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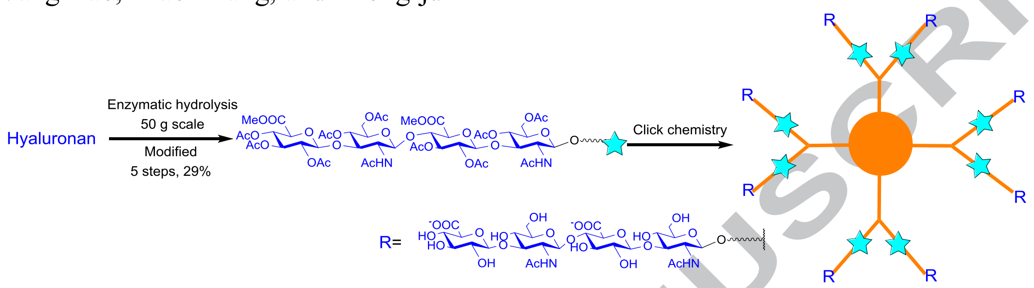

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Synthesis of structure-defined branched hyaluronan tetrasaccharide glycoclusters

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ABSTRACT

Hyaluronan is a glycosaminoglycan with a large number of biological activity. Hyaluronan of different molecular weight often shows different biological activity, sometimes even completely opposite, but the mechanism is not clear. Herein, the hyaluronan tetrasaccharide glycoclusters using hyaluronan tetrasaccharide obtained by enzymolysis of natural hyaluronan were firstly synthesized in high yield. The structurally determined and diverse glycoclusters were of wide molecular weight range and might be used for mimicking the biological activity of natural hyaluronan and facilitating the mechanism study.

The hyaluronan (HA) is a linear and unbranched polymer constituted by disaccharide units of D-glucuronic acid (GlcA) linked to *N*-acetyl-D-glucosamine (GlcNAc) with a β (1→3) linkage between GlcA and GlcNAc and a bond β (1→4) between GlcNAc and GlcA, as showed in Fig 1. Hyaluronan is synthesized by hyaluronan synthases *in v ivo* and could be degraded by several kinds of hyaluronidases (Hyals)[1]. As a result, the natural hyaluronan has a wide range of molecular weight distribution. The hyaluronan with the molecular weight more than 100 kDa is called high molecular weight hyaluronan (HMW-HA), about tens kDa is called low molecular weight hyaluronan (LMW-HA), and a few kDa is called hyaluronan oligosaccharides (o-HA) [2].

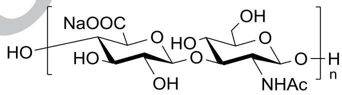

Fig 1 The structure of hyaluronan.

Hyaluronan could interacts with many proteins, which are termed hyaladherins (often called HA binding proteins, HABPs) and most of them are cell-surface receptors[3].The hyaladherins contain lots of proteins, mainly are CD44, Receptor for Hyaluronan Mediated Motility (RHAMM, also known as CD168), Hyaluronan Receptor for Endocytosis (HARE), Lymphatic Vessel Endothelial hyaluronan receptor 1 (LYVE 1, also known as Cell Surface Retention Sequence Binding Protein-1, CRSBP1) and Toll Like Receptors (TLRs)[4]. Hyaluronan regulates an array of biological and pathological processes. Also, hyaluronan have extraordinarily wide-ranging and sometimes opposing biological functions related with molecular weight[5, 6]. The possible explanation for those is that different molecular weight hyaluronan can bind to different numbers of receptors, which is called the multivalent interactions[7], and can yield different processes and biological responses[8, 9,10]. However, there is no clear and precise relationship between the bioactivity of hyaluronan and its molecular weight, and the reason was reviewed by Robert Stern[5]. Especially for hyaluronan with moderate molecular weight (several tens to hundreds of disaccharides units), the literature often reports completely opposite results[5]. A major underlying reason is the difficulty of gaining the HA materials that are homogenous in molecular weight, which is limited by the extraction and purification techniques. Recently, glycoclusters have been artificially synthesized to mimic the multivalent interactions of natural carbohydrates and proteins[11]. Thus, we envisioned that o-HA based glycoclusters was quite a suitable molecules to solve the challenges above. Though synthetic glycoconjugates generally would not reproduce exactly the valency and topology of the natural ligands, the binding properties obtained in most cases are in line with possible applications *in vivo* and as therapeutics[7].

