Regular Article*Highlighted Paper selected by Editor-in-Chief***An Activatable Fluorescent γ -Polyglutamic Acid Complex for Sentinel Lymph Node Imaging**Masayori Hagimori,^{a,b} Eri Hatabe,^a Kohei Sano,^{a,c,d} Hirotaka Miyazaki,^a Hitoshi Sasaki,^e Hideo Saji,^d and Takahiro Mukai^{*,a}

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Sentinel lymph nodes (SLN) are the first lymph nodes (LN) where cancer cells metastasize from the primary tumor. We designed fluorophore-quencher-based activatable nanoparticles for SLN imaging. We selected TAMRA as a fluorophore and BHQ2 or QSY7 as a quencher. Ternary anionic complexes were constructed with generation 4th polyamidoamine dendrimer (G4) modified with TAMRA and *p*-SCN-Bn-DTPA (DTPA), polyethyleneimine (PEI) modified with BHQ2 or QSY7, and γ -polyglutamic acid (γ -PGA) by the electrostatic self-assembly system. TAMRA-G4-DTPA/PEI-BHQ2/ γ -PGA and TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complexes had a particle size of about 40 nm and a ζ -potential of -50 mV, and showed fluorescence resonance energy transfer (FRET) quenching. Fluorescence microscopy studies demonstrated that TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex produced intracellular fluorescent signals in the lysosome. During *in vivo* fluorescent imaging, TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex enabled the detection of mouse popliteal LN. The fluorophore-quencher conjugated γ -PGA complex based on FRET quenching would be useful for fluorescence-based optical imaging of SLN.

Key words fluorescence-based optical imaging; sentinel lymph node; activatable; self-assembly; nanoparticle

Fluorescence-based optical imaging is a powerful tool to visualize biological events associated with disease at the molecular level *in vivo*. Compared to other imaging modalities used for diagnosis, fluorescence-based optical imaging enables real time, precise, and multicolor visualization of targets by fluorescence imaging probes.^{1,2} Recently, activatable fluorescent probes that can turn on and off in response to targets by pH, enzyme activity, and redox potential have attracted attention because they could offer highly selective and sensitive information of molecular targets.² There are several known approaches for the design of activatable fluorescent probes such as self-quenching systems, photon-induced electron transfer-based fluorophores, and fluorescence resonance energy transfer (FRET) quenching by the fluorophore-quencher interaction.^{3,4}

Sentinel lymph nodes (SLN) are the first lymph nodes (LN) where cancer cells are most likely to spread from the primary tumor.^{5,6} The accurate identification of SLN by imaging is useful for SLN biopsy in that it could reduce the need for unnecessary LN dissection and improve the quality of life for patients with early stage breast cancer.⁷⁻¹¹ In a previous study, we reported that the indium-111-labeled γ -polyglutamic acid (γ -PGA) complex composed of generation 4th polyamidoamine (PAMAM) dendrimer (G4) conjugated with *p*-SCN-Bn-DTPA (G4-DTPA), polyethyleneimine (PEI), and γ -PGA visualized rat popliteal LN with single photon emission computed tomography/computed tomography (SPECT/CT).¹² G4 dendrimer has 64 amine terminal groups which are easily modified by functional groups including DTPA and has shown

potential applications in nanomedicine. G4-DTPA electrostatically interacts with PEI to form polyplex. The surface charge of nanoparticles is one of the essential factors that dictate biodistribution and cytotoxicity.¹³⁻¹⁶ The γ -PGA with negative charge contributed to the low cytotoxicity and the high biocompatibility.^{12,17-19} In general, negatively-charged complexes exhibit low cellular uptake because of electrostatic repulsion against the negatively-charged cellular membrane.^{20,21} However, the indium-111-labeled γ -PGA complex underwent high uptake by macrophage cells (high populations in LN) *via* γ -PGA-specific pathway and phagocytosis.¹²

Recently, SLN navigation surgery guided by the cyanine fluorophore, indocyanine green (ICG), has been shown to enable real-time detection of the correct site for exploring SLN.²² It has been reported that a higher detection rate of SLN were obtained by the combination of ICG and radiotracer, and dual-modality approaches using ICG and radiotracer also enabled rapid detection of SLN.^{23,24} Because the γ -PGA complex has many amino groups in G4 and PEI that can be chemically modified with radioisotopes (In-111) and dye molecules, it has the potential to be a dual-modality (SPECT and fluorescence) probe for SLN imaging.

