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공학석사학위논문

**Urokinase–Gold Nanoparticle
Conjugates as Theranostic Agents for
Thrombotic Diseases**

혈전증의 영상화 및 치료가 동시에 가능한
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ABSTRACT

Urokinase-Gold Nanoparticle Conjugates as Theranostic Agents for Thrombotic Diseases

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The thrombotic disease is one of the leading cause of death worldwide. Although thrombolytic drugs such as various plasminogen activators have been used to dissolve a thrombus, their use has been limited by their short half-life and hemorrhage side effects. There have been several studies to combine nanoparticle with plasminogen activators, but there has not been combination with the CT imaging agents. Because CT imaging is suitable for imaging of the thrombus with a fast scan time, the combination of the thrombolytic agents and the CT imaging agents will provide efficient treatment. In this study, we developed theranostic agents combining gold nanoparticles for CT imaging and urokinase for therapeutic effect. The

formation and surface modification of nanoparticles were confirmed by measurement of TEM, DLS, UV, and FT-IR. Urokinase and GC-AuNP combined UK-GC-AuNPs had a core size of 20 nm and a hydrodynamic diameter of about 190 nm. *In vitro* release test, it was confirmed that urokinase was well attached to UK-GC-AuNPs. *In vitro* thrombolysis test, UK-GC-AuNPs dissolved the thrombus within 30 minutes. *In vivo* thrombolysis test, UK-GC-AuNPs were caught in the mesh structure of thrombus and directly imaged the thrombus. After UK-GC-AuNPs were caught in the thrombus, UK on GC-AuNPs continuously dissolved the thrombus and after 48 hours, almost completely dissolve the thrombus. Consequently, we synthesized a theranostic agent by combination of urokinase and gold nanoparticles, and confirmed its imaging ability and thrombolysis effect *in vitro* and *in vivo*. This research will provide platform for combined CT imaging and therapy of thrombotic diseases.

Keywords: Gold Nanoparticles (AuNPs), Computed tomography imaging, Urokinase, Ischemic stroke, Thrombolysis, Nanoparticle-protein conjugates

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1. INTRODUCTION

One in four people dies from ischemic heart disease and ischemic stroke worldwide¹. Thrombosis like ischemic heart disease and ischemic stroke causes problems by preventing circulation of normal blood flow. Accurate and rapid diagnosis and treatment using effective thrombolytic agents are important to reduce mortality².

Thrombolytic agents are used to dissolve the thrombus. Usually, plasminogen activators are used, which change plasminogen to plasmin to dissolve fibrin. Among the various plasminogen activators, urokinase (UK), streptokinase (SK), and tissue plasminogen activator (tPA) are representative of the FDA approved. However, high dose injection is required because of their short half-life, which causes hemorrhage side effect³. Urokinase is relatively safe because it causes a lower hemorrhagic complication. Moreover, urokinase has a low cost compared to tPA⁴, making it suitable therapeutic agents for research.

Direct thrombus imaging has been studied to detect thrombus accurately and detect recurrence after treatment. Among the various imaging devices such as magnetic resonance imaging (MRI), positron emission tomography (PET), ultrasound (US), and computed tomography (CT), CT is usually used

as a first imaging modality for rapid detection of thrombus⁵. Gold nanoparticles (AuNPs) have been studied as contrast agents because gold has a higher atomic number and X-ray absorption coefficient than currently used iodine-based contrast agents. Also, since gold is inert, gold nanoparticles are suitable for use in the body^{6, 7}. Especially, it was confirmed that Glycol chitosan (GC) coated gold nanoparticles can directly image thrombus⁸. Gold nanoparticles stay in the fibrin matrix for a long time and show the thrombus with CT.

Various plasminogen activator loaded nanomedicine have been studied^{2, 3}. Especially, urokinase loaded nanomedicines using chitosan particles⁹, dextran-coated magnetic nanoparticles¹⁰, perfluorocarbon (PFC) nanoparticles¹¹, and microbubble¹² as carriers have been studied. However, gold nanoparticles with plasminogen activators have not been studied.

In this study, we designed urokinase-gold nanoparticle conjugates. After surface modification of GC-AuNPs, urokinase and aldehyde-modified gold nanoparticles were conjugated using aldehyde-amine reaction¹³. Urokinase-GC-AuNPs (UK-GC-AuNPs) conjugates showed CT imaging efficiency by gold nanoparticles and proved therapeutic effect by urokinase. We have developed theranostic agents for simultaneous diagnosis and treatment of thrombotic diseases.

