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# One-pot bifunctionalization of silica nanoparticles conjugated with bioorthogonal linkers: application in dual-modal imaging†

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Covalent surface modification of silica nanoparticles (SNPs) offers great potential for the development of multimodal nanomaterials for biomedical applications. Herein, we report the synthesis of covalently conjugated bifunctional SNPs and their application to *in vivo* multimodal imaging. Bis(methylallyl)silane **15** with cyclopropene and maleimide, designed as a stable bifunctional linker, was efficiently synthesized by traceless Staudiger ligation, and subsequently introduced onto the surface of monodispersed SNPs *via* Sc(OTf)<sub>3</sub>-catalyzed siloxane formation. The bifunctional linker-grafted SNP **20** underwent both thiol-conjugated addition and tetrazine cycloaddition in *one pot*. Finally, positron emission tomography/computed tomography and fluorescence imaging study of dual functional SNP [<sup>125</sup>I]**28** labeled with NIR dye and <sup>125</sup>I isotope showed a prolonged circulation in mice, which is conducive to the systemic delivery of therapeutics.

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## Introduction

Nanomaterials have been used in a wide range of biomedical applications, including drug delivery, diagnostics, and therapy.<sup>1</sup> In recent years, well-designed nanoparticles including gold nanoparticles, quantum dots, polymer nanoparticles, liposomes, and carbon nanotubes<sup>2</sup> have been developed, many of which showed selective, high efficacy delivery of a drug or an imaging agent into its target. Among the various nanocomposites, silica nanoparticles (SNPs) have been investigated extensively as nanomedicinal platforms owing to advantages such as controllable sizes and shapes, easy surface modification, biocompatibility, and biodegradability.<sup>3</sup> In general, functionalized SNPs are prepared by either physical absorption

or covalent bonding. Various functional molecules are easily introduced into the inner space of SNPs by physical doping, but this method is not useful because its loading efficiency is low owing to the easy desorption of the doped material.<sup>4</sup> Alternatively, covalent conjugation of cargo molecules with SNPs enhances nanoparticle stability and prevents SNP cargo from being unexpectedly released.<sup>5</sup> Typically, cargo molecules are prefunctionalized with siloxanes, which are incorporated into the SNPs by conjugation with surface silanol groups. In this process, it is very common to use organoalkoxysilanes containing terminal nucleophilic groups, such as (3-aminopropyl)triethoxysilane (APS) or (3-mercaptopropyl)trimethoxysilane (MPS), as precursors (Fig. 1a). Although various diagnostic or therapeutic agents have been successfully integrated into the surface of SNPs *via* covalent bonding, applicable reactions for the synthesis of functionalized organosilanes are limited because of the easy hydrolysis of alkoxy silane. In addition, this process is unsuitable for the preparation of multimodal SNPs because different types of alkoxy silanes cannot be grafted onto the surface of SNPs in a single synthetic step.

Recently, allylsilanes have been used as precursors for the surface modification of silica by several research groups.<sup>6,7</sup> Compared to alkoxy silanes, allylsilanes are highly stable and can be purified by column chromatography on silica gel, and they are efficiently introduced into silica under refluxing conditions or in the presence of Lewis acid catalysts. In particular, various functional groups such as halides, aldehydes, cyano-

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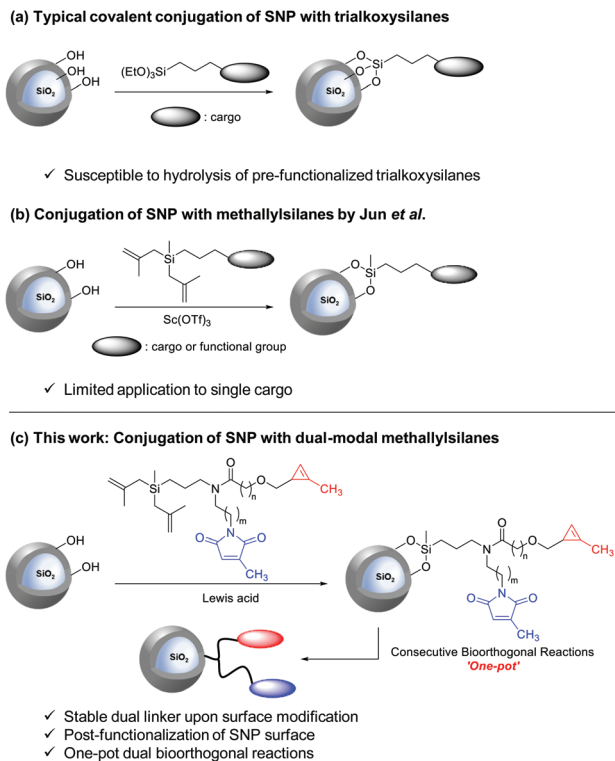