Herein, we present the synthesis of branched hyaluronan tetrasaccharide (HA4) glycoclusters based on copper-catalyzed Huisgen 1, 3 cycloaddition (CuAAC) reaction. The products obtained have a wide spectrum of molecular weight ranging from 3 kDa to 30 kDa.

In order to obtain the structure of the determination and diversity of branched o-HA clusters, three aspects need to be considered. Firstly was the technic to afford a large amount of o-HA to assemble the glycoclusters[12]. Secondly, branched molecules with multiple identical modifiable sites should be chosen as the "scaffold" of the glycoclusters. Also chemical

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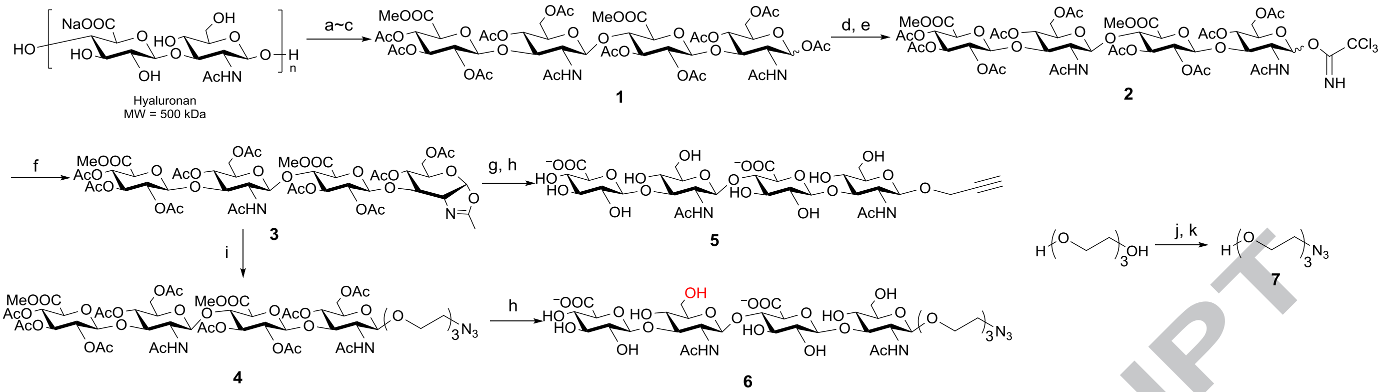


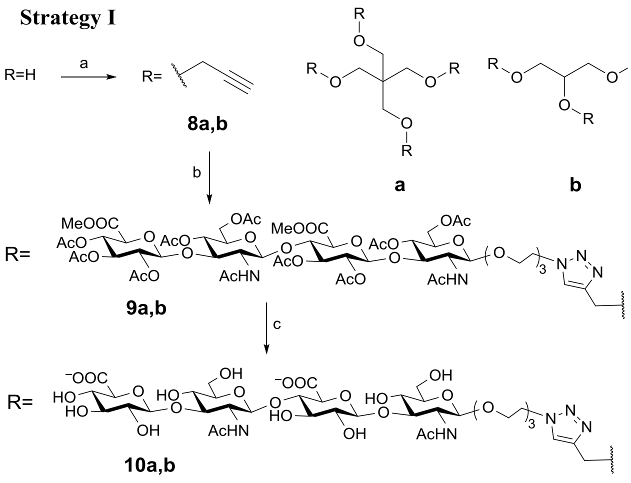
Scheme 1. Synthesis of HA4 building blocks **4-6**. (a) BTH, 37 °C, pH=5.0; (b) AcCl, MeOH, 4 °C; (c) Ac_2O, Pyridine, rt, 42% for a-c. (d) DMAPA, dry THF, 0 °C →rt, ; (e) $CNCCl_3$, DBU, CH_2Cl_2, 0 °C →rt, 84.9% for d-e; (f) TMSOTf, CH_2Cl_2, 0 °C →rt, 81.5%. (g) propynol, $CuCl_2$, $CHCl_3$, reflux, 88.1% . (h) LiOH(aq.) / H_2O_2, THF, 0 °C→rt, then 4M NaOH, MeOH 81.4% for **5** and 92.1 % for **6**; (i) **7**, $CuCl_2$, $CHCl_3$, reflux, 88.1%; (j)TsCl, Et_3N, CH_2Cl_2, 0 °C →rt; (k) NaN_3, DMF, 65 °C, 64.1% for two steps.

reactions with high efficiency was needed to connect o-HA to the scaffolds.

