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Research paper

Essence of affinity and specificity of peanut agglutinin-immobilized fluorescent nanospheres with surface poly(N-vinylacetamide) chains for colorectal cancer

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ABSTRACT

We have designed a novel colonoscopic imaging agent that is composed of submicron-sized fluorescent polystyrene nanospheres with two functional groups – peanut agglutinin (PNA) and poly(N-vinylacetamide) (PNVA) – on their surfaces. PNA is a targeting moiety that binds to β-D-galactosyl-(1-3)-N-acetyl-D-galactosamine (Gal-β(1-3)GalNAc), which is the terminal sugar of the Thomsen–Friedenreich antigen that is specifically expressed on the mucosal side of colorectal cancer cells; it is anchored on the nanosphere surface via a poly(methacrylic) acid (PMAA) linker. PNVA is immobilized to enhance the specificity of PNA by reducing nonspecific interactions between the imaging agent and normal tissues. The essential nature of both functional groups was evaluated through in vivo experiments using PNA-free and PNVA-free nanospheres. The imaging agent recognized specifically tumors on the cecal mucosa of immune-deficient mice in which human colorectal cancer cells had been implanted; however, the recognition capability disappeared when PNA was replaced with wheat germ agglutinin, which has no affinity for Gal-β(1-3)GalNAc. PNA-free nanospheres with exclusively surface PNVA chains rarely adhered to the cecal mucosa of normal mice that did not undergo the cancer cell implantation. In contrast, there were strong nonspecific interactions between normal tissues and PNA-free nanospheres with exclusively surface PMAA chains. In vivo data proved that PNA and PNVA were essential for biorecognition for tumor tissues and a reduction of nonspecific interactions with normal tissues, respectively.

1. Introduction

Colorectal cancer is a major cause of mortality and morbidity in developed countries. Currently, surgical removal is the primary treatment of choice, and early detection and resection are indispensable tools for curing colorectal cancer [1]. Colonoscopy is used for screening colorectal cancer with its ability to provide a definitive diagnosis. The cancer that remains in the mucous membrane or only minimally invades the submucosal tissues without vessel invasion is often treated by endoscopic mucosal resection (EMR). This technique is a minimally invasive operation that can serve as a viable alternative to surgical resection in the early stage of the cancer [2–4]. While colonoscopy remains critically important, the standard white-light colonoscopy has a significant limitation: it can only detect tumor tissues that are larger than ca. 1 cm in size. Notably, tumors of this size have a relatively high probability of metastasis [5]. Magnifying endoscopy has contributed to the detection of small-sized colorectal cancer; however, accurate differentiation of neoplastic mucosal changes in real-time remains a significant challenge [6,7]. Thus, there is a great need for developing an endoscopic imaging agent for the early detection of small-sized colorectal cancer that has few risk of metastasis [8], as well as novel imaging strategies for endoscopy such as narrow band imaging and autofluorescence imaging [6–11].

Colorectal cancer initially develops in the mucous membrane of the large intestine. In view of this characteristic, we designed a nonabsorbable colonoscopic imaging agent that is administered intracolonically and recognizes tumor-derived changes on the mucosal surface with high affinity and specificity [12–15]. The imaging agent is peanut (*Arachis hypogaea*) agglutinin

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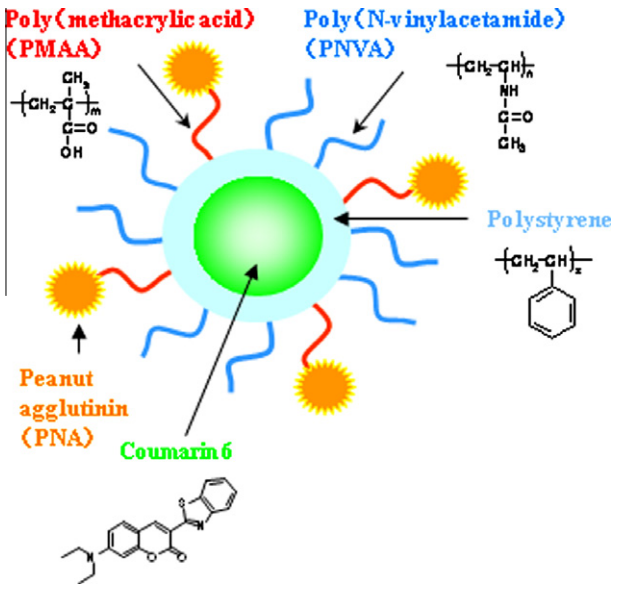

Fig. 1. Schematic representation of the imaging agent.

