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Research article

Poly-ion complex (PIC) formation of heparin and polyamines: PIC with tetrakis (3-aminopropyl) ammonium allows sustained release of heparin

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ABSTRACT

Physical mixtures of cationic polymers and heparin have been developed to overcome the limitations of unfractionated heparin. In this study, we found that heparin associates with natural polyamines in water, resulting in the generation of a poly-ion complex (PIC). PIC formation (or stability) was influenced by the concentration and ratio of heparin and polyamines, molecular weight of heparin, nature of polyamines, and pH conditions. Interestingly, the PIC obtained when heparin and tetrakis (3-aminopropyl) ammonium (Taa) were mixed exhibited stability and was sticky in nature. PIC formation was due to an electrostatic interaction between heparin and Taa. Heparin-Taa PIC was administered subcutaneously to mice, and the time to maximum heparin concentration within the therapeutic range of heparin was markedly increased compared to that after a single dose of heparin. These results suggest that the quaternary ammonium structure of Taa is critical for the preparation of a stable PIC, thereby allowing the sustained release of heparin into the blood.

1. Introduction

Heparin is mainly used as an anticoagulant for the prevention of venous thrombosis and pulmonary embolism in patients undergoing surgery [1, 2]. Heparin is a linear polysaccharide comprised of 16–160 repeating disaccharides of glucosamine (GlcN) and iduronic acid (IdoA) and has an average ~2.7 sulfo groups per disaccharide attached to both the amino and hydroxyl groups of the polysaccharide [3]. Heparin can bind to the serine protease inhibitor antithrombin (AT), causing it to undergo a conformational change [4, 5], markedly accelerating its ability to inactivate the coagulation proteases, including thrombin (factor IIa) and factor Xa [1]. Pentasaccharide sequences containing a 3-*O*-sulfated glucosamine moiety in heparin are critical for the binding and conformational change of AT [6, 7, 8]. In addition to its anticoagulant activity, heparin has other activities, such as reducing the proliferation of vascular smooth muscle cells (VSMCs) [9, 10]. Heparin can induce apoptotic cell death when successfully delivered into cell nucleus [11, 12, 13]; the use of heparin for intimal hyperplasia and cancer therapy has been explored. Oral and subcutaneous administration of heparin is limited because of its relatively high molecular weight and its highly negative charge [14]. Furthermore, heparin administered via the parenteral route is promptly cleared by endothelial cells and macrophages; requiring careful patient monitoring [14]. Therefore, physical mixtures of cationic polymers and heparin have been developed to prepare complexes, based on their electrostatic interactions, for *in vitro* and *in vivo* evaluation. Encapsulation of heparin in hydrogels or hydrophobic polymer matrices can facilitate the controlled release of heparin over several days, resulting in the prevention of VSMC proliferation in culture [9, 10]. Intracellular delivery of heparin, accomplished using nanoparticles, prepared by the mixing of heparin and poly (β-amino esters), polyethylene glycol (PEG) or chitosan-g-PEG, can effectively induce cell death [11, 12, 13]. Chitosan and synthetic cationic polymers have also been used for the condensation of heparin to entrap bovine serum albumin (BSA) or fibroblast growth factor (FGF) on the surface of nanoparticles [15, 16].

Polyamines are present at millimolar concentrations in all living cells and exist mainly as polyamine-RNA complexes [17]. Polyamines are synthesized in many cells, but their types and structures depend on the species. In eukaryotic cells, three types of linear polyamines, putrescine (PUT), spermidine (SPD), and spermine (SPM) are synthesized, and these are essential for the normal cell growth and differentiation [18].