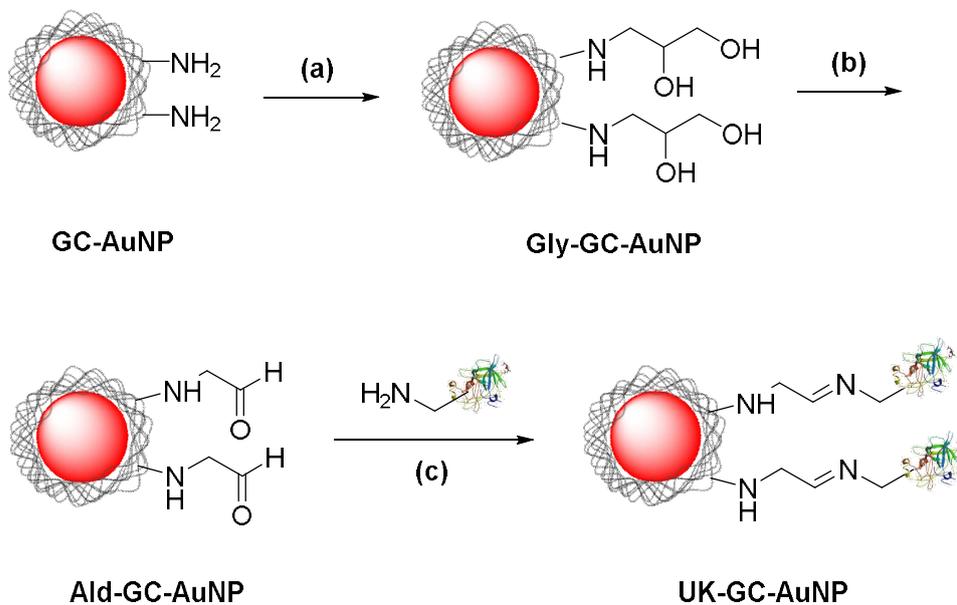


Figure 1. Scheme for synthesis of UK-GC-AuNPs

(a) Glycidol, NaBH_3CN , TWEEN[®] 80, 0.2 M MES buffer solution (pH 5.1), r.t., overnight, (b) NaIO_4 , TWEEN[®] 80, 0.2 M MES buffer solution (pH 5.1), r.t., 2.5 h, (c) UK, TWEEN[®] 80, 0.2 M MES buffer solution (pH 5.1), r.t., 1.5 h, N_2

2. EXPERIMENTS

2.1 Materials

Gold(III) chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, $\geq 99.9\%$), Sodium citrate dihydrate ($\geq 99\%$), Glycol chitosan ($\geq 99\%$), Glycol chitosan (GC) ($\geq 60\%$), Glycidol (96%), Sodium periodate ($\geq 99.8\%$), Sodium cyanoborohydride (95%), MES ($\geq 99\%$), TWEEN[®] 80 were purchased from Sigma Aldrich (St. Louis, MO). Urokinase (500,000 I.U.) was purchased from Green Cross (Korea). Pierce BCA protein assay kit was purchased from Thermo Fisher Scientific (USA). AG 4000 (pyroGlu-Gly-Arg-pNA \cdot HCl, $> 95\%$) was purchased from Merck Millipore (USA). Water used in the experiment was triple distilled water and all other chemicals were used as received without any purification.

2.2 Instruments

The size and zeta potential of particles were measured using Zetasizer ZS90 (Malvern, UK). Ultraviolet-Vis (UV-Vis) absorption spectra were recorded using UV-2450 (Shimadzu, Japan). Centrifugation of AuNPs was performed using Combi-514R (Hanil, Korea). Transmission electronic

microscopy (TEM) images were obtained from HR-TEM at 300 kV using JEM-3010 (JEOL, Japan). Fourier transform infrared (FTIR) measurements were carried out using TENSOR27 (Bruker, USA). Fluorescence microscopy images of thrombus dissolution were obtained using LAMBDA (LS, Korea). *In vivo* microCT (mCT) images were obtained using NFR Polaris-G90 (NanoFocusRay, Korea).

2.3 Preparation of AuNPs using GC as a reducing/stabilizing agent (GC-AuNPs)

Synthesis of GC-AuNPs was performed as previously reported⁸. 300 mg of GC was dissolved in 300 ml of water and stirred for over 24 h. The GC solution was boiled to 70 °C and 100 ml of H₂AuCl₄ · 3H₂O solution (1 mM) was added quickly. The heat source was removed after 24 h and the solution was cooled down to room temperature. The excess of GC was removed by repetitive centrifugation and washing with water.

2.4 Preparation of UK-GC-AuNPs

UK-GC-AuNPs were synthesized through surface modification of GC-AuNPs (Figure 1). GC-AuNPs (15 mg Au) were dispersed in 120 ml of 0.2 M MES buffer solution (pH 5.1). 48 mg of TWEEN[®] 80 was added to GC-

AuNPs solution and stirred for 30 min. 768.36 mg of sodium cyanoborohydride and 0.36 ml of glycidol was added to the solution and stirred overnight at room temperature. The excess chemicals were removed by centrifugation and Gly-GC-AuNPs (10 mg Au) were dispersed in 80 ml of 0.2 M MES buffer solution (pH 5.1). 32 mg of TWEEN[®] 80 was added to Gly-GC-AuNPs solution and stirred for 30 min. 160 mg of NaIO₄ was added to the solution and stirred for 2 h at room temperature. Ald-GC-AuNPs (5 mg Au) solution was washed by centrifugation and dispersed in 40 ml of 0.2 M MES buffer solution (pH 5.1). 16 mg of TWEEN[®] 80 was added to Ald-GC-AuNPs solution and stirred for 30 min. 30 mg of UK was added to the aldehyde-modified AuNPs (Ald-GC-AuNPs) solution and stirred for 1 h at room temperature under nitrogen atmosphere. UK-GC-AuNPs solution was washed by centrifugation and dispersed in 300 μ L of 10 mM PBS (pH 7.4). It kept in 4 °C before use.

