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Galactosyl Human Serum Albumin-NMP1 Conjugate: A Near Infrared (NIR)-Activatable Fluorescence Imaging Agent to Detect Peritoneal Ovarian Cancer Metastases

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Abstract

Patient survival depends on the completeness of resection of peritoneal ovarian cancer metastases (POCM) and therefore, it is important to develop methods to enhance detection. Previous probe designs based on activatable galactosyl human serum albumin (hGSA)-fluorophore pairs, which target lectin receptors expressed on POCM, have used only visible range dyes conjugated to hGSA. However, imaging probes emitting fluorescence in the NIR range are advantageous because NIR photons have deeper *in vivo* tissue penetration and result in lower background autofluorescence than those emitting in the visible range. A NIR-activatable hGSA fluorophore was synthesized using a bacteriochlorin-based dye, NMP1. NMP1 has two unique absorption peaks, one in the green range and the other in the NIR range, but emits at a NIR peak of 780 nm. NMP1, thus, has two different Stokes shifts that have the potential to allow imaging of POCM both at the peritoneal surface and just below it.

hGSA was conjugated with 2 NMP1 molecules to create a self-quenching complex (hGSA-NMP1). The activation ratio of hGSA-NMP1 was measured by the fluorescence intensity before and after exposure to 10% SDS. The activation ratio of hGSA-NMP1 was ~100-fold *in vitro*. Flow cytometry, fluorescence microscopy, and *in vivo* spectral fluorescence imaging were carried out to compare hGSA-NMP1 with hGSA-IR800 and hGSA-ICG (two always-on control agents with similar emission to NMP1) in terms of comparative fluorescence signal and the ability to detect POCM in mice models. The sensitivity and specificity of hGSA-NMP1 for POCM implant detection were determined by co-localizing NMP1 emission spectra with red fluorescent protein (RFP) expressed constitutively in SHIN3 tumor implants at different depths below the peritoneal surface. *In vitro*, SHIN3 cells were easily detectable after 3 hours of incubation with hGSA-NMP1. *In vivo* submillimeter POCM foci were clearly detectable with spectral fluorescence imaging using hGSA-NMP1. Among 555 peritoneal lesions, hGSA-NMP1, using NIR and green excitation light, respectively, detect 75% of all lesions and 91% of lesions ~0.8 mm or greater in diameter. Few false positives were encountered. Nodules located at a depth below the small bowel surface were only depicted with hGSA-NMP1.

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We conclude that hGSA-NMP1 is useful in imaging peritoneal ovarian cancer metastases, located both superficially and deep in the abdominal cavity.

Keywords

fluorescence imaging; activatable; near infrared; multiple excitations

Introduction

Ovarian cancer is the second most common gynecologic cancer in the United States, with 21,650 estimated new cases in 2008; however, it is the most deadly gynecologic malignancy, resulting in 15,520 estimated annual deaths. Due to its nonspecific symptoms, the majority of patients (60–70%) present at an advanced stage, and peritoneal carcinomatosis with ascites is often the first presentation of ovarian malignancy.¹

Long-term cure rates of ovarian cancer range between 20 and 30%, and the most important prognostic factors for patients are primary surgical outcome and patients' clinical response to postoperative platinum-based chemotherapy. However, attempts to detect peritoneal metastases using only the surgeon's unaided eye often results in persistent disease even after maximal debulking. Thus, improving the imaging and detection and hence, resection of peritoneal metastases at the patient's first surgery could improve outcomes.

Fortunately, the uniquely large surface area and shallow depth of peritoneal ovarian cancer metastases (POCM) are well-suited to optical imaging, which could be easily incorporated into laparoscopic resections. Fluorescence imaging allows the detection of POCM at the surface of the peritoneum.² As a result, fluorescence imaging has been proposed as a practical means of guiding surgical resection. Additional advantages include the ability to quickly scan large surface areas with high resolution, its relatively low cost and portability and its lack of ionizing radiation exposure.³

Although many candidate fluorescent imaging agents have been proposed, they usually suffer from one or more shortcomings. For instance, "always on" probes require a prolonged interval for the unbound but fluorescing probe to be cleared to enable sufficient tumor to background (TBR) ratios for visualization. Activatable probes can be imaged more quickly after injection and produce signal only at the tumor site yielding higher TBR. However, many previous probes have emitted light only in the visible range thus, limiting the depth at which POCM can be detected. This can be corrected by using fluorescence probes that emit in the NIR where tissue penetration is greater.