Herein, to develop the γ -PGA complex as a fluorescence probe for SLN detection, we synthesized and evaluated the fluorophore-quencher conjugated activatable fluorescent γ -PGA complex based on FRET quenching. In this system, fluorescence quenching depended on the fluorophore-quencher interaction and was activated by the dissociation of the fluorophore-quencher pair. A highly selective and sensitive *in vivo*

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imaging for SLN can be expected by using activatable fluorescent γ -PGA complex.

MATERIALS AND METHODS

Materials and Reagents PAMAM dendrimer (generation 4th, G4) and PEI (branched form, average molecular weight of 25000) was purchased from Sigma-Aldrich Co. (Milwaukee, WI, U.S.A.). *p*-SCN-Bn-DTPA (1-(4-isothiocyanatobenzyl)-diethylenetriamine pentaacetic acid) was obtained from Macrocytics, Inc. (Dallas, TX, U.S.A.). TAMRA succinimidyl ester and QSY7 succinimidyl ester were purchased from Invitrogen Corporation (Carlsbad, CA, U.S.A.). BHQ2 succinimidyl ester was purchased from Biosearch Technologies, Inc. (Petaluma, CA, U.S.A.). The γ -PGA was provided by Yakult Pharmaceutical Industry Co., Ltd. (Tokyo, Japan). All other chemicals were of the highest purity available.

Synthesis of TAMRA Conjugated G4 Dendrimer (TAMRA-G4) A solution of TAMRA succinimidyl ester (1.2 mg, 2.2 μ mol) in dimethyl sulfoxide (DMSO) (148 μ L) was added to a solution of G4 dendrimer (4.6 mg, 1.1 μ mol) in 0.1 mol/L phosphate buffer (PB, pH 8.6, 320 μ L) at room temperature (r.t.) for 24 h. The mixture was purified by dialysis membrane (Spectra/Por 2 Dialysis Tubing, 12–14 kD MWCO) for 24 h and lyophilized. The concentration of TAMRA was measured after the conjugation step by absorption with the UV-Vis system (UV-2450, Shimadzu Co., Kyoto, Japan) to confirm the number of fluorophore molecules conjugated with G4 dendrimer.

Synthesis of DTPA Conjugated TAMRA-G4 (TAMRA-G4-DTPA) *p*-SCN-Bn-DTPA (5.9 mg) in dry *N,N*-dimethylformamide (DMF) (50 μ L) was added to the solution of TAMRA-G4 (2.0 mg) in 0.1 mol/L PB (pH 8.6, 320 μ L) at 37°C, adjusted to pH 8.6 to 8.0 with 1 mol/L NaOH, for 24 h. The number of DTPAs loaded on a G4 dendrimer was calculated according to a previously reported method.²⁵⁾

Synthesis of BHQ2 or QSY7 Conjugated PEI (PEI-BHQ2, PEI-QSY7) A solution of BHQ2 succinimidyl ester (0.4 mg, 0.6 μ mol) in DMSO (96 μ L) or QSY7 succinimidyl ester (0.5 mg, 0.6 μ mol) in DMSO (63 μ L) was added to a solution of PEI (8.0 mg, 0.3 μ mol) in PB (pH 8.6, 160 μ L) at r.t. for 24 h. The mixture was purified by dialysis membrane for 24 h and lyophilized. The concentrations of BHQ2 or QSY7 were measured after the conjugation step by absorption with the UV-Vis system to confirm the number of quencher molecules conjugated with PEI.

