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A potential of peanut agglutinin-immobilized fluorescent nanospheres as a safe candidate of diagnostic drugs for colonoscopy

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Summary

There is a reasonable likelihood that peanut agglutinin-immobilized fluorescent nanospheres will be developed as a colonoscopic imaging agent from a safety perspective.

Keywords

Imaging; Endoscopic imaging agent; Colonoscopy; Colorectal cancer; Safety

1. Introduction

Nanotechnologies are currently undoubted driving force for innovative progresses in sciences; particularly, successful development of nanomaterials has largely contributed to the medical innovation. We have also investigated the potential of polymeric nanospheres composed of graft copolymers having a hydrophobic polystyrene backbone and hydrophilic polyvinyl branches as carriers for oral peptide delivery [Sakuma et al., 1997a, 1997b, 1999, 2001a, 2002a, 2002b]. The submicron-sized nanosphere is shaped like the sun, and the core

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made of hydrophobic polystyrene is decorated with coronae made of hydrophilic polyvinyl chains [Akashi et al., 1985, 1989]. Diverse properties of nanospheres are primarily attributed to surface polyvinyl chains; macromolecules immobilized on the nanosphere surface through chemical reactions with functional groups of polyvinyl chains confer unique properties that nanospheres do not originally possess [Akashi et al., 1998]. The amphiphilic structure enables to incorporate hydrophilic and hydrophobic small molecules such as drugs/probes into the hydrophobic core and onto the hydrophilic surface layer, respectively, through physicochemical interactions.

In the process of researches, we found that nanospheres whose surface was modified with poly(N-vinylacetamide) (PNVA) chains did not enhance the absorption of peptide drugs at all [Sakuma et al., 1997a]. The lack of the absorption-enhancing function was probably due to PNVA-prevented mucoadhesion of nanospheres incorporating peptide drugs [Sakuma et al., 1999, 2002b]. This character was fatal to carriers for oral peptide delivery; however, one bright idea occurred to us. Disease-induced changes in molecular structures are often observed in the affected sites [Akashi et al., 1998; Ørntoft et al., 1990], and there are endogenous and exogenous molecules that recognize those structural changes with high affinity [Sharon and Lis, 1989; Dabelsteen, 1996; Corfield and Warren, 1996]. The biorecognizable interactions between these molecules (ligands) and their targets are frequently utilized for the diagnosis of diseases. However, nonspecific interactions between ligands and non-targets that reduce the specificity for targets sometimes cause ambiguous or false diagnosis.

Assuming that PNVA enhances the specificity of ligands immobilized on the nanosphere surface by reducing nonspecific interactions, we started the research on a novel endoscopic imaging agent for the early detection of small-sized colorectal cancer [Hiwatari et al., 2008; Sakuma et al., 2009, 2010]. Colorectal cancer initially develops in the mucous membrane of the large intestine. Currently, surgical removal is the primary treatment of choice, and early detection and resection are indispensable tools for curing colorectal cancer [Balch et al., 2006]. However, the initial tumor-derived change is very small throughout the entire large intestine; thus, it is a significant challenge for early detection using an endoscopic approach. In order to amplify signals from such a small change, we designed an imaging agent that possesses a strong affinity for targets (i.e., cancer tissues) with minimal nonspecific interactions with non-targets (i.e., normal tissues). As illustrated in Fig. 1, the imaging agent is peanut (*Arachis hypogaea*) agglutinin (PNA)-immobilized polystyrene nanospheres with surface PNVA chains encapsulating coumarin 6. Lectins are proteins that recognize and bind reversibly to specific carbohydrate residues expressed on the cell surface [Bies et al., 2004]. PNA was 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 [Boland and Roberts, 1988; Campbell et al., 1995; Samuel et al., 1990; Schneider et al., 2001; Wróblewski et al., 2001; Sakuma et al., 2001b]. Based on our assumption, PNVA enhances the specificity of PNA by reducing nonspecific interactions with normal tissues, which cover the majority of the large intestinal mucosa. Coumarin 6 was used as the fluorescent dye that provides an endoscopically detectable fluorescence intensity. The dye was encapsulated into the polystyrene core of nanospheres through their hydrophobic interactions. Here, we expected that intracolonic (enema) administration of the imaging agent would lead to the specific accumulation on the mucosal surface of tumor 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 a series of research, we first optimized the chemical structure of the imaging agent [Hiwatari et al., 2008; Sakuma et al., 2009]. Nanospheres with different chemical structures were prepared, and the activity of PNA immobilized on the nanosphere surface was evaluated. By adjusting the molecular weight of PNVA, we consequently obtained the imaging agent whose affinity and specificity of immobilized PNA for Gal- β (1–3)GalNAc detection were equivalent and superior, respectively, to those of intact PNA. Successive in vitro cell studies demonstrated that the imaging agent specifically bound to TF antigen-expressing cancer cells, presumably due to its recognition of Gal- β (1–3)GalNAc [Sakuma et al., 2009]. The in vivo performance of the imaging agent was evaluated using a human colorectal cancer orthotopic mouse model. Imaging analysis and histological evaluation revealed that bright areas with fluorescence derived from the imaging agent and dark areas without the fluorescence well denoted the presence and absence, respectively, of the tumors on the mucosal surface. This good correlation showed that the imaging agent recognized millimeter-sized tumors on the intestinal mucosa with high affinity and specificity [Sakuma et al., 2009, 2010].