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Polyamines having unusual structures are synthesized in thermophiles, plants and bacteria; allowing these organisms to adapt to their external environment [19]. An extreme thermophile, an *eubacterium* called *Thermus thermophilus*, produces various kinds of linear polyamines, including thermine, thermospermine, caldopentamine (Cdp) and caldohexamine (Cdh), as well as branched polyamines (tris (3-aminopropyl) amine (mitsubishine) and tetrakis (3-aminopropyl) ammonium (Taa)). Taa, in particular, stabilizes nucleic acid and protects it against depurination more effectively than SPM [20, 21]. We have previously shown that poly-ion complex (PIC), generated in aqueous solutions through the electrostatic interaction of chondroitin sulfate (CS) and polyamines, improves the oral bioavailability of CS [22].

In the present study, we have prepared PICs from heparin and polyamines. The formation efficiency (or stability) of PIC from heparin and polyamines was found to depend on the mixing ratio of polyamines and heparin, the molecular weight of heparin, the nature of polyamines, and the pH conditions. PIC generated by mixing heparin and Taa exhibited stability and was sticky in nature. The PIC extended the time to maximum heparin concentration within the therapeutic range of heparin in a mouse model. These results suggest that the nature, shape, and stability of a PIC with heparin depend on the structure of the polyamine. Particularly, the quaternary ammonium structure of Taa was critical for the preparation of a stable PIC, thereby allowing sustained release of heparin into the blood.

2. Materials and methods

2.1. Chemicals and materials

Unfractionated heparin (average molecular weight 13 kDa, with a disaccharide composition of 3.1% of ΔDi-0S, 1.8% of ΔDi-NS, 2.1% of ΔDi-6S, 5.7% of ΔDi-NS6S, 5.0% of ΔDi-UA2SNS, and 82.3% of ΔDi-TriS) from porcine intestinal mucosa was purchased from New Zealand Pharmaceuticals Ltd (NZP; Palmerston North, New Zealand). Putrescine dihydrochloride (PUT· 2HCl), spermidine trihydrochloride (SPD· 3HCl), and spermine tetrahydrochloride (SPM· 4HCl) were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Tris (3-aminopropyl) amine was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Tetrakis (3-aminopropyl) ammonium, caldopentamine and caldohexamine were chemically synthesized as reported previously [19]. Titanium dioxide (anatase type, average particle size, 50 μm) was obtained from Wako Pure Chemical Co. (Osaka, Japan). All other chemicals were of analytical reagent grade.

2.2. PIC formation

Heparin and several kinds of polyamines at specified concentrations were separately dissolved in double-distilled water. Equivalent volumes of solutions of heparin and polyamines were then mixed to generate the PIC. The PIC formation efficiency, effect of pH value on the stability of PIC, determination of particle size and ζ-potential, surface structure analysis were evaluated as described previously [22]. Low molecular weight heparin was obtained by photochemical depolymerization with titanium dioxide (TiO_2) [23].

2.3. Anticoagulation activity after subcutaneous administration of PIC from heparin and Taa

All animal experiments were approved by the Institutional Animal Care and Use Committee of Chiba University and conducted according to the guidelines for Animal Research of Chiba University. The ddY mice (female, weighing 28–30 g, Japan SLC, Inc, Shizuoka, Japan) were housed at 25 °C and 55% RH. A 12 h dark/light cycle was maintained throughout the study. Mice had free access to a standard pellet diet (MF, Oriental Yeast Co., Ltd, Tokyo, Japan) and tap water. The mice were fasted overnight before the experiment. PIC was generated by mixing equivalent solutions of 20 mg/mL of heparin and 80 mM Taa and centrifuged (800 × g, 10 min). After centrifugation, the supernatant was removed, and the resulting pellet was suspended with cone oil. Thus, PIC (30 mg/kg body weight of heparin) or unfractionated heparin (4 or 30 mg/kg body weight) was suspended with 1.7 mL/kg (body weight) of cone oil; resulting samples were administered subcutaneously to the back of the mice. After administration of PIC with Taa, blood samples were

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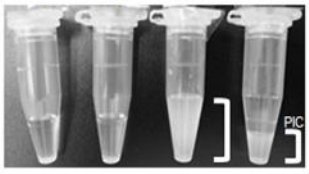