(PNA)-immobilized polystyrene nanospheres with surface poly(N-vinylaceamide) (PNVA) chains encapsulating coumarin 6 (Fig. 1). Lectins are proteins that recognize and bind reversibly to specific carbohydrate residues expressed on the cell surface [16]. PNA is immobilized on the nanosphere surface as a targeting moiety that binds to β-D-galactosyl-(1-3)-N-acetyl-D-galactosamine (Gal-β(1-3) GalNAc), which is the terminal sugar of the Thomsen–Friedenreich (TF) antigen that is specifically expressed on the mucosal side of colorectal cancer cells [17–22]. On the other hand, the initial tumor-derived change is very small throughout the entire large intestine. To detect such small changes accurately, the imaging agent should have a strong affinity for targets (i.e., tumor tissues) with minimal nonspecific interactions with nontargets (i.e., normal tissues). Previously, we have demonstrated that PNVA rarely adhered to the intestinal mucosa due to its strong hydrophilicity [23,24]. This polymer is immobilized on the nanosphere surface to enhance the specificity of PNA by reducing the nonspecific interactions between the imaging agent and normal tissues. Coumarin 6 is used as the fluorescent dye that provides an endoscopically detectable fluorescence intensity. The dye is encapsulated into the polystyrene core of nanospheres through their hydrophobic interactions. We expect that intracolonic (enema) administration of the imaging agent will lead to the specific accumulation on the mucosal surface of cancer tissues in the large intestine through recognition of the tumor-specific TF antigen. Real-time and accurate diagnosis of small-sized early colorectal cancer can be achieved through observations of a pronounced fluorescence contrast between the normal and tumor tissues using the fluorescence endoscopy.

In vivo experiments using a human colorectal cancer orthotopic mouse model demonstrated that the imaging agent recognized millimeter-sized tumors on the intestinal mucosa with high affinity and specificity, irrespective of implanted cancer cell type [13,14]. When mice that did not undergo the cancer cell implantation were used, nonspecific interactions between the imaging agent and normal mucosa were hardly ever observed. Our latest study further indicates that the imaging agent is a safe and stable probe that remains in the large intestine without systemic exposure [15]. It is obvious that PNA-immobilized fluorescent nanospheres with surface PNVA chains are a potential candidate for use as a colonoscopic imaging agent. Researches on the productivity of the imaging agent under conditions specified by good manufacturing practice (GMP) and the biorecognition capability for human tissues removed from cancer patients are being advanced with the aim of realizing clinical trials of the imaging agent in the near future.

As illustrated in Fig. 1, PNA and PNVA are immobilized on the nanosphere surface with the expectation that the former and the latter increase the affinity and specificity, respectively, of the imaging agent for colorectal cancer. In a series of research, an in vitro hemagglutination test, which is frequently used for the assay of lectin activities [19], was initially performed to optimize the chemical structure of the imaging agent, and the imaging agent with high affinity and specificity for Gal-β(1-3)GalNAc exposed on the erythrocyte surface was successfully obtained [12,13]. It appears that the performances of PNA and PNVA are consistent with our expectations; however, no evidence for the indispensability of both functional groups has been discovered through in vivo experiments. This paper describes that PNA and PNVA are prerequisite for biorecognition for tumor tissues and a reduction of nonspecific interactions with normal tissues, respectively, through animal experiments that involved several types of nanospheres including PNA- and PNVA-free ones.

2. Materials and methods

2.1. Materials

N-vinylacetamide (NVA) monomers were donated by Showa Denko Co. (Tokyo, Japan). Coumarin 6, PNA, and wheat germ (*Triticum vulgaris*) agglutinin (WGA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were commercial products of reagent grade.