It is obvious that PNA-immobilized fluorescent nanospheres with surface PNVA chains are a potential candidate for use as a colonoscopic imaging agent. We report herein our current approaches to assess the safety of the imaging agent.

2. Materials and methods

2. 1. Materials

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

Methyl cellulose (#400, 350–550 mPa·s) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). TritonX-100 and LabAssay ALP (kits to measure alkaline phosphatase (ALP)) were obtained from Wako Pure Chemical Industry Co., Ltd. (Osaka, Japan). LDH-Cytotoxicity Assay kit II, which is used to measure lactate dehydrogenase (LDH), was purchased from BioVision, Inc. (Mountain View, CA). Preserved rabbit blood and neuraminidase (sialidase, 1 unit/ml, extracted from *Arthrobacter ureafaciens*) were obtained from Nippon Bio-Test Laboratories Inc. (Tokyo, Japan) and Roche Diagnostics (Indianapolis, IN), respectively. Dulbecco's Phosphate Buffered Saline (PBS with CaCl₂ and MgCl₂) was furnished by Sigma-Aldrich.

The human colorectal adenocarcinoma cell line, Caco-2 (passage: 17 times), was purchased from American Type Culture Collection (Rockville, MD). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Sigma-Aldrich. Non-essential amino acids (10 mM), fetal bovine serum (FBS), L-glutamine (200 mM), trypsin-EDTA (trypsin: 0.25%, EDTA: 1 mM), and antibiotic-antimycotic mixture (penicillin: 10,000 U/ml, streptomycin: 10 mg/ml, amphotericin B: 25 μ g/ml, dissolved in 0.85% NaCl solution) were purchased from Gibco Laboratories (Lenexa, KS). Cell culture inserts with polyethylene terephthalate filters (pore size: 3.0 μ m, growth area: 0.9 cm²) was supplied by Becton Dickinson Bioscience (Bedford, MA).

2. 2. Preparation and characterization of the imaging agent

Preparation procedures for the imaging agent have been described previously [Hiwatari et al., 2008; Sakuma et al., 2009]. 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) 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). The unreacted substances and unencapsulated coumarin 6 were removed by centrifugation. PNA was subsequently bound to the fluorescent nanospheres 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. The resulting PNA-immobilized fluorescent nanospheres with surface PNVA chains (i.e., the imaging agent) were purified, and then dispersed in purified water at a concentration of 20 mg/ml.

Routine characterization of the imaging agent was performed as described previously [Hiwatari et al., 2008; Sakuma et al., 2009]. Briefly, weight- and number-average molecular weights (M_w and M_n) of PNVA and PMAA were determined by gel permeation chromatography. The size (weight-average diameter) of nanospheres was measured by dynamic light-scattering spectrophotometry and scanning electron microphotography. The amount of coumarin 6 encapsulated into the nanosphere core was measured by spectrophotometry. The amount of PNA immobilized on the nanosphere surface was measured by the ninhydrin method. The affinity and specificity of immobilized PNA for recognition of Gal- β (1-3)GalNAc were evaluated using the hemagglutination test.

