



A model ternary heparin conjugate by direct covalent bond strategy applied to drug delivery system

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ABSTRACT

A model ternary heparin conjugate by direct covalent bond strategy has been developed, in which modified heparin using active mixed anhydride as intermediate conjugates with model drug molecule and model specific ligand, respectively. Designed ester bonds between model drug and heparin facilitate hydrolysis kinetics research. The strategy can be extended to design and synthesize a targeted drug delivery system. The key point is to use mixed anhydride groups as activating intermediates to mediate the synthesis of the ternary heparin conjugate. Formation of mixed anhydride is detected by the conductimetry experiment. The ternary heparin conjugate is characterized by ¹³C NMR, FT-IR and GPC, respectively. The decreased trend on degree of substitution (DS) is consistent with that of introduced anticancer drug and specific ligand in drug delivery system. Moreover, their anticoagulant activity is evaluated by measuring activated partial thromboplastin time (APTT) and anti-factor Xa activity. The results show that model ternary heparin conjugate with reduced anticoagulant activity may avoid the risk of severe hemorrhagic complication during the administration and is potential to develop a safe and effective drug delivery system on anticancer research.

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Heparin is a biocompatible, biodegradable and water-soluble natural polysaccharide and rich in animal tissues. It has complicated structure (Fig. 1). In recent years, heparin has attracted intensive attention because it demonstrates a variety of biological activities, such as anticoagulant activity, inhibition of angiogenesis, tumor development, and of proliferation of arterial smooth muscle cells, and so forth.¹ To further extend the applications of heparin, most researches focus on chemical modification of its functional groups or modular synthesis of heparin fragments to obtain desired biological compounds.^{2–4} However, one major problem is that chemical modification may induce unpredictable side reactions due to the complex, heterogeneous structure of heparin. Therefore, most researches are limited to binary heparin conjugate by chemically bonding one functional group to heparin, which largely restricts the wide applications of heparin.^{5–7}

It was reported that heparin derivatives with reduced anticoagulant activity have a great potential as a drug on the tumor research, which made tumor cells released from the primary tumor for cancer patients during surgical manipulation.⁸ However, hepa-

rin therapy can be difficult to manage and of limited use due to its anticoagulant potency and potential for inducing hemorrhagic complications. Therefore, introduction of chemical modification to the heparin moiety to reduce its anticoagulant activity is a promising approach.

In this study, we have developed a model ternary heparin conjugate by direct covalent bond strategy, in which modified heparin using active mixed anhydride as intermediate conjugate with model drug molecule (benzyl alcohol) and model specific ligand (butylamine), respectively (Fig. 2). Ester bonds linked model drug and carrier facilitate the release of drug from carrier. In contrast, amide bonds between model specific ligand and heparin show greater stability. The ternary conjugate strategy can be extended to design and synthesize a multifunctional drug–drug carrier (heparin)–specific ligand, ternary drug delivery system, in which hydroxyl groups and primary amine groups of model molecules have similar to the drug and specific ligand, such as paclitaxel and folate-ethylenediamine. To explore the possibility of the ternary heparin conjugate as a safe drug delivery system, their anticoagulant activity has been investigated.

To improve its solubility in organic solvents, we firstly converted heparin sodium salt into tributylammonium heparin salt. It has been determined that 1 g heparin salts contains 4 mequiv of hydroxyl groups and 1.7 mequiv carboxyl groups. Therefore,

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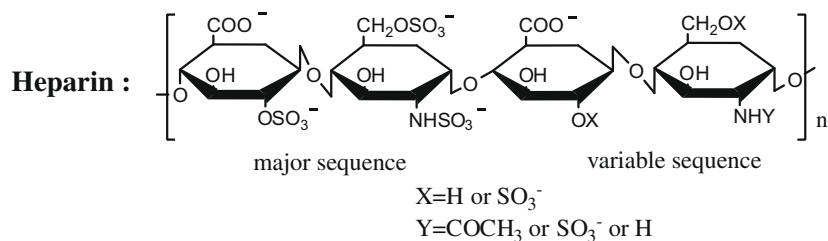


Figure 1. Structure of heparin.

we used 7 fold of acylating reagent and 0.1–0.3 mequiv of DMAP to sufficiently acylate the hydroxyl groups and the carboxyl groups. It reacted with acetic anhydride in the presence of DMAP and Et_3N at the room temperature and continued different reaction time, such as 12, 24 and 48 h. O-acetylated heparins with different DS of acetyl were obtained. Then, O-acetylated heparin reacted with benzyl alcohol under the catalyst of $HClO_4$ for 12 h at 50 °C. By controlling the amount ratio of carboxyl/benzyl group (1.2:1), benzyl alcohol reacted with only a part of active groups. The binary heparin conjugate was obtained as white powders. During the preparation of ternary heparin, the molar amount of butylamine was slightly higher than that of the remaining active groups to facilitate the reaction completely. The above product conjugated with butylamine in the presence of triethylamine and reaction was continued at 50 °C for 12 h. The ternary heparins with various DS were obtained.