The human colorectal adenocarcinoma cell line, HT-29, was purchased from Dainippon-Sumitomo Pharma Biomedical Co. Ltd. (Osaka, Japan). McCoy's 5A Medium, Modified (with sodium bicarbonate, without L-glutamine) and Dulbecco's Phosphate Buffered Saline (PBS with $CaCl_2$ and $MgCl_2$) were obtained from Sigma–Aldrich. Fetal bovine serum (FBS), penicillin (10,000 U/mL), streptomycin (10 mg/mL), L-glutamine (200 mM), and trypsin–EDTA (0.25% trypsin and 1 mM EDTA) were purchased from GIBCO Laboratories (Lenexa, KS, USA). Preserved rabbit blood and neuraminidase (sialidase, 1 U/mL, extracted from *Arthrobacter ureafaciens*) were obtained from Nippon Bio-Test Laboratories Inc. (Tokyo, Japan) and Roche Diagnostics (Indianapolis, IN, USA), respectively.

2.2. Preparation of nanospheres

2.2.1. Preparation of lectin-free nanospheres

As shown in Table 1, three types of lectin-free nanospheres were prepared in the same manner described previously [12,13]. Briefly, PNVA and poly(*tert*-butyl methacrylate) (PBMA) were prepared by the free radical polymerization of NVA and butyl methacrylate (BMA) monomers, respectively, in the presence of 2-mercaptoethanol. The resulting hydroxyl group-terminated PNVA and PBMA were reacted with *p*-chloromethyl styrene. Vinylbenzyl group-terminated poly(methacrylic acid) (PMAA) was obtained by hydrolyzing vinylbenzyl group-terminated PBMA in the presence of hydroquinone. Fluorescent nanospheres with surface chains of PNVA and PMAA (a precursor of the imaging agent, Run 1) were prepared by dispersion copolymerization between vinylbenzyl group-terminated PNVA, vinylbenzyl group-terminated PMAA, and styrene at a weight ratio of 1:1:2 in an ethanol/water mixture containing 2,2′-azobisisobutyronitrile and coumarin 6 (0.1% of the total monomers). Either vinylbenzyl

Table 1
Characterization of lectin-free and lectin-immobilized fluorescent nanospheres prepared in this study.

Characterization		Lectin-free fluorescent nanospheres			Lectin-immobiized fluorescent nanospheres	
		Run l Nanospheres with PNVA and PMAA chains	Run 2 Nanospheres with PNVA chains	Run 3 Nanospheres with PMAA chains	Run 4 PNA-immobilized nanospheres with PNVA chains	Run 5 WGA-immobilized nanospheres with PNVA chains
Average molecular weight of PNVA (Mw/Mn)		9500/4000	9500/4000	–[i]	9500/4000	9500/4000
Average molecular weight of PMAA (Mw/Mn)		10,000/5600	–[i]	10,000/5600	10,000/5600	10,000/5600
NVA/MAA[a]		0.52/0.48	1.0/0.0	0.0/1.0	0.52/0.48	0.52/0.48
Particle size[b] (nm)		302 ± 80	384 ± 90	512 ± 148	–[h]	–[h]
Zeta potential[c] (mV)		−21.8	−2.8	−36.1	–[h]	–[h]
Encapsulated coumarin 6[d] (μg/mg)		0.8	1.0	1.1	0.8	0.8
Immobilized lectin (μg/mg)[e]		–[i]	–[i]	–[i]	6.60	3.53
Agglutination of Gal-P(l-3)GalNAc-exposed erythrocytes[f] (μg/mL)	Lectin equivalent	–[i]	–[i]	–[i]	0.0320	>17.7
	Nanosphere equivalent	19.5	>20,000	6.25	4.85	>5014
Agglutination of plain erythrocytes[g] (μg/mL)	Lectin equivalent	–[i]	–[i]	–[i]	>33.0	0.0345
	Nanosphere equivalent	39.1	>20,000	6.25	>5000	9.77

[a] Ratio of the number of NVA units to that of MAA units on the nanosphere surface calculated from spectra of electron spectroscopy for chemical analysis (ECSA).
[b] Weight-average diameter (mean ± s.d.).
[c] Measured in neutral PBS at 25 °C.
[d] Encapsulated amount (μg) of coumarin 6 per milligram of nanospheres.
[e] Immobilized amount (μg) of lectin (PNA or WGA) per milligram of nanospheres.
[f] Mirrimum concentration of immobilized lectins/nanospheres that induced agglutination of neurarrrinidase-treated (Gal-P(l-3)GalNAc-exposed) erythrocytes.
[g] Minimum concentration of immobilized lectins/nanospheres that induced the agglutination of neurarrrinidase-untreated (plain) erythrocytes.
[h] Not tested.
[i] Not required.

group-terminated PNVA or vinylbenzyl group-terminated PMAA was copolymerized with styrene at a weight ratio of 1:1 to obtain fluorescent nanospheres with the respective surface chains solely (Runs 2 and 3). After the unreacted substances and unencapsulated coumarin 6 were removed by centrifugation, lectin-free nanospheres were dispersed in purified water at a concentration of 20 mg/mL.