2. 3. Stability of the imaging agent exposed to the environment of the large intestine

2. 3. 1. The activity of PNA immobilized on the nanosphere surface—Animal experiments were approved by the Ethical Review Committee of Setsunan University. Sodium pentobarbital (Dainippon-Sumitomo Pharma Co., Ltd., Osaka, Japan) was injected intraperitoneally to ddY mice (5-week-old, female) at a dose of 15 mg/mouse. The abdomen was opened, and the descending colon was observed. An approximately 4-cm loop of the colon was prepared in the abdomen by ligating both ends of the colon after washing its luminal side with saline solution. The imaging agent was dispersed in PBS at a concentration of 20 mg/ml and 0.3 ml of the dispersion was injected into the loop. At 30 min after injection, the dispersion was drained from the intestinal lumen by air and its volume was measured. After centrifugation of the collected imaging agent dispersion (18,400 g, 15 min), the precipitate was re-dispersed in fresh PBS at a concentration of 20 mg/ml. The supernatant was used for the experiments described in sections 2. 3. 2. and 2. 4. 1.

The activity of PNA immobilized on the surface of nanospheres re-dispersed in PBS was evaluated using the hemagglutination test used in our previous study [Hiwatari et al., 2008]. 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 imaging agent dispersion 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, agglutination of the erythrocytes was observed with the naked eye. The minimum concentration of immobilized PNA that induced erythrocyte agglutination, which was defined as the minimum agglutination concentration (MAC), was measured. As a control, the unused imaging agent that was not exposed to the environment of the large intestine was tested in the same manner described above.

2. 3. 2. Leakage of coumarin 6 from the nanosphere core—Coumarin 6 in the supernatant was analyzed by high-performance liquid chromatography (HPLC). An

acetonitrile/water mixture (8/2, v/v) was used as a mobile phase. A column of 75 mm × 4.6 mm filled with octadecylsilyl silica gel of 5 µm mean particle size (J'sphere ODS-H80, YMC, Kyoto, Japan) was used. The injection volume was 20 µl, the flow rate was 1.0 ml/min, and the column temperature was 40°C. Coumarin 6 was detected using a fluorescence detector at an excitation wavelength of 480 nm and an emission wavelength of 530 nm with an 18 nm bandwidth. The lower limit of quantification was 0.35 ng/ml. As a control, the dispersion of the unused imaging agent (20 mg/ml) was prepared, and the concentration of coumarin 6 in the supernatant was evaluated using the same procedure described above.

2. 4. Irritation of the imaging agent against biological membranes

2. 4. 1. Damage of the large intestinal membrane treated with the imaging agent

—The enzymatic activity of ALP and LDH in the supernatant was determined using the respective commercial kits. For evaluation of the steady state, the same set of mouse experiments was performed by substituting the imaging agent dispersion with an equivalent volume of the agent-free PBS. Damage of the large intestinal membrane treated with methyl cellulose and TritonX-100 dissolved in PBS at a concentration of 20 mg/ml was also evaluated as negative and positive controls, respectively, as described above.

2. 4. 2. Effect of the imaging agent on the tight junction—Caco-2 cells were cultured in DMEM supplemented with 10% (v/v) of FBS, 1% (v/v) of non-essential amino acids, 1% (v/v) of L-glutamine, and 0.5% (v/v) of the antibiotic-antimycotic mixture in a flask of adequate volume at 37°C in a humidified atmosphere of 95% O₂/5% CO₂. Cells were routinely passaged when they became 90% confluent, and the narrow passage cell line (passage: ≤ 10 times) was used for all studies. Caco-2 cells were harvested by trypsin-EDTA. Collected cells were seeded on the cell culture inserts at a density of 6.4×10^4 cells/insert. The fresh culture medium (0.8 ml in the insert and 2.0 ml in the well) was replenished every 48 h during the first 6 days and every 24 h after that period. On the 18th through the 21st day after seeding, the monolayer of Caco-2 cells was obtained.

Hank's balanced salt solution (KCl: 5.36 mM, NaCl: 136.89 mM, Na₂HPO₄: 0.34 mM, KH₂PO₄: 0.44 mM, NaHCO₃: 4.17 mM, CaCl₂: 1.26 mM, MgCl₂: 0.49 mM, MgSO₄: 0.41mM, D-glucose: 19.45 mM) was prepared, and its pH was adjusted to 7.4 with HEPES. The Caco-2 cell monolayer on the filter was pre-incubated in the cell culture by exchanging the culture medium with an equivalent volume of the Hank's balanced salt solution at 37°C for 20 min. After pre-incubation, the apical and basal sides of the monolayer were immersed in 0.8 ml of PBS containing the imaging agent at a concentration of 20 mg/ml and 2.0 ml of PBS, respectively. The transepithelial electric resistance (TEER) of the monolayer was monitored for 90 min. As a control, the same set of cell experiments was performed for PBS, methyl cellulose-dissolving PBS (20 mg/ml), and TritonX-100-dissolving PBS (20 mg/ml).

