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The effect of complexation of Cu(II) with P6A peptide and its analogs on their thrombolytic activities

Mei Yang[a], Guohui Cui[b], Ming Zhao[b,*], Chao Wang[a], Lili Wang[b,c], Hu Liu[b,c,d,**], Shiqi Peng[a,b,***]

[a] College of Pharmaceutical Sciences, Peking University, Beijing 100083, PR China
[b] College of Pharmaceutical Sciences, Capital Medical University, Beijing 100069, PR China
[c] School of Pharmacy, Memorial University of Newfoundland, St. John's, NL, Canada A1B 3V6
[d] Department of Biochemistry, Faculty of Science, Memorial University of Newfoundland, St. John's, NL, Canada

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ABSTRACT

The complexation of Cu(II) with previously synthesized thrombolytic peptides, Pro-Ala-Lys (**6**), Arg-Pro-Ala-Lys (**7**), Ala-Arg-Pro-Ala-Lys (**8**), Gly-Arg-Pro-Ala-Lys (**9**) and Gln-Arg-Pro-Ala-Lys (**10**), resulted in the formation of complexes, Cu(II)-(Pro-Ala-Lys) (**6-Cu**), Cu(II)-(Arg-Pro-Ala-Lys) (**7-Cu**), Cu(II)-(Ala-Arg-Pro-Ala-Lys) (**8-Cu**), Cu(II)-(Gly-Arg-Pro-Ala-Lys) (**9-Cu**) and Cu(II)-(Gln-Arg-Pro-Ala-Lys) (**10-Cu**), which was confirmed by UV-Vis, circular dichroism and ESI-MS analyses. Complexes (**6–10**)-**Cu** in normal saline (NS) were found to be able to assemble into aggregates, and the size of the aggregates were measured for the next eight consecutive days. It was found that on the 8th day, the diameters of (**6–10**)-**Cu** aggregates ranged from 179.21 ± 38.33 nm to 293.46 ± 51.07 nm. The Zeta potentials of (**6–10**)-**Cu** aggregates in NS were also examined and it was found that aggregates of (**6**, **8**, **10**)-**Cu** were negatively charged, and **7-Cu** and **9-Cu** were positively charged. The powders of (**6–10**)-**Cu** were analyzed by transmission electron microscopy and were found to have mean particle sizes of 8–15 nm. The *in vitro* euglobulin lysis, vasodilation and thrombolytic assays indicated that complexation with Cu(II) resulted in a significant increase in the activities of **6–10**. The *in vivo* thrombolytic assays revealed that complexation with Cu(II) resulted in a significant enhancement in the *in vivo* thrombolytic activities for **6**, **7**, **8** and **10** at 10 μmol/kg, and **9** at 1 μmol/kg and 0.1 μmol/kg, respectively. These findings suggested that the self-assembly of the Cu(II)–peptide complexes into nano-scale aggregates was beneficial in improving the thrombolytic activity of the peptides.

1. Introduction

A pentapeptide, Ala-Arg-Pro-Ala-Lys (ARPAK, **8**) known as P6A, was isolated from products degraded by plasmin from fibrin B β-chain and it was shown to possess a microvascular permeability-increasing effect in rat and human skin (Belew et al., 1978; Gerdin and Saldeen, 1978). Later it was also found that P6A induced significant local vasodilation of bovine mesenteric arteries and affected coronary blood dynamics in canine coronary thrombosis (Anderson et al., 1983; Mehta et al., 1989; Saldeen et al., 1991a,b). To examine its clinical potentials, the effect of P6A on coronary thrombosis in canine model was compared to that of tissue plasminogen activator (t-PA) (Mehta et al., 1989; Nichols et al., 1991). It was revealed that in dogs with electronically induced thrombus, the effects of P6A on the thrombolysis and the reestablishment of the coronary blood flow were similar to that of t-PA, while the reestablished blood flow with P6A was shorter than that with t-PA (Mehta et al., 1989). It is well known that thromboses cause a variety of heart conditions such as heart attack, stroke and other peripheral vascular diseases. However, the currently used thrombolytic agents, such as t-PA, urokinase (UK) and streptokinase (SK), are all large proteins. The severe side effects such as hemorrhagenic tendency and immunogenic reactions seen with large proteins substantially limited their therapeutic benefits (Banerjee et al., 2004; Hellebrekers et al., 2000;

Abbreviations: ARPAK, Ala-Arg-Pro-Ala-Lys; CD, circular dichroism; SK, streptokinase; TEM, transmission electron microscopy; t-PA, tissue plasminogen activator; UK, urokinase; NS, normal saline; NE, noradrenaline.

* Corresponding author. Tel.: +86 10 8391 1535; fax: +86 10 8391 1535.
** Corresponding author at: School of Pharmacy, Memorial University of Newfoundland, St. John's, NL, Canada A1B 3V6. Tel.: +1 709 777 6382; fax: +1 709 777 7044.
*** Corresponding author at: College of Pharmaceutical Sciences, Capital Medical University, Beijing 100069, PR China. Tel.: +86 10 8391 1528; fax: +86 10 8391 1528.
E-mail addresses: maozhao@126.com (M. Zhao), hliu@mun.ca (H. Liu), sqpeng@bjmu.edu.cn (S. Peng).

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Khan and Gowda, 2003; Rouf et al., 1996; Wardlaw et al., 1997). Thus, thrombolytic oligopeptides have attracted a lot of interests.

P6A (**8**), a small thrombolytic peptide, attracted our interests and we have developed many analogs of P6A, among which Gln-Arg-Pro-Ala-Lys (**9**) and Gly-Arg-Pro-Ala-Lys (**10**) were found to be more potent than P6A (**8**) (Zhao et al., 2004). *In vivo* metabolism studies showed that Pro-Ala-Lys (**6**) and Arg-Pro-Ala-Lys (**7**) were active metabolites of P6A (**8**) (Zhao et al., 2003).