2.5 Activity assay of UK-GC-AuNPs

Activity assay was carried out using colorimetric substrate AG4000 (pyroGlu-Gly-Arg-pNA · HCl). 800 μ L of Tris buffer solution (pH 8.8) was incubated at 37 °C for 5 min. 100 μ L of UK-GC-AuNPs or UK standard was added to the buffer and incubated at 37 °C for 2 min. 100 μ L of preheated

AG4000 was added and incubated at 37 °C for 5 min. 100 uL of 20 % acetic acid was added and incubated at 37 °C for 5 min for stopping the reaction. Samples were collected and UV absorbance at 405 nm was measured.

2.6 Release test of UK-GC-AuNPs

UK-GC-AuNPs or UK was dispersed in 2 ml of 10 mM PBS (pH 7.4) and the solution was transferred into a dialysis membrane bag (Spectra/Por[®], MWCO = 100 kDa, Spectrum Laboratories, Inc., USA). The closed bag was put into 10 ml of 10 mM PBS (pH 7.4) and incubated in a water bath at 37 °C under 80 rpm. 10 ml of solution was periodically removed and replaced with fresh 10 ml of 10 mM PBS (pH 7.4). The collected samples were analyzed using BCA assay.

2.7 *In vitro* thrombolysis test

In vitro thrombolysis test was carried out using Cy5.5 contained thrombus. UK-GC-AuNPs and UK (10,000 IU) were added to 2 ml of PBS and 120 ul of mouse serum respectively. The well plate was incubated at 37 °C at 80 rpm. The amount of remained thrombus was measured by fluorescence microscopy every 10 minutes.

2.8 *In vivo* thrombolysis test

In vivo thrombolysis test was carried out as previously reported¹⁰. Thrombi were formed in C57BL/6 male mice by applying 1 x 1 mm² filter paper soaked in FeCl₃ to the exposed carotid artery for 10 minutes. After 30 minutes of intravenous infusion, the mice were imaged by mCT.

3. RESULTS AND DISCUSSION

3.1 Characterization of AuNPs

UK-GC-AuNPs were synthesized through surface modification of GC-AuNPs (figure 1). GC-AuNPs were synthesized by reduction of HAuCl_4 with glycol chitosan. GC also acted as a stabilizer to form nanoparticles. The formation of nanoparticles was determined using TEM. (Figure 2). Glycol chitosan coated GC-AuNPs had spherical and uniform morphologies. (Figure 2(a)). The size of GC-AuNPs analyzed by TEM was 20 nm. UK-GC-AuNPs also formed uniform and spherical morphologies. Urokinase conjugation was successful without any morphology modification of gold nanoparticles. The hydrodynamic diameters of AuNPs in media were measured by DLS (Figure 3(a)). Hydrodynamic diameters largely increased compared to the particle size analyzed by TEM because of the large size of GC. GC-AuNPs, Gly-GC-AuNPs, Ald-GC-AuNPs, UK-GC-AuNPs had hydrodynamic diameters of 128.9 nm, 187.3 nm, 185.9 nm, 192.5 nm respectively. UV spectra also showed the formation of AuNPs. (Figure 3(b)). GC-AuNPs, Gly-GC-AuNPs, Ald-GC-AuNPs, UK-GC-AuNPs had peaks of 523 nm, 525 nm, 529 nm, 536 nm, respectively. The peaks were red-shifted as the surface modification progressed and the graphs also showed that no

aggregations between AuNPs had occurred. The modification of the particle surface was confirmed by zeta potential change (Table 1). The zeta potential of GC-AuNPs, Gly-GC-AuNPs, Ald-GC-AuNPs, UK-GC-AuNPs were 35.3 mV, 22.4 mV, -16.4 mV, -6.23 mV, respectively. When GC-AuNPs were converted to Gly-GC-AuNPs, the absolute value of zeta potential decreased due to the decrease of amine functional groups. When the urokinase was reacted with Ald-GC-AuNPs, the absolute value of zeta potential decreased because urokinase had a positive charge. The conjugation of urokinase was confirmed by FTIR (Figure 4). The increase of about 1700 cm^{-1} peak showed the formation of C=N and the decrease of broad peak of about 3300 cm^{-1} is observed. The stability of UK-GC-AuNPs in the biological environment was also confirmed (Figure 5). GC-AuNPs are stable in PBS due to the glycol chitosan on the surface while Cit-AuNPs are aggregated in PBS. When urokinase was conjugated to GC-AuNPs, the particles were still stable in PBS.