2. 5. Non-absorbability of the imaging agent through biological membranes

2. 5. 1. In vitro permeation of the imaging agent through the Caco-2 cell monolayer

—A basal medium was collected at the final sampling point (90 min) in the cell experiment mentioned above when the imaging agent dispersion was used as an apical medium. The basal medium (0.1 ml) was mixed with 0.9 ml of acetonitrile, the mixture was heated at 60°C for 30 min, and the supernatant (0.9 ml) was collected after centrifugation (18,400 g, 15 min). After removal of the solvent under vacuum at 40°C, residues were dissolved in 0.1 ml of the acetonitrile/water mixture (8/2, v/v). Coumarin 6 dissolved in the solution was analyzed using the HPLC method described above.

2. 5. 2. In vivo permeation of the imaging agent through the large intestinal membrane

—Animal experimental protocols were approved by Vanderbilt University's

Institutional Animal Care and Use Committee and Use of the Medical Center. Gold mice (20–25 g, male) were fasted for 18 h with free access to water before the experiments (n=3). Mice were anesthetized with a ketamine/xylazine solution and the anesthetic condition was maintained during the experiment using inspired gas comprised of isoflurane (1.0%), oxygen (2.0%) and air (97%). The abdominal cavity of each mouse was opened, and a colonic loop of approximately 5 cm was prepared in the abdomen by ligating one end of the colon after washing its luminal side with PBS. The imaging agent was dispersed in PBS at a concentration of 20 mg/ml and coumarin 6 was dissolved in PBS containing DMSO (1%, v/v) at a concentration of 10 µg/ml. After either the imaging agent dispersion or coumarin 6 solution was injected into the loop at a dose of 0.1 ml, another end of the colon was ligated. At 2 h after injection, mice were sacrificed and blood (approximately 0.2 ml) was collected from the abdominal artery. The colonic loop with luminal contents, the liver, and the kidneys were removed and weighed. Individual whole tissues were homogenized in PBS at a concentration of 1 g of tissue/3 ml of PBS. The blood was diluted with a triple volume of PBS.

Each sample solution (0.1 ml) was mixed with 0.9 ml of acetonitrile, and then the mixture was heated at 60°C for 30 min. The supernatant (0.8 ml) was collected after centrifugation of the mixture at 16,000 g for 30 min. After removal of the solvent under vacuum at room temperature, the residue was dissolved in 0.15 ml of the acetonitrile/water mixture (8/2, v/v). Coumarin 6 in the sample was analyzed using the same HPLC procedures described in the section 2. 3. 2., except for the following conditions: the injection volume was 100 µl, the column temperature was room temperature, and coumarin 6 was detected with a UV-VIS detector at 458 nm. The lower limit of quantification was 0.4 ng/ml.

Recovery rates of the imaging agent and coumarin 6 were expressed as percentages of their accumulation in the respective organs at 2 h after injection into the colonic loop. It was assumed that the blood volume of mouse was 58 ml/kg [Riches et al., 1973]. The minimal detectable level of coumarin 6 was calculated to be 2.4 ng/gram of the tissue.

3. Results

3. 1. Characterization of the imaging agent

Fig. 1 shows a schematic representation of the imaging agent. Coumarin 6 was encapsulated physicochemically into the polystyrene core of nanospheres with surface chains of PNVA and PMAA to obtain the precursor of the imaging agent. PNA was anchored chemically to the polymeric platform via the PMAA linker located on the nanosphere surface. Table 1 summarizes the characteristics of the imaging agent. Compared with previous data [Hiwatari et al., 2008; Sakuma et al., 2009, 2010], good reproducibility of the preparation was observed. The scanning electron microphotograph also revealed that the size of the imaging agent was ca. 300 nm (Fig. 2).