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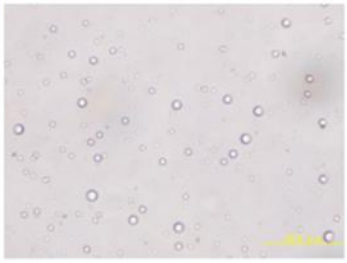


C

		SPM (mM)*			SPD (mM)*			PUT (mM)*		
		10	50	100	10	50	100	10	50	100
Heparin (mg/mL)* (Mw. 13000)	0.1	+	+	–	+	–	–	–	–	–
	0.5	+ +	+	+	+ +	+	+	–	–	–
	1.0	+ +	+ +	+	+ +	+ +	+	–	–	–
	5.0	+ + +	+ + +	+ +	+ +	+ + +	+ +	–	–	–
	10	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	–	–	–

D

		SPM (mM)*		
		10	50	100
Heparin (mg/mL)* (Mw. 13000)	0.5	+ +	+	+
	1.0	+ +	+ +	+
	5.0	+ + +	+ + +	+ +
LMWH-7 (mg/mL)* (Mw. 7000)	0.5	+	+	–
	1.0	+ +	+	+
	5.0	+ + +	+ + +	+ +
LMWH-3.5 (mg/mL)* (MW: 3500)	0.5	+	–	–
	1.0	+ +	+	–
	5.0	+ + +	+ + +	+

Figure 1. PIC formation efficiency was influenced by mixing concentration ratio of heparin and polyamines, the molecular weight of heparin. (A) Photograph of (1) 20 mg/mL of heparin, (2) 20 mM of SPM, (3) PIC, (4) PIC that separated into two layers after standing for 20 min. (B) Microscopic image of PIC generated from heparin and SPM. The scale-bar indicates 20.0 μm. (C) PIC formation efficiency under different conditions of mixing ratios between heparin and polyamine (SPM, SPD or PUT). (D) Aqueous solutions of SPM and heparin with different molecular weights were prepared at different concentrations and their equivalent volumes were mixed with each other to generate PIC. PIC formation was evaluated by measuring the transmittance (%T): -, PIC formation could not be observed (≧90%T); +, PIC formation was poor (30–89%T); ++, PIC formation was good (10–29%T); +++, PIC formation was very good (1–9%T) and exhibited strongly turbid white solution conditions after 24 h (<1%T), but PIC precipitated very quickly (within several seconds). *, final concentration.

Table 1. The size and surface charge of PIC particle generated under the optimal mixing ratio conditions between heparin and polyamines.

PIC formation		MV (μm)	ζ (mV)
Heparin (10 mg/mL)*	SPM (10 mM)* in water	0.57 ± 0.25	-25.3 ± 0.22
	SPM (10 mM)* in water ajusted to pH 3.0		-6.49 ± 0.66
	SPM (10 mM)* in water ajusted to pH 5.0		-20.6 ± 0.56
	SPM (10 mM)* in water ajusted to pH 9.0		-19.4 ± 0.44
	Thermine (10 mM)*	0.45 ± 0.03	-35.8 ± 1.28
	Caldopentamine (20 mM)*	1.88 ± 0.18	4.11 ± 1.41
Heparin (5 mg/mL)*	Mitsubisine (20 mM)*	0.78 ± 0.08	-10.0 ± 0.23

Mean ± S.D., n = 3.
* final concentration.

collected from the tail vein at time intervals of (0, 2, 4, 8, 12, 16, 20, and 24 h) after subcutaneous administration. The blood samples were then anticoagulated with 3.2% trisodium citrate solution at the ratio of 9:1. Each collected sample was immediately centrifuged (800× g, 15 min) to obtain the plasma, which was then transferred to a new Eppendorf tube and stored at -20 °C. Anti-factor Xa activity and anti-factor IIa (thrombin) activity were measured as described previously [23]. Clexane (2000IU/0.2 mL) (Sanofi) was used as a standard.