It is well recognized that rigid conformations of oligopeptides may be obtained via constructing metal–oligopeptide complexes, especially transition metal–oligopeptide complexes. Among the transition metal–oligopeptide complexes, Cu(II)–oligopeptide complexes are known to be excellent scaffold to decrease the conformational flexibility (Facchin et al., 2000, 2002; Inomata et al., 2005; Nascimento et al., 2001), to simulate enzyme systems (Holm et al., 1996; Mylonas et al., 2005; Shtyrlin et al., 2005; Todorova-Balvaya et al., 2005; Weder et al., 2002), to alter conformation (Łodyga-Chruscinska et al., 2004), to reveal magnetic interactions (Sanchiz et al., 2006) and to exhibit therapeutic potentials (Kong et al., 2007). Complexation of oligopeptides with metal ions would not only help define their secondary and tertiary structures but also enhance their interactions with enzymes due to the tremendous potential of complexes for self-assembly. The self-assembled oligopeptides will not only be able to access an array of three-dimensional structures rapidly (Gao and Matsui, 2005; Rajagopal and Schneider, 2004; Reches and Gazit, 2006; Scheibel, 2005; Zanuy et al., 2006; Zhao and Zhang, 2004), but also to exhibit an obvious tendency to form a nanosystem, which in turn gives the oligopeptides unique properties (Carny et al., 2006; Montet et al., 2006; Patolsky et al., 2004; Sarikaya et al., 2003).

In the present paper, the Cu(II) complexes of previously synthesized P6A analogs, **6**, **7**, **8**, **9** and **10**, were prepared. ESI-MS, UV–vis and circular dichroism (CD) were used to reveal the formation of the complexes, **(6–10)-Cu**. It is not uncommon for Cu(II) complexes to form self-assembled aggregates (Braunecker and Matyjaszewski, 2007; Ghosh and Verma, 2007; Ma et al., 2006; Tiliakos et al., 2003). The **(6–10)-Cu** complexes formed were examined for the sizes of the self-assembled aggregates in normal saline (NS). Transmission electron microscopy (TEM) was used to analyze the sizes of **(6–10)-Cu** in their solid state. Bioassays, including *in vitro* euglobulin clot lysis assay, *in vitro* vasodilation assay, *in vitro* thrombolytic assay and *in vivo* thrombolytic assay, were performed to reveal the effects resulted from Cu(II) complexation on peptides **6–10**.

2. Experimental

2.1. Chemical synthesis

2.1.1. General

The protected amino acids of L-configuration used were purchased from Sigma Chemical Co. All the coupling and removing of protective groups were carried out under anhydrous conditions. Reactions were monitored using TLC. The purity of the intermediates was also checked using TLC (Merck silica gel plates of type 60 F_{254}, 0.25 mm layer thickness), and the purity of the final products determined by HPLC (Waters, C_{18} column 4.6 mm × 150 mm) was higher than 95%. IR spectroscopy was determined using 330 FT-IR spectrometer (Nicolet Avatar Thermo Electron Corporation). ESI-MS spectroscopy was determined using Waters HPLC/MS (Quattro micro™ API). Optical rotation was determined on a polarimeter (P-1020, Jasco, Japan). The elemental analysis was recorded on Elementar Vario EL III (Germany).

2.1.2. Synthesis of peptides ***6–10***

Peptides **6–10** were synthesized according to Scheme 1 as previously reported (Zhao et al., 2003, 2004).

*2.1.2.1. Preparation of Cu(II)-(Pro-Ala-Lys) (**6**-Cu).* To the solution of 72 mg (0.23 mmol) of peptide **6** in 2 mL of distilled water, the solution of 50 mg (0.29 mmol) of $CuCl_2{\cdot}2H_2O$ in 0.2 mL water was added. The solution was treated using 1 mol/L Na_2CO_3 with stirring at room temperature for 6 h. After filtration, the filtrate was subjected to evaporation under reduced pressure and the residue was purified by size-exclusion chromatography (Sephadex G10) to provide 21 mg (20% yield) of the title compound as a blue powder. ESI-MS (*m*/*z*) 376 $[M-H]^+$. $[\alpha]_D^{20} = -66.60$ (*c* = 0.01, CH_3OH). IR(KBr) 3252, 3097, 2950, 2860, 1611, 1567, 1382, 1299 cm^{-1}. Anal. Calcd. for $C_{14}H_{25}N_4O_4Cu{\cdot}2H_2O$: C 40.72, H 7.08, N 13.57. Found: C 40.53, H 7.22, N 13.39.

*2.1.2.2. Preparation of Cu(II)-(Arg-Pro-Ala-Lys) (**7**-Cu).* Using the procedure above and from 108 mg (0.23 mmol) of peptide **7**, 21 mg (15% yield) of the title compound was obtained as a blue powder. ESI-MS (*m*/*z*) 532 $[M-H]^+$. $[\alpha]_D^{20} = 51.20$ (*c* = 0.01, CH_3OH). IR(KBr) 3396, 2921, 1655, 1432, 889, 683 cm^{-1}. Anal. Calcd. for $C_{20}H_{37}N_8O_5Cu{\cdot}2H_2O$: C 42.21, H 7.26, N 19.69. Found: C 42.41, H 7.38, N 19.50.

*2.1.2.3. Preparation of Cu(II)-(Ala-Arg-Pro-Ala-Lys) (**8**-Cu).* Using the procedure above and from 125 mg (0.23 mmol) of peptide **8**, 29 mg (19% yield) of the title compound was obtained as a blue powder. ESI-MS (*m*/*z*) 603 $[M-H]^+$. $[\alpha]_D^{20} = 75.30$ (*c* = 0.01, CH_3OH). IR(KBr) 3261, 2938, 1654, 1580, 1454, 1399, 560 cm^{-1}. Anal. Calcd. for $C_{23}H_{42}N_9O_6Cu{\cdot}2H_2O$: C 43.15, H 7.24, N 19.69. Found: C 43.33, H 7.11, N 19.88.