2.2.2. Preparation of lectin-immobilized nanospheres

A couple of lectin-immobilized nanospheres were prepared (Runs 4 and 5, Table 1). PNA was bound to the fluorescent nanospheres with surface chains of PNVA and PMAA (Run 1) through the coupling of the amino groups of PNA with the carboxyl groups of PMAA activated by pre-incubation with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide [12,13]. The resulting PNA-immobilized fluorescent nanospheres with surface PNVA chains (i.e., the imaging agent, Run 4) were purified and then dispersed in purified water at a concentration of 20 mg/mL. PNA was replaced with WGA, and WGA-immobilized fluorescent nanospheres with surface PNVA chains (Run 5) were prepared in the same manner described above.

2.3. Characterization of nanospheres

Routine characterization of lectin-free and lectin-immobilized fluorescent nanospheres was performed as described previously [12,13]. Briefly, weight- and number-average molecular weights (Mw and Mn) of PNVA and PMAA were determined by gel permeation chromatography. The ratio of the number of NVA units to that of MAA units on the nanosphere surface was evaluated by electron spectroscopy for chemical analysis (ESCA). The nanosphere size was measured by dynamic light-scattering spectrophotometry. The zeta potential of nanospheres was measured by electrophoretic light-scattering spectrophotometry in PBS. The amount of coumarin 6 encapsulated into the nanosphere core was measured by spectrophotometry. The amount of lectin immobilized on the nanosphere surface was measured by the ninhydrin method.

The affinity and specificity of immobilized lectin for recognition of Gal-β(1-3)GalNAc were evaluated using the hemagglutination test, as done in our previous research [12]. Briefly, rabbit erythrocytes were treated with neuraminidase to expose Gal-β(1-3)GalNAc on their surface. The neuraminidase-treated and untreated erythrocytes were suspended in PBS. Separately, a twofold dilution series of the dispersion of lectin-immobilized fluorescent nanospheres was prepared in a 96-well microtiter U-plate. An equivalent volume of the suspension containing either neuraminidase-treated or untreated erythrocytes was added to each well. After incubation of the mixture at room temperature for 1 h, erythrocyte agglutination was observed. The minimum concentration of immobilized lectin that induced erythrocyte agglutination, which was defined as the minimum agglutination concentration (MAC), was measured. A similar experiment was carried out by substituting lectin-immobilized fluorescent nanospheres with lectin-free ones to evaluate polymer chains-induced nonspecific interactions with erythrocytes. For this evaluation, MAC was expressed as a nanosphere equivalent.

2.4. Animal experiments

2.4.1. Human colorectal cancer orthotopic mouse model

Orthotopic implantation of human colorectal cancer cells was conducted as described previously [14]. HT-29 cells were seeded at a density of 2×10^4 cells/mL in a flask of adequate volume (25–75 mL). McCoy's 5A Medium, Modified supplemented with 10% (v/v) FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, and 1.5 mM L-glutamine, was used as a culture medium. The cells were grown as a monolayer in the media and were maintained at 37 °C in a humidified atmosphere of 95% O_2/5% CO_2. Cells were then routinely passaged when they became 100% confluent, and the low passage cell lines (passage: ⩽10 times) were used.

Animal experiments were approved by the Ethical Review Committee of Setsunan University. Severe combined immunodefi-

ciency (SCID) mice, 6-week-old C.B-17/Icr SCID/SCID female mice, were used for cancer cell implantation. After cultured HT-29 cells were harvested through the treatment with trypsin–EDTA, they were suspended in PBS at a concentration of 4×10^7 cells/mL. An incision approximately 1.5 cm long was made along the left lateral abdomen of the SCID mouse anesthetized with sodium pentobarbital. The cell suspension (ca. 0.025 mL) was injected into cecal serosa of the SCID mouse, and the abdomen was then closed. SCID mice bearing cecal tumors obtained were maintained until implanted cancer cells invaded the mucosal side of epithelial cells.