Galactosyl human serum albumin (hGSA) is a galactose-conjugated human protein that binds to Lectin receptors on the surfaces of POCM. hGSA-fluorophore conjugates bind the β -D galactose (a lectin) receptor on ovarian cancer cells at physiologic pH. Upon internalization, the hGSA-fluorophore conjugates are "dequenched," thus emitting light.^{4–7} Activatable fluorophores that emit in the near-infrared range (NIR) are advantageous because they have deeper *in vivo* tissue penetration and yield lower background autofluorescence than those emitting in the visible range. The newly synthesized bacteriochlorin-based NIR dye, NMP1, has two unique absorption peaks [in the green ($\lambda_1 = 550$ nm) and NIR ($\lambda_2 = 750$ nm) wavelength ranges], with a single NIR emission peak of 780nm (Figure 1A).⁸ NMP1 has the potential to allow separate excitation of tumor nodules at the surface (green excitation) and at a depth below the surface (NIR excitation).

Herein, we describe our findings with a NIR-activatable hGSA-NMP1, and we compare it to two probes with similar emission wavelengths ("always-on" hGSA-ICG and hGSA-IR800

probes, with peak emission wavelengths of 830 nm and 800nm, respectively)⁹ to determine its advantages in detecting cancer foci.

Materials and Methods

Synthesis chemical activation, and stability in serum of hGSA-NMP1 conjugate

The synthesis method of NMP1 has been published.¹⁰ Briefly, bacteriochlorin possessing two phenylethynyl substituents was prepared by sequential functionalization of the corresponding dibromobacteriochlorin¹¹ with palladium-catalyzed Sonogashira reaction. The NHS ester was obtained by basic hydrolysis of the corresponding methyl ester (NaOH in MeOH/THF) and reaction of the resulting acid with *N*-hydroxysuccinimide, in the presence of EDCI and *N,N*-dimethylpyridine in DMF. The final product was obtained in 96% yield, after purification with column chromatography.¹⁰ Synthesis of hGSA was performed with direct amidation reaction with human serum albumin and β -galactosamine. The details of synthesis and characterization were previously published.¹²

hGSA (0.25 mg, 3.5 nmol) and HSA were incubated with NMP1-NHS ester (27.5 μ g, 35 nmol) in 0.1 M Na₂HPO₄ (pH 8.6) at room temperature for 1 h, followed by purification with a size exclusion column (PD-10; GE Healthcare, Piscataway, NJ). ICG-Sulfo-OSu (Dojindo Molecular Technologies, Rockville, MD) and IRDye 800CW (IR800; LI-COR Biosciences, Lincoln, NE) labeling of hGSA was also performed by reacting hGSA with ICG and IR800 at a ratio of 1:10 and 1:5, respectively, in the same manner as hGSA-NMP1. The concentrations of each dye were calculated by measuring the absorption with the UV-Vis system (8453 Value UV-Vis system; Agilent Technologies, Santa Clara, CA) to confirm the number of fluorophore molecules conjugated with each hGSA molecule. The protein concentration was also determined by measuring the absorption at 280 nm with a UV-Vis system.

The quenching abilities of each conjugate were investigated by denaturing each with 1% SDS as described previously.⁵ Briefly, the conjugates were incubated either with 1% SDS in PBS or PBS alone for 15 min at room temperature. The change in fluorescence intensity of NMP1 was investigated with an *in vivo* imaging system (Maestro, CRi Inc., Woburn, MA) using two distinct band pass filters [(i) 671–705nm; (ii) 503–555nm] and one long-pass filter over 750nm for excitation and emission light, respectively. The fluorescence signal intensity of ICG and IR800 was measured by using 710–760 nm excitation and 800 nm long-pass emission filters.

hGSA-NMP1 was added to mouse serum collected from female nude mice (National Cancer Institute Animal Production Facility, Frederick, MD), and the serum samples were incubated at 37°C for 0, 1.5, and 3 h. After incubation, the change in fluorescence intensity was evaluated on the Maestro camera.