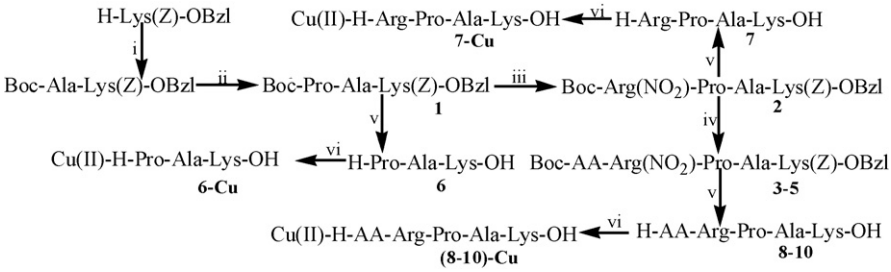
*2.1.2.4. Preparation of Cu(II)-(Gly-Arg-Pro-Ala-Lys) (**9**-Cu).* Using the procedure above and from 108 mg (0.23 mmol) of peptide **9**, 32 mg (21% yield) of the title compound was obtained as a blue powder. ESI-MS (*m*/*z*) 589 $[M-H]^+$. $[\alpha]_D^{20} = 31.10$ (*c* = 0.01, CH_3OH). IR(KBr) 3277, 2941, 2860, 1654, 1581, 1397, 1318, 672 cm^{-1}. Anal. Calcd. for $C_{22}H_{40}N_9O_6Cu{\cdot}2H_2O$: C 42.20, H 7.08, N 20.13. Found: C 42.39, H 7.19, N 20.31.

*2.1.2.5. Preparation of Cu(II)-(Gln-Arg-Pro-Ala-Lys) (**10**-Cu).* Using the procedure above and from 138 mg (0.23 mmol) of peptide **10**, 34 mg (20% yield) of the title compound was obtained as a blue powder. ESI-MS (*m*/*z*) 660 $[M-H]^+$. $[\alpha]_D^{20} = 28.80$ (*c* = 0.01, CH_3OH). IR(KBr) 3260, 2910, 1645, 1550, 1439, 1382, 1270, 1112, 990, 653 cm^{-1}. Anal. Calcd. for $C_{25}H_{45}N_{10}O_7Cu{\cdot}2H_2O$: C 44.21, H 6.97, N 20.62. Found: C 44.40, H 6.53, N 20.80.

2.2. Characterization

2.2.1. UV–vis and CD spectral analysis

Aqueous solutions (1 mM) of peptides **6–10** and **(6–10)-Cu** were prepared. The pH of **6–10** was 5.86, 8.72, 6.03, 5.31 and 4.96, respectively, while the pH of **(6–10)-Cu** was 8.61, 8.41, 7.25, 7.01 and 7.66, respectively. The UV–vis spectra of the preparations were recorded on a UV–vis spectrophotometer (UV-2550, Shimadzu, Tokyo, Japan) over the range of 200–800 nm at 25 °C and the CD spectra of the same preparations were recorded on a spectropolarimeter with JASCO Canvas Program (Model J-810, Jasco, Tokyo, Japan) over the range of 200–800 nm at 25 °C.



Scheme 1. Preparation of Cu(II) complexes of compounds **6–10**. Wherein AA = Ala (for **3** and **8**), Gly (for **4** and **9**), and Gln (for **5** and **10**). (i) Boc-Ala-OH, Dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBt), *N*-methylmor-pholine (NMM); (ii) Hydrogen chloride in ethyl acetate (4N), Boc-Pro, DCC, HOBt, NMM; iii) Hydrogen chloride in ethyl acetate (4N), Boc-Arg(NO_2), DCC, HOBt, NMM; (iv) Hydrogen chloride in ethyl acetate (4N), Boc-Ala or Boc-Gly or Boc-Gln, DCC, HOBt, NMM; (v) H_2, Pd/C, C_{18} column; (vi) $CuCl_2$, pH 8–9, Sephadex G10 column.

*2.2.2. Size and zeta potential of the aggregates of (**6–10**)-**Cu** assembled in NS*

To characterize the aggregates of **6–10** and **(6–10)-Cu** assembled in solution, 1 mg/mL of preparations of **6–10** and **(6–10)**-**Cu** were prepared in NS, and the size and zeta potential of the aggregates were measured using a particle size analyzer (Malvern Zeta Sizer, Nano-ZS90, Worcestershire, UK) and a Zeta Potential Analyzer (ZetaPlus, Brookhaven Instruments Corporation, Holtsville, NY, USA), respectively. The size measurements were carried out at 25 °C every 24 h for 8 days, and zeta potential was measured at 1 h and on the 8th day.

*2.2.3. Morphology and TEM images of (**6–10**)-**Cu** powders*

Samples for TEM analysis were prepared according to the method used by others (Gibson et al., 2007). In brief, one drop of the solutions (1 mg/mL) of **(6–10)** or **(6–10)-Cu** in tri-distilled water was added to a copper supported mesh membrane (150 mesh) and the excess solution was removed with filter paper. The samples were dried in open air at room temperature to prepare the corresponding powders. The TEM analysis of the powders of **6–10** or **(6–10)**-**Cu** was performed using an electron microscope (JEM-1230, JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

2.3. Bioassays

2.3.1. In vitro euglobulin lysis assay

Blood collected from pig was centrifuged at 3000 × *g* for 10 min and the platelet poor plasma (PPP) was removed. The diluted plasma (1:20 in distilled water) was precipitated at pH 5.7 with acetic acid (0.1%). After 10 min the supernatant was centrifuged at 3000 × *g* for 10 min at 4 °C and the precipitate was lyophilized to obtain euglobulin. To 7 mL of borax buffer (3.5 μM, pH 9.28), 25 mg of euglobulin was dissolved, to which 0.7 mL of $CaCl_2$ solution (25 mM) were added and the mixture was immediately extended onto a 10 cm × 10 cm glass board to form a 1 mm layer. Onto the layer, 10 μL of NS, UK (28,000 IU/mL) in NS, peptides **6–10** in NS (10 mM), **(6–10)-Cu** in NS (10 mM) or $CuCl_2$ in NS (10 mM) were added. The diameter of the lysis ring was measured after 4 h.

