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Polycationic γ-Cyclodextrin with Amino Side Chains for a Highly Efficient Anti-Heparin Coagulant

Wen-Jin An, Zhuo Lei, Xiao-Yong Yu, Chu-Han Liu, Chen Zhang, Yong Chen, and Yu Liu**

Multicharged cyclodextrins have attracted significant attention because of their applications in biology and pharmaceuticals. This study reports an aminoethoxy-phenyl-pyridinium-modified γ-cyclodextrin (PyA-γ-CD) as a highly efficient coagulant for heparin through multivalent interactions. The UV titration experiment is performed to obtain apparent binding constants (K_{obs}) between PyA-γ-CD and heparin as high as 9.85×10^6 M^{-1}. The activated partial thromboplastin time (aPTT) experiment in porcine plasma indicates that PyA-γ-CD not only exhibits nearly complete neutralization activity for unfractionated heparin (UFH), but more importantly, it also effectively neutralizes three LMWHs (dalteparin (Dalte), enoxaparin (Enoxa), and nadroparin (Nadro)) with a broader therapeutic window compared to protamine. The top neutralization activity of PyA-γ-CD for UFH, Dalte, Enoxa, and Nadro is 94%, 91%, 99%, and 85%, respectively. Interestingly, in vivo assays in mice further suggest that PyA-γ-CD significantly reverses the severe bleeding caused by heparin overdose while exhibiting remarkable biocompatibility. Therefore, PyA-γ-CD holds significant potential as a heparin antidote for clinical applications.

1. Introduction

In the development of modern medicine, many prescription drugs and medications have been designed to treat complex diseases.[1] However, drug overdose can lead to serious side effects and may even threaten life.[2] Currently, cyclodextrins (CDs), a class of cyclic oligosaccharides formed from d-pyranose glucose units linked by α-1,4-glycosidic bonds, are widely used in the fields of biology and pharmaceuticals due to their significant advantages, including low cost, ease of chemical modification, and excellent biocompatibility.[3] Moreover, multicharged cyclodextrins have been effectively applied to mitigate the adverse effects related to drug overdose and toxicity, thereby promoting a return to normal metabolic levels.[4] For example, Zhang et al. designed a carboxylated γ-CD derivative (Org 25 969) to reverse the effect of neuromuscular blocks (NMBs).[5] The negatively charged groups of Org 25 969 would bind to the positively charged rocuronium by electrostatic interactions, allowing rocuronium to be completely encapsulated into the cavity of Org 25 969. Furthermore, Org 25 969 exhibited good reversal activity for rocuronium-induced NMBs both in vitro and in vivo. Liu et al. reported that tyramine-modified β-CD can inhibit and reverse the inherent cytotoxicity of bile acids.[6] Compared to the corresponding natural β-CD, l- and d-Tyr-β-CDs, tyramine-modified β-CD formed a more stable supramolecular complex with deoxycholic acid (DCA). In vivo experiments in mice indicated that excess DCA can be eliminated via the urinary system, helping maintain normal blood concentrations of DCA.

It is well known that heparin, a naturally occurring anionic polysaccharide with a high negative charge density, is commonly used as an anticoagulant in surgical procedures.[7] Heparin can inhibit the activity of antithrombin-III by binding to it, thus hindering the coagulation process. To preserve fluidity during blood dialysis, unfractionated heparin (UFH) is frequently employed, in contrast to low-molecular-weight heparins (LMWHs) and the synthetic fondaparinux, which are used to prevent thrombotic conditions such as deep vein thrombosis and pulmonary embolism.[8] Protamine is currently the only FDA-approved antagonist for quickly restoring coagulation function after surgical procedures involving heparin.[9] However, the clinical application of protamine is limited because it only partially neutralizes LMWHs and has almost no effect on fondaparinux.[10] It may also cause side effects, including hypotension, bradycardia, thrombocytopenia, and allergic reactions.[11] Additionally, the production and quality of protamine is influenced by factors such as the source, climate, and environmental conditions.[12] Therefore, there has been significant interest in the search for anti-heparin coagulants that exhibit strong neutralization effects, are synthesized through chemical methods, and do not result in severe adverse effects.[13]

To meet these needs, various potential anti-heparin coagulants have been developed, including small molecules,[14] cationic polymers,[15] proteins, and peptides.[16] Li et al. constructed two porous organic polymers with abundant positive charges, supramolecular organic frameworks (SOFs) and porous organic polymers (POPs), as novel heparin antidotes.[17] Due to the intrinsic nanoscaled porosity, these porous polymers can effectively adsorb heparin within their interior. Wang et al. designed oligoethylene glycol functionalized guanidinocalixarene (GC4AOEG),

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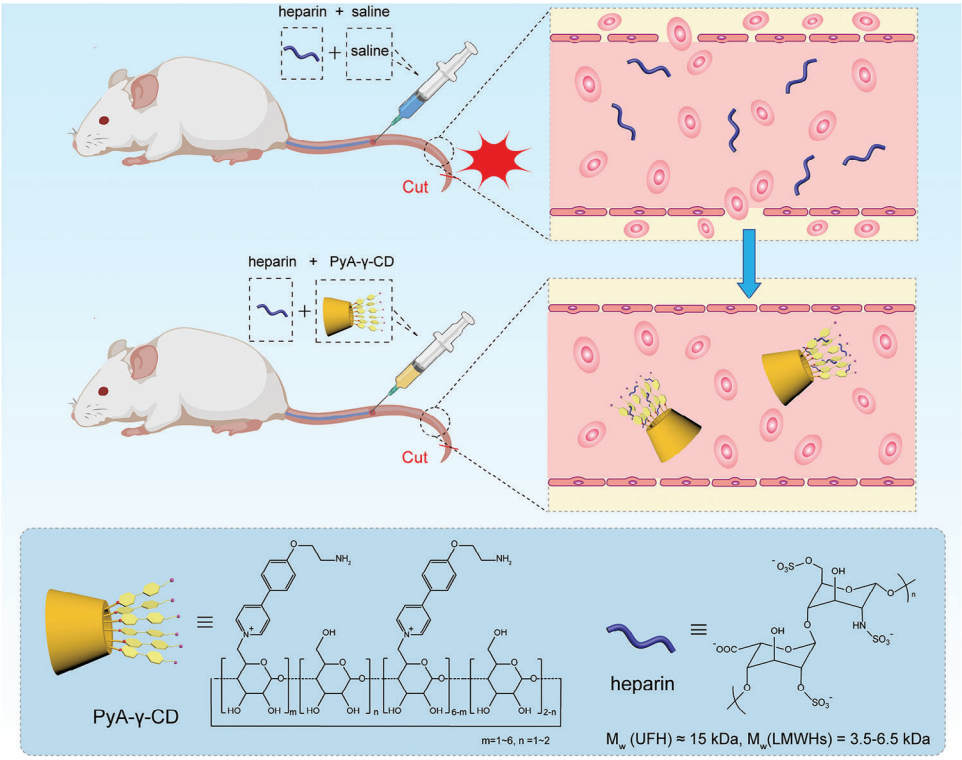