2.4.2. Interactions between nanospheres and cecal mucosa with or without tumors

The interactions were examined using a minor modification of the procedures reported previously [14]. SCID mice bearing cecal tumors (24-h fasting) were anesthetized with ether, the abdomen was opened, and the cecum, in which HT-29 cancer cells had been implanted, was exposed. An approximately 3-cm loop of the tumor-bearing cecum was prepared in the abdomen by ligating both junctions of the cecum to the ileum and ascending colon after washing the luminal side of the cecum with PBS. Nanospheres (Runs 2–5, Table 1) were separately dispersed in PBS at a concentration of 20 mg/mL, and 0.5 mL of the dispersion was injected into the loop. Ten minutes after injection, the loop was removed and its luminal side was washed with 2 mL of PBS. The cecum was cut longitudinally and a fluorescence microphotograph of the mucosal surface was taken using a fluorescence microscope (IX71-22FL/PH; Olympus Co., Ltd., Tokyo, Japan; excitation: 470–495 nm; emission: 510–550 nm; exposure: 1/60 of a second). As a control, normal SCID mice that did not undergo cancer cell implantation were tested in the same manner as described above.

3. Results and discussion

3.1. Characterization of nanospheres

Table 1 summarizes the characterization of lectin-free and lectin-immobilized fluorescent nanospheres. Since our previous research revealed that PNA immobilized on the nanosphere surface possessed high affinity and specificity for Gal-β(1-3)GalNAc when the molecular weight of PNVA was nearly equal to that of PMAA [12], we used PNVA with an Mw/Mn of 9500/4000 and PMAA with an Mw/Mn of 10,000/5600 in this study. The ESCA analysis proved that PNVA and PMAA chains were introduced on the nanosphere surface in proportion to the amount loaded in the copolymerization with styrene. Neither nanosphere size nor the amount of coumarin 6 encapsulated in the nanosphere core was affected by chemical composition of nanospheres. The zeta potential of nanospheres with exclusively nonionic PNVA chains (Run 2) was approximately 0 mV in neutral PBS. Nanospheres with anionic PMAA chains were negatively charged (Runs 1 and 3), and the absolute value of the zeta potential was reduced when PNVA chains were introduced to the anionic nanospheres.

Either PNA or WGA was immobilized on the surface of nanospheres with PNVA and PMAA chains (Run 1) through the chemical reaction with PMAA. Regardless of lectin type, the immobilized amount was approximately 5 μg/mg, which was comparable to the amount observed in our previous researches [12–15]. It is known that PNA and WGA specifically recognize Gal-β(1-3)GalNAc and N-acetyl-D-glucosamine, respectively [16]. The latter lectin also possesses carbohydrate-binding specificity for N-acetyl-D-neuraminic acid (sialic acid) that is found on the mucosal surface of healthy epithelial cells in the intestine. The recognition of lectins for specific carbohydrates can be evaluated using the hemagglutination test [12,13,19]. Since a lectin molecule possesses several sites that bind to carbohydrate residues on the erythrocyte surface, a cross-linking network is established between the lectins and erythrocytes, thereby inducing erythrocyte agglutination. Here, Gal-β(1-3)GalNAc is specifically exposed on the surface of the erythrocytes pretreated with neuraminidase. The minimum concentration of lectins that induces agglutination of neuraminidase-treated erythrocyte decreases with an increase in the affinity of lectins for Gal-β(1-3)GalNAc. As shown in Table 1, the MAC of PNA-immobilized fluorescent nanospheres with surface PNVA chains (i.e., the imaging agent, Run 4) for Gal-β(1-3)GalNAc-exposed erythrocytes was 0.0320 μg/mL as a lectin equivalent. The replacement of PNA with WGA resulted in a 500-fold increase in the MAC, indicating that immobilized WGA did not recognize Gal-β(1-3)GalNAc. The specificity of PNA for Gal-β(1-3)GalNAc can be estimated by comparing the MACs for neuraminidase-treated and neuraminidase-untreated erythrocytes. The ratio of MAC for neuraminidase-untreated erythrocytes to MAC for neuraminidase-treated erythrocytes increases with an increase in the specificity. Agglutination of neuraminidase-untreated erythrocytes did not occur even when the concentration of PNA-immobilized nanospheres was set to 33.0 μg/mL as a lectin equivalent, which was the maximal concentration used in this study (Table 1). A comparison between both MAC values revealed that PNA was actively immobilized on the nanosphere surface, as observed in our previous researches [12–15]. Neuraminidase-untreated erythrocytes agglutinated when the concentration of WGA-immobilized nanospheres reached 0.0345 μg/mL as a lectin equivalent. This agglutination was induced presumably because the immobilized WGA recognized sialic acid on the surface of plain erythrocytes.