2.3.2. In vitro vasodilation assay

A constant temperature of the buffer solution was kept using a water-bath (CS501, Chongqing Yinhe Experimental Apparatus Ltd., Chongqing, China). A tension transducer (JZ101, Beidian Xinghang Machine and Equipment Ltd. of China) and a two-channel physiological recorder (LMS-2B, Chengdu Apparatus Manufacturing Ltd., Chengdu, China) were used to evaluate the vascular relaxation activity. Male Wistar rats weighing 250–300 g (the Animal Center of Peking University) were used.

Immediately after decapitation, rat aortic strips were taken and put in a perfusion bath with 15 mL of oxygenated (95%O_2/5%CO_2) Kreb's solution (pH 7.4) at 37 °C. The aortic strip was connected to a tension transducer, and the relaxation constriction curve of muscles was registered. Administration of 0.59 μM noradrenaline (NE) was used to induce hypertonic constriction of the vessel strip. As the constriction reached its maximum, NE was washed out and the vessel strip was stabilized for 30 min. After renewal of the solution, NE (0.59 μM) was added. When the hypertonic constriction value of the aortic strip reached its peak, peptides **6–10** in NS (final concentrations ranged from 500 μM to 0.1 μM) or **(6–10)-Cu** in NS (final concentrations ranged from 50 μM to 0.1 μM) or $CuCl_2$ in NS (final concentrations ranged from 1 mM to 1 μM) were administrated to observe the vascular relaxation activity of the test compounds.

2.3.3. In vitro thrombolytic activity assay

Male Wistar rats weighing 250–300 g (purchased from the Animal Center of Peking University) were anesthetized with pentobarbital sodium (80.0 mg/kg, ip). The right carotid artery of the animals was separated. To a glass tube filled with artery blood (about 0.5 mL) obtained from the right carotid artery of the animal, a stainless steel filament helix (15 circles; *L*, 18 mm; *D*, 1.8 mm) was put in immediately. After 40 min the helixes with thrombus were carefully taken out and were suspended in tri-distilled water for 1 h. They were weighted and were dipped into 8 mL of NS or 8 mL of UK in NS (100 IU/mL) or peptides **6–10**, **(6–10)-Cu** or $CuCl_2$ in NS (100 nM/L). After 2 h, the helixes were taken out and weighted. The reduction of thrombolytic mass was recorded.

2.3.4. In vivo thrombolytic activity assay

Male Wistar rats weighing 200–250 g (purchased from the Animal Center of Peking University) were anesthetized with pentobarbital sodium (80.0 mg/kg, ip). The right carotid artery and left vein jugular of the animals were separated. To a glass tube filled with artery blood (about 0.1 mL) obtained from the right carotid artery of the animal, a stainless steel filament helix (15 circles; *L*, 15 mm; *D*, 1.0 mm) was put in immediately. After 15 min the helix with thrombus was carefully taken out and weighed, and put into a polyethylene tube. The polyethylene tube was filled with heparin sodium (50 IU/mL in NS) and one end was inserted into the left jugular vein. Heparin sodium was injected via the other end of the polyethylene tube as the anticoagulant, and this end was inserted into the right carotid artery, following which NS (3 mL/kg), $CuCl_2$ (10 μmol/kg) in NS, UK (20,000 IU/kg) in NS, **6–10** (10 μmol/kg) in NS or **(6–10)-Cu** (10 μmol/kg) in NS was injected. The blood was circulated through the polyethylene tube for 60 min, after which the helix was taken out to record weight. The reduction of thrombus weight was calculated.

The thrombolytic activities of **9** and **9-Cu** at 10, 1 and 0.1 μmol/kg were measured by the same method as described above. The respective preparations were injected to rats and the reduction of thrombus weight was calculated.

2.4. Statistic analysis

All results are expressed as mean ± standard deviation (S.D.) from at least three experiments ($n = 3–10$). The Student's t test and one-way analysis of variance (ANOVA) were used for evaluating statistical significance. Statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Synthesis of *6–10* and (*6–10*)-*Cu*

According to previously reported methods (Zhao et al., 2003, 2004), which is shown in Scheme 1, Boc-Pro-Ala-Lys(Z)-OBzl (**1**, 94.8% yield), Boc-Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl (**2**, 80.6% yield), Boc-Ala-Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl (**3**, 69.3% yield), Boc-Gly-Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl (**4**, 72.5% yield) and Boc-Gln-Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl (**5**, 40.3% yield) were obtained. Compounds **1–5** were converted into **6** (75% yield), **7** (72% yield), **8** (73% yield), **9** (74% yield) and **10** (71% yield), respectively. At pH 8–9 peptides **6–10** were mixed with $CuCl_2$ resulting in **6-Cu**, **7-Cu**, **8-Cu**, **9-Cu** and **10-Cu** in 20%, 15%, 19%, 21% and 20% yield, respectively. ESI-MS results revealed that the peaks corresponding to the respective **(6–10)-Cu** were equivalent to the theoretical molecular weight of the complexes (M) minus 1 (proton) or [M−1]. Based on the results of UV and CD analysis (see below) which revealed d–d transitions in the complexes, the general coordination model of **(6–10)-Cu** could be postulated as {NH_2, $2N^-$, CO} (Osz et al., 2002). According to this 3N coordination model, one proton in the peptide is displaced upon the complexation with Cu(II).