Scheme 1. The neutralization process of PyA-γ-CD for heparin and the corresponding structures of PyA-γ-CD and heparin.

where the abundant guanidinium groups and the OEG groups achieved specific recognition and biocompatibility.[18] Therefore, GC4AOEG significantly reversed the effects in mice administered heparin. Liu et al. synthesized a series of amphiphilic multicharged β-CD (AMCD) assemblies as agents against heparin.[19] With this special structure, the positively charged imidazolium units and the cyclodextrin cavity on the AMCD assemblies can bind heparin through multivalent interactions. Meanwhile, the strategy of co-assembling AMCD with vitamin K (VK) can effectively capture heparin and release VK, thereby achieving a synergistic coagulation effect. Although research on heparin reversal agents has attracted significant attention, there are few reports regarding multicharged cyclodextrins as coagulants, to the best of our knowledge.

Herein, we reported aminoethoxy-phenyl-pyridinium-modified γ-cyclodextrin (PyA-γ-CD) as a highly efficient anti-heparin coagulant. The molecule of PyA-γ-CD is multicharged and can be easily prepared by modifying octakis-(6-iodo-6-deoxy)-γ-CD with 4-phenylpyridinium units. Additionally, the 2-aminoethoxyl chain was introduced at the rim of the cavity to enhance binding to heparin through electrostatic and hydrogen bonding interactions. The zeta potential titration experiment indicated that PyA-γ-CD had the ability to neutralize heparin through electrostatic interactions, and the UV titration experiment further confirmed its high binding affinity for heparin. Activated partial thromboplastin time (aPTT) assays confirmed that PyA-γ-CD effectively neutralized four heparins in porcine plasma at a wide therapeutic dose range. Although the neutralization activity of PyA-γ-CD for heparin slightly decreased after reaching the top neutralization, the therapeutic window was significantly broader than that of protamine. Furthermore, the evaluations of in vivo antagonistic efficacy and biocompatibility showed that PyA-γ-CD can completely reverse bleeding caused by anticoagulant overdose, with no adverse effects observed in vivo. Therefore, this novel heparin antidote holds the potential application value in clinical treatment (**Scheme 1**).

2. Results and Discussion

2.1. Synthesis and Characterization

A supramolecular γ-cyclodextrin derivative (PyA-γ-CD) was developed as a heparin antidote. Given the easily modifiable characteristics of CDs, we choose a larger γ-CD host as the base scaffold instead of α-CD or β-CD. Briefly, the product PyA-γ-CD was obtained through a nucleophilic substitution reaction of octakis-(6-iodo-6-deoxy)-γ-CD and **2**, followed by the removal of protecting groups using 2 M hydrogen chloride in ethyl acetate (**Figure 1a**). The characterization of intermediates (Figures S1 and S2, Supporting Information) and PyA-γ-CD (Figures S3–S5, Supporting Information) were depicted in the Supporting Information. By comparing the integral ratios of H-1 protons at 5.00–5.22 ppm with the b/b' protons at 8.03–8.35 ppm in ^{1}H NMR spectrum, the degree of substitution of PyA-γ-CD was determined to be 6 (Figure S4, Supporting Information). An online prediction platform was developed through a machine learning approach to predict the pK_a of the conjugate acid of PyA-γ-CD.[20] The predicted pK_a value was determined to be 7.54, which is close to the

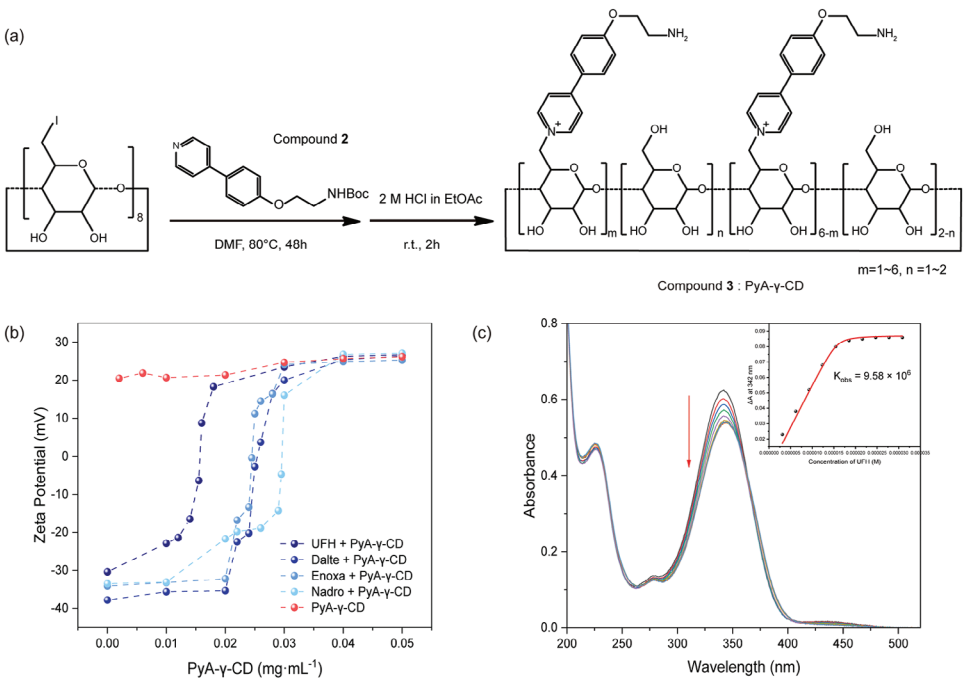

Figure 1. a) Synthetic route of PyA-γ-CD. b) Zeta potential variations with the addition of PyA-γ-CD solution (0–0.05 mg mL^{-1}) to UFH, Dalte, Enoxa, or Nadro solution (2 IU mL^{-1}). c) UV spectral variations with the addition of UFH solution (0–30.85 μM) to PyA-γ-CD solution (6.17 μM). (Inset) The absorbance of PyA-γ-CD solution (6.17 μM) at 342 nm with the addition of UFH solution (0–30.85 μM) to calculate K_{obs}.