3. 2. Stability of the imaging agent exposed to the environment of the large intestine

In terms of the stability of the imaging agent after its intracolonic administration, the activity of PNA immobilized on the nanosphere surface and leakage of coumarin 6 from the nanosphere core were examined. After incubation of the imaging agent in the closed colonic loop for 30 min, approximately 0.3 ml of the dispersion was collected. The amount represented a volume nearly equal to the original injection. The PNA activity of the imaging agent after incubation was first compared with that of the unused imaging agent before incubation. As shown in Table 2, the agglutination of neuraminidase-treated erythrocytes occurred when the average concentration of the imaging agent exposed to the environment of the large intestine reached 0.064 µg/ml as a PNA equivalent. In contrast, neuraminidase-

untreated erythrocytes did not agglutinate at all even when the concentration of the imaging agent was set to 16.3 $\mu\text{g/ml}$ as a PNA equivalent, which was the maximal concentration in this study. Consequently, the average ratio of MAC was calculated to be more than 255. No change in the PNA activity of the imaging agent before and after its incubation in the colonic loop was observed.

Leakage of coumarin 6 from the imaging agent incubated in the colonic loop for 30 min was next examined. After centrifugation of the luminal content, coumarin 6 in the supernatant was assayed. No peak of coumarin 6 was detected at the corresponding retention time in the chromatogram. Based on the assay conditions (the lower limit of quantification: 0.35 ng/ml, the encapsulated amount of coumarin 6 per milligram of the imaging agent: 0.8 $\mu\text{g/mg}$ (Table 1), the estimated concentration of the imaging agent collected: approximately 20 mg/ml), the undetectable leakage of coumarin 6 was estimated to be less than 0.0022% of the amount of coumarin 6 encapsulated into the nanosphere core. The absence of measurable leakage of coumarin 6 from the unused imaging agent that was not exposed to the environment of the large intestine was also observed.

3. 3. Irritation of the imaging agent against biological membranes

The imaging agent is designed as a non-absorbable diagnostic drug. Since the imaging agent is administered to the luminal side of the large intestine, the local cytotoxicity against the large intestinal membrane was evaluated through assays of ALP and LDH in the luminal content of the large intestine treated with the agent. Control experiments were performed simultaneously using the imaging agent-free PBS, methyl cellulose, and TritonX-100. As shown in Fig. 3, the ALP activity in the intestinal lumen after incubation with PBS was 1080 U/L on an average. This value obtained in the steady state remained constant when PBS was substituted with PBS containing either the imaging agent or methyl cellulose at a concentration of 20 mg/ml. However, an equivalent amount of TritonX-100, which was used as a positive control, induced an extreme increase in the ALP activity. A similar pattern of the enzymatic activity in the intestinal lumen after incubation with the respective media was also observed for LDH.

The effect of the imaging agent on the tight junction was subsequently examined through *in vitro* experiments using the Caco-2 cell monolayer. The same set of media described above was used. Fig. 4 shows the time course of the transepithelial electric resistance (TEER) of the Caco-2 cell monolayer during a 90-min experiment. The TEER value reached 0 within 10 min when the monolayer was immersed in TritonX-100, which indicated the complete destruction of the monolayer. No significant change in the TEER values was observed whenever residual 3 media were tested.

3. 4. Non-absorbability of the imaging agent through biological membranes

Several experiments were performed with the aim of proving the non-absorbability of the imaging agent. We first examined the *in vitro* membrane permeation of the imaging agent through the Caco-2 cell monolayer. The apical and basal sides of the monolayer were immersed in the imaging agent dispersion (20 mg/ml, 0.8 ml) and the agent-free PBS (2.0 ml), respectively. At 90 min after incubation, the collected basal medium was treated with heated acetonitrile to extract coumarin 6 from the nanosphere core. Before the experiment, we confirmed that coumarin 6 was almost completely extracted through this process (data not shown). Based on the experimental conditions, it was estimated that the minimal detection level of coumarin 6 corresponded to 0.0055% of the imaging agent; however, no fluorescent dye was observed in the basal medium (data not shown).

Finally, in vivo membrane permeation of the imaging agent was examined using the closed loop method. At 2 h after injection of the imaging agent dispersion into the colonic loop, the recovery of coumarin 6 from the colonic loop with the luminal content, the liver, kidneys, and blood was monitored. Before the experiment, we confirmed that coumarin 6-based recovery rate of the imaging agent from the tissue homogenate was approximately 95% and the calibration curve of standard solutions presented a linear regression ($R^2 > 0.99$), irrespective of tissue type (data not shown). As shown in Table 3, the imaging agent-derived coumarin 6 was observed only in the large intestine. Insignificant detection of coumarin 6 in 3 residual tissues strongly indicated that the imaging agent remained in the large intestinal lumen without systemic exposure. The same set of animal experiments was performed for intact coumarin 6. During the 2-h incubation, a half amount of coumarin 6 disappeared from the administration site. Coumarin 6 was clearly observed in the liver, kidneys and blood, which indicated that the fluorescent dye was delivered to the systemic circulation.