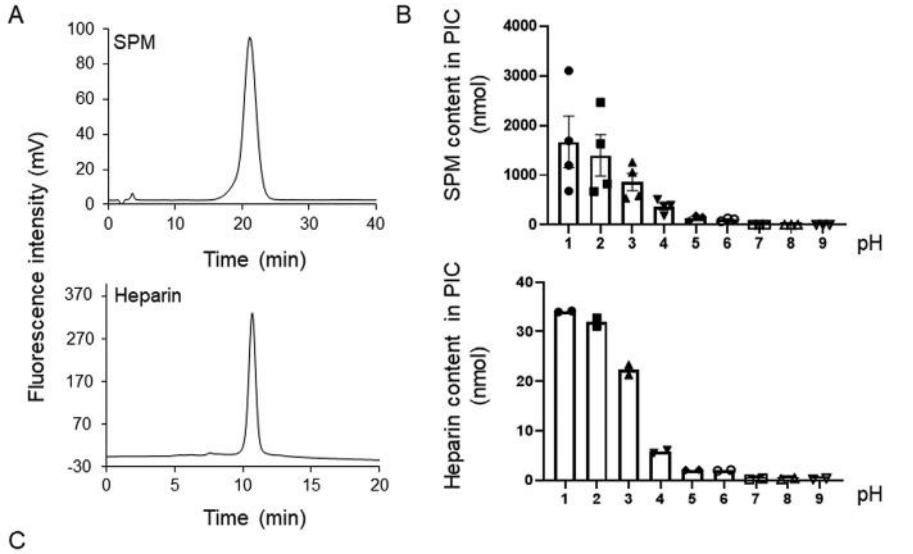
3. Results and discussions

3.1. Preparation of poly-ion complex from heparin and polyamines

Initially, we examined whether polyamines associated with unfractionated porcine intestinal mucosa heparin (Mw. 13 kDa) dissolved in water. An aqueous solution containing heparin (20 mg/mL) or SPM (20 mM) was transparent; however, a turbid white suspension of PIC was generated when equivalent volumes of 20 mg/mL of heparin and 20 mM of SPM solution were mixed (Figure 1A). The turbid white suspension separated into two layers after approximately 20 min of settling. If the two layers were thoroughly mixed again using a vortex mixer, the original PIC suspension could be restored. Morphological characterization of the PIC suspension from heparin (10 mg/mL) and 10 mM SPM showed that it was spherical in shape and in a micrometer size range (Figure 1B), correlating closely with the particle size (0.57 ± 0.25 μm) measured by dynamic light scattering (Table 1) [22].

As PIC formation efficiency was influenced by the mixing concentration ratio of heparin and SPM (Figure 1C), we hypothesized that a hydrophobic PIC was formed by the neutralization of positively charged SPM with negatively charged heparin molecules. The effect of the chain length of polyamines on the formation efficiency of the PIC was also investigated using different mixing ratios of heparin and polyamines solutions. Putrescine (1, 4-butanediamine) has two amino groups, spermidine (*N*-(3-aminopropyl) butane-1, 4-diamine) has three amino groups, and SPM (*N*, *N*′-Bis (3-aminopropyl) butane-1, 4-diamine) has four amino groups. As a result, PIC formation efficiency depends on the concentration ratio of heparin and polyamines. The polyamine chain length is an important parameter as 100 mM PUT was unable to produce PIC with unfractionated heparin (Figure 1C). Furthermore, chain length of heparin was critical for the formation of PICs (Figure 1D). These results indicate that the molecular chain lengths of both heparin and polyamines are important for PIC formation.