physiological pH of 7.4. In the conditions, it was anticipated that ≈50% of the amino groups of PyA-γ-CD would be protonated, while the other 50% would exist as the free amino groups. Therefore, the strong binding between PyA-γ-CD and heparin was achieved through multivalent interactions. The abundant positively charged pyridinium units and partially protonated amino groups (NH_3^+) of PyA-γ-CD would interact with the negatively charged sulfonate and carboxylate anions of heparin through electrostatic interactions. Additionally, the unprotonated amino groups of PyA-γ-CD may contribute to forming intermolecular hydrogen bonds with heparin.

2.2. The Binding Behavior of PyA-γ-CD for Heparin

At the concentration of 2 IU mL^{-1}, the solution of UFH, Dalte, Enoxa, and Nadro exhibited a negative zeta potential of −30.4, −37.1, −34.1, and −33.5 mV, respectively (Figure 1b). An evident phenomenon was that a continuous decrease in the zeta potential of the heparin solution was observed with the addition of the PyA-γ-CD solution. At the concentration of 0.03, 0.04, 0.03, and 0.04 mg mL^{-1} for PyA-γ-CD, the zeta potential of UFH, Dalte, Enoxa, and Nadro solution was +26.3, +25.4, +24.1, and +26.8 mV, respectively. These values corresponded to the free PyA-γ-CD solution and remained stable with further increases in concentration, suggesting that PyA-γ-CD could neutralize heparin by electrostatic interactions.

The UV titration experiment was conducted to derive the apparent binding constants (K_{obs}) between PyA-γ-CD and heparin. The absorbance continuously decreased with the addition of heparin solution to the PyA-γ-CD solution and then remained constant. The K_{obs} values between PyA-γ-CD and UFH, Dalte, Enoxa, and Nadro derived from UV titration experiment were 9.58×10^6 M^{-1}, 9.85×10^6 M^{-1}, 5.60×10^6 M^{-1} and 3.82×10^6 M^{-1}, respectively (Figures 1c and S6–S8, Supporting Information). The high binding affinity of PyA-γ-CD for heparin can be attributed to multivalent interactions. On one hand, the cationic pyridinium units and partially protonated amino groups (NH_3^+) of PyA-γ-CD interacted with the sulfonate and carboxylate anions of heparin through electrostatic interactions. On the other hand, the enhanced binding affinity was contributed by the additional intermolecular hydrogen bonds formed between the neutral amino groups of PyA-γ-CD and heparin.

2.3. Neutralization Activity Assays In Vitro

Given the strong binding affinity of PyA-γ-CD for heparin, as demonstrated by the above zeta potential and UV titration experiments, in vitro neutralization activity of PyA-γ-CD for heparin was further performed using aPTT assays in porcine plasma. A high dose of heparin (2 IU mL^{-1}) was considered an anticoagulant overdose situation. The heparinized plasma was treated with increasing doses of PyA-γ-CD to evaluate its neutralization activity for heparin, while the neutralization activity of protamine for heparin was assessed as a reference. The aPTT assays showed that PyA-γ-CD displayed high neutralization activity for heparin in porcine plasma (**Figures** 2a–d and S9 and Table S1, Supporting Information). Meanwhile, the negligible neutralization activity of free PyA, obtained by directly removing the protecting groups from compound **2** for heparin under the same conditions, further supported the significant role of PyA-γ-CD in heparin antag-