Cell Culture

The established ovarian cancer cell line, SHIN3 was used for *in vitro* fluorescence microscopy, flow cytometry, and *in vivo* optical imaging of POCM.¹³ Cell lines were grown in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum (Gibco), 0.03% L-glutamine at 37°C, 100 U/mL penicillin, and 100 μ g/mL streptomycin in 5% CO₂.

Confirmation of red fluorescent protein (DsRed2) transfection in SHIN3 cells

The red fluorescent protein (RFP DsRed2)-expressing plasmid (Clontech Laboratories) was previously transfected into the SHIN3 cell line and served as the standard of reference for

cancer location.³ Flow cytometry and fluorescence microscopy was carried out as described below to confirm continued successful expression of RFP in the SHIN3_DsRed cell line.

Fluorescence Microscopy

In order to study and compare the temporal sequence of intracellular fluorescence activation of hGSA-NMP1 with that of hGSA-ICG and hGSA-IR800, SHIN3 cells (2×10^4) were plated on a cover glass bottom culture well and incubated for 10 h. Either hGSA-NMP1, hGSA-ICG, hGSA-IR800, or no dye at all was added to the media at $1 \mu\text{g/mL}$. For the blocking study, $100 \mu\text{g}$ of non-conjugated hGSA was added together with hGSA-NMP1. In order to confirm the continued successful expression of RFP in the SHIN3_DsRed cell line, 2×10^4 transfected cells were plated on a cover glass and also incubated for 10h. The non-transfected SHIN3 cells were incubated with each dye for either 1.5h or 3h and then washed once with PBS. Fluorescence microscopy was carried out on an Olympus BX61 microscope (Olympus America, Inc.) for hGSA-NMP, hGSA-ICG, and hGSA-IR800 using the filters settings: excitation wavelength 672.5nm-747.5nm and an emission wavelength range 765–855nm. For the SHIN3 cells transfected with RFP, a band pass filter of 530–570nm and one long-pass filter over 590 nm were used for excitation and emission light, respectively. Transmitted light differential interference contrast (DIC) images were obtained as well.

Animal model of peritoneal metastases

All procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animal Resources (1996), the National Research Council, and approved by the local Animal Care and Use Committee. Intraperitoneal xenografts were established by i.p. injection of 2×10^6 SHIN3 cells suspended in $200 \mu\text{L}$ PBS into the peritoneum of female nude mice (National Cancer Institute Animal Production Facility, Frederick, MD). Imaging was performed at 14–21 days after injection of the cells.

In vivo spectral fluorescence imaging

To compare the utility of hGSA-NMP1, hGSA-ICG and hGSA-IR800 in detecting peritoneal tumor implants, aliquots of hGSA-NMP1 ($25 \mu\text{g}$), hGSA-ICG ($25 \mu\text{g}$), hGSA-IR800 ($25 \mu\text{g}$), or control HSA-NMP1 ($25 \mu\text{g}$) were suspended in $300 \mu\text{L}$ PBS and injected into the peritoneal cavities of tumor bearing female nude mice. For each sample, either 1.5h or 3h after i.p. injection of each probe, mice were euthanized by carbon dioxide inhalation. Several mice were allocated to each dye and corresponding time point group as follows: hGSA-NMP1 ($n=5$ for 1.5h; $n=15$ for 3h), hGSA-ICG ($n=5$ for 1.5h; $n=5$ for 3h), hGSA-IR800 ($n=5$ for 1.5h; $n=13$ for 3h), and HSA-NMP1 ($n=3$ for 3h). After euthanasia, the mouse abdominal wall was excised, and the abdominal cavity was exposed. Optical images of the whole abdomen were first obtained. Following this, the small bowel mesentery was extracted and close-up images were obtained. Spectral fluorescence images were acquired using the Maestro In-Vivo Imaging System. Two distinct band pass filters [(i) 671–705nm; (ii) 503–555nm] and one long-pass filters over 750nm were used for emission and excitation light, respectively. The tunable filter was automatically stepped in 10nm increments from 650 to 950 nm for time course experiments, while the optical imaging camera captured images at each wavelength interval with constant exposure. The spectral fluorescence images consisting of autofluorescence spectra (from the small bowel, the black plate, and the mouse's skin) and the spectra specific to each probe were obtained. Using commercial software (Maestro software) and saved fluorescence spectra, the original spectral fluorescence images were unmixed to show the fluorescent signal unique to each injected probe and autofluorescence. To calculate the tumor-to-background ratio (TBR), two regions of interest (ROIs) were placed at the largest tumor and its adjacent intestine or mecenteric membrane (background) in an unmixed image, which was produced based on the respective spectra of fluorophores.

