ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2014, **50**, 7004

Received 28th January 2014,
Accepted 2nd April 2014

DOI: 10.1039/c4cc00708e

www.rsc.org/chemcomm

Efficient access to the non-reducing end of low molecular weight heparin for fluorescent labeling†

Zongqiang Wang, Chen Shi, Xuri Wu and Yijun Chen*

A novel thiol fluorophore was synthesized to be selectively attached to the non-reducing end of low molecular weight heparin (LMWH) *via* a Michael addition. Double labeling of LMWH was demonstrated to be a feasible approach for the determination of heparinase II activity by FRET.

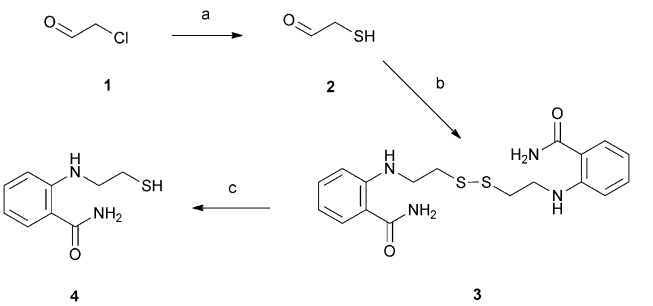
The glycosaminoglycans (GAGs) are a family of linear, anionic polysaccharides comprised of a repeating disaccharide, which is found on the surface of animal cells and within extracellular matrices. There are four groups of GAGs: (1) hyaluronan, (2) keratin sulfate, (3) chondroitin sulfate, and (4) heparan sulfate, the most structurally complex GAG.[1,2] In particular, low molecular weight heparin (LMWH) has been widely used and intensively investigated among GAGs. LMWH possesses a variety of biological activities, including being an anticoagulant, having anti-cancer activity, and affecting regulation of the biological functions of morphogens, growth factors, cytokines, chemokines and enzymes.[3,4]

Fluorescent labeling of proteins or nucleic acids has been a well-established approach for studying protein–protein interactions,[5,6] and DNA and RNA folding.[7] To date, it has not been possible to utilize this approach for GAGs due to the lack of suitable methodology to selectively label the non-reducing ends of GAGs with fluorophores.[8,9] Thus, developing a method to selectively functionalize the non-reducing ends of GAGs, such as LMWH, is attractive for various applications.

In general, specific enzymes selectively hydrolyze GAGs to produce oligosaccharides containing a $\Delta^{4,5}$-unsaturated carboxylic acid at the non-reducing terminus.[10] This unique feature in the heavily sulfated GAG chain provides an opportunity for selective functionalization *via* Michael addition. However, the carboxylic acids on the non-reducing ends of GAGs are relatively poor Michael acceptor,[11] and the lack of a suitable fluorophore makes this selective chemical functionalization of the unsaturated uronic acid (ΔUA) double bond on GAGs extremely difficult.

In order to develop a selective method for the functionalization of the non-reducing ends of GAGs, we aimed to design a new fluorophore to selectively attack the $\Delta^{4,5}$-unsaturated carboxylic acid. After screening various conjugated systems, we found that a thiol group can be conjugated to the $\Delta^{4,5}$-unsaturated carboxylic acid of a GAG *via* Michael addition with high efficiency.[12,13] To obtain a fluorophore with a reactive thiol group, we then synthesized sulfhydryl acetaldehyde (**2**) by the condensation of chloroacetaldehyde (**1**) and sodium bisulfide. Next, reductive amination of **2** by 2-aminobenzamide gave the dimeric compound **3**. The structure of **3** was confirmed by ^{1}H-NMR (Fig. S1, ESI†), ^{13}C-NMR (Fig. S2, ESI†) and IR (Fig. S3, ESI†). Finally, reduction of **3** by dithiothreitol in DMSO resulted in the monomeric fluorophore 2-(2-mercaptoethylamino)benzamide (**4**, 2-MEAB) in an isolated yield of 75%. The structure of **4** was confirmed by ^{1}H-NMR (Fig. S4, ESI†), ^{13}C-NMR (Fig. S5, ESI†) and IR (Fig. S6, ESI†). This newly synthesized fluorophore contained a strong nucleophilic thiol group, and exhibited a high fluorescence intensity with excitation and emission wavelengths of 340 nm and 440 nm respectively (Scheme 1).

LMWH (MW 2000–8000) **5** bearing a $\Delta^{4,5}$-unsaturated carboxylic acid at the non reducing end was used as a model for the



Scheme 1 Synthetic route to 2-(2-mercaptoethyl-amino)benzamide. Reagents and conditions: (a) NaSH, H_2O; (b) 2-aminobenzamide, $NaCNBH_3$, $ZnCl_2$, MeOH, 3 Å sieves; (c) dithiothreitol, DMSO.