The hemagglutination test in the presence of lectin-free fluorescent nanospheres (Runs 1–3) was also conducted to evaluate nonspecific interactions between erythrocytes and polymer chains (PNVA and PMAA) located on the nanosphere surface. Plain erythrocytes did not agglutinate at all when they were incubated with nanospheres with surface PNVA chains at a concentration of 20 mg/mL as a nanosphere equivalent, which was the maximum concentration used in this study due to turbidity. A similar pattern was also observed for Gal-β(1-3)GalNAc-exposed erythrocytes. These data strongly indicated that nanospheres with surface PNVA chains rarely interacted with any carbohydrate residue on the erythrocyte surface. In contrast, when PNVA was replaced with PMAA, a precipitous MAC reduction was observed, irrespective of erythrocyte pretreatment, indicating that strong nonspecific interactions were induced by PMAA. The pattern of erythrocyte agglutination induced by fluorescent nanospheres with surface chains of PNVA and PMAA (i.e., the precursor of the imaging agent, Run 1) was similar to that exhibited by nanospheres with exclusively surface PMAA chains. Since nanospheres with surface chains of PNVA and PMAA are negatively charged (Table 1), it seems that anionic PMAA has priority over nonionic PNVA on the nanosphere surface. This property may be favorable to the immobilization of PNA on the nanosphere surface because it becomes increasingly difficult for PNVA to interfere with the chemical reaction between the amino groups of lectins and the carboxyl groups of PMAA.

3.2. Essence of affinity and specificity of the imaging agent for colorectal cancer

Carbohydrates are found on the surface of most cells as the form of glycoproteins, glycolipids, and polysaccharides [25–27]. The TF antigen is specifically expressed on the mucosal side of cancer cells in the early stage of colorectal cancer; however, its terminal sugar, Gal-β(1-3)GalNAc, is masked by oligosaccharide side chain extension or sialylation in normal cells [17,18]. Disease-induced changes in molecular structures are often observed in the affected sites [28,29], and there are endogenous and exogenous molecules that