Phantom “Proof of Principle” Experiments

To evaluate the utility of hGSA-NMP1 in detecting implants located superficially and deep in the abdominal cavity, the abdominal wall of female nude mice were excised ($n = 3$). A “phantom” sample consisting of an Eppendorf tube wrapped in optical black lab tape leaving only the tip exposed, with approximately 20 μL of fully activated (with 1% SDS) hGSA-NMP1, was placed atop a piece of small intestine. Spectral fluorescent images were obtained using two distinct band pass filters [(i) green spectral range filter, 503–555nm and (ii) near infra-red spectral range filter, 671–705nm] and one long-pass filter over 750 nm, as described above. Then, the phantom sample was placed beneath the same section of small intestine, and spectral fluorescent images were again obtained in both settings.

Image J software (National Institutes of Health) was used to subtract the spectral image obtained using the green spectral range band pass filter (presumed to depict only surface fluorescence) from that obtained using the NIR-spectral range filter presumed to detect both surface and deeper fluorescence. The resulting images represented the fluorescence signal present at a shallow depth below the surface ($\sim 2\text{mm}$).

“Tumor Depth” Model

To compare the utility of hGSA-NMP1 and hGSA-IR800 in imaging peritoneal implants located both superficially and deep in the abdominal cavity, peritoneal xenografts were established 14d after i.p. injection of SHIN3 cells (2×10^6) suspended in 200 μL PBS in female nude mice. At day 14 each mouse received either 25 μg of hGSA-NMP1 or hGSA-IR800 diluted in 300 μL PBS. Three hours later, the abdominal wall was excised, and a subdiaphragmatic tumor nodule was removed ($n=13$ for hGSA-NMP1, $n=9$ for hGSA-IR800). A standard reference solution of hGSA-NMP1 or hGSA-IR800 was placed adjacent to the mouse. The extracted nodule was placed atop a piece of small intestine, and spectral fluorescent images were obtained using two distinct band pass filters (i) a green spectral range filter (503–555nm) and (ii) a near-infra-red spectral range filter (671–705nm) and one long-pass filter over 750nm. The extracted nodule was then placed beneath the same section of small intestine, and spectral fluorescent images were again obtained.

The intensities between the two images obtained using the two different sets of band pass filters were normalized based on standard reference solutions. The green spectral image (presumed to depict fluorescence only at the surface of the abdominal cavity) was subtracted from the NIR-spectral range filter (presumed to depict fluorescence at and below the surface of the abdominal cavity). The resulting images represent tumor nodules located at a shallow depth below the surface (thickness $\sim 2\text{mm}$).

Assessment of the sensitivity and specificity of hGSA-NMP1 in the detection of peritoneal metastases

To verify that there was no spectral overlap between NMP1 and DsRed, tumor models were established 14d after i.p. injection of non-transfected SHIN3 cells ($n=8$) and RFP-transfected SHIN3 cells (2×10^6) suspended in 200 μL PBS into female nude mice ($n=10$). Mice with non-transfected SHIN3 tumors ($n=3$) were injected with 25 μg of hGSA-NMP1 suspended in 300 μL PBS ($n=3$) or 300 μL PBS alone ($n=5$). Mice with RFP-transfected SHIN3 tumors were injected with 25 μg of hGSA-NMP1 suspended in 300 μL PBS ($n=5$) or 300 μL PBS alone ($n=5$). Three hours after injection of the dyes, spectral fluorescent images were obtained with the appropriate filter settings, and spectral unmixing demonstrated no overlap between NMP1 or DsRed fluorescence.