Several approaches towards o-HA such as chemical synthesis strategy[13-16] and enzymatic synthesis strategy[17] have been reported. Also, the direct digestion of HMW-HA by the hyaluronidases was widely used[18, 19]. In this paper, bovine testicular hyaluronidases (BTH) was used to gain o-HA[19]. As HA disaccharide and its derivatives exhibit little biological activities[20], we focused on HA4, which was the smallest unit with biological activity[5]. It was also the major degradation products of BTH. The degradation process was carried out in a NaOAc/NaCl buffer (pH=5.0). The degree of polymerization was quickly decreased after the enzyme was added and o-HA was appeared in the system. When the amount of HA4 was gradually increased, the degradation rate decreased[21]. The enzymatic hydrolysis process was continued for 2 weeks to afford the completed degradation products when the HMW-HA starting material was 50 g. After the hydrolysis process, hyaluronidase was successfully removed from the reaction mixture by heating and filtering procedure (see SI). To avoid the time-consuming anion exchange or size exclusion chromatography that was usually utilized for the o-HA purification[18], the mixture of degradation products were directly protected[20] and the fully-protected HA4 **1** was obtained in 42% for 3 steps from the HA polysaccharide in 23 grams scales.

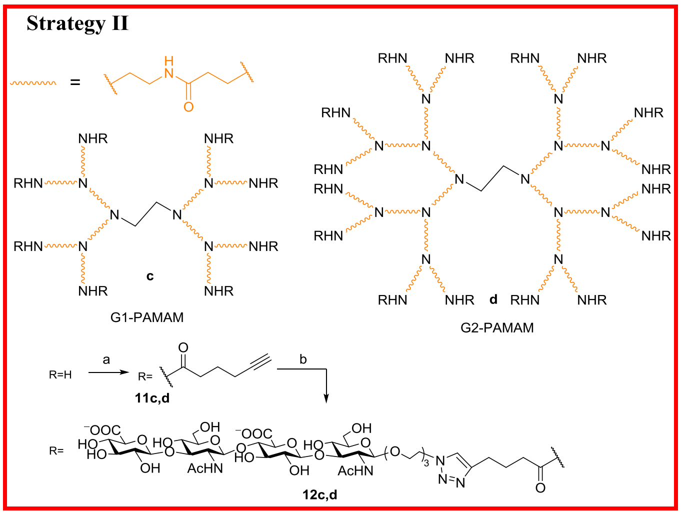
As the hyaluronan containing carboxyl group which is its active group, amide condensation reaction should be avoided in the next linkages. In this work, the linker was designed to connect onto the reducing end of HA4 via *O*-glycosidic bond and connected to the scaffold by copper-catalyzed azide-alkyne Huisgen 1, 3-cycloadditions (CuAAC) [22]. Therefore, the linker molecules should contain both a hydroxyl group to connect with o-HA and an azido or alkynyl group that could be coupled to core scaffolds. To achieve these goals, azido-alcohol **7** was ~~firstly~~ synthesized from triethylene glycol in two steps. Challenges came from the *O*-glycosylation at the reducing end of the tetrasaccharide **1**. Since the low-reactivity of an acetyl protected N-acetyl saccharide donor, the direct glycosylation at C-1 has proven to be a great difficulty. The exchange of the NHAc group to NHPhth, NHTca, NHTroc $N(Ac)_2$ or NHTFA was always utilized to eliminate the formation of the oxazoline, which was sluggish to transform into the desired glycosylated product under the classical condition. However, in our synthetic approach, the transformation of already existed NHAc groups required multiple steps, thereby, a novel approach was developed. Firstly, the acetyl protect group at the reducing end was removed by 3-dimethyl aminopropyl amine (DMAPA) in THF. The exposed hydroxyl was then converted to trichloroacetimidate **2**. After treating with catalytic amount of TMSOTf in cold CH_2Cl_2, oxazoline **3** was obtained in 85% from **1**. As shown in **Scheme 1**, the successfully glycosylation occurred when oxazoline **3** was refluxed with linker **7** in chloroform under the catalytic of copper chloride that yielded the desired building blocks **4** in 88%. In order to obtain a alkynyl linked tetrasaccharide, **3** was treated with propargyl alcohol under the same $CuCl_2$ catalyzed glycosylation reaction, and then saponification, which yield compound 5 in 81% yield.