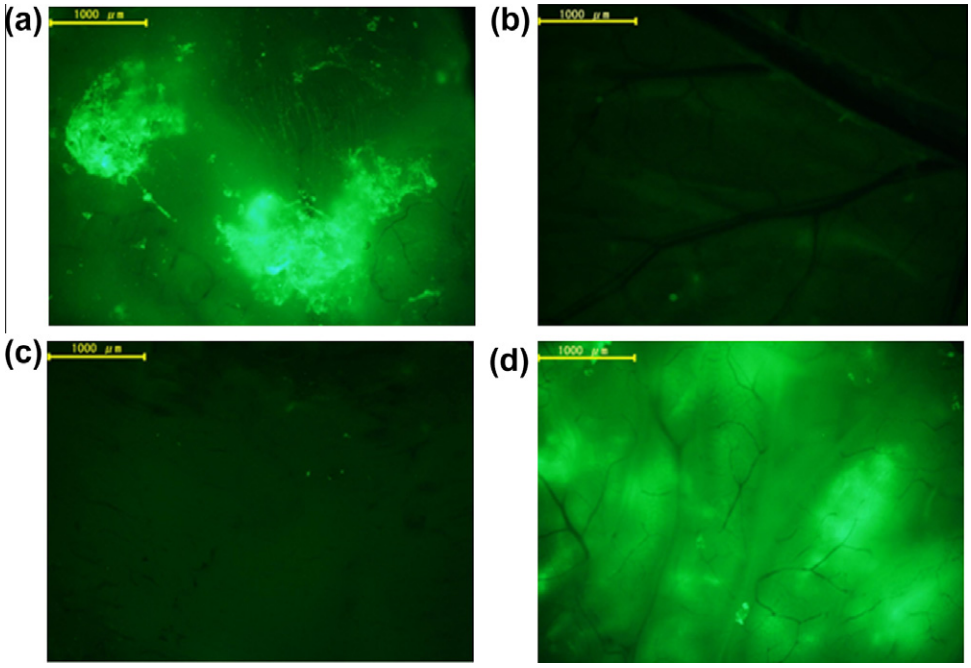

Fig. 2. Fluorescence microphotographs of the cecal mucosa in HT-29 cell-implanted (a and c) and normal (b and d) SCID mice treated with either PNA-immobilized fluorescent nanospheres with surface PNVA chains (a and b) or WGA-immobilized ones (c and d) at a concentration of 20 mg/mL for 10 min. The cancer cell-implanted mice were sacrificed on the 50th day after orthotopic implantation. A 3-cm loop of the tumor-bearing cecum was then prepared. The luminal side of the cecum was treated with lectin-immobilized fluorescent nanospheres, washed with PBS, and then observed under fluorescence microscopy (magnification: 40×; excitation: 470–495 nm; emission: 510–550 nm; exposure: 1/60 of a second).

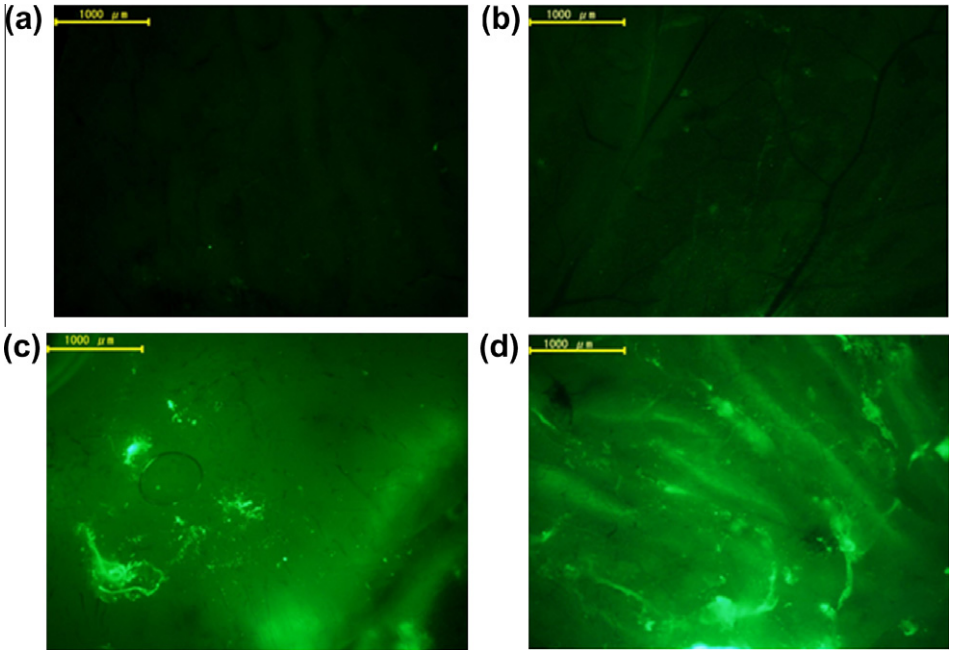

Fig. 3. Fluorescence microphotographs of the cecal mucosa in HT-29 cell-implanted (a and c) and normal (b and d) SCID mice treated with lectin-free fluorescent nanospheres at a concentration of 20 mg/mL for 10 min. The cancer cell-implanted mice were sacrificed on the 50th day after orthotopic implantation. A 3-cm loop of the tumor-bearing cecum was then prepared. The luminal side of the cecum was treated with lectin-free fluorescent nanospheres, washed with PBS, and then observed under fluorescence microscopy (magnification: 40×; excitation: 470–495 nm; emission: 510–550 nm; exposure: 1/60 of a second). Images were obtained when the cecal mucosa were treated with nanospheres with surface PNVA chains (a and b) and nanospheres with surface PMAA chains (c and d).