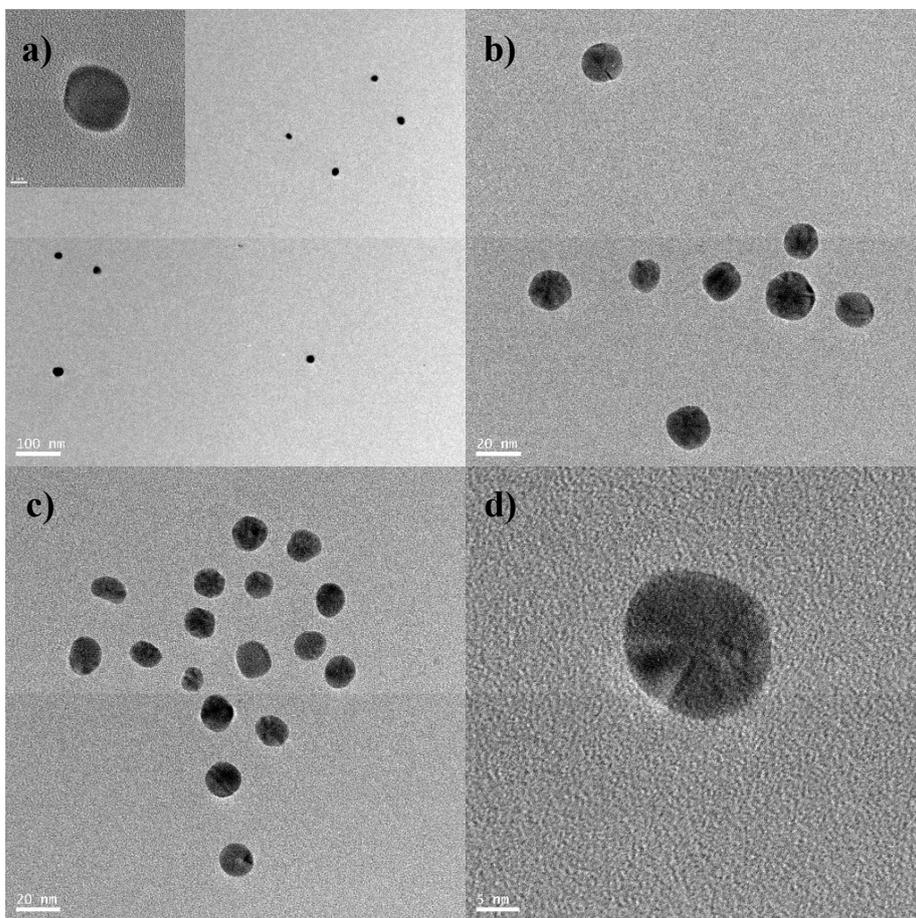


Figure 2. TEM image of (a) GC-AuNPs, scale bar: 100 nm (Inset: GC-AuNPs, scale bar: 5 nm) (b) GC-AuNPs, scale bar: 20 nm, (c) UK-GC-AuNPs, scale bar: 20 nm (d) UK-GC-AuNPs, scale bar: 5 nm

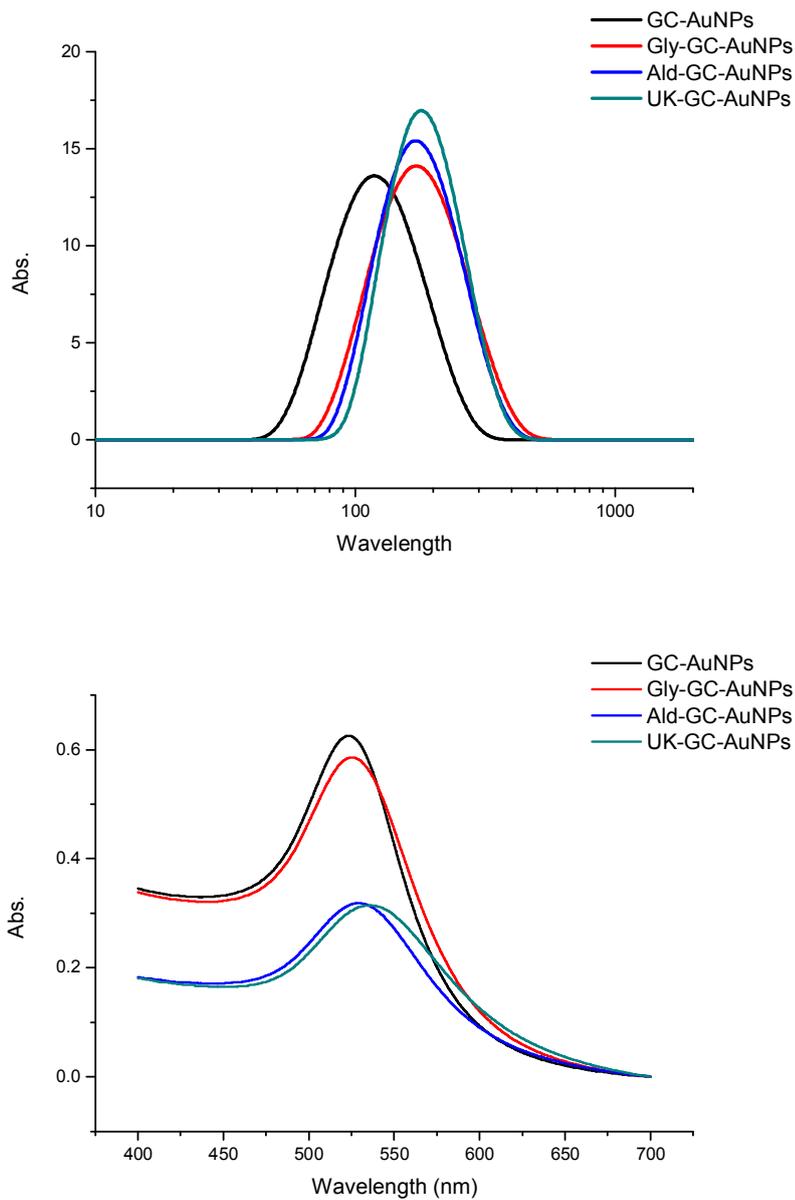


Figure 3. (a) Size distribution based on DLS and (b) UV spectra of GC-AuNPs, Gly-GC-AuNPs, Ald-GC-AuNPs, and UK-GC-AuNPs