To examine whether the building blocks **4, 5** and **6** was suiltable for the glycoclusters assembling, a variety of scaffolds was designed, which containing propargylated polyols **8a** and **8b**, propargylated poly (amidoamine) (PAMAM) [23] dendrimers **11c** and **11d**, and GATG (gallic acid-triethylene glycol) dendrimers **13e, 13f** and **13g**.



Scheme 2. Synthesis of polyol derivatives HA glycoclusters **10a-b**. (a) propargyl bromide, KOH, DMF, 0 →50 °C, 80.2 % (**8a**), 89.0 % (**8b**). (b) **4**, $CuSO_4$, Na ascorbate, $MeOH/H_2O$, rt, 97.9% (**9a**), 91.0% (**9b**); (c) 1M LiOH(aq.)/H_2O_2, THF, 0 °C→rt, then 4 M NaOH, MeOH, 99% (**10a**), 99% (**10b**).

A dendrimer is a polymeric molecule composed of multiple perfectly branched monomers that emanate radially from a central core, reminiscent of a tree, whence dendrimers derive their name (Greek, dendra). The dendrimers are defined by generation (G1, G2, G3⋯) and dendrimers of higher generations are larger, more branched and have more end groups at their periphery than dendrimers of lower generations[24]. The first series of dendrimers we chose was poly (amidoamine) (PAMAM) which was used frequently to assemble glycoclusters[25, 26] and offered the significant advantage of ease of preparation. Also, the PAMAM dendrimers of varying size are commercially available. In this research, G1-PAMAM (8 amino groups) and G2-PAMAM (16 amino groups) were used as scaffold. Since the end of PAMAM is an amino group, modification is required to perform click chemistry. Thus 1- hexynoic acid was connected to PAMAM with classic amide condensation.

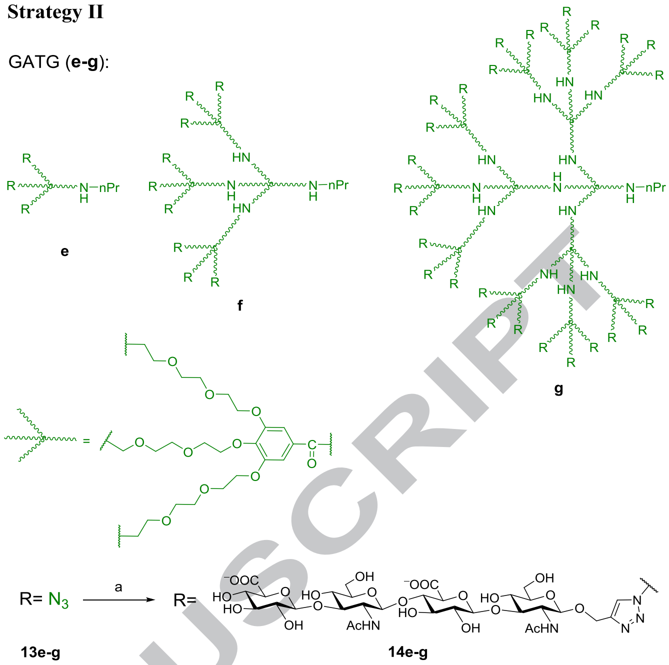


Scheme 3. Synthesis of (G1-G2)-PAMAM-HA4 dendrimers **12c-d**. (a) 5-Hexynoicacid, EDC, HOBt, DMF, 0 °C→rt, 88.4% (**11c**), 91.1% (**11d**); (b) **6**, CuI, MeOH/H_2O, rt, 91.0% (**12c**), 87.0% (**12d**).