Preparation of Activatable Fluorescent Ternary Complexes TAMRA-G4-DTPA and PEI-BHQ2 or PEI-QSY7 in 5% glucose were mixed by pipetting thoroughly and left for 15 min at r.t., and then γ -PGA in 5% glucose were mixed with TAMRA-G4-DTPA/PEI-BHQ2 or TAMRA-G4-DTPA/PEI-QSY7 complexes by pipetting and left for another 15 min at r.t. In this study, we constructed ternary complexes without purification at a theoretical charge ratio of 1:8:16 corresponding to carboxyl groups of TAMRA-G4-DTPA:amino groups of PEI-BHQ2 or PEI-QSY7:carboxyl groups of γ -PGA.¹²⁾

Physicochemical Property of Ternary Complexes The particle sizes and ζ -potentials of each ternary complex in 5% glucose were measured using a Zetasizer Nano ZS (Malvern Instruments, Ltd., Malvern, U.K.). The number-fractionated mean diameter was measured. Fluorescent properties of

each ternary complex in 5% glucose were measured using a fluorescence spectrophotometer (F7000, Hitachi High-Tech, Tokyo, Japan).

Fluorescence Microscopy Study RAW264 cells (2×10^5) were plated on a Lab-Tek II System Glass 2 well chamber slide (Thermo Scientific, NY, U.S.A.) and incubated for 16 h. TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex (5 μ g TAMRA-G4-DTPA) in Dulbecco's modified Eagle's medium (DMEM) without phenol red was added in each well and incubated for 24 h. Imaging was performed using a fluorescence microscope (Eclipse Ti, Nikon, Tokyo, Japan) without removing medium from the chamber slide ($n=3$). The Cy3 filter cube, excitation wavelength from 513 to 556 nm, emission wavelength from 570 to 613 nm, was employed for imaging.

Lysosomal marker (LysoTracker[®] Green DND-26, Invitrogen Co.) was used to investigate the intracellular localization of TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex (5 μ g TAMRA-G4-DTPA) ($n=3$). After 24 h, the medium was removed and cells were washed once with phosphate buffered saline (PBS). Lysosomal marker was added and incubated for 0.5 h. Imaging was performed using the fluorescence microscope equipped with the fluorescein isothiocyanate (FITC) filter cube (excitation wavelength from 467 to 498 nm, emission wavelength from 513 to 556 nm).

In Vitro Imaging Study TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA (5 μ g TAMRA-G4-DTPA) was added to RAW264 cells. After 0, 6, and 24 h incubation, the cells were washed and collected into tubes. Fluorescence images of cell pellets (2×10^6 cells) were acquired by IVIS Lumina XR (Perkin-Elmer, Inc., Waltham, MA, U.S.A.) (excitation wavelength: 520 nm, emission wavelength: 570 nm, exposure time 1 s) ($n=4$). Fluorescence signal intensity was analyzed by Living Image software (PerkinElmer, Inc.).

In Vivo Fluorescence Imaging in Normal Mice Animal studies were conducted in accordance with the institutional guideline of Kyoto University, and the experimental procedures were approved by the Kyoto University Animal Care Committee. *in vivo* fluorescence imaging was performed in six-week-old male BALB/c-nu/nu mice (Japan SLC Inc., Hamamatsu, Japan). TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex (5 μ g TAMRA-G4-DTPA) was injected into the normal mouse footpad. At 6 h after the injection, the imaging data was acquired using Maestro *in vivo* imaging system (Maestro 2, CRi Inc., Woburn, MA, U.S.A.) ($n=2$). After *in vivo* imaging study, the popliteal LNs were excised from both injected and non-injected sides, and then *ex vivo* fluorescence imaging study was performed. The contralateral popliteal LN was used as a control. A band-pass filter from 503 to 548 nm and a long-pass filter over 560 nm were used for excitation and emission light, respectively. The tunable filter was automatically stepped in 10 nm increments from 560 to 750 nm at constant exposure (100 and 500 ms for *in vivo* and *ex vivo* imaging, respectively) while the camera sequentially captured images at each wavelength interval. The spectral fluorescence images consisting of autofluorescence and TAMRA were unmixed based on their spectral patterns with Maestro software (CRi Inc.) as previously reported.²⁶⁾

RESULTS AND DISCUSSION

TAMRA (rhodamine-core fluorophore) exhibits bright fluo-

rescence at around 580nm, and has good properties such as pH insensitivity, high photostability, and high fluorescence quantum yield.²⁷⁾ Therefore, TAMRA is widely used to label macromolecules such as peptides and proteins.^{28,29)} To obtain the effective FRET quenching, the absorption spectrum of the quencher should overlap the emission spectrum of the fluorophore and when the distance between quencher and fluorophore is typically less than 100 Å.³⁰⁾ BHQ2 and QSY7 are non-fluorescent dyes, and have absorption peaks from 550 to 650nm and 500 to 600nm, respectively.^{31–34)} For these reasons, we selected TAMRA as the fluorophore to conjugate G4 dendrimer and BHQ2 and QSY7 as quencher dyes to conjugate PEI. Fluorophore could be brought close to quencher by the formation of a complex, and the FRET quenching is expected.