Sensitivity and specificity were determined by comparing sites of fluorescence from hGSA-NMP1 with sites of fluorescence from RFP-transfected SHIN3 tumors ($n=10$). Three hours

after i.p. injection of 25 μ g of hGSA-NMP1, the mouse abdominal wall was excised and the small bowel mesentery extracted. A nonfluorescent optically black plate was placed under the extracted small bowel mesentery, and a ruler and reference solution (of either hGSA-NMP1 or hGSA-IR800) were placed adjacent to the specimen. Magnified *in vivo* spectral fluorescence imaging was performed using the Maestro In-Vivo Imaging System. In these experiments, the tunable filter was automatically stepped in 10nm increments from 450 to 950 nm. To isolate the fluorescent signal from DsRed, an excitation filter of 503–555 nm and a long-pass filter over 580 nm were used. The detection size limit for the tumor implants was determined visually using an unmixed composite image.

The spectral fluorescence images were unmixed, and regions of interest (ROI) were assigned using automated software based on a predetermined threshold. All visible nodules with short axis diameters ≥ 0.8 mm on RFP images were included based on the minimum ROI size. True positives for hGSA-NMP1 were defined as ROIs with average fluorescence intensity of > 3 a.u., whereas as true negatives for hGSA-NMP1 were defined as ROIs with average fluorescence intensity < 3 a.u. on the spectrally unmixed images. False positives for hGSA-NMP1 were defined as ROIs in which fluorescence was seen only on the NMP1 image and not the RFP image.

The sensitivity of hGSA-NMP1 for the detection of peritoneal cancer foci was defined as the number of foci positive for both hGSA-NMP1 and RFP divided by the number of foci positive for just RFP. In contrast, the specificity of hGSA-NMP1 was defined as the number of peritoneal foci negative for both RFP and hGSA-NMP1 divided by the number of peritoneal foci negative for RFP.

Results

Optical characteristics of hGSA-NMP1 conjugate

The number of NMP1, ICG, and IR800 dyes conjugated with each hGSA molecule was 1.8 ± 0.3 , 0.9 ± 0.3 , 1.6 ± 0.4 , respectively. The quenching capacities measured by adding 1% SDS to dye-conjugated antibody were >50 , 5, and 2-fold for hGSA-NMP1, hGSA-ICG, and hGSA-IR800, respectively. No measurable dequenching of hGSA-NMP1 was observed in mouse serum at 37°C for 3 hrs. As expected, hGSA-NMP1 yielded a single NIR emission peak after green or NIR excitation (Figure 1B).

Intracellular activation of hGSA-NMP1 after cellular internalization

Microscopic images of the SHIN3 cells incubated with 1 μ g/mL of hGSA-NMP1, hGSA-ICG, and hGSA-IR800 demonstrated diffuse and vague fluorescence but showed no clustering of signal in the cytoplasm 1.5h after incubation with hGSA-NMP1; however, several large and intense clusters of fluorescence representing probe accumulation in lysosomes were seen 3h after incubation with hGSA-NMP1 (Figure 2). Clear blocking of binding with excess hGSA demonstrated the specific binding of hGSA to the β -D galactose receptor (Supplemental fig 1A). These results suggest that hGSA-NMP1 is activated after internalization and lysosomal processing (Supplemental fig 1B), which leads to complete dequenching by 3h.

hGSA-NMP1 produces a high *in vivo* tumor-to-background fluorescence

To determine the optimal timing for *in vivo* imaging and also to evaluate the feasibility of using hGSA-NMP1 for the detection of peritoneal metastases, spectral fluorescent images were obtained 1.5h and 3h after intraperitoneal injection of 25 μ g of each dye into live nude mice.