We also chose GATG dendrimers as scaffold because presence of terminal azides in them could directly perform click chemistry. GATG dendrimers which are composed of a repeating unit carrying a gallic acid core and hydrophilic triethylene glycol arms with terminal azide groups, was firstly synthesized by Roy and his group[27]. The (G1-G3) GATG (**13e-g**) was obtained by constant catalytic hydrogenation and amide condensation as described in ref.[28].

Table 1. The synthesized hyaluronan tetrasaccharide glycoclusters.

Compound	Name	M.W. (Calc.)	MALDI-TOF	SEC-MALS Mw (g/mol)
9a	Pent-HA4-4	4023.6	4042.8 $[M+Na]^+$	N/A
9b	Gly-HA4-3	3007.7	3028.7 $[M+Na]^+$	N/A
12c	G1-PAMAM-HA4-8	9653.3	9656.3 $[M+H]^+$	N/A
12d	G2-PAMAM-HA4-16	22403.0	4448.12 $[M+5Na]^{5+}$	2.168×10^4 (±4.135%)
14e	G1-GATG-HA4-3	3120.8	3144.4 $[M+Na]^+$	N/A
14f	G2-GATG-HA4-9	9807.8	-	1.933×10^4 (±2.956%)
14g	G3-GATG-HA4-27	29967.3	-	6.018×10^5 (±2.160%)



Scheme 4. Synthesis of G1-G3-GATG-HA4 dendrimers (**14e-g**). (a) **5**, $CuSO_4$, Na ascorbate, MeOH / H_2O, rt, 86% (**14e**), 91% (**14f**), 90% (**14g**).

The last step was connect the HA4 with the linker to the scaffold. CuAAC was the perfect reaction due to its high efficiency, no side reactions and mild conditions[22, 29-31]. There were two strategies for assembling glycoclusters, strategy I was using the fully-protected HA4 to synthesis the clusters and then the protective groups were removed while strategy II was removing the protective groups firstly and then assemble glycoclusters. Procedure for global deprotection was followed the method provided by J-C Jacquinet to eliminate the formation of the Δ4,5 GlcA[32]. Strategy I was used for the synthesis of compound **9a,b** and the results were satisfactory (**Scheme 2**), while compound **6** did not react with **9a** and **9b** at the same condition (data was not shown). But the strategy I was not suitable to all situations because the PAMAM series scaffolds were not tolerated under H_2O_2 condition. Thus, an alternative strategy (II) was utilized that yielded **12c** and **12d** in 91% and 87%, respectively (**Scheme 3**). The strategy II could be also applied to the GATG series of scaffolds as reported[28] and **14e-g** was synthesized with high yield (**Scheme 4**). The glycoclusters were characterized by Matrix-Assisted Laser Desorption / Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS), however, as molecular weight increases, glycoclusters fly with more difficulty in MALDI-TOF MS and fragmentation appears[33]. As a supplement, size exclusion chromatography coupled to multiangle light scattering (SEC-MALS) method was used to confirm the molecular weights of **12d**, **14f** and **14g**. For **14f** the Mw tested by SEC-MALS was twice of the calculated value and for **14g** was 20 times, maybe polymerization occurs due to the GATG scaffolds. The results were shown in **Table 1**.

In summary, hyaluronan tetrasaccharide was obtained by enzymatic hydrolysis of high molecular weight hyaluronan with large scale and high yield, and a new method of direct reaction from the degradation products to the building blocks and then separation and purification is the first report. The HA4 building blocks was used for assembling glycoclusters in this research and could be used for some other applications. The structurally determined and diverse HA4 glycoclusters with wide molecular weight range (3 kDa to 30 kDa) were synthesized, which were the first reported structurally determined HA4 glycoclusters and also large molecular weight rare tetrasaccharide glycoclusters. These compounds can be used to study the mechanism of hyaluronan biological properties and could be applied in the field of biomedicine. Also this research could provide methods for the synthesis of hyaluronan and other glycosaminoglycan glycoclusters.

Acknowledgments

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Highlights

1. An efficient method of obtaining HA4 building block has been developed.
2. HA4 glycoclusters with wide molecular weight range (3k to 30 k) were synthesized.
3. Strategy for synthesis of glycosaminoglycan glycoclusters has been established.