State Key Laboratory of Natural Medicines and Laboratory of Chemical Biology, China Pharmaceutical University, 24 Tongjia Street, Nanjing, Jiangsu Province, 210009, People's Republic of China. E-mail: yjchen@cpu.edu.cn; Fax: +86 25 83271031; Tel: +86 25 83271031

† Electronic supplementary information (ESI) available: Detailed methods and supplementary figures. See DOI: 10.1039/c4cc00708e

GAG oligosaccharides. To address the poor reactivity of the carboxylic acid at the non-reducing end of LMWH, a three-step process, including protection, a 1,4-Michael addition, and deprotection, to transform the carboxylic acid of LMWH to a carboxylic benzyl ester was implemented.[14] First, LMWH sodium salt was changed to the form of benzethonium chloride salt. Then, $PhCH_2Cl$ was used to esterify the LMWH benzethonium chloride salt to give LMWH benzyl ester **6** with a $\Delta^{4,5}$-unsaturated benzyl ester at the non-reducing end. Next, LMWH benzyl ester **6** was reacted with 2-MEAB and boric acid in formamide at 50 °C for 24 h, and the resulting mixture was dialyzed (MWCO 500) against deionized water to remove any unreacted 2-MEAB and formamide, and then lyophilized to yield the 2-MEAB labeled LMWH benzyl ester **7** (Scheme 2).

LMWH benzyl ester **6** and 2-MEAB labeled LMWH benzyl ester **7** were then separated by HPLC, and compounds **6** and **7** were monitored by UV (232 nm) and a fluorescence detector (340/440 nm) as shown in Fig. 1. Before the thia-Michael addition, LMWH benzyl ester **6** at 1.7 min showed UV absorbance from the double bond at the non-reducing end (Fig. 1a).[15] However, when LMWH benzyl ester **6** was dissolved in water and detected by the fluorescence detector, only a small amount of impurity from the solvent was observed (Fig. 1b). After the thia-Michael addition, the 2-MEAB labeled LMWH benzyl ester **7** exhibited both UV absorbance (232 nm) and fluorescence absorbance (340/440 nm). In addition, the chromatogram at 232 nm indicated that nearly all of the unreacted 2-MEAB and formamide had been removed, and that the relatively pure 2-MEAB labeled LMWH benzyl ester **7** had been obtained (Fig. 1c). Moreover, Fig. 1d clearly confirmed that the conjugation between 2-MEAB and the non-reducing end of LMWH benzyl ester **7** had been accomplished to show strong fluorescence compared to Fig. 1b. To cleave the benzyl protecting group from **7**, 0.1 N NaOH was used to hydrolyze **7** at 60 °C for 1 h, and product **8** was

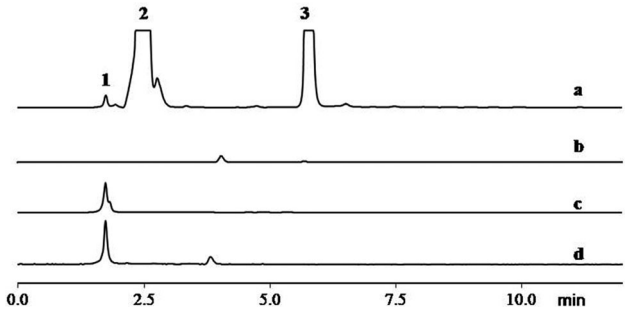
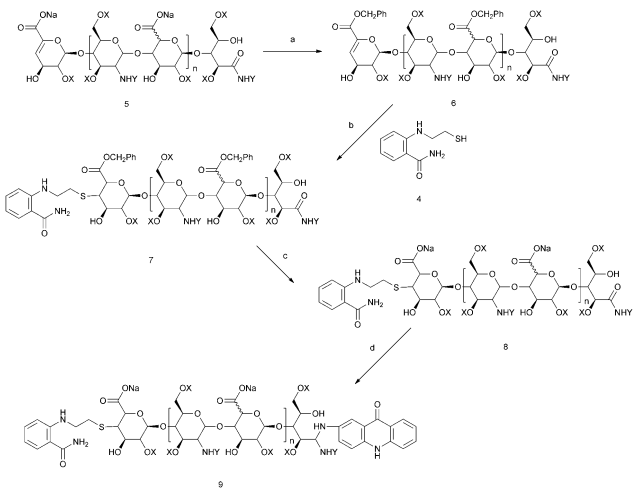