In this study, we modified the carboxyl group of heparin with acetyl group of the acetic anhydride via the O-acylation reaction to form a mixed anhydride group. The activating intermediate could be effectively applied to synthesize binary heparin conjugate via the esterification occurred at the mixed anhydride groups. Then, butylamine was bonded to the residual mixed anhydride groups via the N-acylation reaction. At last, a ternary heparin conjugate was synthesized. As shown in Table 1, we prepared ternary heparin with various DS and investigated influence on the anticoagulant activity of the product. It was found that DS of benzyl in

binary heparin increased with the increase of DS of acetyl. However, the DS of butylamine in ternary heparin had no obvious difference with the increased trend. It was demonstrated that the activating mixed anhydride groups largely improved the activity and selectivity toward the following esterification. The formation of the mixed anhydrides was observed by an increase in the sulfate-to-carboxyl ratio of the O-acetylated heparin by conductimetric titration. The trend on DS of acetyl, benzyl, and butylamine coupled on the ternary heparin conjugate was gradually attenuated. The decreased substitution trend was consistent with that of introduction of anticancer drug and specific ligand in drug delivery system. The quantity of drug conjugated with carrier should be much more than that of specific ligand, which could specifically delivery drug to tumor tissue. The molecular weight and molecular weight distributions were measured by GPC, and the molecular weight distribution for the heparin derivatives were about 1.7–1.8.

Direct proofs confirming the formation of the ternary heparin conjugates came from ^{13}C NMR experiments in Figure 3. Because of the interference of $-NHCOCH_3$ in heparin, the presence of $-COCH_3$ and $-NHCH_2CH_2CH_2CH_3$ of ternary heparin conjugate was overlaid near δ 22 ppm, meanwhile the signal of $-COCH_3$ was similar to that of $-NHCOCH_3$ near 177 ppm in ^{13}C NMR spectrum. It was observed the corresponding carbon peaks of $-OCH_2C_6H_5$ appeared at δ 61.4 ppm and δ 132.0 ppm in ^{13}C NMR. The carbon peaks of $-NH(CH_2)_3CH_3$ was shown in δ 15.8 ppm, δ 21.9 ppm, δ 31.7 ppm and δ 42.6 ppm, respectively. Glucosamine