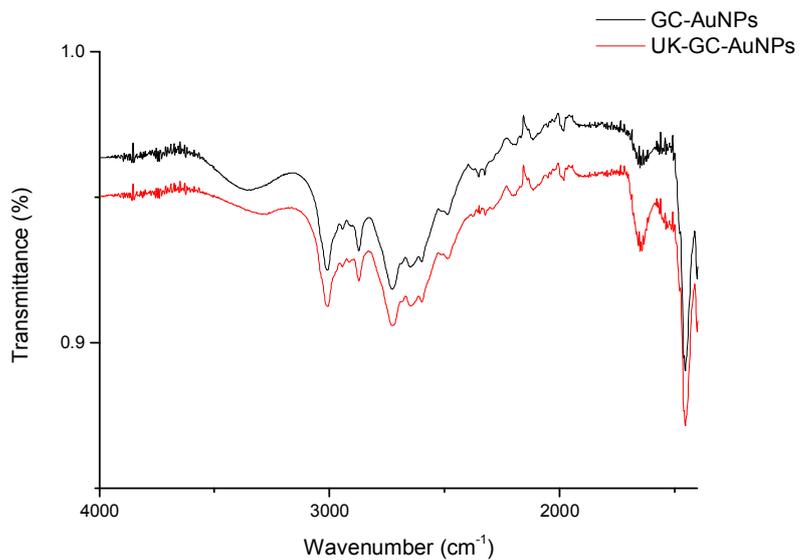


Figure 4. FT-IR spectra of GC-AuNPs (black line) and UK-GC-AuNPs (red line)

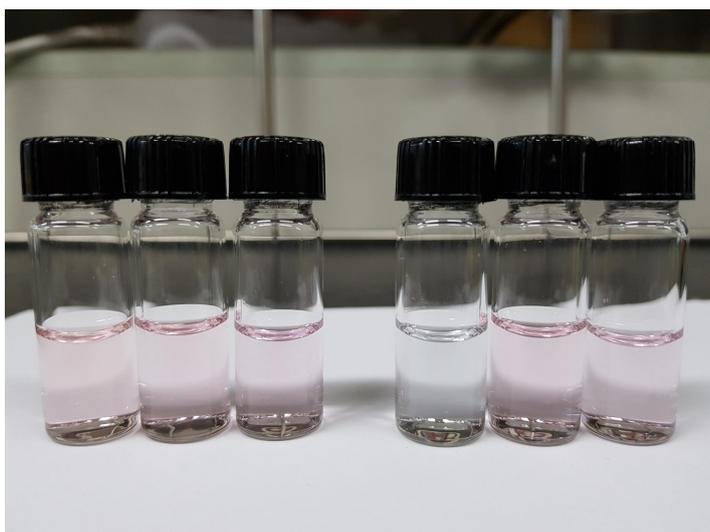


Figure 5. Particle stability of Cit-AuNPs (left), GC-AuNPs (middle), UK-GC-AuNPs (right) in (a) DW and (b) PBS

	GC-AuNPs	Gly-GC-AuNPs	Ald-GC-AuNPs	UK-GC-AuNPs
Size (nm)	128.9 ± 47.4	187.3 ± 70.1	185.9 ± 62.1	192.5 ± 45.7
Zeta potential (mV)	35.3 ± 5.8	22.4 ± 7.6	-16.4 ± 5.2	-6.2 ± 3.5
UV (nm)	523	525	529	536

Table 1. Characterization of GC-AuNPs, Gly-GC-AuNPs, Ald-GC-AuNPs, and UK-GC-AuNPs

3.2 Activity assay of UK-GC-AuNPs

The activity of UK-GC-AuNPs was analyzed using urokinase colorimetric substrate¹⁴. pGlu-Gly-Arg-pNA + H₂O turned to pGlu-Gly-Arg-OH + pNA when urokinase was reacted with the substrate. After stopping the reaction using acetic acid, the absorption at 405 nm was measured. A standard curve was obtained using various concentrations of UK solution (Figure 6). A control sample was obtained by adding acetic acid directly without any incubation. The activity of UK-GC-AuNPs (0.125 mg Au/ml) was similar to that of about 122 IU of urokinase. Therefore, it was confirmed that UK-GC-AuNPs exhibited 9,800 IU of UK activity per 1 mg Au.