One and a half hours after i.p injection of hGSA-NMP1, hGSA-IR800, and hGSA-ICG, fluorescence imaging of the small bowel mesentery began to reveal peritoneal metastases with hGSA-NMP1 (Supplemental fig. 2). However, by 3h after i.p. injection, the fluorescence intensity of hGSA-NMP1 had increased leading to higher activation ratios. hGSA-IR800 and hGSA-ICG produced comparable signal after NIR excitation. However, images using hGSA-NMP1 with green excitation were sharper and there was less background than other images due to the longer Stokes shift (Figure 3A). HSA-NMP1 did not show specific accumulation to SHIN3 tumors (Supplemental fig. 3). Submillimeter cancer foci were clearly detectable with close-up imaging of the small bowel-mesentery (Figure 3B). The mean tumor-to- Intestine ratios with green and NIR excitation light in the abdomen *in situ* was only 3.17 $\pm$ 2.02 and 2.95 $\pm$ 1.46 1.5h after injection with hGSA-NMP1, respectively; but by 3h, the tumor-to-Intestine ratios increased to 22.21 $\pm$ 10.24 and 13.32 $\pm$ 8.27. Comparatively, the Tumor-to-Intestine ratios for hGSA-ICG and hGSA-IR800 in labeling of SHIN3 POCM in the abdomen *in situ* was much lower at 3.30 $\pm$ 1.54 and 4.65 $\pm$ 2.01, respectively, after 1.5h of incubation,. The Tumor-to-Intestine ratios for each of those dyes increased to 7.89 $\pm$ 4.70 and 3.93 $\pm$ 1.90 3h after injection, respectively. (See also Supplemental Fig. 2 and Supplemental Table 1).

Phantom “Proof of Principle” Experiments

When the phantom sample was placed atop the small intestine, the resulting image clearly depicted the signal (Figure 4). However, when the phantom sample was placed below the small intestine, it was not visible in the green range but was clearly seen using the NIR filter set.

“Tumor Depth” Model

In the mice injected with hGSA-NMP1 (n=13) in which a tumor nodule was placed atop the small intestine, the nodule was clearly visualized with either the green or NIR image (Figure 5). However, when the tumor nodule was placed below the small intestine, the lesion could only be detected with the NIR-Green subtraction image.

In contrast, in mice injected with hGSA-IR800 (n=9) only the superficial implants were detected. (Figure 5). The deeper tumor nodules were not visualized. Furthermore, since hGSA-IR800 lacks an absorption peak in the green spectrum, the original images acquired using the band-pass filter in the green spectral range depicted only blurry fluorescent signal.

Sensitivity and Specificity of hGSA-NMP1

Analysis of unmixed images showed no spectral overlap between hGSA-NMP1 and DsRed (Supplementary Figure 4). The sensitivity and specificity of hGSA-NMP1 in detecting peritoneal cancer nodules were calculated and compared, using RFP-positive nodules as the reference for true positive tumors. A separate sensitivity and specificity was calculated for each of the hGSA-NMP1 dye's two unique absorption peaks and the corresponding optical filter settings.

RFP-positive nodules were defined as having an average fluorescence intensity > 100 a.u. (scaled a.u./s) on images unmixed for the DsRed spectra (>3 a.u. on images unmixed for the hGSA-NMP1 spectra) (Figure 6). One hundred-eleven foci showed hGSA-NMP1 fluorescence among the 149 RFP-positive foci 0.8 mm with the NIR images ($\lambda_2 = 750$ nm). Twenty foci showed hGSA-NMP1 fluorescence with intensity >3 a.u. among the 237 RFP-negative foci. Thus, hGSA-NMP1 using NIR excitation light was found to have a sensitivity of 74.5% (111 of 149) and a specificity of 92% (217 out of 237).

In contrast, 135 foci showed hGSA-NMP1 fluorescence among the 149 RFP-positive foci 0.8 mm with green images ($\lambda_1 = 550$ nm). Twenty foci were found to show hGSA-NMP1 fluorescence with intensity > 3 a.u. among the 237 RFP-negative foci. Thus, hGSA-NMP1 using green light excitation had a sensitivity of 91% (135 out of 149) and specificity of 92% (217 out of 237) in detecting peritoneal implants 0.8 mm.

Discussion

Improving the completeness of resection of POCM is likely to improve survival. The unaided human eye cannot detect small or hidden lesions and therefore, POCM has been a target of research in optically enhanced surgery.

