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Thrombin-activatable fluorescent peptide incorporated gold nanoparticles for dual optical/computed tomography thrombus imaging

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ABSTRACT

Thrombosis is an important pathophysiologic phenomenon in various cardiovascular diseases, which can lead to oxygen deprivation and infarction of tissues by generation of a thrombus. Thus, direct thrombus imaging can provide beneficial in diagnosis and therapy of thrombosis. Herein, we developed thrombin-activatable fluorescent peptide (TAP) incorporated silica-coated gold nanoparticles (TAP-SiO_2@AuNPs) for direct imaging of thrombus by dual near-infrared fluorescence (NIRF) and micro-computed tomography (micro-CT) imaging, wherein TAP molecules were used as targeted thrombin-activatable peptide probes for thrombin-specific NIRF imaging. The freshly prepared TAP-SiO_2@AuNPs had an average diameter of 39.8 ± 2.55 nm and they showed the quenched NIRF signal in aqueous condition, due to the excellent quenching effect of TAP molecules on the silica-gold nanoparticle surface. However, 30.31-fold higher NIRF intensity was rapidly recovered in the presence of thrombin *in vitro*, due to the thrombin-specific cleavage of quenched TAP molecules on the gold particle surface. Furthermore, TAP-SiO_2@AuNPs were successfully accumulated in thrombus by their particle size-dependent capturing property, and they presented a potential X-ray absorption property in a dose-dependent manner. Finally, thrombotic lesion was clearly distinguished from peripheral tissues by dual NIRF/micro-CT imaging after intravenous injection of TAP-SiO_2@AuNPs in the *in situ* thrombotic mouse model, simultaneously. This study showed that thrombin-activatable fluorescent peptide incorporated silica-coated gold nanoparticles can be potentially used as a dual imaging probe for direct thrombus imaging and therapy in clinical applications.

1. Introduction

Thrombosis is pathophysiological phenomena, which are the formation of thrombus in a blood vessel. The thrombus formation can obstruct the flow of blood, resulting in tissue oxygen deprivation and infarction. Therefore, angiostenosis and the oxygen deprivation by thrombosis are a major cause of morbidity and death [1]. When the vessel wall or endothelium is damaged, thrombus formation is induced by the interaction between collagen and tissue factor in the blood [2]. During the thrombus formation,

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tissue factor which exposed to the blood activates the fibrin formation as well as the thrombin generation. Importantly, thrombin is a serine protease which proteolytically cleaved from of coagulation factor II (prothrombin) [3]. In addition, thrombin is one of the principal mediators that converts fibrinogen into fibrin during the thrombus formation. Although the exact mechanism of thrombin activation and thrombus formation *in vivo* has been well investigated *in vitro,* direct diagnosis of thrombosis has been limited by the lack of the appropriate imaging probes *in vivo*. Since 1974, conventional imaging probes of the thrombus have been developed to fibrin- and platelet-related imaging [4]. Furthermore, conventional thrombus imaging probes were limited to monitor aged clots or clots after anticoagulation treatment [5].

Until now, magnetic resonance (MR) and computed tomography (CT) images with various venography techniques have been used to directly image thrombus in the clinical field [6]. In particular, CT venography with X-ray irradiation is the most commonly used for imaging of thrombus because it provides prompt, accurate and detailed information of thrombotic lesion [7]. For example, cerebral venous sinus thrombosis (CVT) and deep vein thrombosis (DVT) were often scanned using non-contrast CT as a first-line investigation in the emergency in the clinical field [8]. However, non-contrast CT is difficult to obtain a precise distribution of thrombus, because that density of the thrombus in blood vessels is similar to adjacent blood [9]. In this regard, development of thrombus-specific contrast agents which can directly visualize the thrombus and thrombin activity in the blood vessels will contribute to a prompt and accurate diagnosis of thrombosis. Thrombosis also may commonly be confronted by various clinicians due to the diversity of causes, including surgery, trauma, cancer, pregnancy, and inflammatory bowel disease [10–12]. In addition, the symptoms and signs of thrombosis are not enough to thrombus-specific diagnosis. It can cause a fatal problem such as pulmonary embolism, heart attack, or stroke [11]. Once the diagnosis is performed, adequate therapeutic treatment which combined anticoagulants with symptomatic treatment started as soon as possible to decrease post-thrombotic morbidity [13]. Therefore, early and precise diagnosis of thrombosis is important to restore venous patency and preserves valvular function, resulting in increasing survival rate of a patient. It has been reported that gold nanoparticles (AuNPs) have been studied extensively as CT contrast agents, due to their high X-ray absorption coefficient property [9,14]. Furthermore, bismuth (Bi), ytterbium (Yb) or gold (Au)-based nanoparticles which have K-edge values in the X-ray energy band have developed as a novel imaging agent for the imaging of thrombosis by CT [9,15–17]. In addition, various types of nanoplatforms such as thrombin inhibitor-conjugated nanoparticle and fibrin-target peptide conjugated nanoparticles have developed for early and precise diagnosis of thrombosis using MRI or CT [18–21]. Although these thrombosis imaging nanoplatforms can provide thrombus-specific imaging with high spatiotemporal resolution, imaging of thrombosis-related enzyme activity is still challenging.