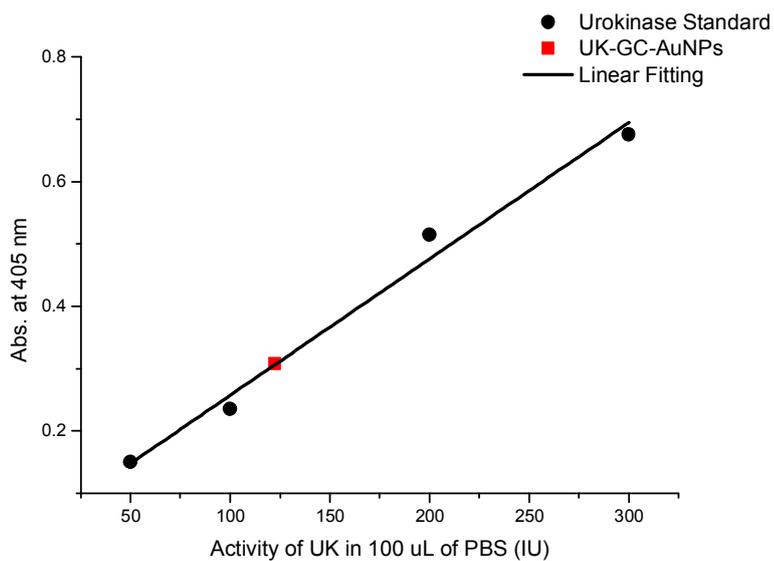
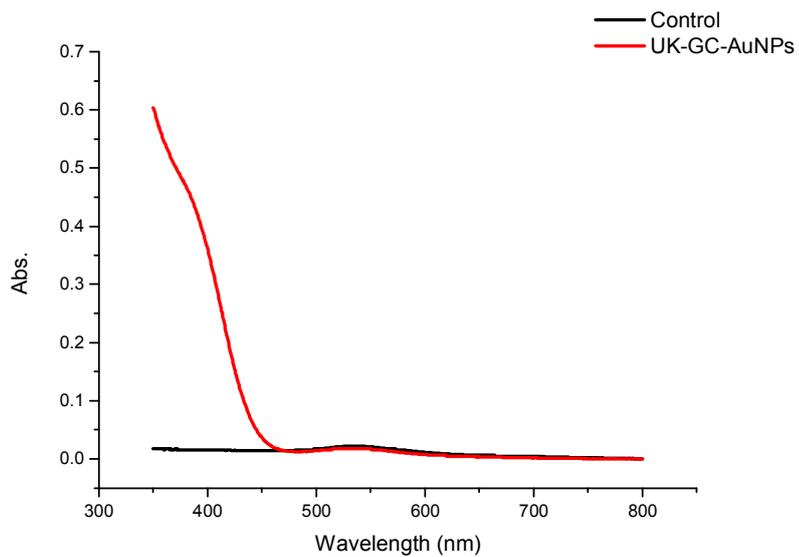


Figure 6. The activity of UK-GC-AuNPs. UK-GC-AuNPs solution was diluted and UK-GC-AuNPs (0.125 mg Au/ml) was measured.

3.3 Release test of UK-GC-AuNPs

In vitro release test of UK-GC-AuNPs was done using regular dialysis method¹⁵. Free urokinase was released in one day, while UK-GC-AuNPs were not released after 8 days. AuNPs and urokinase appeared to be well attached without separation and UK-GC-AuNPs are expected to reach clots without the loss of UK *in vivo*.

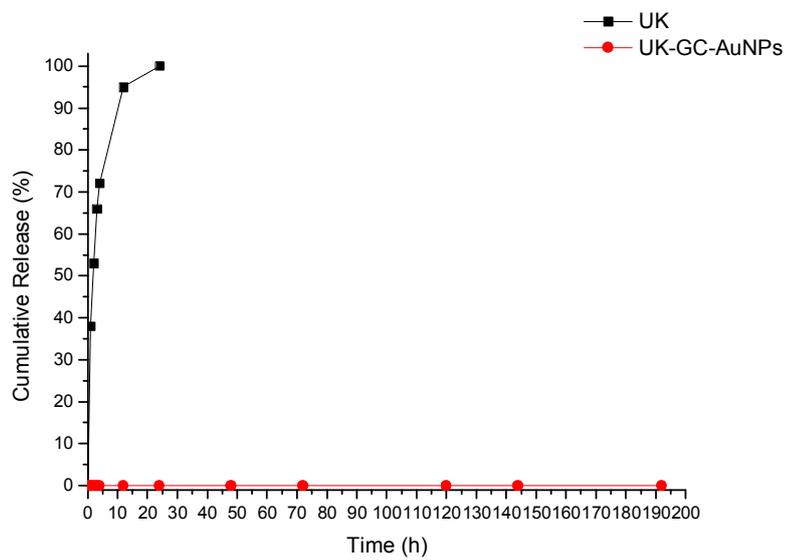


Figure 7. Cumulative release of UK-GC-AuNPs and UK

3.4 *In vitro* thrombolysis test

Dissolution of 2 cm thrombus contained Cy 5.5 was observed every 10 minutes. Control sample containing only saline retained thrombus shape over 30 minutes and remained unchanged. 10,000 IU of urokinase completely dissolved thrombus in 20 minutes. UK-GC-AuNPs gradually dissolved the thrombus and completely removed the clot in 30 minutes. 60 ul of UK-GC-AuNPs (16.7 mg/ml) showed somewhat similar thrombolysis ability with 10,000 IU of urokinase *in vitro*.