Stability of PIC under various pH conditions was next investigated by analyzing the contents of heparin and SPM in the PIC. The purified PIC, obtained under various pH conditions, were dissociated by the addition of sodium hydroxide, and then SPM was separated from heparin using a



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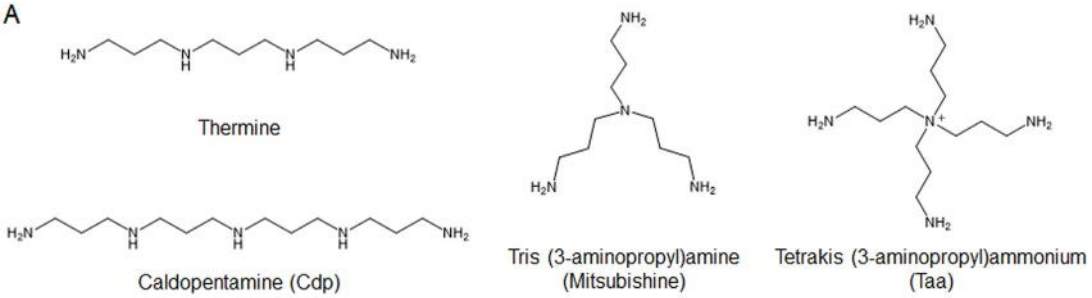
pH	1	2	3	4	5	6	7	8	9
SPM (nmol/nmol heparin)	38	32.5	29	38.3	44.5	35.2	5	1.7	3.9

Figure 2. The contents of SPM and heparin in PIC at diverse pH values. (A) Chromatograms of SPM and heparin. (B) The contents of SPM and heparin in PIC at diverse pH values. (C) The number of SPM molecules/heparin at diverse pH values. PIC stability under various pH conditions and the determination of heparin and SPM in PIC were investigated as described under "Materials and Methods." Values are indicated as means ± SE.

A



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Heparin (mg/mL)* (Mw. 13000)	Thermine (mM)* 1	10	20	Caldopentamine (mM)* 1	10	20
1	+ +	+ + +	+ +	+ +	+ +	+
5	+	+ + +	+ + +	+	+ + +	+ + +
10	–	+ + +	+ + +	–	+ + +	+ + +
	Tris (3-aminopropyl)amine (Mitsubishine) (mM)*			Tetrakis (3-aminopropyl)ammonium (Taa) (mM)*		
	1	10	20	1	10	20
1	+	+ +	+ +	+ +**	+ +**	+ +**
5	–	+	+ + +	+**	+ + +**	+ + +**
10	–	–	+ +	–**	+ + +**	+ + +**

C

Figure 3. Effect of unusual polyamines on the PIC formation efficiency. (A) The chemical structures of unusual polyamines. (B) Aqueous solutions of unusual polyamines and heparin were prepared at different concentrations and their equivalent volumes were mixed to generate PIC. The PIC formation efficiencies were evaluated by turbidimetric assay as described in Figure 1(C) The photograph shows the PIC generated from heparin + Taa. **, Visual evaluation.

10 K cut-off spin column. The resulting free heparin and SPM were quantitatively determined by HPLC (Figure 2). The content of heparin and SPM in the PIC, and PIC stability increased in a pH-dependent manner. Under various pH conditions, surface charge of the PIC was measured, and it was found that ζ-potential of the PIC increased under acidic conditions. These results support the idea that neutralization of the surface charge of the PIC is critical for its stability.