The preparation scheme of ternary complexes is summarized in Fig. 1. The reaction of TAMRA succinimidyl ester and G4 dendrimer in DMSO at r.t. afforded TAMRA-G4. The mean number of TAMRA fluorophores per G4 dendrimer was estimated to be 0.91 by absorption with the UV-Vis system. Negative charge was given to TAMRA-G4 by conjugated with *p*-SCN-Bn-DTPA, and TAMRA-G4-DTPA showed -20.5 ± 0.9 mV ζ -potential. The number of DTPAs conjugated to a TAMRA-G4 was about fifty. BHQ2-labeled PEI (PEI-BHQ2) and QSY7-labeled PEI (PEI-QSY7) were synthesized by the reaction of PEI with BHQ2 succinimidyl ester or QSY7 succinimidyl ester in 0.1 mol/L PB. The mean number of BHQ2 or QSY7 quenchers per PEI was estimated

to be 1.8 and 0.5, respectively. The TAMRA-G4-DTPA/PEI-BHQ2 complex and TAMRA-G4-DTPA/PEI-QSY7 complex were obtained at a 1:8 charge ratio of the carboxyl group of TAMRA-G4-DTPA:amino group of PEI-BHQ2 or PEI-QSY7 with approximately +50 mV ζ -potential. For the preparation of ternary complexes, γ -PGA solution were mixed with TAMRA-G4-DTPA/PEI-BHQ2 or TAMRA-G4-DTPA/PEI-QSY7 complexes and left for 15 min. Always-on fluorescent TAMRA-G4-DTPA/PEI/ γ -PGA complex was also obtained from TAMRA-G4-DTPA with PEI and γ -PGA. As shown in Table 1, each complex had an average diameter of 40 nm within the preferred range for lymphatic uptake (10 to 50 nm)³⁵⁾ and was strongly negatively charged (ζ -potential: approximately -50 mV) in 5% glucose. These properties for each complex were similar to that of previously reported γ -PGA acid complex (G4-DTPA/PEI/ γ -PGA complex) for sentinel LN imaging.¹²⁾ In addition, these results demonstrated that conjugated molecules (TAMRA, BHQ2, and QSY7) did not influence the electric interaction of complexes.

The fluorescence spectrum of each complex is shown in Fig. 2. The fluorescence intensity of TAMRA-G4-DTPA/PEI-BHQ2/ γ -PGA complex at 580 nm was 52% decreased from that of TAMRA-G4-DTPA/PEI/ γ -PGA complex, indicating that FRET quenching occurred. In TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex, it was also observed that the fluorescence intensity at 580 nm was 63% decreased. The number of BHQ2 molecules conjugated to PEI was more than that of QSY7 molecules, however, the higher quench-

Fig. 1. Reaction Scheme for the Synthesis of TAMRA-G4-DTPA/PEI-BHQ2/ γ -PGA and TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA Complexes with Negative ζ -Potential

Table 1. Particle Size and ζ -Potential of Each Complex

Complex	Particle size (nm)	ζ -Potential (mV)
G4-DTPA/PEI/ γ -PGA	33.2 ± 4.5	-47.4 ± 0.9
TAMRA-G4-DTPA/PEI/ γ -PGA	41.4 ± 0.5	-50.5 ± 2.1
TAMRA-G4-DTPA/PEI-BHQ2/ γ -PGA	40.7 ± 4.3	-48.3 ± 1.2
TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA	41.2 ± 5.0	-48.8 ± 3.0

Each value represents the mean $\pm$ S.D. ($n=3$).

ing effect of fluorophore-quencher pairs was observed in the TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex. BHQ2 is an azo-based compound, whereas QSY7 is a rhodamine-based compound as well as TAMRA.^{30,31} Therefore, it is supposed that the stacking interaction of fluorophore-quencher in the TAMRA-QSY7 pair should be stronger than that in the TAMRA-BHQ2 pair. For these reasons, further experiments including *in vitro* and *in vivo* fluorescence imaging were

performed using the TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex.