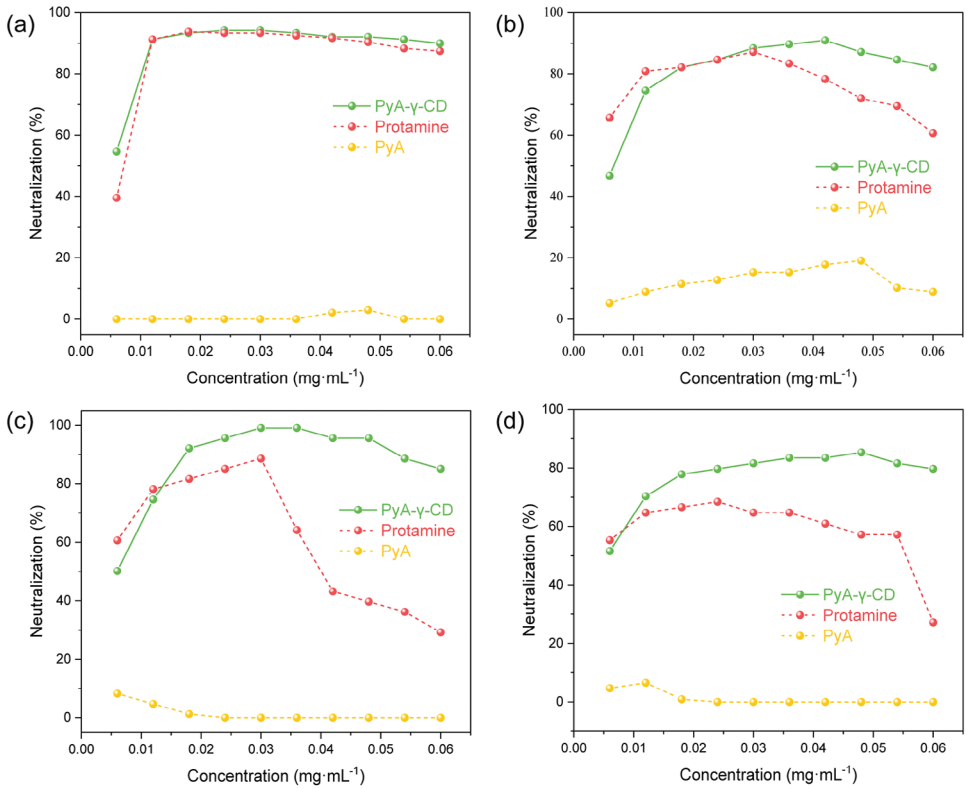

Figure 2. The neutralization activity (%) of a) UFH, b) Dalte, c) Enoxa, and d) Nadro plasma (2 IU mL^{-1}) treated with increasing doses of PyA-γ-CD, protamine, or PyA (0.006–0.06 mg mL^{-1}).

onism (Figure 2a–d). The top neutralization activity of PyA-γ-CD for UFH was 94%, and it could retain over 90% neutralization activity in the dose range of 0.012 to 0.06 mg mL^{-1} (Figure 2a). More importantly, PyA-γ-CD also showed a wide therapeutic window for the three LMWHs. The top neutralization activity of PyA-γ-CD for Dalte, Enoxa, and Nadro was 91%, 99%, and 85%, respectively, after which the neutralization activity slightly decreased. At a dose of up to 0.06 mg mL^{-1}, the neutralization activity of PyA-γ-CD for Dalte, Enoxa, and Nadro remained at 82%, 85%, and 80%, respectively (Figure 2b–d). In contrast, protamine only had a neutralization activity of over 90% for UFH (Figure 2a). The top neutralization activity of protamine for Dalte, Enoxa, and Nadro was 87%, 89%, and 69%, respectively, following a rapid decrease in the neutralization activity. At a dose of up to 0.06 mg mL^{-1}, the neutralization activity of protamine for Dalte, Enoxa, and Nadro decreased to only 61%, 29%, and 27%, respectively, indicating that the risk of bleeding may be associated with high dosage (Figure 2b–d). All the results suggested that PyA-γ-CD not only performed nearly complete antagonism for UFH, but, more importantly, also effectively antagonized three LMWHs with a broader therapeutic window as compared to protamine.

2.4. Hemolysis and Cell Viability Evaluation

Hemolysis and cell viability were further investigated to assess the biocompatibility of PyA-γ-CD at the cellular level. Previous research has demonstrated that the negatively charged surfaces of red blood cells (RBCs) can interact with cationic groups on polymers through electrostatic interactions, which may cause damage to RBCs.[21] Therefore, evaluating the hemolysis of PyA-γ-CD is a critical parameter. The hemolysis of PyA-γ-CD was assessed in 5% rabbit RBCs, indicating that PyA-γ-CD exhibited nearly negligible hemolysis (<5%) within the concentration range of 0.02–0.2 mg mL^{-1}, which was significantly higher than the dosage of PyA-γ-CD used for antagonizing heparin in aPTT assays (**Figure 3**a,b). The cytotoxicity of PyA-γ-CD was also evaluated using L929 cells (a mouse fibroblast cell line) and 293T cells (a human embryonic kidney cell line) through a CCK-8 staining assay. The cell viability of L929 and 293T cells was over 87% and 83%, respectively (Figure 3c,d), within the concentration range of 0.025–0.2 mg mL^{-1}, indicating that PyA-γ-CD exhibited very low cytotoxicity to these cells.

2.5. Neutralization Efficacy Assays In Vivo

Encouraged by the low hemolysis and high cell viability of PyA-γ-CD, we further investigated its in vivo neutralization efficacy for heparin in mice. The tail transection model in mice has long been regarded as a reliable method for antagonizing heparin in vivo (**Figure 4**a).[22] UFH and Enoxa were selected as representatives, which based on the neutralization ability of PyA-γ-CD and protamine for UFH or Enoxa, showed a more significant difference compared to Dalte or Nadro in aPTT assays. A high dose of heparin (200 IU kg^{-1}) was considered an anticoagulant overdose

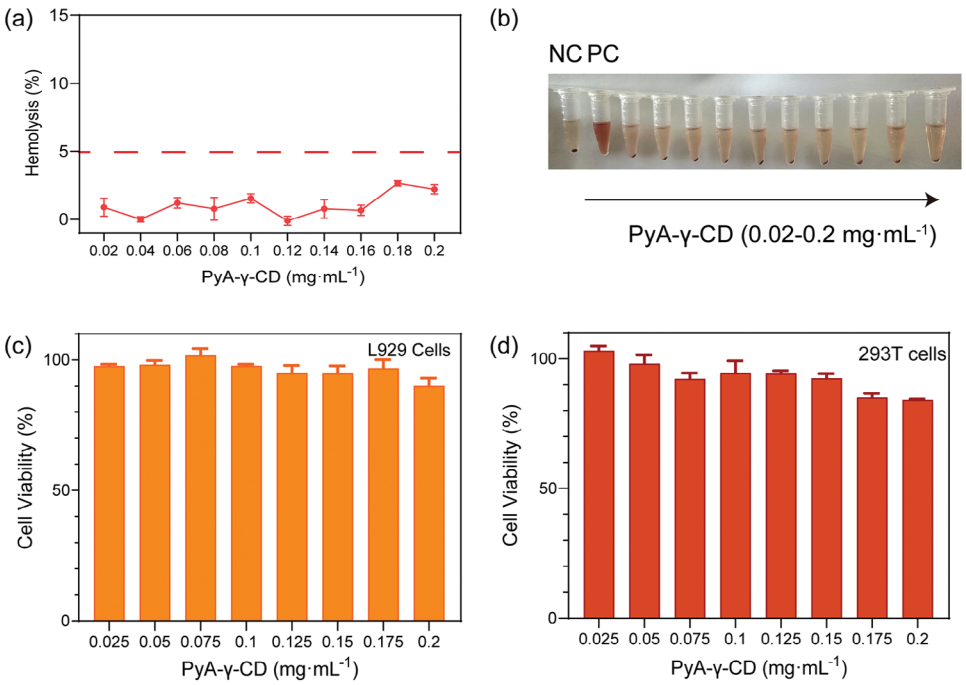

Figure 3. a,b) The hemolysis (%) of PyA-γ-CD at various concentrations (0.02–0.2 mg mL^{-1}) in 5% MRBCs. Cell viability (%) of c) L929 cells and d) 293T cells incubated with PyA-γ-CD at various concentrations (0.025–0.2 mg mL^{-1}) for 24 h. Each result represented the mean ± S.E.M. (n = 3).