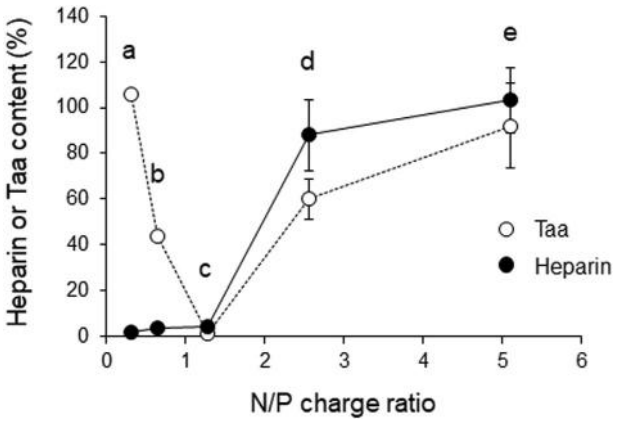

Figure 4. Effect of negative/positive (N/P) ratio on PIC formation with Taa. Heparin or Taa in supernatant of PIC solution was analyzed to determine the optimal concentration mixing ratio. Heparin (10 mg/mL) and Taa at the specified concentrations were mixed and centrifuged, resulting supernatant but not PIC pellet was submitted to HPLC. Concentrations of Taa in each sample were 40 mM (a), 20 mM (b), 10 mM (c), 5 mM (d) and 2.5 mM (e), respectively. N/P ratio was calculated using number of sulfonate and carboxy groups/heparin disaccharide and of amino group/Taa. Based on the result of disaccharide analysis of heparin (see Materials and Methods), average number of sulfo and carboxy groups of heparin disaccharide repeating unit was determined to be ~3.38, while molecular weight of average molecular weight of disaccharide repeating unit was also determined to be 534. The number of amino groups of Taa was calculated to be +5. When 10 mM of Taa was mixed with 10 mg/mL of heparin, N/P ratio was calculated to be 1:3.

3.2. *Effect of unusual polyamines on PIC formation efficiency*

The effect of branched polyamines on PIC formation with heparin was examined. Linear and branched polyamines having same molecular weights were used to exclude the possibility that the effect was solely influenced by the parameter of molecular weight. Thus, the chemical compositions of thermine and caldpentamine (Cdp) are same as that of mitsubisine and Taa, respectively (Figure 3A). As shown in Figure 3B, the optimal conditions for PIC formation were different among the unusual polyamines. Particle size and ζ-potential of each PIC is shown in Table 1. Interestingly, the configuration and the yellow color of the PIC generated from Taa were significantly different from the PICs generated from other polyamines. The PIC generated from Taa aggregated and sedimented soon in the water (Figure 3C); while a turbid white suspension of particles was observed for PICs generated from other polyamines, including mitsubisine. PIC pellets generated from Taa were very sticky and a turbid suspension was not obtained even with stirring. Additionally, the pellets did not dissociate on the addition of sodium hydroxide. In general, heparin may exist as a linear form in neutral pH and the electrostatic repulsion of each sulfonate/carboxy group of the heparin chain must affect their coagulation. It may have been possible to change their forms in solution as a complex, after some parts of the negatively charged acidic groups of heparin were electronically neutralized by amines. Although PIC particles of heparin (13 kDa, 7 kDa and 3.5 kDa) and linear polyamines such as SPD, SPM, thermine and Cdp were successfully formed, they were unstable in basic conditions. Thus, the association of linear polyamines and linear heparin in parallel positions could not keep their particles in basic conditions. In the case of branched polyamines, particularly, Taa showed a different PIC formation with linear heparin. Considering that the chemical composition of Taa is same as that of Cdp (Figure 3A), Taa may have caused structural changes in heparin (or a bridge connection between heparin), and thereby associates with heparin more effectively with a stable form.