Fig. 1 A strategy for the construction of dual functional SNPs by covalent conjugation.

nides, and azides, which are preinstalled on allylsilanes, are grafted on the surface of silica and further applied to the incorporation of a desired biomolecule onto the silica by bioorthogonal reactions. For example, Jun *et al.*<sup>8</sup> demonstrated that methallylsilanes containing NHS-ester groups were integrated on silica surfaces in the presence of  $\text{Sc}(\text{OTf})_3$  to afford NHS-ester functionalized silica, which allowed the immobilization of glucose oxidase on silica *via* amide formation (Fig. 1b).

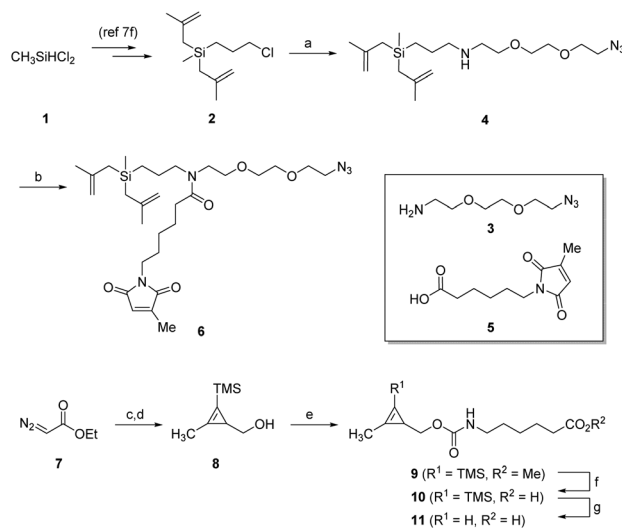
Owing to the high stability and versatility of allylsilanes, they are suitable substrates for preparing multifunctional SNPs *via* post-grafting. Accordingly, a novel methallylsilane conjugated with two bioorthogonal functional groups, methylcyclopropene and methylmaleimide, was designed (Fig. 1c). Methylcyclopropene, an electron-rich and strained alkene, can react with tetrazines *via* the inverse-electron demand Diels–Alder reaction (IEDDA),<sup>9,10</sup> whereas methylmaleimide acts as an effective Michael acceptor to undergo conjugate addition with reactive nucleophiles such as thiols and amines.<sup>11</sup> Accordingly, it is challenging to synthesize a reactive allylsilane with both bioorthogonal alkenes within one molecule and prove its effectiveness in SNP post-surface modification. Furthermore, if bioorthogonal reactions on the SNP surface occur independently without scrambling their functionality, this method will provide easy access to biomedical applications such as multimodal imaging or theranostics through covalent conjugation.<sup>12</sup>

In this study, we report the synthesis of bifunctional SNPs using methallylsilanes with a novel bioorthogonal dual linker as a post-grafting precursor and its application to multimodal *in vivo* imaging.

## Results and discussion

The current study began with the synthesis of maleimide-conjugated methallylsilane **6** and cyclopropene **11**, as shown in Scheme 1. According to a previously reported procedure,<sup>7f</sup> we prepared chloroalkyl bismethallylsilane **2**, which was converted to tertiary amide **4** by alkylation with azido amine **3**, followed by an amide coupling reaction of the corresponding amine **4** with maleimide-conjugated acid **5**. The use of methyl-substituted maleimide is crucial for preventing undesirable cycloaddition between azide and maleimide during amide coupling.<sup>13</sup> Next, cyclopropenation of TMS-propyne with diazoacetate in the presence of  $\text{Rh}(\text{OAc})_4$  and subsequent DIBAL reduction yielded methylcyclopropenyl methyl alcohol **8**, which was treated with carbonyl diimidazole (CDI) and aminoalkyl ester to yield methylcyclopropenyl ester **9**. Finally, hydrolysis of **9** under basic conditions and concomitant removal of the trimethylsilyl group produced acid **11** in high yield.