situation. In the experiment, forty ICR mice were randomly assigned to seven groups to study the in vivo neutralization efficacy of PyA-γ-CD for heparin: i) Saline normal control group: saline + saline; ii) Heparin negative control group: UFH (200 IU kg^{-1}) + saline or Enoxa (200 IU kg^{-1}) + saline; iii) Experimental group: UFH (200 IU kg^{-1}) + PyA-γ-CD (2.6 mg kg^{-1}) or Enoxa (200 IU kg^{-1}) + PyA-γ-CD (2.6 mg kg^{-1}); iv) Protamine positive control group: UFH (200 IU kg^{-1}) + protamine (2.6 mg kg^{-1}) or Enoxa (200 IU kg^{-1}) + protamine (2.6 mg kg^{-1}). All groups were given intravenous administration via the mice tail vein at 0 min

(a)

Group	the first injection (0 min)	the second injection (7 min)
Saline	Saline	Saline
UFH	UFH	Saline
UFH + PyA-γ-CD	UFH	PyA-γ-CD
UFH + Protamine	UFH	Protamine
Enoxa	Enoxa	Saline
Enoxa + PyA-γ-CD	Enoxa	PyA-γ-CD
Enoxa + Protamine	Enoxa	Protamine

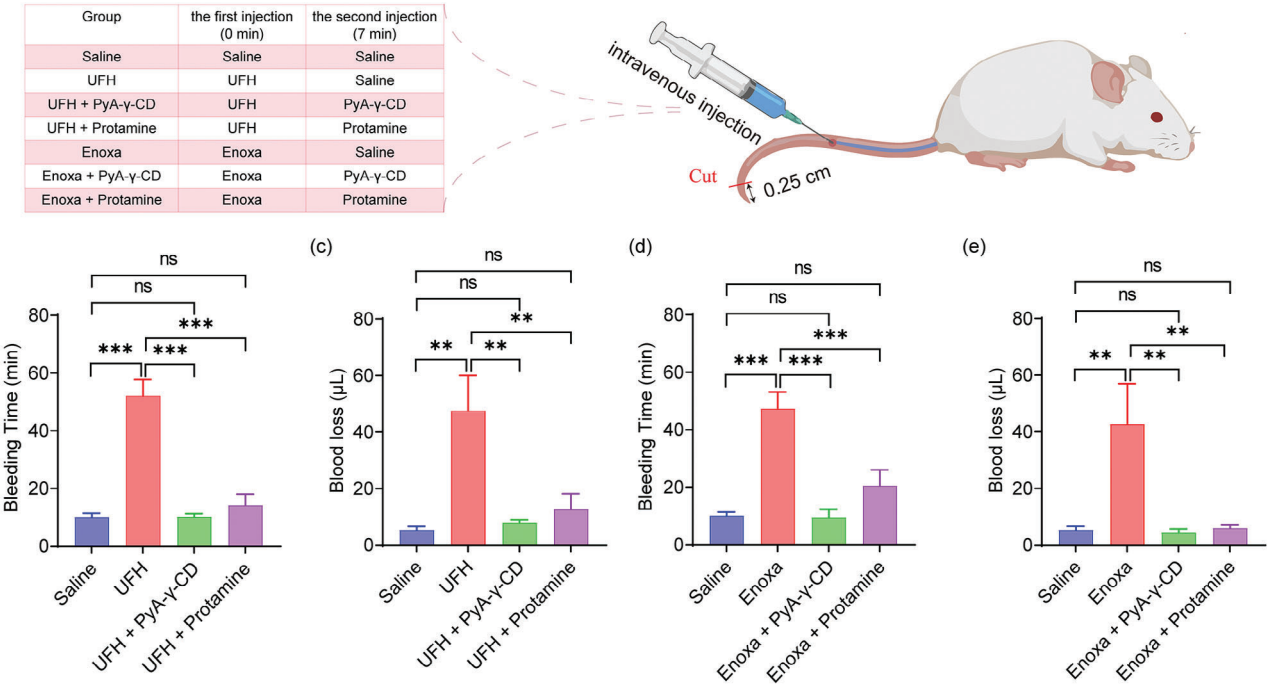

Figure 4. a) A schematic representation of the tail transection model in mice. b,d) The blood loss time (min) and c,e) blood loss volume (μL). Each result represented the mean ± S.E.M. (n = 5). *$p < 0.05$, ***$p < 0.001$, and ns represent "no significant difference".