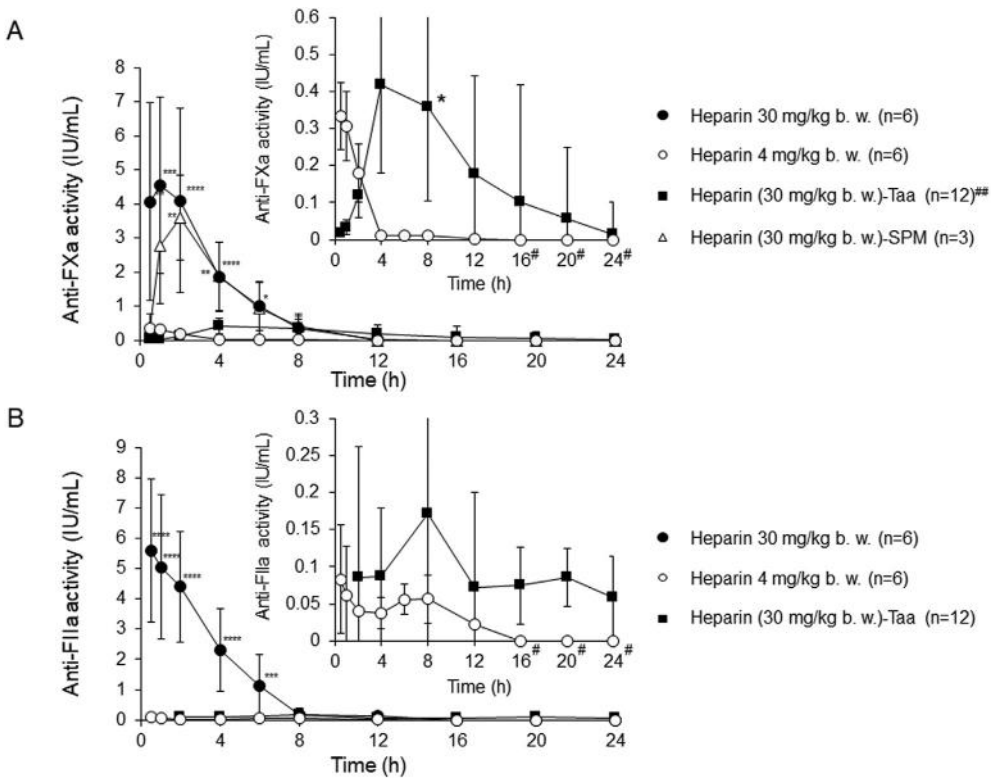

Figure 5. PIC with Taa allows sustained release of heparin. Activities of anti-FXa (A) anti-FIIa (B) of heparin released from PIC in subcutaneous tissue. PIC was generated by mixing equivalent solutions of 20 mg/mL of heparin and 80 mM Taa, which were then centrifuged (800× g, 10 min). After centrifugation, supernatant was removed and resulting pellet was suspended with cone oil. Thus, unfractionated heparin (4 or 30 mg/kg body weight) or PIC (30 mg/kg body weight of heparin) was administered subcutaneously to the back of the mice. After administration of PIC with Taa, blood samples were collected at each time point. Values are indicated as means ± SD. Statistical analysis was performed using GraphPad Prism 8.4.3, using one-way ANOVA followed by Dunnett's test, comparing heparin (30 mg/kg) vs. heparin (4 mg/kg), heparin (30 mg/kg)-SPM vs. heparin (4 mg/kg), heparin (30 mg/kg)-Taa vs. heparin (4 mg/kg). $^{****}p < 0.001$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ against heparin (4 mg/kg). #: Activities of anti-FXa and anti-FIIa were not detected in mice administered heparin (30 mg/kg), heparin (4 mg/kg) and heparin (30 mg/kg)-SPM. ##: anti-FXa activity in mice (n = 6) was measured after 0.5 and 1 h.

Next, we tried to analyze the contents of Taa and heparin in PIC by HPLC to determine the optimal mixing ratio, as transmittance (%*T*) of PIC from Taa was not measurable. However, it was difficult to determine the Taa and heparin contents in PIC, as it was very stable under basic conditions. Therefore, Taa and heparin supernatant obtained by the centrifugation of PIC was analyzed. If Taa and heparin are used up for formation of PIC, their concentrations might have decreased. As shown in Figure 4, heparin and Taa contents in the supernatant drastically decreased when the negative/positive (N/P) charge ratio is 1:3. These results suggest that PIC formation from heparin and Taa is dependent on electrostatic interactions.