Fig. 1 HPLC chromatograms of unlabeled and labeled low molecular weight heparin. (a) LMWH ester **6** and 2-MEAB were dissolved in formamide and monitored at UV 232 nm. (b) LMWH ester **6** was dissolved in water and monitored by a fluorescence detector at 340/440 nm. (c) After dialysis, 2-MEAB-LMWH **7** was monitored at UV 232 nm. (d) After dialysis, 2-MEAB-LMWH **7** was monitored by a fluorescence detector at 340/440 nm. Peak 1: LMWH derivatives; peak 2: formamide; peak 3: 2-MEAB.

precipitated in methanol. The fluorescence intensity of the 2-MEAB labeled LMWH **8** was detected to be very strong (Fig. S7, ESI†). The high fluorescence intensity of the 2-MEAB labeled LMWH clearly demonstrated that the conjugation between the novel fluorophore 2-MEAB and the non-reducing end of the LMWH *via* thia-Michael addition is a feasible approach for the fluorescent labeling of LMWH.

To further confirm the utility of this novel fluorophore, LMWH with double fluorescence labeling was prepared in order to investigate the interaction between GAGs and proteins by fluorescence resonance energy transfer (FRET) as illustrated in Fig. 2a. Given that 2-MEAB possesses a new emission wavelength of 440 nm, with a 90% overlap with the excitation wavelength of a commercially available fluorophore, 2-aminoacridone (2-AMAC), and labeling at the reducing end was easier to achieve (Fig. S8, ESI†),[16] double labeling of LMWH on the reducing and non-reducing ends by these two fluorophores would create a donor and acceptor pair for FRET in order to determine the heparinase activity because the length of the LMWH is less than 10 nm.[17] Subsequently, to obtain the double fluorophore labeled LMWH **9**, 2-MEAB-LMWH **8** was dissolved in deionized water and mixed with a solution of 2-AMAC dissolved in 85% (v/v) acetic acid–DMSO. The mixture was incubated at 37 °C for 16 h and dialyzed



Scheme 2 Synthetic route to the double fluorescent labeling of low molecular weight heparin. Reagents and conditions: (a) $C_{27}H_{42}NO_2$, $PhCH_2Cl$, CH_2Cl_2; (b) boric acid, formamide, 50 °C; (c) NaOH, 60 °C; (d) 2-aminoacridone, $NaCNBH_3$, acetic acid–DMSO (1 : 3, v/v); X = SO_3Na or H; Y = $COCH_3$, SO_3Na or H.

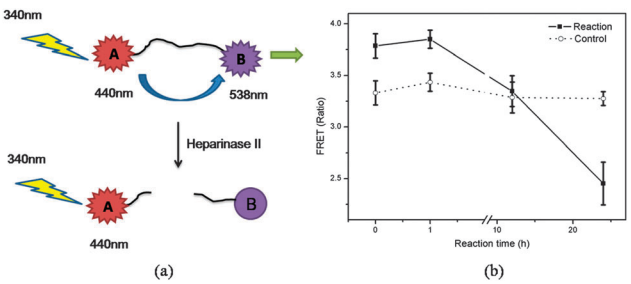


Fig. 2 Heparinase II activity assay by FRET. (a) Schematic illustration of the activity assay of heparinase II. (b) Time courses of the FRET. FRET ratios (340–538 nm/340–440 nm) of the control (dotted line) and the activity assay (solid line). Data are an average of 3 independent determinations with standard deviations.

against deionized water to remove any unreacted 2-AMAC, and then lyophilized to yield **9**. Since heparinase II (HepII), from *Pedobacter heparinus*, is able to degrade heparin from a long chain to short chains in an endolytic manner,[18] recombinant HepII was then prepared for an activity assay by FRET. The gene encoding his-tagged HepII was synthesized and transformed into *E. coli* BL21 (DE3) cells. One liter cultures were grown at 37 °C in a Luria-Bertani (LB) medium supplemented with 50 μg ml^{-1} kanamycin and protein expression was induced by the addition of 500 μM isopropyl 1-thio-β-D-galactopyranoside for 8–12 h at 15 °C. Purification of the rHepII was performed as described in the literature.[19] Briefly, recombinant HepII was purified by two chromatographic steps, including immobilized metal-chelate chromatography using a 1 ml His-trap FF column (GE Healthcare) and a cation exchange column using a 1 ml Hitrap-SP column. The protein purity was assessed by SDS-PAGE as shown in Fig. S9 (ESI†). For the determination of the HepII activity by FRET, LMWH was double labeled with two fluorophores on the reducing- and non-reducing ends. Then, the degradation of the LMWH double labeled with fluorophores was measured by time-resolved fluorescence after the addition of the purified HepII. The control consisted of 100 μl of **9** (1 mg ml^{-1}) and 100 μl potassium phosphate buffer (pH 7.4) in a 96 black well plate. After incubating at 37 °C for 24 h, the FRET ratio (340–538 nm/340–440 nm) did not change (Fig. 2b, dotted line), indicating that no LMWH had been degraded. However, when **9** and HepII were mixed and incubated under the same conditions, the FRET ratio decreased in a time-dependent manner (Fig. 2b, solid line). The results demonstrated that 2-MEAB had been selectively conjugated to the non-reducing end of the LMWH, and can be used as a FRET donor for a heparinase activity assay. Given the fact that non-selective labeling can damage the recognition site of an enzyme,[20] the present assay is more beneficial and useful.