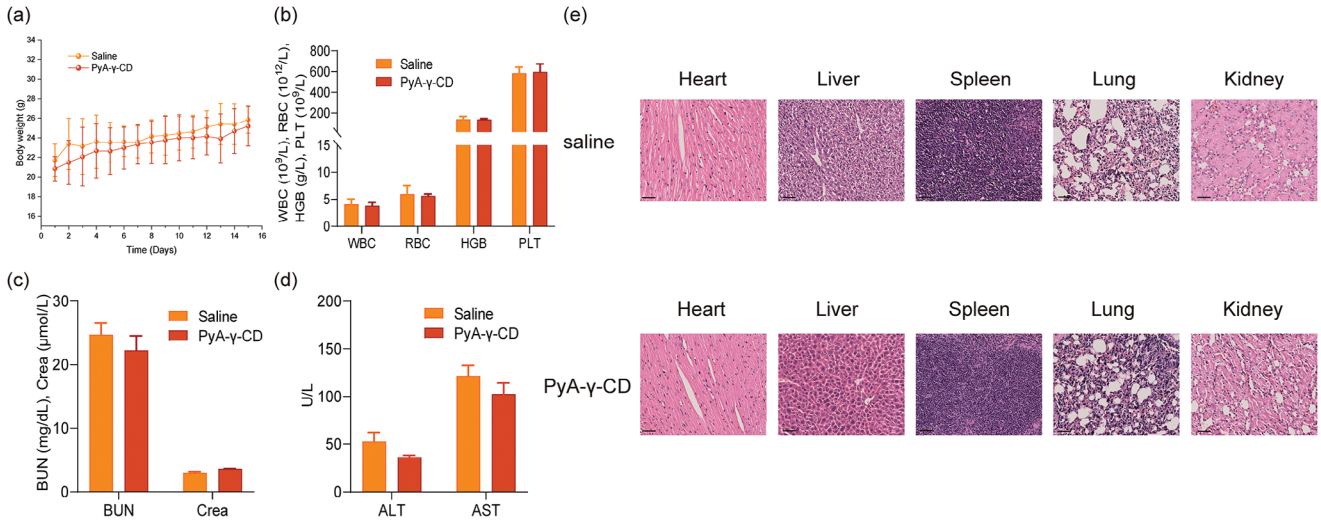

Figure 5. a) The body weight of mice in fifteen days was recorded following the intravenous administration of PyA-γ-CD (2.6 mg kg^{-1}) or saline. Each result represented the mean ± S.E.M. (n = 5). b) The hematological parameters, including WBCs, RBCs, HGB, and PLT in mice five days after the intravenous administration of PyA-γ-CD (2.6 mg kg^{-1}) or saline. c) Renal and d) hepatic function biomarkers, including BUN, Crea, ALT, and AST in mice five days after the intravenous administration of PyA-γ-CD (2.6 mg kg^{-1}) or saline. Each result represented the mean ± S.E.M. (n = 3). e) Histopathological morphology of major organs in mice five days after intravenous administration of PyA-γ-CD (2.6 mg kg^{-1}) or saline. (scale bar = 50 μm).

for the first injection (saline, UFH or Enoxa) and at 7 min for the second injection (saline, PyA-γ-CD or protamine), and then the distal 0.25 cm of the mice tail was transected after another 7 min. The bleeding time was recorded until no additional blood appeared, and the blood collected with filter paper was dissolved in 10 wt% NaOH solution. The blood loss volume was determined by assessing the absorbance at 405 nm according to a standard curve (Figure S10, Supporting Information). For the saline control group, the average bleeding time and blood loss volume were 10.03 ± 2.95 min and 5.22 ± 3.40 μL, respectively (Figure 4b–d; Table S2, Supporting Information). After administration with UFH or Enoxa (200 IU kg^{-1}), the average bleeding time and blood loss volume increased to 52.02 ± 12.85 min and 47.62 ± 27.78 μL and to 47.34 ± 12.82 min and 42.74 ± 31.99 μL, respectively. Interestingly, for the UFH or Enoxa (200 IU kg^{-1}) group further treated with PyA-γ-CD (2.6 mg kg^{-1}), the average bleeding time and blood loss volume were significantly reduced to 10.17 ± 2.42 min and 7.98 ± 2.16 μL and to 9.49 ± 6.22 min and 4.38 ± 2.59 μL, respectively (Figure 4b–d; Table S2, Supporting Information). These values were comparable to those of the saline group, indicating that PyA-γ-CD can completely neutralize UFH and Enoxa. In contrast, for the UFH or Enoxa (200 IU kg^{-1}) group further treated with protamine (2.6 mg kg^{-1}), the average bleeding time and blood loss volume were reduced to 14.21 ± 8.39 min and 12.70 ± 12.12 μL and to 20.60 ± 12.18 min and 5.90 ± 2.98 μL, respectively (Figure 4b–d; Table S2, Supporting Information). Importantly, the average bleeding time in Enoxa (200 IU kg^{-1}) group treated with protamine (2.6 mg kg^{-1}) was not completely reversed to the level of the saline group, indicating that PyA-γ-CD exhibited higher in vivo neutralization efficacy for Enoxa compared to protamine (Figure 4c). All results in mice further supported the significant antagonistic efficacy of PyA-γ-CD for heparin, both in vitro and in vivo.

2.6. Biocompatibility Evaluation In Vivo

As in vivo studies have demonstrated that PyA-γ-CD can effectively neutralize heparin, further investigations were conducted to evaluate the in vivo biocompatibility of PyA-γ-CD at the effective dose. After intravenous administration of PyA-γ-CD at a dose of 2.6 mg kg^{-1} or saline via the tail vein of mice, the weight changes, survival rate, or any abnormal signs of mice were continuously monitored for fifteen days. All the treated mice exhibited normal growth comparable to the saline group (**Figure 5a**; Table S3, Supporting Information). The mice were euthanized, and blood and the major organs (heart, liver, spleen, lungs, and kidneys) were collected five days after the administration of PyA-γ-CD (2.6 mg kg^{-1}). The hematological parameters of mice treated with PyA-γ-CD (2.6 mg kg^{-1}), including white blood cells (WBCs), RBCs, hemoglobin (HGB), and platelets (PLT), were comparable to those of the saline group (Figure 5b). Additionally, the liver and kidney injury markers of mice treated with PyA-γ-CD (2.6 mg kg^{-1}), such as blood urea nitrogen (BUN), creatinine (Crea), alanine aminotransferase (ALT), and aspartate aminotransferase (AST), also displayed negligible difference compared to the saline group (Figure 5c,d). Hematoxylin and eosin (H&E) staining results indicated that no abnormalities were observed in the tissue sections, suggesting that PyA-γ-CD did not cause any damage to the major organs (Figure 5e). In conclusion, these results demonstrated that PyA-γ-CD, at the effective dose used for treating heparin in vivo, is biocompatible.