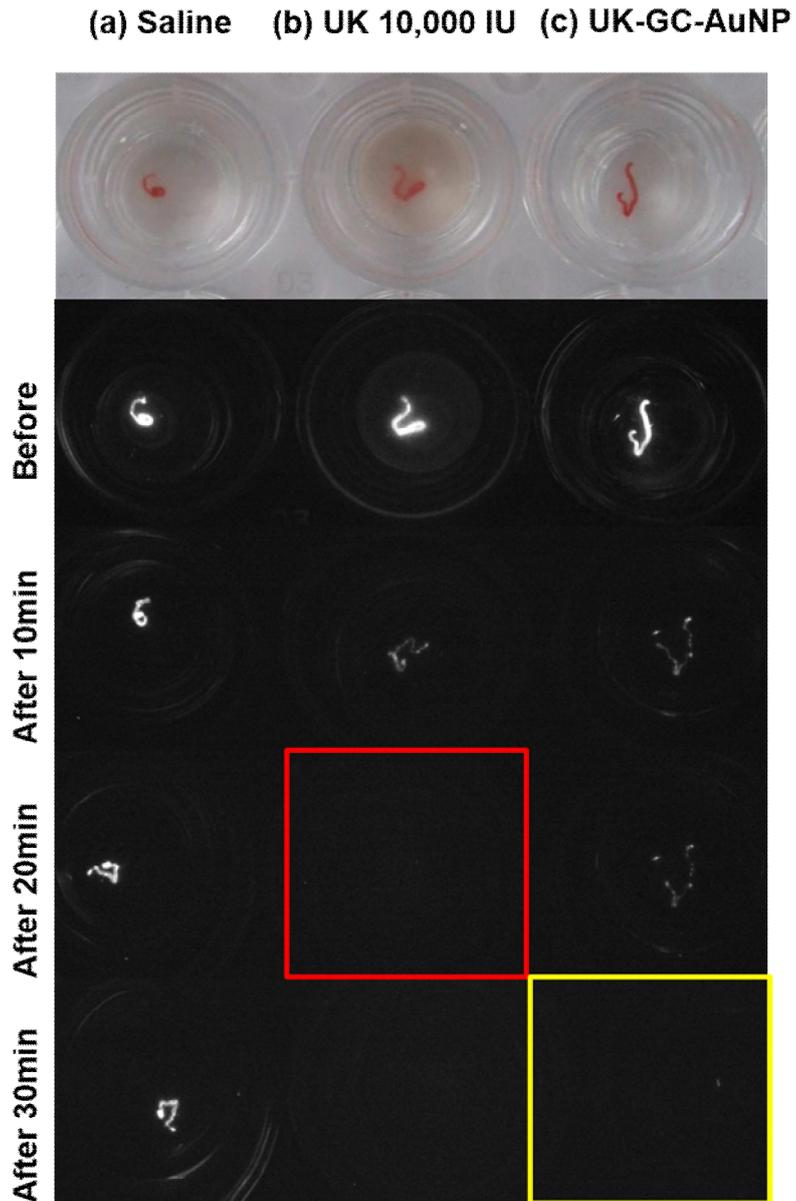


Figure 8. *In vitro* thrombolysis test with time (a) Saline, (b) UK 10,000 IU, (c) UK-GC-AuNPs (16.7 mg Au/ml, 60 μ l)

3.5 *In vivo* thrombolysis test

Three experimental groups were tested. After formation of thrombus, three samples were injected with intravenous infusion. When GC-AuNPs and UK-GC-AuNPs were injected, thrombi were imaged by gold nanoparticles due to the mesh structure of thrombus. When GC-AuNPs alone was injected, the shape of the thrombus remained unchanged even after 24 hours. A simple mixing of GC-AuNPs and urokinase showed imaging ability due to the AuNPs and dissolved the thrombus at 24 hours. In the case of UK-GC-AuNPs, the thrombus was also well imaged and the thrombus was almost completely dissolved at 48 hours. It has been confirmed that after urokinase on gold nanoparticles was caught in the thrombus, the thrombus is continuously dissolved.