Many fluorescent agents and designs have been proposed for this task. These designs have evolved from the first nontargeted fluorophores, which nonspecifically leaked into abnormal areas, to newer targeted “always on” probes, which bind disease-specific molecular targets and on to activatable “smart probes,” which fluoresce only when binding to the targets. Early on, our laboratory explored targeting probes to POCM using the glycoprotein avidin, a ligand for lectins like the β -D-galactose receptor. Unfortunately, since avidin is a hen egg protein, its immunogenicity precludes its clinic use. Seeking alternative lectin-targeted moieties, we subsequently conjugated green fluorescent dyes like rhodamine green to a non-immunogenic ligand to human using galactose-conjugated human albumins.¹⁴ Here, we have reported on the newly synthesized bacteriochlorin-based NIR fluorescence agent, hGSA-NMP1, which has several favorable properties for optical imaging of POCM. The hGSA component is highly biocompatible and binds ovarian cancer with high specificity when administered intraperitoneally. Furthermore, the D-galactose receptor is an optimal target for other peritoneal metastases since it is expressed in a wide range of cancers prone to metastases.¹⁵ In addition, hGSA has already been used as a clinical diagnostic agent for measuring hepatic reserve in Japan.

The NMP1 component also demonstrates desirable features. Its two unique absorption peaks (one in the green and one in the NIR spectrum) have the potential to allow separate imaging of tumor nodules located both superficially and deeper in the abdominal cavity. Although we have covalently conjugated ICG with antibodies to successfully synthesize activatable imaging probes,⁵ this chemistry did not work similarly with ICG and albumin-derivatives because ICG can non-covalently bind to albumin and yield brighter emission. IR800 is a hydrophilic variant of pentamethines, which is designed resist quenching when conjugated with proteins due to less interaction with aromatic rings associated with hydrophobic amino acids. On the other hand, our lab has also synthesized other activatable imaging probes, but these were limited to emissions in the green range.³ Therefore, activatable versions of hGSA probes which emit near infrared light, presented challenges. In this study, hGSA-NMP1 demonstrated activatable emission of NIR light with very high activation ratios (~ 100). By combining an “activatable” quenching mechanism with lectin-targeting in the design of hSA-NMP1, we improved the TBR by minimizing background, and we also improved sensitivity and specificity for tumor foci using a highly fluorescent dye.

NMP1 has similar structure to a second-generation photosensitizer with photodynamic properties, and it has already entered phase I studies for the treatment of colorectal liver metastases.^{16, 17} Thus, beyond its diagnostic capabilities, hGSA-NMP1 and its derivatives, have potential as phototherapeutic agents. Moreover, due to the high efficiency of NMP1, a relatively low dose of hGSA-NMP1 (25 μ g), containing less than 1 μ g of NMP1 (estimated adult human dose ~ 3 mg), is required to visualize POCM suggesting that this probe may have an acceptable toxicity profile for clinical use. For comparison, the typical adult dose of fluorescein 10% is 500 mg via intravenous administration.

A potential alternative will be the use of fluorescent proteins, which are excellent endogenous fluorescence emitters to be used for depicting various biological processes both *in vitro* and *in vivo*.^{18, 19} A recently reported alternative technology to this method is the tumor-specific imaging using telomerase promoter-regulated expression of fluorescent proteins, which are induced with the adenovirus-mediated gene transfection *in vivo*.^{20, 21} However, for the medical application, fluorescence proteins require virus-mediated *in vivo* gene transfection, which is unlikely to be permitted in humans at least in the near term. In contrast, one concern of this hGSA-NMP1 probe for clinical translation is whether suitable equipment is available. Not only must such cameras be capable of imaging in the NIR but they also must deliver near-real time imaging. Recently, several major optical camera manufacturers have introduced cameras that could potentially be introduced into operating rooms. With the proper equipment, fluorescence imaging could be safe, low cost and highly efficient.^{22, 23}

A limitation of this and other optical fluorescence imaging probes is that even NIR light can only penetrate to a depth of a maximum of a few centimeters. The detection of deeper lesions is very blurred due to light scattering. However, most ovarian cancer metastases are at or near the surface and can be made more visible by moving bowel and mesentery out of the way so that it is closer to the camera. One concern relating to the detection of metastases at varied depths is that the apparent size of the peritoneal tumor nodule could appear larger than its actual size: an intensely fluorescing tiny mass buried deep in tissue could theoretically yield the same appearance as a larger tumor with lower fluorescence located closer to the surface. The correct size of the lesion would become clear as the lesion was resected and became closer to the surface.²⁴