3. Conclusion

In this study, we introduced the potential of PyA-γ-CD as a novel and efficient antidote for heparin antagonism. The efficient neutralization of heparin anticoagulants was achieved through multivalent interactions. The cationic pyridinium units and the

partially protonated amino groups (NH_3^+) of PyA-γ-CD can bind to the sulfonate and carboxylate anions of heparin through electrostatic interactions. Additionally, the neutral amino groups of PyA-γ-CD should also form extra hydrogen bonds with heparin to enhance binding affinity. The binding behavior of PyA-γ-CD for heparin was demonstrated in zeta potential and UV titration experiments. The high binding affinity of PyA-γ-CD for heparin was estimated to reach 10^6 M^{-1}. The aPTT results indicated that PyA-γ-CD not only displayed nearly complete reversal activity for UFH in porcine plasma, but more importantly, it also effectively reversed three LMWHs with a broader treatment window compared to the clinically used protamine. The efficacy of PyA-γ-CD in neutralizing heparin was further verified through in vivo evaluations using a tail transection model in mice. Furthermore, PyA-γ-CD exhibited low hemolysis and high cell viability at the cellular level, and its excellent biocompatibility in vivo has also been demonstrated through dose safety evaluations and histopathological examinations. Therefore, the present study provides a new approach for using supramolecular macrocyclic compounds as coagulants through multivalent interactions.

4. Experimental Section

General Procedures: The ^{1}H NMR and 2D COSY spectra were obtained on Bruker AV400 spectrometers. The chemical shifts were referenced to the solvent peak at 25 °C. High resolution mass spectrometry (HRMS) was performed on a Bruker Solarix scimax MRMS spectrometer. Zeta potential measurements were performed on a Malvern Panalytical Zetasizer Nano ZS. UV spectrum measurements were recorded on a Shimadzu UV-3600 spectrophotometer. Hemolysis, cell viability, and blood loss volume assays were conducted on an AMR-100 microplate reader.

*Synthesis of compound **1**, 4-(pyridin-4-yl)phenol*: 4-Hydroxyphenylboronic acid (17.3 g, 123 mmol), 4-bromopyridine hydrochloride (20 g, 103 mmol), and potassium carbonate (42.6 g, 309 mmol) were added to a mixed solution of DMF/H_2O (2:1, 300 mL) under N_2 atmosphere. Then $Pd(PPh_3)_4$ (100 mg, 0.09 mmol) was added, and the mixture was stirred at 110 °C overnight. After the solution was cooled to room temperature, H_2O (60 mL) was added ropwise. The precipitate formed was collected by filtration, recrystallized and dried to yield a white product, compound **1**. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.83 (s, 1H), 8.54 (d, J = 6.2 Hz, 2H), 7.66 (d, J = 8.6 Hz, 2H), 7.61 (d, J = 6.2 Hz, 2H), 6.89 (d, J = 8.6 Hz, 2H).

*Synthesis of Compound **2**, tert-butyl (2-(4-(pyridin-4-yl)phenoxy)ethyl)carbamate*: Compound **1** (10 g, 58.4 mmol) and potassium carbonate (16.1 g, 117 mmol) were dissolved in a suitable reaction vessel with DMF (150 mL) and then 2-(Boc-amino)-ethyl bromide (26.2 g, 117 mmol) was added to the solution. The mixture was stirred at room temperature overnight and then added dropwise to ice water (300 mL). The resulting precipitate formed was collected by filtration, washed with H_2O, and dried to yield a white product, compound **2**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.58 (d, J = 4.6 Hz, 2H), 7.77 (d, J = 8.8 Hz, 2H), 7.69-7.63 (m, 2H), 7.06 (dd, J = 10.4, 7.3 Hz, 3H), 4.02 (t, J = 5.8 Hz, 2H), 3.32-3.27 (m, 2H), 1.38 (s, 9H).

*Synthesis of Compound **3**, PyA-γ-CD*: Octakis-(6-iodo-6-deoxy)-γ-cyclodextrin (1.0 equiv) and compound **2** (16.0 equiv) were dissolved in an appropriate reaction vessel with DMF (500 µL) at 100 °C for 48 h under N_2 atmosphere. Then, the solution was cooled to room temperature and added dropwise to a vessel with cold acetone (100 mL). The precipitate formed was collected by filtration, washed with acetone, and dried to obtain Boc-PyA-γ-CD. Then, Boc-PyA-γ-CD was dissolved in 2 M hydrogen chloride in ethyl acetate (5 mL) and then stirred at room temperature for 2 h. The resulting precipitate formed was collected by filtration, washed with EtOAc, and dried to yield a yellow-brown product, compound **3** (PyA-γ-CD). ^{1}H NMR (400 MHz, D_2O): δ 8.86 (s, 2H), 8.72–8.37 (m, 10H), 8.35-8.03 (m, 12H), 7.99 (s, 5H), 7.85 (s, 2H), 7.71 (d, J = 7.0 Hz, 2H), 7.61 (d, J = 8.2 Hz, 3H), 7.23 (s, 2H), 7.01 (d, J = 6.8 Hz, 4H), 6.89 (s, 6H), 5.11 (d, J = 8.2 Hz, 8H), 4.51-4.20 (m, 12H), 4.18-3.42 (m, 48H), 3.28 (d, J = 30.0 Hz, 12H). HRMS (ESI) *m/z*: Calcd for $[M - 3I]^{3+}$, 953.60; Found, 953.63.

Zeta Potential Titration Experiment: Different concentrations of PyA-γ-CD solution (0.01–0.05 mg mL^{-1}) were gradually added to a fixed concentration of heparin solution (2 IU mL^{-1}) to measure the zeta potential values. Different concentrations of free PyA-γ-CD solution (0.002–0.05 mg mL^{-1}) were also measured as a reference.

UV Titration Experiment: Due to heparin did not have a conjugated functional group, heparin solution with different concentrations (0–30.85 µM) were gradually added to a fixed concentration of PyA-γ-CD solution (6.17 µM) to obtain a series of absorbance spectra. The variations in absorbance at 342 nm were analyzed and fitted to calculate K_{obs}.

aPTT Assays In Vitro in Porcine Plasma: *Preparation of a Series of Heparinized Plasma*: The heparinized plasma (2 IU mL^{-1}) was prepared by diluting a heparin solution (200 IU mL^{-1}, dissolved in saline) with porcine plasma. Different concentrations of heparinized plasma were prepared using the halving dilution method, with the concentrations of heparin being from 0.03125 to 2 IU mL^{-1}.