To evaluate the quenching ability and cell membrane permeability *in vitro*, we obtained fluorescence microscope images of the TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex in mouse macrophage-like cells, RAW264. Upon incubation of TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex in RAW264 cells, a time-dependent increase in intracellular fluorescence

Fig. 2. Fluorescence Emission Spectra of Complexes

The fluorescence intensities of TAMRA-G4-DTPA/PEI-BHQ2/ γ -PGA complex and TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex at 580 nm was decreased by 52 and 63%, respectively, from that of TAMRA-G4-DTPA/PEI/ γ -PGA complex.

Fig. 3. Fluorescence Microscopy Images of Macrophages (RAW264) ($n=3$)

(A) Non-treated cells, (B) cells treated with TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex. Cells were observed without removing medium including TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex from the chamber slide.

Fig. 4. *In Vitro* Fluorescence Imaging Study

(A) *In vitro* fluorescence images of cell pellets 0, 6, and 24 h after incubation with TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA. (B) Time-dependent change of fluorescence signal intensity of TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA taken up by RAW264 cells ($n=4$).

Fig. 5. Subcellular Localization of TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA in Macrophages (RAW264) ($n=3$)

(a) DIC, (b) TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex (TAMRA fluorescent signal: red), (c) LysoTracker fluorescent signal (green), and (d) Merge: yellow signals indicate colocalization of TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex and LysoTracker.

Fig. 6. *In Vivo* Fluorescent Imaging after Injection of TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA Complex into Mouse Footpads (Arrows)

TAMRA fluorescence signal (red) was clearly visualized in mouse popliteal LN (arrow heads). *Ex vivo* fluorescent image acquired after the excision of lymph nodes (A: injected side, B: non-injected side (control)).

intensity of TAMRA was observed, whereas fluorescence was not observed in medium (Fig. 3). A quantitative evaluation of the cellular uptake of TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA was performed by fluorescence imaging. Fluorescence signal was increased in a time-dependent manner as determined by *in vitro* fluorescence microscopy study (Fig. 4). These results indicated that this complex is activatable; that is, stable and quenched in the medium, and dissociated and dequenched inside the cells. It has been reported that nanoparticles entering cells *via* endocytic pathways are internalized in lysosome and degraded by acidic lysosomal pH.³⁶⁾ Then, as shown in Fig. 5, the fluorescence of TAMRA was co-localized with the fluorescence of LysoTracker, demonstrating that the dissociation of TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex was occurred in the lysosome.

In vivo fluorescence imaging was conducted by administration of TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex into mouse the footpad intradermally. The popliteal LN (SLN in this model) was clearly visualized by the TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex after 6h (Fig. 6). Fluorescence signal was also observed in injection site, suggesting the probe uptake by inflammatory cells. To analysis the localization of TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex, *ex vivo* experiment was performed by *ex vivo* imaging the excision of lymph nodes. These results indicate that the TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex was internalized within the popliteal LN. On the other hand, the background signal was weak, suggesting that the TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex did not leak to the capillary vessels, but was retained within the lymph nodes.

CONCLUSION

We designed and prepared two fluorophore-quencher conjugated γ -PGA complexes, TAMRA-G4-DTPA/PEI-BHQ2/ γ -PGA complex, and TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex by the electrostatic self-assembly system. In this system, fluorescence was quenched by a fluorophore-quencher pair-based FRET quenching. The fluorescence of TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex was quenched more by a strong stacking interaction of fluorophore-quencher, and dequenched by the dissociation of this complex in the lysosome. During the *in vivo* fluorescent imaging study, the TAMRA-G4-DTPA/PEI-QSY7/ γ -PGA complex clearly visualized the mouse popliteal LN. DTPA was utilized to introduce negative charges to G4 dendrimer in this study functions as a chelation site with indium-111.¹²⁾ Therefore, this complex could be applicable to optical and SPECT dual-modality probes. We believe that this novel complex will have wide applications in SLN imaging.

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Conflict of Interest The authors declare no conflict of Interest.

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