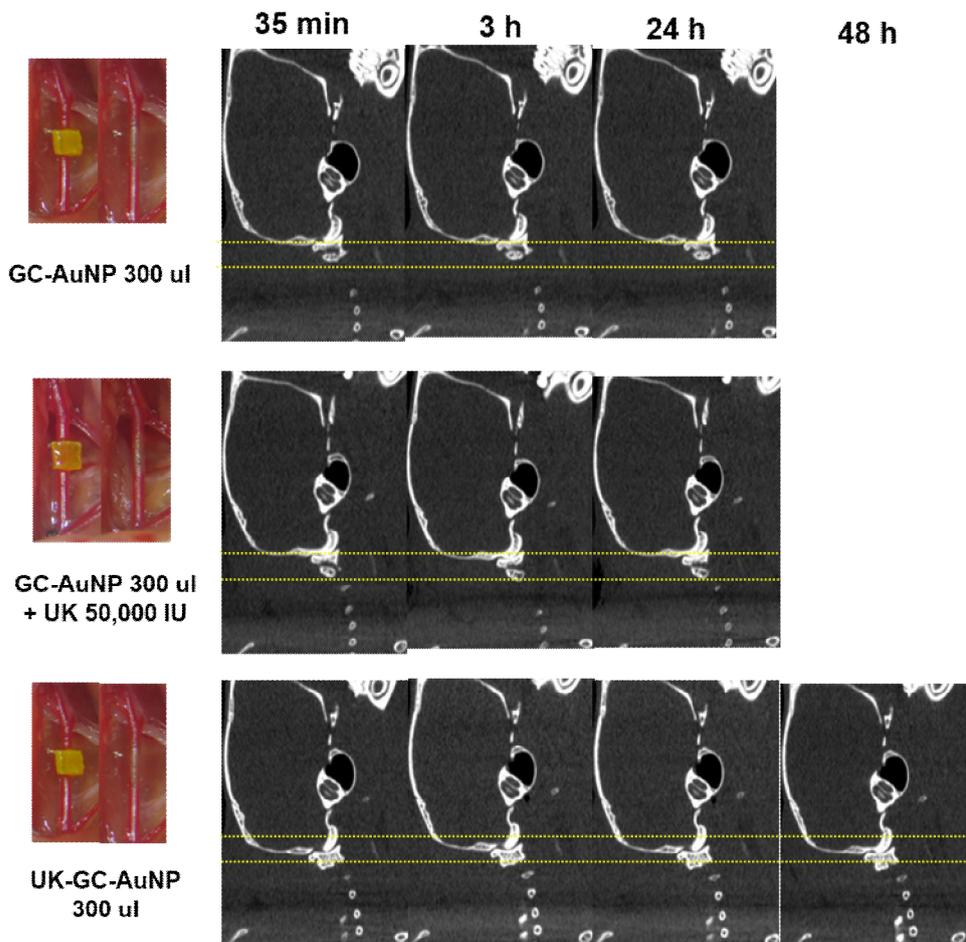


Figure 9. *In vivo* thrombolysis test of UK-GC-AuNPs

4. CONCLUSION

In this study, we designed theranostic agents for thrombotic diseases by combining gold nanoparticles and urokinase. Urokinase-gold nanoparticle conjugates were characterized by DLS, UV-Vis spectroscopy, TEM, and FT-IR. UK-GC-AuNPs were synthesized without change of morphology of gold nanoparticles. The stability of UK-GC-AuNPs in the physiological environment was also confirmed. *In vitro*, UK was not released from UK-GC-AuNPs for 8 days and the stability of binding was confirmed. The activity assay using urokinase colorimetric substrate was found that of UK-GC-AuNPs had an activity of 9,800 IU UK per 1 mg Au. *In vitro* thrombolysis test, UK-GC-AuNPs completely dissolved thrombus within 30 minutes. *In vivo* thrombolysis test, UK-GC-AuNPs imaged thrombus because gold nanoparticles are caught in the mesh structure of thrombus. UK-GC-AuNPs were caught in the thrombus and consistently dissolved the thrombus, and the thrombus was almost completely dissolved after 48 hours. Consequently, we synthesized a theranostic agent by chemically binding of urokinase to gold nanoparticles and confirmed its effect *in vitro* and *in vivo*. This research will provide platform for combined CT imaging and therapy

of thrombotic diseases. Subsequent studies with a targeting moiety are expected to show further improved effect.

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요약문

혈전증은 전세계적으로 가장 큰 사망 원인 중 하나이다. 혈전의 용해를 위해 다양한 플라스미노겐 활성화제와 같은 혈전용해제가 사용되어왔지만, 반감기가 짧고 출혈 부작용이 존재하여 사용이 제한되었다. 나노입자와 플라스미노겐 활성화제를 결합시키는 몇 가지 연구가 있었지만 CT 영상 제제와의 결합은 없었다. CT 영상은 빠른 스캔 시간으로 혈전을 영상화하는 데 적합하기 때문에 혈전 용해제와 CT 영상 제제의 결합은 효과적인 치료를 제공할 수 있을 것이다. 본 연구에서는 CT 영상화를 위한 금 나노입자와 치료 효능을 위한 유로키나제를 결합하여 진단과 치료를 동시에 할 수 있는 입자를 개발하였다. 나노 입자의 형성 및 표면 개질은 TEM, DLS, UV 및 FT-IR 의 측정에 의해 확인되었다. 유로키나제와 글리콜키토산-금나노입자가 결합된 입자는 20 nm 의 코어 크기를 가지며 수용액 상에서 약 190 nm 의 직경을 가졌다. 유로키나제 방출 실험에서 UK-GC-AuNPs 에 유로키나제가 잘 부착되어 있음을 확인하였다. 체외

혈전 용해 실험에서 UK-GC-AuNPs 는 30 분 내에 혈전을 용해시켰다. 생체 내 혈전 용해 실험에서 UK-GC-AuNPs 는 혈전의 메쉬 구조에 잡혀 직접적으로 혈전을 영상화하였다. UK-GC-AuNPs 가 혈전에 걸린 후 지속적으로 혈전을 용해 시켰고 48 시간 후에 혈전을 거의 다 녹였다. 결과적으로 우리는 유로키나제와 금 나노입자의 결합으로 진단과 치료를 동시에 하는 제제를 합성하였고, 체내와 체외에서 영상 능력과 혈전 용해 효과를 확인하였다. 이 연구는 혈전증의 CT 영상화와 치료를 위한 플랫폼을 제공할 것이다.

주요 어: 금 나노입자, 컴퓨터 단층 촬영, 유로키나제, 허혈성 뇌졸중, 혈전 용해, 나노 입자-단백질 복합체

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