To conjugate the azide moiety of **6** with acid **11** using amide coupling reagents, the reduction of azide to primary amine was required. However, we suspected that the direct formation of the corresponding amine would cause an intra- or intermolecular conjugate addition of the resulting amine to



Scheme 1 Synthesis of maleimide conjugated allylsilane **6** and cyclopropene conjugated acid **11**. Reagents and conditions: (a) amine **3**,  $\text{K}_2\text{CO}_3$  (2.0 equiv.),  $80^\circ\text{C}$ , 58%; (b) acid **5**, EDCI (1.2 equiv.), DMAP (1.0 equiv.),  $\text{CH}_2\text{Cl}_2$ , rt, 78%; (c)  $\text{Me}-\text{C}\equiv\text{C}-\text{TMS}$ ,  $\text{Rh}_2(\text{OAc})_4$  (0.01 equiv.),  $\text{CH}_2\text{Cl}_2$ , rt, 44%; (d) DIBAL (2.0 equiv.),  $\text{Et}_2\text{O}$ ,  $0^\circ\text{C}$ , 85%; (e) (i) CDI (1.1 equiv.), THF, rt; (ii)  $\text{MeO}_2\text{C}(\text{CH}_2)_5\text{NH}_3\text{Cl}$ ,  $\text{Et}_3\text{N}$  (2.0 equiv.), DMF,  $50^\circ\text{C}$ , 51%; (f) KOH (3.0 equiv.),  $\text{MeOH}/\text{H}_2\text{O}$  (4 : 1),  $0^\circ\text{C}$ ; (g) TBAF (3.0 equiv.), THF, rt, 95% (2 steps).

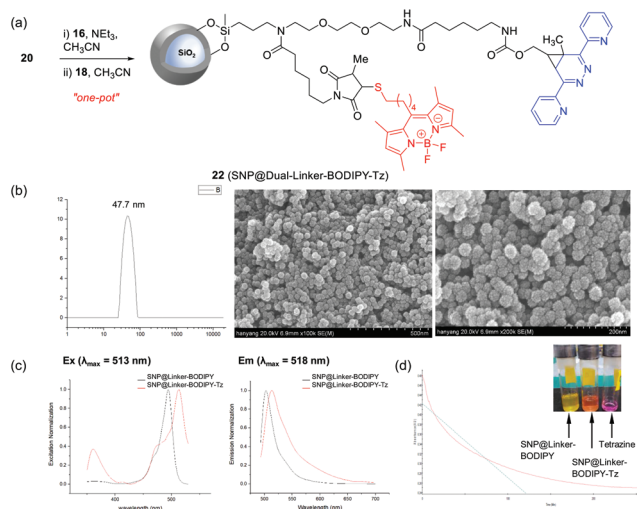


gation, and the supernatant was removed. The isolated SNP **20** was washed with water and redispersed in acetonitrile three times, which produced a colloidal solution of **20** without aggregation. The size and shape of the surface-modified SNP **20** were characterized using DLS and SEM (Fig. 2). To further characterize the covalently bound linker on SNP **20**, NMR experiments<sup>18</sup> were performed, as shown in Fig. 2. The dried SNP **20** was dispersed in D<sub>2</sub>O and treated with NaOD to disrupt silica nanoparticles to release silanol **22** in solution. No observation of particles in the DLS experiment indicated that silica nanoparticle **20** fully decomposed. The structure of compound **21** was determined by <sup>1</sup>H NMR and MS analyses. Interestingly, the <sup>1</sup>H NMR spectrum showed that the maleimide group was transformed to enoic acid under basic hydrolysis, and the proton on the cyclopropane ring was replaced by deuterium.<sup>19</sup> The proton signal at the silicon atom  $\alpha$ -position was used to estimate the loading efficacy of the linker on the silica surface. The integrated intensity of the methyl protons attached to silicon was compared with that of the protons derived from potassium phthalate, an internal standard. The result indicated that the surface coverage of **20** by organic linker is 0.75 molecules per nm<sup>2</sup> (see the ESI† for calculation).