To date, the selective labeling of heparin with fluorescent probes has proven to be a challenging issue. Although a number of approaches have been attempted, they are generally non-selective, conjugating the fluorophore onto all of the carboxylic acid groups in GAG oligosaccharides. In the case of selective conjugations, they are not suitable for FRET due to the negative impact on the protein function and the unavoidable opening of another sugar ring by Hg^{2+}.[21] In conclusion, we have synthesized a novel fluorophore and selectively conjugated it with the non-reducing end of LMWH; this approach could be used to investigate the conformational changes of GAG oligosaccharides and the interactions between GAGs and proteins.

This work was supported by the ''111 Project'' from the Ministry of Education of China and the State administration of Foreign Expert Affairs (No. 111-2-07) and the research grant from the State Key Laboratory of Natural Medicines, China Pharmaceutical University (SKLNMZZCX No. 201301).

Notes and references

1 R. J. Linhardt and T. Toida, *Acc. Chem. Res.*, 2004, **37**, 431–438.
2 N. S. Gandhi and R. L. Mancera, *Chem. Biol. Drug Des.*, 2008, **72**, 455–482.
3 J. Nishioka and S. Goodin, *J. Oncol. Pharm. Pract.*, 2007, **13**, 85–97.
4 C. R. Parish, *Nat. Rev. Immunol.*, 2006, **6**, 633–643.
5 D. Maurel, L. C. Argar, C. Brock, M. L. Rives, E. Bourrier, M. A. Ayoub, H. Bazin, N. Tinel, T. Duuroux, L. Prezeau, E. Trinquet and J. P. Pin, *Nat. Methods*, 2008, **5**, 561–567.
6 H. E. Rajapakse, N. Gahlaut, S. Mohandessi, D. Yu, J. R. Turner and L. W. Miller, *PNAS*, 2010, **107**, 13582–13587.
7 D. Klostermeier and D. P. Millar, *Biopolymers*, 2002, **61**, 159–179.
8 E. Gemma, O. Meyer, D. Uhrín and A. N. Hulme, *Mol. Biosyst.*, 2008, **4**, 481–495.
9 E. Gemma, A. N. Hulme, A. Jahnke, L. Jin, M. Lyon, R. M. Müller and D. Uhrín, *Chem. Commun.*, 2007, 2686–2688.
10 S. Ernst, R. Langer and C. L. Cooney, *Crit. Rev. Biochem. Mol. Biol.*, 1995, **30**, 387–444.
11 S. Gao, T. Tzeng, M. N. V. Sastry, C. M. Chu, J. T. Liu, C. Lin and C. F. Yao, *Tetrahedron Lett.*, 2006, **47**, 1889–1893.
12 C. L. Dickinson, J. K. Williams and B. C. McKusick, *J. Org. Chem.*, 1964, **29**, 1915–1919.
13 M. K. Chaudhuri and S. Hussain, *J. Mol. Catal.*, 2007, **269**, 214–217.
14 B. E. Rossiter and N. M. Swingle, *Chem. Rev.*, 1992, **92**, 771–806.
15 A. Linker and P. Hovingh, *Methods Enzymol.*, 1972, **28**, 902–911.
16 J. Peter, *Anal. Biochem.*, 1991, **2**, 196–244.
17 S. Khan, J. Gor, B. Mulloy and S. J. Perkins, *J. Mol. Biol.*, 2010, **395**, 504–521.
18 D. Shaya, W. J. Zhao, M. L. Garron, Z. P. Xiao, Q. Z. Cui, Z. Q. Zhang, T. Sulea, R. J. Linhardt and M. Cygler, *J. Biol. Chem.*, 2010, **285**, 20051–20061.
19 D. Shaya, A. Tocilj, Y. Li, J. Myette, G. Venkataraman, R. Sasisekharan and M. Cygler, *J. Biol. Chem.*, 2006, **281**, 15525–15535.
20 K. Enomoto, H. Okamoto, Y. Numata and H. Takemoto, *J. Pharm. Biomed. Anal.*, 2006, **41**, 912–917.
21 M. A. Skidmore, S. J. Patey, N. T. K. Thanh, D. G. Ferning, J. E. Turnbull and E. A. Yates, *Chem. Commun.*, 2004, 2700–2701.