4. Discussion

Our past researches evidently demonstrate that PNA-immobilized fluorescent nanospheres with surface PNVA chains are suitable as an imaging agent for fluorescence colonoscopy [Hiwatari et al., 2008; Sakuma et al., 2009, 2010]. The evidence encourages us to evaluate the clinical potential of the imaging agent. Drug candidates should possess potentials for efficacy and safety; however, the safety of the imaging agent has been hardly ever examined because the efficacy evaluation (the PNA-induced tumor recognition and the PNVA-reduced nonspecific interactions with normal tissues) preceded the safety evaluation, as did generally in the drug research. The latter was initiated while details of in vivo tumor recognition were examined using several types of orthotopic mouse models [Sakuma et al., 2010].

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 resection using endoscopy. This minimally invasive operation, known as endoscopic mucosal resection (EMR), can serve as an alternative to surgical resection in the early stage of the cancer [Ponchon, 2001; Kudo et al., 2001; Rembacken et al., 2001]. While the colonoscopy procedure is critically important, standard white-light colonoscopy has a major limitation: it can only detect tumor tissues that are larger than ca. 1 cm in size. Tumors of this size have a relatively high probability of metastasis [Kashida and Kudo, 2006]. 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 [Tischendorf et al., 2007; DaCosta et al., 2007]. Thus, there is a great need for developing an endoscopic imaging agent that can provide early detection of small-sized colorectal cancer [Kelly et al., 2004], as well as novel imaging strategies for endoscopy such as narrow band imaging and autofluorescence imaging [Tischendorf et al., 2007; DaCosta et al., 2007; Kelly et al., 2004; Anandasabapathy, 2008; Van der Broek et al., 2007; Hirata et al., 2007].

Carbohydrates are found on the surface of most cells as the form of glycoproteins, glycolipids, and polysaccharides [Sharon and Lis, 1989; Dabelsteen, 1996; Corfield and Warren, 1996]. Surface glycoproteins are integral components that regulate cell-to-cell interactions and act as receptors for a number of different ligands such as lectins and antibodies. Taking note that colorectal cancer initially develops in the mucous membrane of the large intestine, we designed a non-absorbable colonoscopic imaging agent that recognizes tumor-derived changes in the large intestinal mucosa with high affinity and specificity. 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 [Boland and

Roberts, 1988; Campbell et al., 1995]. Our imaging agent is composed of submicron-sized fluorescent polystyrene nanospheres with two functional groups- peanut agglutinin (PNA) and poly(N-vinylacetamide) (PNVA) -on their surfaces (Fig. 1). The former binds to Gal- β (1-3)GalNAc with high affinity, while the latter enhances the specificity of PNA for the carbohydrate.

When the potential of PNA-immobilized fluorescent nanospheres with surface PNVA chains as a drug candidate is considered from a safety perspective, it is essential that their chemical structure and formation are maintained at the administration site. Fig. 5 shows the chemical structure of a fluorescent dye-free imaging agent. The graft copolymer, which is a platform of the imaging agent, is a stable polymer that does not degrade in physiological conditions. However, the stability of PNA itself and its binding site to the polymer remains uncertain, although lectins are relatively stable in the gastrointestinal tract [Wróblewski et al., 2001; Sakuma et al., 2001b]. Another possible instability of the imaging agent is the physicochemical interaction-based encapsulation of coumarin 6 (Fig. 1). Therefore, we examined changes in the PNA activity and the coumarin 6 encapsulation of the imaging agent before and after its incubation in the closed colonic loop. The incubation time was set to 30 min-interval which corresponded to the period confirmed to be sufficient for PNA-induced biorecognition in our previous research [Sakuma et al., 2010]. As shown in Table 2, the PNA activity was constant, and no leakage of coumarin 6 was observed after incubation. Data showed that the imaging agent was stable in the luminal side of the large intestine.