3.2. UV–vis and CD spectra of (*6–10*)-*Cu*

To confirm the formation of **(6–10)-Cu**, the UV–vis and CD spectra of **6–10** and **(6–10)-Cu** were obtained and compared. The UV–vis spectra of **(6–10)-Cu** as shown in Fig. 1 displayed a characteristic absorption band in the 520–638 nm range. This characteristic absorption was the result of d–d transitions of Cu(II) in the visible light region (Shtyrlin et al., 2005) with the exception of **7-Cu**, which had an additional shoulder in its spectrum. The difference demonstrated by **7-Cu** is likely due to the N-terminus in peptide **7**. Unlike the other peptides (**6**, **8**, **9** and **10**) which contain neutral amino acids at their N-termini, the N-terminus of **7** is arginine residue which is a highly basic amino acid and contains an additional guanido group as its side-chain. Guanidinium in arginine exhibits a much higher pK_a value (12.47) than ordinary $-^+NH_3$. Therefore, arginine residue may use its guanido nitrogen as well as its amino nitrogen to coordinate with Cu(II) while the other peptides (**6**, **8**, **9** and **10**) may only use their amino nitrogen to coordinate with Cu(II).

The CD spectra of **(6–10)-Cu** as shown in Fig. 2 showed a characteristic absorbance in the 550–650 nm range, which is consistent with the absorbance corresponding to the d–d transition of Cu(II) reported (Gao and Matsui, 2005; Tiliakos et al., 2003). Based on the results of UV and CD analysis which revealed d–d transitions in the complexes and the results of ESI-MS which showed (M−1) peaks, the general coordination model of **(6–10)-Cu** could be postulated as {NH_2, $2N^-$, CO}. As such, the complexation with Cu(II) will decrease the pH and consequently change the isoelectric point of peptides **6–10** to a certain extent. To achieve better complexation, however, the pH of the mixture was initially adjusted to 8 using Na_2CO_3 during the preparation of **(6–10)-Cu**. The final pH values of **(6–10)-Cu** were measured to be 8.61, 8.41, 7.25, 7.01 and 7.66, respectively.

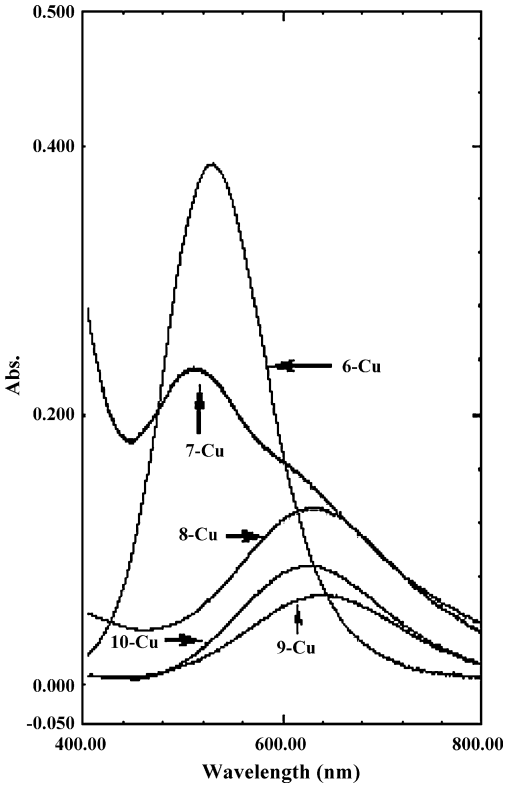


Fig. 1. Visible light absorption spectra of **(6–10)-Cu** in water.

3.3. Size and zeta potential of the aggregates of (*6–10*)-*Cu* assembled in NS

The results of size of aggregates of **(6–10)-Cu** assembled in NS are shown in Fig. 3 and the values were averages of eight measurements. It was found that the average size (in diameter) of **(6–10)-Cu** in NS was less than 250 nm in the 8 days monitored. This observation demonstrated that **(6–10)-Cu** possessed good assembling properties and the assembled nano-particles were stable for at least 8 days. The results of zeta potential demonstrated that the

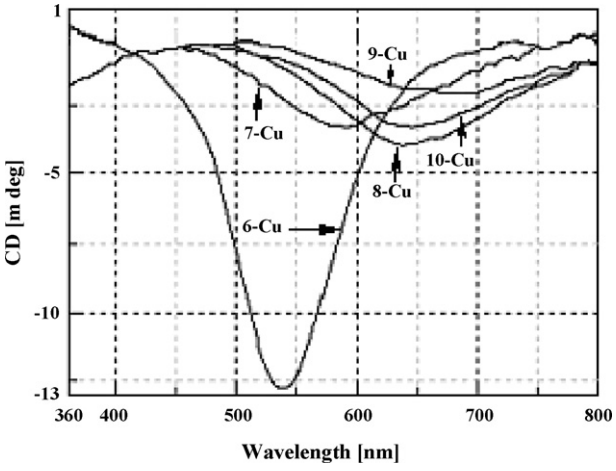


Fig. 2. CD spectra of **(6–10)-Cu** in water.

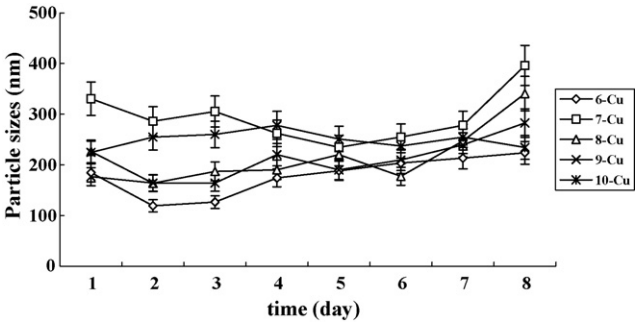


Fig. 3. Particle sizes of **(6–10)-Cu** aggregates assembled in NS (1 mg/mL), monitored for 8 days. $N = 8$.

surfaces of **6-Cu**, **8-Cu** and **10-Cu** were negatively charged, and the surfaces of **7-Cu** and **9-Cu** were positively charged. At 1 h following complexation, the zeta potentials of **(6–10)-Cu** were found to be −1.60, 4.25, −2.30, 0.14 and −4.79 mV, respectively. On the 8th day, the zeta potentials of **(6–10)-Cu** were found to be −1.52, 9.33, −2.60, 0.12 and −5.27 mV, respectively. The results demonstrated that there was little change in the electrical properties of the complexes formed between the first hour and on the 8th day. All the data show that chelating with Cu(II) helped the peptides to self-assemble into nano-scale particles.