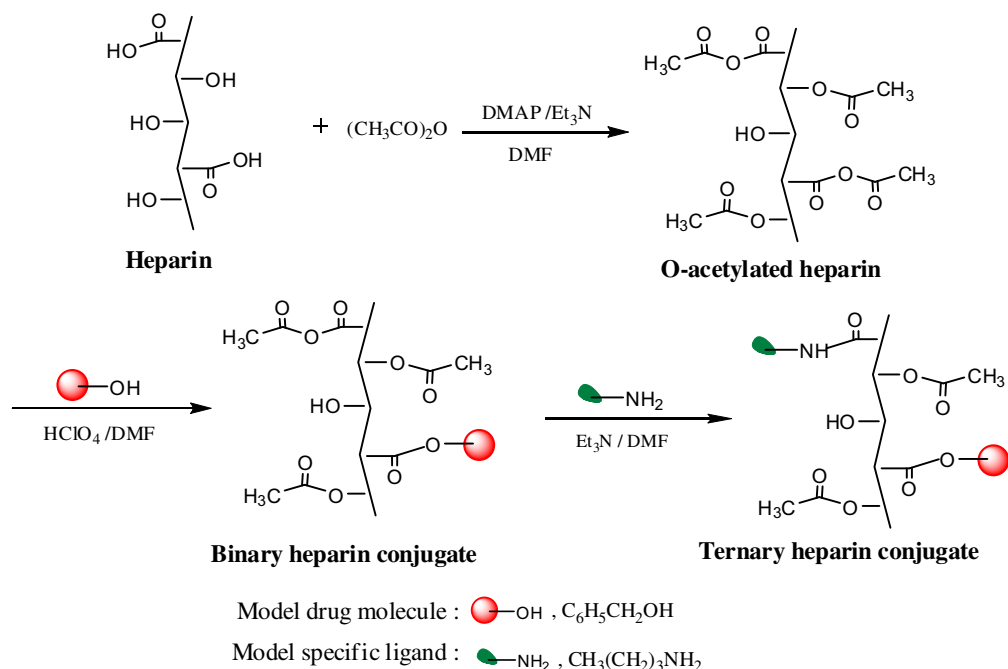
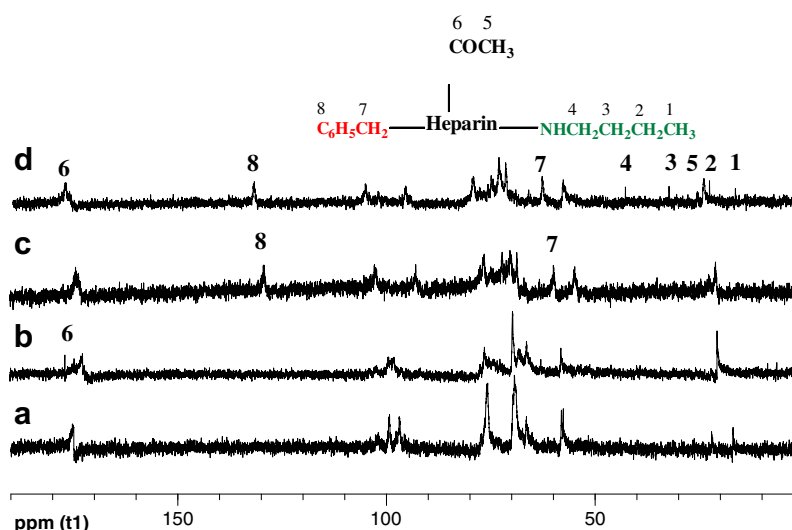
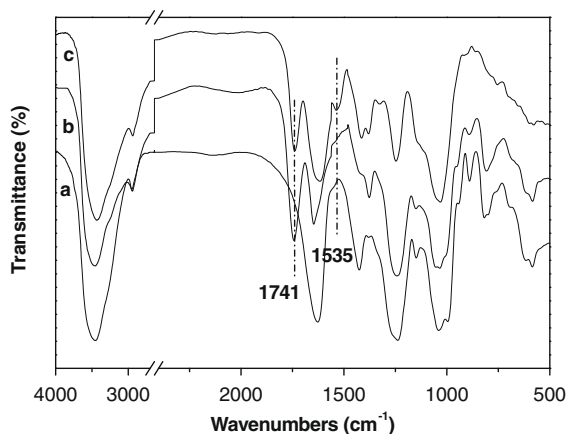


Figure 2. Synthetic route of the ternary heparin conjugate.

Table 1

Analytical characteristics of the heparin derivatives.

Derivatives	DS ^a (%) (acetyl, benzyl, butylamine groups)			SO ₃ ⁻ /COO ⁻	M _n ^b	PDI ^b	Anticoagulant activity ^c	
							APTT	Anti-factor Xa
Heparin	0			2.22	12,000		150	150
O-acetylated heparin (1, 2 and 3)	7.8			2.41	12,697	1.79	135 ± 8	129 ± 9
	8.5			2.52	12,795	1.83	127 ± 15	125 ± 11
	9.2			2.58	13,012	1.81	121 ± 10	119 ± 7
				ND ^d	13,114	1.82	124 ± 7	95 ± 2
Binary heparin (1, 2 and 3)	7.8		2.9	ND	13,345	1.73	116 ± 8	89 ± 12
	8.5		3.2	ND	13,420	1.77	108 ± 4	76 ± 11
	9.2		3.4	ND	13,364	1.82	74 ± 5	69 ± 8
Ternary heparin (1, 2 and 3)	7.8	2.9	1.2	ND	13,483	1.74	68 ± 6	64 ± 4
	8.5	3.2	1.3	ND	13,541	1.73	56 ± 10	59 ± 7
	9.2	3.4	1.0	ND				

^a DS, the degree of substitution indicates the weight percentage of acetyl groups, benzyl groups and butylamine groups introduced heparin, determined by ¹H NMR.^b M_n, PDI, determined by GPC.^c Anticoagulant activity determined by APTT assay and anti-factor Xa activity assay (the results are mean ± SEM, n = 3), respectively.^d ND, not determined.**Figure 3.** ¹³C NMR spectra of (a) heparin, (b) O-acetylated heparin, (c) Binary heparin conjugate, and (c) Ternary heparin conjugate in D₂O.**Figure 4.** FT-IR spectra of (a) heparin, (b) O-acetylated heparin and (c) binary heparin conjugate.

skeleton of heparin in ternary heparin conjugate attributed about from 57 to 104 ppm, which contains hexosamine residue and uronic acid residues with different groups. Due to the introduction of organic agent, it was observed the change of conformation resulting in the movement of chemical shift.