We subsequently examined the irritation of the imaging agent against the membrane of the large intestine. Enzymatic activities of ALP and LDH, which are widely used as markers in cytotoxicity studies, in the luminal content of the large intestine treated with the imaging agent for 30 min were measured. Both enzymes are rapidly released when the membrane is damaged. Except for the case of TritonX-100, the constant enzymatic activities observed indicated that there was no imaging agent-induced membrane damage (Fig. 3). We also examined the effect of the imaging agent on the tight junction. It is known that some compounds induce the modulation of the tight junction [Hayashi et al., 1999; Tavelin et al., 2003]. Changes in the TEER values of the Caco-2 cell monolayer were monitored in the presence of the imaging agent. The TEER value is reduced when the tight junction is modulated; however, the constant TEER values of the Caco-2 cell monolayer in the presence of the imaging agent indicated that the agent did not cause any significant change in the conjunction of cells (Fig. 4). The local toxicity of the imaging agent likely depends on the surface properties of nanospheres. PNA is expected to be safe because it is extracted from foods. Regarding with PNVA, Showa Denko Co., a supplier of NVA monomers, has developed the polymer as an adhesive for transdermal applications [Chow et al., 2008]. Our previous research also revealed that nanospheres with surface PNVA chains caused no damage to the intestinal mucosa [Sakuma et al., 2002b]. As expected, data indicated that the cytotoxicity of the imaging agent was acceptable.

The imaging agent is designed as a non-absorbable diagnostic drug for local administration into the luminal side of the large intestine. We finally examined the non-absorbability of the agent through biological membranes. The basal medium of the Caco-2 cell monolayer was taken at the final sampling point of the in vitro cytotoxicity studies mentioned above. Since coumarin 6 is seldom leaked from the nanospheres core under physiological conditions (Table 2), the detection of coumarin 6 in the basal medium strongly supports that the imaging agent penetrates the monolayer. The coumarin 6-derived fluorescence was not detected at all even though the fluorescent dye was analyzed using the HPLC method with high sensitivity. Data revealed that there was no significant permeation of the imaging agent through the Caco-2 cell monolayer. Furthermore, the distribution of the imaging agent and intact coumarin 6 was examined. As shown in Table 3, the imaging agent-derived coumarin

6 was almost completely recovered from the large intestine where the agent was administered. Alternatively, when intact coumarin 6 was incubated in the colonic loop for 2 h, a half amount of the dye disappeared from the loop, and its systemic exposure was clearly observed. Coumarin 6 was highly absorbed from the intestine, presumably because the strong hydrophobicity resulted in the high membrane permeability [Lipinski et al., 2001]. Coumarin 6 would be detected in the liver, kidneys, and blood if the significant amount was leaked from the nanosphere core in the colonic loop. Data obtained from the distribution studies demonstrated that the imaging agent remained in the luminal side of the large intestine without systemic exposure and that there was no significant leakage of coumarin 6 in the large intestinal lumen.

In this study, the potential of our imaging agent as a candidate of diagnostic drugs for colonoscopy was evaluated from a safety perspective. It is evident that the imaging agent is a safe and stable compound which remains in the large intestine without systemic exposure. However, there are several issues that must be settled for clinical trials of the imaging agent. It is important to estimate the lower limit of tumor detection with the imaging agent on the basis of the expression level of the TF antigen. The productivity, specifications, and the chemical stability of the imaging agent should be examined. A study on the biorecognition of the imaging agent for human tissues removed from cancer patients was also initiated. Further work will be discussed in future reports.

5. Conclusions

There is a reasonable likelihood that PNA-immobilized fluorescent nanospheres with surface PNVA chains will be developed as a colonoscopic diagnostic drug from a safety perspective.

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Fig. 1.
Schematic representation of the imaging agent.

Fig. 2.
Scanning electron microphotograph of the imaging agent.

Fig. 3.

Assays of alkaline phosphatase (ALP, ■) and lactate dehydrogenase (LDH, □) in the luminal content of the large intestine after incubation with the respective media. Concentrations of the imaging agent, methyl cellulose, and TritonX-100 were adjusted to 20 mg/ml, and PBS was used as the solvent. Media were injected into the closed colonic loop. At 30 min after injection, the luminal content was collected and centrifuged. The enzymatic activity in the supernatant was measured using commercial kits. Each value represents the mean $\pm$ s.d. (ALP assay: 8 experiments, LDH assay: 4 experiments).

Fig. 4.

Time course of the transepithelial electric resistance (TEER) of the Caco-2 cell monolayer incubated with the respective media (○: PBS, ●: the imaging agent dispersion (20 mg/ml, dispersed in PBS), ▲: methyl cellulose solution (20 mg/ml, dissolved in PBS), ■: tritonX-100 solution (20 mg/ml, dissolved in PBS)). These media were applied to the apical side of the monolayer, while the basal side was immersed in PBS. The TEER of the monolayer was monitored for 90 min. The change in the TEER value after incubation was expressed as the percentage of the initial TEER value measured before incubation. Each value represents the mean $\pm$ s.d. of 3 experiments.