3.4. Morphology and particle size of (*6–10*)-*Cu* powders

The morphology and particle size of **6–10** or **(6–10)-Cu** in solid state were examined. However, peptides **6–10** did not show any unique TEM morphology. The images of **(6–10)-Cu** powders under TEM as shown in Fig. 4 revealed that all the powders consisted of spherical particles of similar size in the nanometer range, 8–15 nm in diameter, which suggested that chelating with Cu(II) assisted the peptides to self-assemble into nano-scale particles following the evaporation of water.

3.5. In vitro euglobulin lysis activities of *6–10* and (*6–10*)-*Cu*

As shown in Fig. 5, the *in vitro* euglobulin lysis evaluation demonstrated that the lysis areas resulted from 10 mM of **6–10** and **(6–10)-Cu** were significantly larger than that from NS and 10 mM of $CuCl_2$, indicating that both **6–10** and **(6–10)-Cu** are associated with the fibrinolytic activities. The fibrinolytic activities of **(6–10)-Cu** as shown by their lysis areas were found to be stronger than those of **6–10**, suggesting that Cu(II) complexation led to an enhancement of *in vitro* fibrinolytic potency for **6–10**. It was also shown that 10 mM of $CuCl_2$ (2.9 ± 0.6 mm) did not result in increased lysis area compared to NS (3.0 ± 0.6 nm), suggesting that $CuCl_2$ was not fibrinolytic active, which suggested that the increase of the fibrinolytic activities of **(6–10)-Cu** is due to the complexation. It was also found that there were no significant differences among the activities of peptides **6–10**. However, **7-Cu** was the least effective

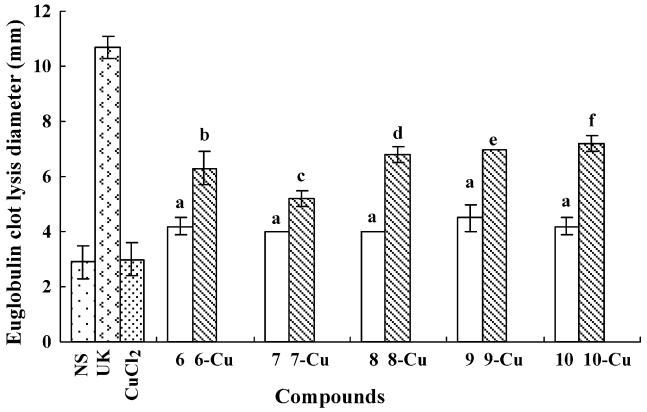


Fig. 5. The diameter of the lysis ring caused by compounds **6–10** and complexes **(6–10)-Cu** after 4 h. NS, negative control; UK, positive control; For each group, $n = 3$; (a) Compared to NS $p < 0.01$; (b) Compared to NS $p < 0.001$, to **6** $p < 0.01$, to **7-Cu**, $p < 0.05$; (c) Compared to NS $p < 0.001$, to **7** $p < 0.01$; (d) Compared to NS and **8** $p < 0.001$, to **7-Cu** $p < 0.01$; (e) Compared to NS, **9** and **7-Cu** $p < 0.001$; (f) Compared to NS and **10** $p < 0.001$, to **7-Cu** $p < 0.01$.

as compared to other complexes, which is likely due to the arginine residue participating in the complexation.

3.6. In vitro vasodilating activities of *6–10* and (*6–10*)-*Cu*

The relaxation effect of **6–10** and **(6–10)-Cu** on NE induced vasoconstriction was expressed by the half maximal effective concentration (EC_{50}) and was determined using GWBASIC EXE program (Montet et al., 2006). The results (as shown in Fig. 6) indicated that the EC_{50} values of **6–10** to NE induced vasoconstriction were 84.5 μM, 57.6 μM, 250.0 μM, 243.0 μM and 131.0 μM, respectively, while for **(6–10)-Cu** were 2.63 μM, 20.3 μM, 2.57 μM, 5.55 μM and 6.17 μM, respectively. The comparison of the EC_{50} values of **6–10** and **(6–10)-Cu** revealed that the *in vitro* vasodilating effect of **6–10** was increased by complexation with Cu(II) by 32, 2.8, 97.3, 43.8 and 21.2 fold, respectively. The EC_{50} value of $CuCl_2$ to NE induced vasoconstriction was higher than 10 mM, indicating that Cu(II) alone had little vasodilating effect. It was also found that the activity of **7** was higher compared to peptides **6**, **8**, **9** and **10**, while that of **7-Cu** was lower compared to those of (**6**, **8**, **9**, **10**)-**Cu** (Fig. 6), which was likely due to the N-terminal arginine residue in **7**. We suppose that the N-terminal arginine residue in peptide **7** would contribute to its vasodilating effect, while after complexation with Cu this contribution was actually diminished.