Figure 4 showed the FT-IR spectra of the heparin, O-acetylated heparin and binary heparin conjugate. A new band at 1741 cm⁻¹ appeared in spectra **b** and **c**, which was assigned to the ester bond vibration.⁹ The result indicated that acetyl groups were bonded to both the hydroxyl and carboxyl groups of heparin and thus mixed anhydride group was formed at the position of the carboxyl group. This new band appeared in 1535 cm⁻¹, which was attributed to the –C=C– stretching mode of benzene ring of the benzyl alcohol. It strongly indicated that benzyloxy group was successfully linked to the O-acetylated heparin, forming a binary heparin conjugate. However, in the final ternary heparin conjugate, the characteristic IR bands of the amido group were not detected, which may be due to the fact that the DS of butylamine was relative low and thus the IR bands of amido group were weak and hidden in the background of the IR bands of heparin itself.

Anticoagulant activity of heparin derivatives with different DS was investigated by APTT and anti-factor Xa activity assays. As a whole, the anticoagulant activity of heparin derivatives decreased as compared with that of heparin. Meanwhile, heparin derivatives with low DS of acetyl (DS1) showed higher anticoagulant activity in Figures 5 and 6. The anticoagulant activity of heparin is partially influenced by the carboxyl groups.¹ With the increased trend on DS, the amount of carboxyl groups were reduced therefore anticoagulant activity of heparin derivatives was decreased. This may be explained that the introduction of organic agents to carboxyl

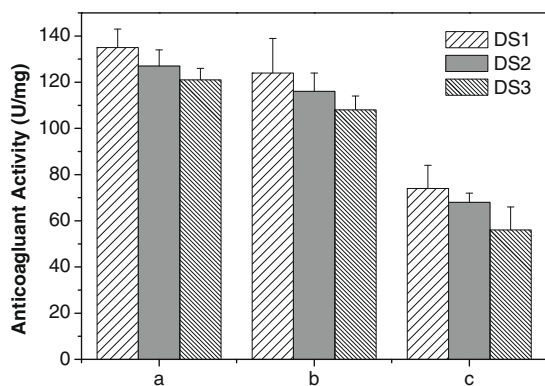


Figure 5. Anticoagulant activity of (a) O-acetylated heparin, (b) binary heparin conjugate and (c) ternary heparin conjugate by APTT assay.

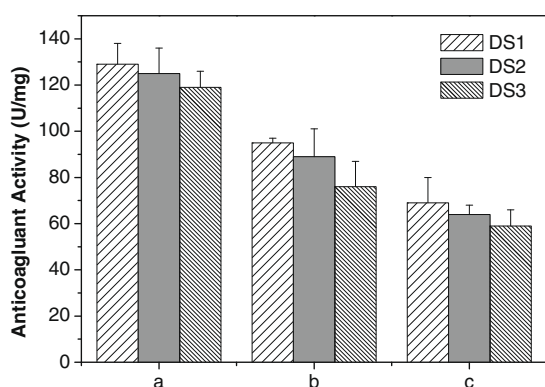


Figure 6. Anticoagulant activity of (a) O-acetylated heparin, (b) binary heparin conjugate and (c) ternary heparin conjugate by anti-factor Xa assay.

groups led to the conformational change of AT III in heparin structure, which affects the underlying anticoagulant mechanism thereby inactivating the coagulation enzymes.¹ Therefore, the model ternary heparin conjugates with reduced anticoagulant might avoid the risk of severe hemorrhagic complication in vivo administration and was potential to further investigate application in anticancer research.

In summary, the model drug molecule and model specific ligand have been successfully introduced to the O-acetylated heparin via

ester bond and amido bond, respectively. The key point is to use mixed anhydride groups as activating intermediates to mediate the synthesis of the model ternary heparin conjugates. ¹³C NMR, FT-IR and GPC characterizations confirm the ternary structure of heparin conjugates. The model ternary heparin conjugate with reduced anticoagulant activity can avoid the risk of severe hemorrhagic complication, at the same time it may be developed a safe and effective drug or drug carrier to apply in drug delivery system. The present study takes an important step toward the design, synthesis, and applications of intelligent heparin-based targeted drug delivery systems if the present model molecules are replaced by drug molecules and target-specific ligands, respectively. Meanwhile, a ternary heparin conjugate for targeted-specific delivery of paclitaxel is under studying in our laboratory now.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bmcl.2008.10.132](https://doi.org/10.1016/j.bmcl.2008.10.132).

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