AtGlcAK) and UDP-sugar pyrophosphorylase from *A. thaliana* (AtUSP). At the same time, the generated UDP-GlcNAc and UDP-GlcA are directly polymerized into HA polymers under the function of PmHAS, which significantly improves the product yield.

One-pot reaction strategy was also employed in the chemical synthesis of HA. van der Marel et al. described an efficient chemical strategy toward a set of HA oligosaccharides ranging in size from three to seven monomers having a glucosamine-reducing end by the combination of chemoselective and one-pot glycosylation strategies [63]. This synthesis is based on a glucuronate ester thioglycoside and a trifluoro-*N*-phenylimidate glucosamine building block (Fig. 6C).

4.8. Functionalized HA preparation from unnatural UDP-sugar donor

Unnatural UDP-sugar donors have great potential as enzymatic substrates in carbohydrate synthesis, as enzyme inhibitors in biochemical studies, as tools for assay development, and as reagents for the study of glycoconjugate biosynthesis. Linhardt et al. reported the synthesis of azido modified UDP-4-N_3-GlcNAc and its successful incorporation into hyaluronan using polysaccharide synthases (Fig. 7) [64]. UDP-4-N_3-GlcNAc is not only served as a chain termination substrate for hyaluronan synthesis to control the size of HA polysaccharide, but also the resulting azido functionalized HA can be readily covalently tagged with imaging probes or epitope using an azide-specific reaction. Compared to the chemical method to introduce multiple azido groups into polysaccharides [65], this enzymatic technique provided an alternative approach to access regioselectively azide-functionalized HA at the non-reducing end of the chain, which would bring better control of the final structure of HA polysaccharide or oligosaccharide.

5. Conclusion and perspective

HA is currently widely used in therapeutic and cosmetic area due to its valuable physicochemical properties. The design and synthesis of homogeneous or structurally defined HA is of major interest for a safer and reliable drug development as well as a better understanding of its structure-activity relationships. Although several research groups have made effort for the synthesis of pure structurally defined HA oligomers since the pioneering work of Jeanloz in 1964, HA has received only a little attention from the synthetic carbohydrate community compared to other glycosaminoglycan family members [7,36]. In this review, we examined the remarkable progress in the chemical or chemoenzymatic synthesis and application thereof, providing a broad spectrum of options to access homogeneous HA. The traditional chemical approaches are still the major ways to prepare homogeneous HA, and there has been steady improvement in this area, including higher yields, easier purification and larger scale. However, it is clear that in particular modern glycochemistry and innovative techniques like automated synthesis or chemoenzymatic approach will play a pivotal role in future applications.

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