For example, we reported that tumor [22,23] or thrombosis [9,24] specific-CT images were successfully carried out using biocompatible polymer coated AuNPs in various animal models. In particular, biocompatible glycol chitosan coated AuNPs showed the substantial accumulation in the thrombus by their particle size-dependent capturing property, allowing the high-resolution CT visualization of primary and recurrent thrombus in mouse carotid artery [9]. Moreover, fibrin targeting peptide modified AuNPs showed the high-resolution *in vivo* CT images of thrombus in carotid artery [24]. Although AuNP-based nanoparticles were well-developed for CT imaging of thrombus, it is difficult to direct imaging of thrombin activity that is one of the key enzymes during the thrombus formation. It is because that direct imaging of thrombin activity might provide an opportunity for rapid and precise diagnosis of thrombotic lesion from peripheral tissue. However, AuNP-based CT contrast agents that directly image the fibrin activity in the thrombus have not been developed so far. Importantly, multimodal imaging based on the nanotechnology and molecular imaging have provided opportunities for the development of activatable probes for detecting of biological changes in living body. In this point of view, AuNPs have attracted a great deal of attention as a multimodal platform due to their intrinsic advantages such as easy fabrication, controllable size and shapes and biocompatibility, etc. [25]. Moreover, the excellent surface area-to-volume ratio and fluorescence quenching properties of AuNPs lead to development of various fluorescence-activatable optical imaging probes [26,27].

Herein, we have developed a novel fluorescence/micro-CT dual imaging probe wherein the optical imaging probe of near-infrared fluorescence (NIRF) dye, Cy5.5, conjugated thrombin-activatable peptide (Cy5.5-Gly-D-Phe-Pip-*Arg-Ser*-Gly-Gly-Gly-Gly-Lys-Cys), in resulting thrombin-activatable fluorescent peptide (TAP) that may be specifically cleavable by thrombin. And then TAP molecules were directly incorporated to silica caped AuNPs (SiO_2@AuNPs), in resulting TAP-SiO_2@AuNPs (Scheme 1a). The freshly prepared dual imaging probe of TAP-SiO_2@AuNPs may present the excellent quenched NIRF signal in a normal blood vessel, because of distance-dependent quenching effect of excited states of Cy5.5 on the surface of TAP-SiO_2@AuNPs [25,28]. However, the quenched fluorescence of Cy5.5 molecules on the surface of TAP-SiO_2@AuNPs can be strongly activated in the presence of thrombin at the thrombotic lesion, due to the rapid cleavage from thrombin specific-cleavable peptide on the surface TAP-SiO_2@AuNPs. In addition, silica caped AuNPs also can be accumulated in thrombus by their particle size-dependent capturing property, and they allow a high-resolution micro-CT imaging of thrombus at the targeted thrombotic lesion, due to their high X-ray absorption coefficient property (Scheme 1b). Finally, for the first time, we demonstrated that activatable fluorescence/micro-CT dual imaging using TAP-SiO_2@AuNPs provided the successful dual imaging of thrombin activity and anatomical information of thrombotic lesion with high sensitivity in a live thrombotic animal model, simultaneously.

2. Material and methods

2.1. Materials

Gold(III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$, 99.9%), trisodium citrate dihydrate, N-(3-dimethylaminopropyl)-N′-ethyl-carbodiimide hydrochloride (EDC; 98%), N-hydroxy-succinimide (NHS; 98%) tetraethylorthosilicate (TEOS, 99%), ammonium hydroxide solution (NH_4OH, 28–30%), (3-aminopropyl)triethoxysilane (APTES; 99%), thrombin (form human plasma) and calcium chloride anhydrous were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methoxy polyethylene glycol sulfhydryl 5000 (mPEG-SH, MW = 5000 Da) was obtained from SunBio (Anyang, Korea). Thrombin-specific cleavable peptide (Gly-D-Phe-Pip-Arg-Ser-Gly-Gly-Gly-Gly-Lys(BOC)-Cys-NH_2; where Pip indicates pipecolic acid) was purchased from Peptron (Dajeon, Korea). Cy5.5 monofunctional N-hydroxysuccinimide ester (Cy5.5-NHS) ($\lambda_{ex}/\lambda_{em}$ = 675/695 nm) was purchased from GE Healthcare (Little Chalfont, United Kingdom). Rabbit polyclonal anti-thrombin (ab92621) antibody and Alexa Fluor® 594 conjugated goat anti-rabbit IgG antibody (ab150080) were purchased from abcam (Cambridge, United Kingdom). All other chemicals were purchased as reagent grade and used without further purification. Ten-weeks-old male C57BL/6 mice were obtained from Orient Bio (Seoul, Korea).