3.7. In vitro thrombolytic activities of *6–10* and (*6–10*)-*Cu*

In vitro thrombolytic activities of **6–10** and **(6–10)-Cu** were evaluated using a reported method with modifications (Braunecker and Matyjaszewski, 2007; Tiliakos et al., 2003). The reduction of thrombus in weight was used as the *in vitro* thrombolytic activity, and the data are presented in Fig. 7. It was demonstrated that incubation

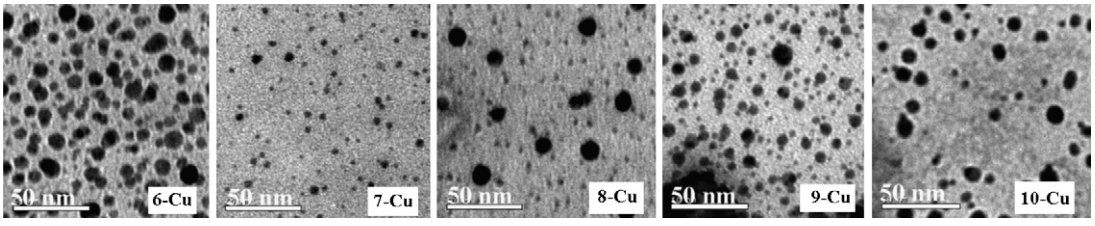


Fig. 4. TEM (Model JEM-1230, JEOL, Japan) images of **(6–10)-Cu** powders.

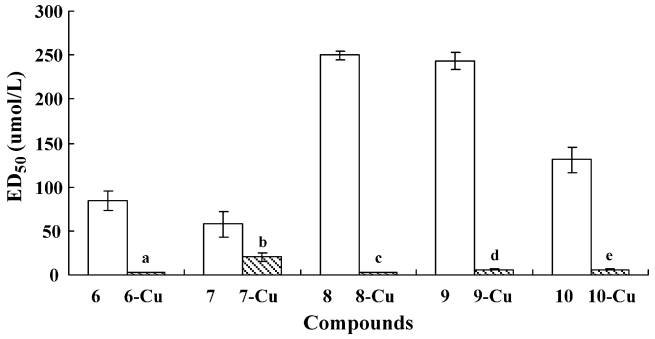


Fig. 6. The EC_{50} values of **6–10** and **(6–10)-Cu** against NE induced vasoconstriction. For each group, $n = 6$, the EC_{50} values of $CuCl_2$ against NE induced vasoconstriction was greater than 100 M; (a) Compared to **6** $p < 0.0001$; (b) Compared to **7** $p < 0.0001$; (c) Compared to **8** $p < 0.0001$; (d) Compared to **9** $p < 0.0001$; (e) Compared to **10** $p < 0.0001$.

with peptides **6–10** (100 nM) for 2 h resulted in reductions ranging from 25.76 mg to 30.44 mg, which were significantly higher than that of NS (20.67 mg at $p < 0.01$) and $CuCl_2$ (22.15 mg at $p < 0.01$). Incubation with **(6–10)-Cu** (100 nM) for 2 h resulted in reductions of thrombus ranging from 30.86 mg to 36.65 mg, which were significantly higher than those of **6–10** ($p < 0.05$). The results showed that Cu(II) complexation led to a significant increase in the *in vitro* thrombolytic activity of **6–10**. The activity of 100 nM of $CuCl_2$ was found to be equivalent to that of NS, suggesting that Cu(II) alone had little *in vitro* thrombolytic activity.

3.8. In vivo thrombolytic activities of *6–10* and (*6–10*)-*Cu*

The results of *in vivo* thrombolytic assays are presented in Fig. 8. It was found that the reductions of thrombus by 10 μmol/kg of **6**, **7**, **8**, **9** and **10** were 18.16, 12.83, 13.34, 19.22 and 15.37 mg, respectively, which were significantly higher than that by NS (6.93 mg, $p < 0.01$). The reductions of thrombus by 10 μmol/kg of **(6**, **7**, **8**, **9**, **10)-Cu** were 19.98, 18.12, 19.28, 19.40 and 18.8 mg, respectively. Among them, peptides **6** and **9** showed a similar *in vivo* thrombolytic activity as UK. The *in vivo* thrombolytic activities of **(6**, **7**, **8**, **10)-Cu** were shown to be similar to that of UK and significantly higher than that of their corresponding counterparts, **6**, **7**, **8** and **10** ($p < 0.05$). The thrombus reduction by 10 μmol/kg of $CuCl_2$ was 7.44 mg which was similar to that of NS (6.93 mg), suggesting Cu(II) alone had little *in vivo* thrombolytic activity. Peptide **9** exhibited a

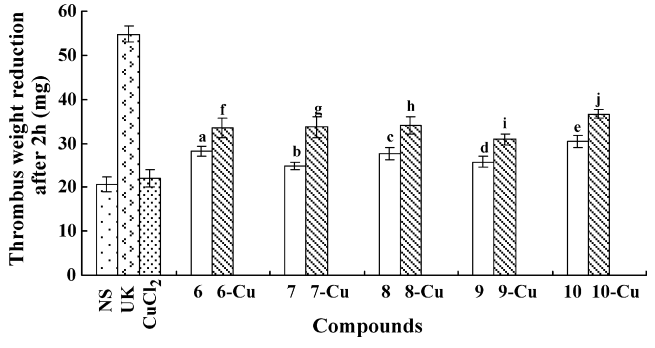


Fig. 7. The *in vitro* thrombolytic activity of **6–10** and **(6–10)-Cu** following 2 h incubation. NS, vehicle; UK, positive control; For each group, $n = 6$; (a) Compared to NS $p < 0.0005$, to **7** $p < 0.05$; (b) Compared to NS and **10** $p < 0.01$, to **8** $p < 0.05$; (c) Compared to NS $p < 0.001$; (d) Compared to NS $p < 0.01$, to **10** $p < 0.05$; (e) Compared to NS $p < 0.0001$; (f) Compared to NS $p < 0.0001$, to **6** $p < 0.05$; (g) Compared to NS $p < 0.0001$, to **7** $p < 0.01$; (h) Compared to NS $p < 0.0001$, to **8** $p < 0.05$; (i) Compared to NS $p < 0.0001$, to **9** $p < 0.01$, to **10-Cu** $p < 0.05$; (j) Compared to NS $p < 0.0001$, to **10** $p < 0.01$.