can recognize those structural changes with high affinity [25–27]. The biorecognizable interactions between these molecules (ligands) and their targets are often utilized for the diagnosis of diseases. However, nonspecific interactions between ligands and nontargets that reduce the specificity for targets sometimes cause ambiguous or false diagnoses. As illustrated in Fig. 1, our imaging agent is composed of submicron-sized fluorescent polystyrene nanospheres with two functional groups – PNA and PNVA – on

their surfaces. We expect that the former binds to Gal-β(1-3)GalNAc with high affinity, while the latter enhances the specificity of the former for the carbohydrate by reducing nonspecific interactions.

We first compared the in vivo performance of PNA-immobilized fluorescent nanospheres with surface PNVA chains (i.e., the imaging agent) with that of WGA-immobilized ones in the human colorectal cancer orthotopic mouse model. HT-29 cells were implanted on the cecal serosa of SCID mice, and the mice were sacrificed for biorecognition studies on the 50th day after orthotopic implantation because our previous research demonstrated that implanted HT-29 cells had definitely invaded the mucosal side when the mice were maintained for 7 weeks after implantation. Fig. 2 shows fluorescence microphotographs of the cecal mucosa in cancer cell-implanted and normal SCID mice treated with lectin-immobilized fluorescent nanospheres with surface PNVA chains. The exposure time was set as 1/60 of a second, which was free from autofluorescence of the cecal mucosa [13,14]. As shown in Fig. 2a, strong nanosphere-derived fluorescence was observed at several sites of the cecal mucosa in cancer cell-implanted SCID mice treated with PNA-immobilized fluorescent nanospheres. No specific accumulation of the nanospheres was observed when SCID mice that did not undergo cancer cell implantation were used (Fig. 2b). High affinity and specificity of the imaging agent for millimeter-sized tumors on the cecal mucosa was observed with good reproducibility [13,14]. In contrast, the recognition capability for tumors disappeared when PNA was replaced with WGA (Fig. 2c). A difference in tumor imaging between PNA-immobilized fluorescent nanospheres and WGA-immobilized counterparts demonstrated that the imaging agent was accumulated on tumor tissues in the cecal mucosa through PNA-induced biorecognition for Gal-β(1-3)GalNAc, which is the terminal sugar of the TF antigen. Nanosphere-derived fluorescence was widely observed on the mucosal surface of the cecum in normal mice treated with WGA-immobilized fluorescent nanospheres (Fig. 2d). This was likely due to the recognition of sialic acid on the healthy cecal mucosa by WGA. It seemed that PNVA did not interfere with the lectin activity.

The in vivo performance of lectin-free fluorescent nanospheres was subsequently evaluated to confirm whether PNVA is prerequisite for the reduction of nonspecific interactions with normal tissues. Fig. 3 shows fluorescence microphotographs of the cecal mucosa in cancer cell-implanted and normal SCID mice treated with lectin-free fluorescent nanospheres. When the surface of the nanospheres was covered exclusively with PNVA chains, no specific accumulation of the nanosphere was observed, irrespective of cancer cell implantation (Fig. 3a and b). The data revealed that nonspecific interactions between PNVA and fresh tissues were so weak that lectin-free fluorescent nanospheres with surface PNVA chains rarely adhered to the cecal mucosa. In contrast, strong nanosphere-derived fluorescence was extensively observed on the cecal mucosa treated with nanospheres whose surface was covered exclusively with PMAA chains (Fig. 3c and d). As observed in the in vitro hemagglutination test, there were strong nonspecific interactions between PMAA and fresh tissues, irrespective of the presence or absence of tumor tissues.

Through the in vivo experiments, we successfully proved that PNA and PNVA are essential for biorecognition for tumor tissues and a reduction of nonspecific interactions with normal tissues, respectively.

4. Conclusions

PNA-immobilized fluorescent nanospheres with surface PNVA chains were designed as a novel colonoscopic imaging agent. The in vivo performances of the imaging agent, PNA-free nanospheres, and PNVA-free nanospheres were evaluated using the human colorectal cancer orthotopic animal model. As expected, the in vivo data demonstrated that PNA and PNVA were indispensable for recognizing tumor tissues and preventing the imaging agent from interacting with normal tissues, respectively.

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