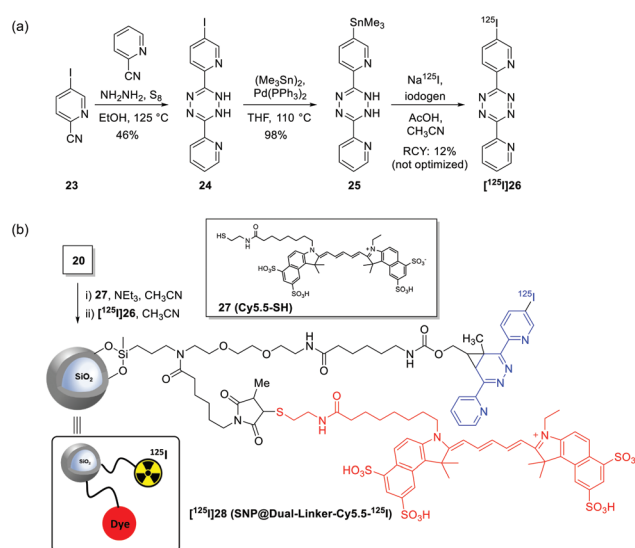
Since the installation of linker **15** on the SNP surface was confirmed, the bioorthogonal reactions used in Scheme 3 were applied to the linker-conjugated SNPs, as shown in Fig. 3. The dispersion of the dual linker-doped silica **20** in CH<sub>3</sub>CN was treated with BODIPY dye **16** in the presence of triethylamine, followed by the consecutive addition of tetrazine **18** in *one pot* to yield dual functional SNP **22**. After the solvent was exchanged with water and acetonitrile three times, the purified SNP dispersion **22** was characterized by DLS, SEM, and fluorescence spectroscopy. The DLS and SEM results showed that the size of the functionalized SNP **22** was consistently main-

tained (40 nm) without particle aggregation. The optical property data of **22** in CH<sub>3</sub>CN exhibited only a small red-shift compared with free silane **19**.<sup>20</sup> Furthermore, a kinetic study revealed that the rate of tetrazine cycloaddition on the SNP surface is slower than that of the reaction with the free linker **17** because of steric interference of the silica surface with the bioorthogonal reactions.

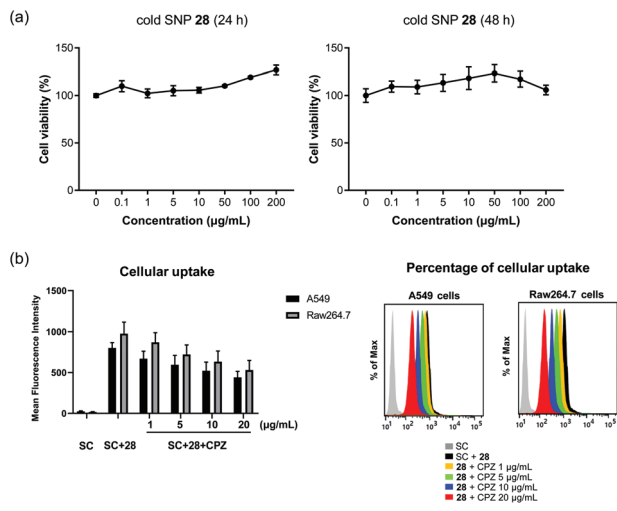
To investigate the biological application of dual linker-conjugated SNPs in an *in vivo* system, a biodistribution study using dual-imaging probes conjugated with a fluorophore and a radioactive isotope was performed (Scheme 4 and Fig. 4). Accordingly, a cyanine dye (Cy5.5) was applied to facilitate optical bioimaging since near-infrared (NIR) fluorescence shows relatively good tissue penetration and low background signal.<sup>21</sup> We also used iodine-125 as a source of the positron emission tomographic (PET) bioimaging probe, which could accurately visualize the biodistribution of SNPs in various tissues or organs *in vivo* regardless of tissue penetration depth. For the synthesis of radioactive tetrazine, we used an oxidative halo destannylation, recently developed by Valliant *et al.*,<sup>22</sup> to introduce <sup>125</sup>I into the pyridine moiety. Starting from 2-cyano-4-iodopyridine **23**, iodopyridinyl dihydrotetrazine **24** was prepared using an excess amount of hydrazine and sulfur, which was transformed to trimethylstannyl-substituted dihydrotetrazine **25** in high yield by a palladium-catalyzed coupling reaction. After we explored the reaction conditions for iodination using cold sodium iodide, radiolabeling was achieved by adding Na<sup>125</sup>I to a mixture of stannane **25** and iodogen under acidic conditions. This reaction was completed within 5 min at room temperature, and the corresponding [<sup>125</sup>I]-iodinated tetrazine **26** was used in the next step without purification. Following the same one-pot procedure used for the synthesis of SNP **22** (Fig. 3a), the dual linker conjugated SNP **20** was first