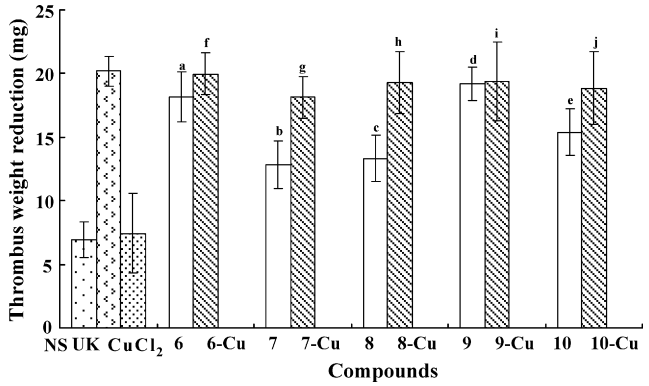


Fig. 8. The *in vivo* effect of **6–10** and **(6–10)-Cu** on the thrombus weight reduction. NS, negative control; UK, positive control; For each group, $n = 10$; UK = 20,000 IU/kg; $CuCl_2$ = 10 μmol/kg; **6–10** = 10 μmol/kg; **(6–10)-Cu** = 10 μmol/kg; (a) Compared to NS, **7** and **8** $p < 0.0001$, to **10** $p < 0.01$; (b) Compared to NS, UK and **9** $p < 0.0001$, to **10** $p < 0.01$; (c) Compared to NS, UK and **9** $p < 0.0001$, to **10** $p < 0.05$; (d) Compared to NS and **10** $p < 0.0001$; (e) Compared to NS $p < 0.0001$, to UK, $p < 0.01$; (f) Compared to NS $p < 0.0001$, to **6** and **7-Cu** $p < 0.05$; (g) Compared to NS and **7** $p < 0.0001$; (h) Compared to NS and **8** $p < 0.0001$; (i) Compared to NS, $p < 0.0001$; (j) Compared to NS $p < 0.0001$, to **10** $p < 0.01$.

higher activity compared to other peptides tested, and no significant difference was observed between **9** and **9-Cu**.

3.9. Dose-dependent in vivo thrombolytic activities of *9* and *9-Cu*

Due to the good *in vivo* thrombolytic activity exhibited by peptide **9** (equally as good as UK) and little difference between **9** and its corresponding complex, **9-Cu**, **9** and **9-Cu** were selected to do a further dose-dependent study. The results as shown in Fig. 9 indicated that peptide **9** at 10, 1 and 0.1 μmol/kg caused reduction of thrombus by 19.20, 9.81 and 7.02 mg, respectively, and the same amounts of **9-Cu** resulted in thrombus reduction of 19.20, 13.98 and 11.20 mg, respectively. The results indicated that the thrombolytic activities of both **9** and **9-Cu** were dose dependent, and the reductions of thrombus by **9-Cu** at 1 and 0.1 μmol/kg were significantly higher than **9** ($p < 0.05$ and 0.0001). The results suggested that the thrombolytic activities of both peptide **9** and its complex **9-Cu** reached a plateau as concentrations increased and no difference was observed at higher concentrations. However, when the dose was decreased to 0.1 μmol/kg, no thrombolytic activity was observed for peptide **9** (7.02 mg) as compared to NS (6.93 mg), while

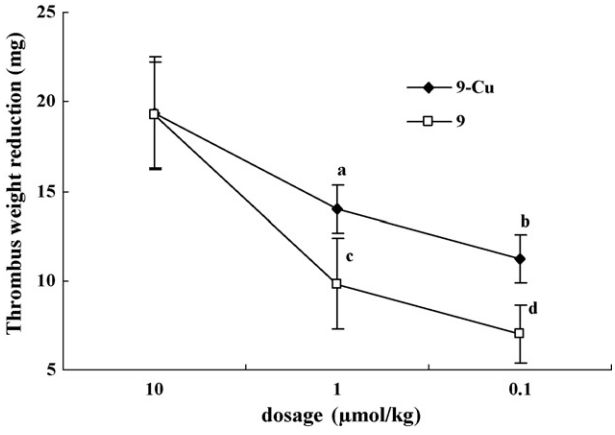


Fig. 9. The dose-dependent thrombolytic activity of **9** and **9-Cu**. $n = 10$; (a) Compared to 1 μmol/kg of **9** $p < 0.0001$, to 10 and 0.1 μmol/kg of **9-Cu** $p < 0.001$; (b) Compared to NS $p < 0.0001$, to 1 and 0.1 μmol/kg of **9** $p < 0.05$; (c) Compared with NS and 0.1 μmol/kg of **9** $p < 0.001$; (d) Compared to NS $p > 0.05$.

9-Cu still showed some activity (11.02 mg). These results suggest that although **9** was the most potent peptide, complexation with Cu(II) further improved the *in vivo* thrombolytic activity.

4. Conclusion

Cu–peptide complexes were successfully synthesized and confirmed by UV, CD and ESI-MS spectra. Measurement of particle size and zeta potential of the resulting Cu–peptide complexes demonstrated the formation of nano-scale aggregates through self-assembly, which was aided by the copper ion. From the *in vitro* and *in vivo* tests, it was clear that Cu–peptide complexes significantly enhanced the euglobulin lysis activity, vasolidating activity and thrombolytic activity as compared to the ligand peptides. Among the Cu–peptide complexes, **7-Cu** is the least effective, which might be due to the participation of the N-terminal arginine residue in the complexation. The ability of **(6–10)-Cu** in forming nano-scale aggregates may be responsible for their increased *in vitro* and *in vivo* thrombolytic activities as compared to the ligand peptides, which was further manifested by the dose-dependent thrombolytic activity of **9-Cu**.

Acknowledgements

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