From the surgeon's perspective, the highly activatable characteristics of hGSA-NMP1 probe can offer significant advantages by allowing more specific visualization enabling more complete surgical resection of surface implants using green excitation light that does not penetrate beyond the surface. Then, after resecting the visible surface tumor implants, NIR excitation could be used to detect deeper lesion utilizing the very high (~100 fold) activation ratio of hGSA-NMP1. The surgeon can then resect down to the subsurface lesion, whereupon excitation can be switched back to green so that these implants can be clearly visualized with high specificity and removed with fluorescence guidance.

In conclusion, we demonstrate that hGSA-NMP1 could be useful in diagnosing peritoneal ovarian cancer implants both at the surface and at a shallow depth in the abdominal cavity with high sensitivity and specificity. This probe binds specifically to the D-galactose receptor on ovarian cancer cells and is activated after internalization that generally requires 3 hours for optimal results. The experience in animal models suggests that hGSA-NMP1 may be useful not only for implant detection during surgical resection of peritoneal metastases but may also be useful for targeted phototherapy or drug delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A. Structures and emission profiles of NMP1 and IR800 are shown. NMP1 has two available excitation peaks in green and NIR wavelengths. B. The fluorescence signals of hGSA-NMP1 with or without chemical activation with adding 1%SDS are shown. hGSA-NMP1 yield similar strength of NIR emission by exciting green or NIR light.

Figure 2.

Sequential microscopic images of SHIN3 cells, which are incubated with 1 μ M of activatable hGSA-NMP1, always on hGSA-IR800 and hGSA-ICG for 1.5 h and 3 h, clearly show fluorescence signal in lysosomes only in cells with 3 h incubation (right column). However, each dye only minimally accumulated in SHIN3 cells at 1.5h. Differential interference contrast and fluorescence images are shown. Scale bar represents 25 μ m (original magnification $\times 200$).

Figure 3.

In situ spectral fluorescence images of abdomen (A) and mesenteric membrane (B) in mice bearing peritoneal SHIN3 ovarian cancer are shown. Images obtained with NIR excitation were comparable for the use of all three agents. However, images using hGSA-NMP1 with green excitation were sharper and less background than other images due to the longer Stokes shift (**A; Right column**). No fluorescence is shown in mice injected hGSA-IR800 and hGSA-ICG with green excitation. Submillimeter cancer foci were clearly detectable with close-up imaging of the small bowel-mesentery (**B**).

Figure 4.

In situ spectral fluorescence images of non-tumor bearing mice with a tube phantom of hGSA-NMP1 are shown. When the phantom sample was placed atop the small intestine, the resulting image clearly indicated that the fluorescent signal was located atop the small intestine. However, when the phantom sample was placed below the small intestine, the resulting image the emission light was not visible in the green range but was clearly seen like a backlight image using the NIR filter set.

Figure 5.

In situ spectral fluorescence images of peritoneal SHIN3-tumor bearing mice with a resected large tumor nodule containing hGSA-NMP1 are shown. In images in which a tumor nodule was placed atop the small intestine, the nodule was clearly visualized with either the green or NIR excitation light. However, when the tumor nodule was placed below the small intestine, the lesion could only be detected with the NIR-Green subtraction image shown like a backlight image. Since hGSA-IR800 lacks an absorption peak in the green spectrum, the original images acquired using the band-pass filter in the green spectral range depicted only blurry fluorescent signal.

Figure 6.

In situ spectral fluorescence images of the mesenteric membrane in mice bearing peritoneal SHIN3-dsRed ovarian cancer are shown. Fluorescence signals of NMP1 and RFP, which are shown separately in different spectrally resolved fluorescence images, are mostly coincident. Sensitivity and specificity of hGSA-NMP1 for detecting SHIN3-dsRed tumors around 1 mm was calculated by examining 386 nodules.