**Fig. 3** Synthesis and characterization of dual functional SNP **22**. (a) One-pot sequential bioorthogonal reactions on the SNP surface; (b) DLS and SEM images; (c) excitation and emission spectra of **22**; (d) UV absorbance intensity at 535 nm in cycloaddition of tetrazine **18** to SNP in CH<sub>3</sub>CN.



**Scheme 4** Synthesis of dual-imaging silica nanoparticle [<sup>125</sup>I]**28**. (a) Synthesis of <sup>125</sup>I-labeled tetrazine **26**; (b) one-pot synthesis of SNP conjugated with Cy 5.5 dye **27** and <sup>125</sup>I-tetrazine **26**.

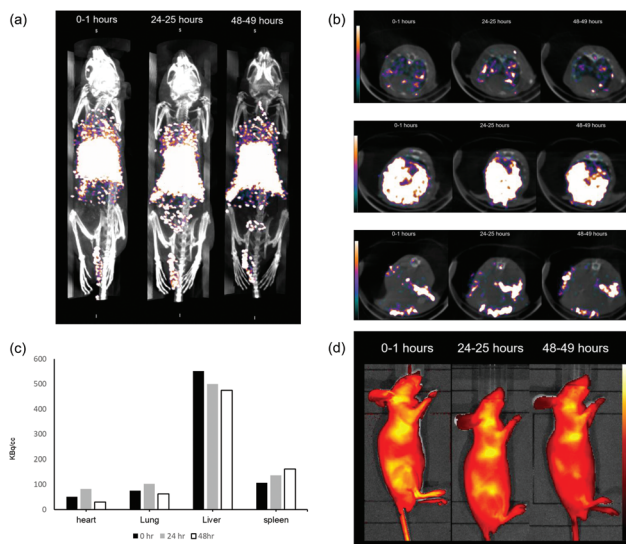


**Fig. 4** Cell viability and cellular uptake of SNP 28. (a) HEK293 T cells were incubated with SNP 28 for 24 h and 48 h respectively at the indicated concentrations; (b) SNP 28 ( $100 \mu\text{g mL}^{-1}$ ) was incubated in A549 and Raw264.7 cells in the presence of the indicated CPZ concentrations (SC: solvent control, 0.1% DMSO).

treated with thioalkyl cyanine dye 27, followed by cycloaddition of [ $^{125}\text{I}$ ]-tetrazine 26 to yield the desired SNP [ $^{125}\text{I}$ ]28, in which both imaging probes were covalently conjugated. On the other hand, the same one-pot procedure was applied to the synthesis of cold SNP 28, which was characterized by fluorescence, DLS and SEM (Fig. S2†).