3.3. PIC with Taa extended the time to maximum heparin concentration in the therapeutic range

Physical mixtures of cationic polymers and heparin have been evaluated *in vitro* and *in vivo* [10, 12, 13, 15, 16, 24]. PIC with Taa was very stable, even though the PIC was suspended in basic conditions. Therefore, the release of heparin from PIC administered subcutaneously to mice was investigated. Plasma heparin concentration was monitored by measuring the anti-FXa activity. The reported therapeutic range for anti-FXa activity and half-life of unfractionated heparin are 0.3–0.7 IU/mL [25, 26] and 63 ± 15 min [27], respectively. In the study, heparin (4 mg/kg body weight) was administered subcutaneously to mice and was absorbed by the mice. Maximum activity was achieved at therapeutic range; 0.5–1 h after injection, while rapid decrease of its activity was observed from 2 to 4 h (Figure 5A). In case of high-dose of heparin (30 mg/kg), significant increase of anti-FXa activity was observed, however, heparin clearance was similar to low-dose heparin (4 mg/kg) and its activity disappeared from 16 to 24 h. Subcutaneous administration of PIC containing heparin (30 mg/kg) and SPM was also examined, however, heparin clearance did not change. However, PIC containing heparin (30 mg/kg) and Taa showed prolonged time to maximum concentration within the therapeutic range at 4–8 h and weak activity of anti-FXa was maintained at 24 h after subcutaneous administration (Figure 5A). In fact, plasma anti-FXa activity of mice administered PIC-Taa from 16 to 24 h was detected in eight mice among a total of 12 mice (66.7% of mice). The sticky PIC material prepared with heparin and Taa made it difficult to prepare a uniform suspension with cone oil for injection, thus, the detection of anti-FXa activity was uneven in the plasma samples of individual mice. It has been reported that anticoagulant activity of heparin was inhibited by SPM *in vitro* [28]. However, plasma Taa was not detected in mice administered PIC (data not shown). This result suggests that heparin might have been released after degradation of PIC (especially Taa by amine oxidase) in the subcutaneous tissue [29]. As mentioned earlier, inactivation of thrombin by AT requires more than 18 saccharides of heparin (molecular weight<5400) [14]. Thus, anti-FIIa activity was also analyzed to confirm the sustained release of heparin with a longer chain length from PIC with Taa (Figure 5B). As a result, maximum anti-FIIa activity was 8 h and its weak activity was retained at 24 h after subcutaneous administration. This result suggests that heparin having more than 18 saccharides was gradually released from the PIC in the subcutaneous tissue.

4. Conclusion

In this study, we prepared PICs from unfractionated heparin and polyamines. PIC formation was influenced by the concentration ratio of heparin and polyamine, the molecular weight of heparin, the nature of polyamines, and the pH conditions. Interestingly, PIC generated from heparin and Taa exhibited stability and had a sticky form. Its formation was based on the electrostatic interactions between heparin and Taa. Additionally, maximum heparin concentration within the therapeutic range of heparin was markedly increased compared to that after a single dose of heparin. Taa was not detected in the plasma of mice administered PIC. These results suggest that PIC with Taa, administered subcutaneously, allows the sustained release of heparin into the blood. Recent reports have shown that heparin can inhibit the entry of COVID-19 through binding with spiked glycoprotein [30, 31]. Furthermore, anti-coagulant activity of heparin reduces the mortality of hospitalized patients with

severe COVID-19; complicated with coagulopathy [32]. Thus, stable sustained release of heparin from PIC might be of use COVID-19 management, if the sticky nature of PIC with Taa is improved. Ongoing research studies aim to improve the configuration of PIC with Taa.

Declarations

Author contribution statement

K. Higashi and T. Toida: Conceived and designed the experiments; Wrote the paper.

D. Ito, D. Ge and H. Manaka: Performed the experiments; Analyzed and interpreted the data.

N. Kogure, Y. Terui, H. Takayama and R.J. Linhardt: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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