Fig. 5.
The chemical structure of a fluorescent dye-free imaging agent.

Table 1

Characteristics of the imaging agent.

Characteristics	
Molecular weight of PNVA (Mw/Mn)	9,500/4,000
Molecular weight of PMAA (Mw/Mn)	10,000/5,600
Particle size (nm)	302
Encapsulated coumarin 6 ($\mu\text{g}/\text{mg}$) ¹⁾	0.8
Immobilized PNA ($\mu\text{g}/\text{mg}$) ²⁾	3.25
MAC for neuraminidase-treated erythrocytes ($\mu\text{g}/\text{ml}$) ³⁾	0.06
MAC for neuraminidase-untreated erythrocytes ($\mu\text{g}/\text{ml}$) ⁴⁾	>16.3

¹⁾ Encapsulated amount (μg) of coumarin 6 per milligram of the imaging agent

²⁾ Immobilized amount (μg) of PNA per milligram of the imaging agent

³⁾ Minimum concentration of PNA immobilized on the nanosphere surface that induced neuraminidase-treated erythrocyte agglutination

⁴⁾ Minimum concentration of PNA immobilized on the nanosphere surface that induced neuraminidase-untreated erythrocyte agglutination

Table 2

Characteristics of the imaging agent before and after its incubation in the closed colonic loop (mean $\pm$ s.d., n=6).

	The imaging agent that was not exposed to the environment of the large intestine	The imaging agent that was exposed to the environment of the large intestine
MAC for neuraminidase-treated erythrocytes ($\mu\text{g}/\text{ml}$) ¹⁾	0.060 $\pm$ 0.031	0.064 $\pm$ 0.051
MAC for neuraminidase-untreated erythrocytes ($\mu\text{g}/\text{ml}$) ²⁾	>16.3	>16.3
Ratio of MAC ³⁾	>271	>255
Leakage of coumarin 6 (%) ⁴⁾	n.d. ⁵⁾	n.d. ⁵⁾

¹⁾ Minimum concentration of PNA immobilized on the nanosphere surface that induced neuraminidase-treated erythrocyte agglutination

²⁾ Minimum concentration of PNA immobilized on the nanosphere surface that induced neuraminidase-untreated erythrocyte agglutination

³⁾ MAC for neuraminidase-untreated erythrocytes/MAC for neuraminidase-treated erythrocytes

⁴⁾ Percentage of coumarin 6 leaked from the nanosphere core

⁵⁾ Not detected (<0.0022% of the amount of coumarin 6 encapsulated into the nanosphere core)

Table 3

Recovery of the imaging agent and coumarin 6 at 2 h after incubation of them in the closed colonic loop (mean $\pm$ s.d., n=3).

	The imaging agent^{1, 2)}		Coumarin 6³⁾	
	Tissue weight (g)	Recovery (% of dose)	Tissue weight (g)	Recovery (% of dose)
Large intestine ⁴⁾	0.241 $\pm$ 0.058	104 $\pm$ 4.3	0.197 $\pm$ 0.041	53.3 $\pm$ 7.5
Liver	0.846 $\pm$ 0.19	n.d. ⁶⁾	1.07 $\pm$ 0.12	2.86 $\pm$ 1.5
Kidney	0.308 $\pm$ 0.035	n.d. ⁶⁾	0.350 $\pm$ 0.015	0.626 $\pm$ 0.21
Blood	— ⁵⁾	n.d. ⁶⁾	— ⁵⁾	2.12 $\pm$ 1.2

¹⁾ The imaging agent was injected into the closed colonic loop at a dose of 2 mg/mouse

²⁾ The dose of imaging agent corresponded to 1.6 μ g/mouse as a coumarin 6 equivalent (refer to "Encapsulated coumarin 6" in Table 1)

³⁾ Coumarin 6 was injected into the closed colonic loop at a dose of 1 μ g/mouse

⁴⁾ Closed colonic loop was collected with luminal contents including the imaging agent and coumarin 6

⁵⁾ A part of the blood (approximately 0.2 ml) was collected from the abdominal artery

⁶⁾ Not detected (Minimal detectable levels of the imaging agent in the liver, kidney, and blood were 0.13%, 0.046%, and 0.20%, respectively, of dose)