Before the synthesized SNP [ $^{125}\text{I}$ ]28 was used in the *in vivo* experiment, the intrinsic cytotoxicity of cold SNP 28 was measured by the MTT assay,<sup>23</sup> which revealed that dual-modal SNP 28 induced no significant toxicity to HEK293 T cells, even at high concentrations (up to  $200 \mu\text{g mL}^{-1}$ ) for 48 h (Fig. 4a). The stability of cold SNP 28 was also tested in DI water,  $1\times$  PBS buffer and 10% FBS solution. The particle size and optical property of SNP 28 were not changed in the presence of the tested solutions, which indicated that SNP 28 is stable under physiological conditions (Fig. S3†). Additionally, the internalization of SNP 28 was investigated using chlorpromazine (CPZ) as an inhibitor of clathrin-mediated endocytosis. We observed that the SNP uptake by A549 and Raw264.7 decreased in dose-dependent manner by the clathrin inhibition (Fig. 4b). The result showed that clathrin-dependent endocytosis is the major mechanism of SNP 28 in cells.

Finally, a dispersed solution of Cy5.5 dye and  $^{125}\text{I}$ -labeled silica nanoparticle [ $^{125}\text{I}$ ]28 in water was tested for systemic circulation *in vivo* (Fig. 5). All protocols were approved by the Institutional Animal Care and Use Committee of the Seoul National University Bundang Hospital (IACUC number BA-2009-304-085-04). The Ji Seok Young Research Center is fully accredited by the AAALAC. All animals were cared for in accordance with the ILAR Guide for the Care and Use of Laboratory Animals 8th Edition. The biodistribution of this SNP was determined at 24 and 48 h post-injection in C57Bl/6 mice. Immediately after the intravenous injection of SNP



**Fig. 5** Sequential *in vivo* biodistribution imaging data after intravenously administration of the Cy5.5 and  $^{125}\text{I}$  covalently-labeled SNP [ $^{125}\text{I}$ ]28 to C57BL/6 mice. (a) Three-dimensional PET/CT images of whole body; (b) PET/CT images of transverse sections: lung (upper), liver (middle), and spleen (lower); (c) quantitative analysis of radioactivity in each organ of interest; (d) fluorescence images.

[ $^{125}\text{I}$ ]28, most SNPs accumulated in the liver, but gradually cleared at 24 and 48 h. The SNPs in the lung and heart increased for 24 h and then decreased at 48 h, whereas the PET/CT signals in the spleen increased up to 48 h. With regard to the safety of SNP [ $^{125}\text{I}$ ]28, the total activity that was detected in the PET/CT images showed no significant change for 48 h. In addition, no uptake of I-125 in the thyroid was observed, which indicated that there was no significant degradation of our dual-imaging SNPs up to 48 h. In the fluorescence experiment, most of the SNPs were detected in the liver. The biodistribution based on the fluorescence images was comparable to that found in the SPECT/CT images. The results established that our dual-modal imaging SNPs were tolerated under *in vivo* conditions for a long period of time.

## Conclusions

We successfully developed new bifunctional silica nanoparticles, which can be covalently conjugated with heteroatom nucleophiles and tetrazines *via* one-pot consecutive bioorthogonal reactions. The synthesis of key methallylsilane 15 with cyclopropenes and maleimide was achieved by traceless Staudinger ligation. Methallylsilane 15, which has two reactive alkenes, was introduced onto SNP surfaces smaller than 50 nm *via* Sc(OTf)<sub>3</sub>-catalyzed siloxane formation. The dual linker-grafted SNP 20 was sufficiently stable to enable both conjugate addition and cycloaddition reactions in *one pot*. In addition, we demonstrated that dual functional SNP [ $^{125}\text{I}$ ]28 labeled with NIR dye and  $^{125}\text{I}$  is applicable to biodistribution studies. Thus, we believe that the current covalent conjugation

method using bioorthogonal dual linkers offers great potential for the development of multifunctional silica nanoparticles for a wide variety of biomedical applications.

## Author contributions

Jaewoon Lee, Jeunghwan Kim, Incheol Heo, Su Jin Kim and Sein Jang collected experimental data and analysed the data. Kwang-Suk Jang, Chul-Su Yang, Youngbok Lee, and Won Cheol Yoo supervised the investigation and co-wrote the manuscript. Sun-Joon Min conceptualized the project methodology, wrote the original draft and supervised the investigation.

## Conflicts of interest

The authors declare no competing financial interest.

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