Preparation of a Series of Antagonistic Plasma: Different concentrations of PyA-γ-CD or protamine solution (0.06–0.6 mg mL^{-1}) were prepared by diluting the original concentration of PyA-γ-CD or protamine solution (0.6 mg mL^{-1}, dissolved in saline). Subsequently, different concentrations of PyA-γ-CD or protamine solution (10 µL, 0.06-0.6 mg mL^{-1}) were added to the heparinized plasma (90 µL, 2 IU mL^{-1}) to obtain the antagonistic plasma.

Neutralization Activity Assays in Vitro: The experimental process is described as follows: The test plasma (100 µL) and aPTT ellagic acid solution (100 µL) were individually placed in a water bath at 37 °C for 5 min. Following the incubation, the two solutions were mixed and incubated at 37 °C for an additional 5 min. After that, warmed $CaCl_2$ (100 µL, 25 mM) was added to the mixture, and the timing was initiated immediately while the solution was gently agitated continuously. Once fibrin filaments were observed, the timing was stopped, and the clotting time was recorded. In the experiment, the clotting time of blank porcine plasma was first tested to determine the normal clotting time. Then, the clotting time of a series of heparinized plasma (0.03125–2 IU mL^{-1}) was tested to obtain a standard curve. Additionally, the clotting time of a series of antagonistic plasma was tested to determine the neutralization effect. The neutralization activity (%) of PyA-γ-CD or protamine for heparin was calculated through the standard curve.

$$\text{Neutralization acitivity } (\%) = \frac{2 - C_{test\ plasma}}{2} \times 100\% \quad (1)$$

where $C_{test\ plasma}$ (IU mL^{-1}) refers to the concentration of remaining heparin that unneutralized by the antidote, as determined from the standard curve.

Hemolysis Evaluation: 2% rabbit RBCs in Alserver's solution were concentrated to 5% using saline for later use. A series of 700 µL solutions were prepared by mixing 5% RBCs (140 µL) and different concentrations of PyA-γ-CD solution (560 µL, 0.02–0.2 mg mL^{-1}) in saline. Subsequently, the mixtures were incubated in an incubator for 1 h at 37 °C. Following incubation, all solutions were centrifuged at 3000 rpm for 5 min. After that, the supernatant (100 µL) was transferred into the 96-well plate to measure the absorbance at 545 nm by a microplate reader. All experiments were performed in triplicate, and each data point was represented as the mean ± S.E.M. of three independent experiments.

$$\text{Hemolysis } (\%) = \frac{A_{samples} - A_{NC}}{A_{PC} - A_{NC}} \times 100\% \quad (2)$$

where the RBCs in saline were defined as the negative control (NC), and the RBCs in distilled water were defined as the positive control (PC).

Cell Viability Evaluation: The CCK-8 staining assay was employed to evaluate the cytotoxicity of PyA-γ-CD. L-929 cells and 293T cells were cultured in high-glucose DMEM with 10% fetal bovine serum and 1% penicillin streptomycin. Scattered cells (100 µL) were seeded into a 96-well plate and incubated in a 37 °C incubator with a 5% CO_2 atmosphere for 24 h. After that, the original culture medium was replaced with DMEM containing PyA-γ-CD at different concentrations (0.025–0.2 mg mL^{-1}). After incubating for 24 h, the old culture was removed, and the DMEM containing 10% CCK-8 was added to each well of the 96-well plate. The cells were incubated in a 37 °C incubator with a 5% CO_2 atmosphere for another 2 h, after which the absorbance of each well was measured at 450 nm by a microplate reader. All experiments were performed in six duplicates, and each data point was represented as the mean ± S.E.M. of three independent experiments.

$$\text{Cell } viability\ (\%) = \frac{A_{samples} - A_{Blank}}{A_{\text{Control}} - A_{Blank}} \times 100\% \quad (3)$$

where 10% CCK-8 DMEM with cells was the control group, while 10% CCK-8 DMEM without cells was the blank group.

Neutralization Efficacy Assays In Vivo in Mice: Forty ICR mice (female, 4–6 weeks, 20–25 g weight) were randomly assigned to eight groups for the tail transection experiment. In the saline normal control group, both the first and second injections were saline. In the heparin negative control group, the difference was that the first injection was UFH or Enoxa solution (200 IU kg^{-1}). In the experimental group, after administering UFH or Enoxa solution (200 IU kg^{-1}), PyA-γ-CD solution (2.6 mg kg^{-1}) was administered as the second treatment. In the protamine positive control group, after administering UFH or Enoxa solution, protamine solution (2.6 mg kg^{-1}) was administered as the second treatment. Heparin solution (20 IU mL^{-1}), PyA-γ-CD solution (0.52 mg mL^{-1}), and protamine solution (0.52 mg mL^{-1}) were prepared with saline. The administration volume was controlled within 0.1–0.15 mL based on the weight of each ICR mice. The experimental process is described as follows: The mice were restrained in holders, but blood circulation should be maintained. Subsequently, the mice were given intravenous administration via the mice tail vein at 0 min for the first injection (saline, UFH, or Enoxa solution, 0.1–0.15 mL) and at 7 min for the second injection (saline, PyA-γ-CD solution, or protamine solution, 0.1–0.15 mL). Following another 7 min, the distal 0.25 cm of the mice's tail was transected with scissors. The bleeding time was recorded until no further blood appeared, and the blood was collected with filter paper. Additionally, a standard curve of blood was established by measuring the absorbance of blood (405 nm) collected from the hearts of normal mice at various volumes (2.5–100 mL) dissolved in 3 mL of 10 wt% NaOH solution. The blood loss volume was calculated by measuring the absorbance of the lysed blood cells dissolved in the same concentration NaOH solution according to the standard curve.

Statistical Analysis: Results were presented as mean ± standard deviation. Statistical analysis of the data was carried out by one-way ANOVA with Duncan post-test (IBM SPSS Statistics 27). P values ≤ 0.05 were considered significant, $*p < 0.05$, $***p < 0.001$, and ns represent "no significant difference" between the experimental group and the control group.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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coagulants, heparin neutralization, multicharged cyclodextrins, supramolecules

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