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Radiolabeled and Near-Infrared Fluorescent Fibrinogen Derivatives Create a System for the Identification and Repair of Obscure Gastrointestinal Bleeding

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Abstract

Background—GI hemorrhage is often difficult to localize. A test that does not depend on active bleeding might prove clinically useful. We tested two novel fibrinogen (FBG)-based contrast agents for their ability to localize GI hemorrhage after bleeding stopped. ¹²⁵I-FBG permits gamma-ray based pre-operative or intraoperative scanning, and near-infrared (NIR) fluorescent FBG (FBG800) permits real-time intraoperative visualization of active clot.

Methods—Bovine FBG was radiolabeled with ¹²⁵I or conjugated to the NIR fluorophore CW800. Sites of bleeding were created by gastrotomy, mucosal resection of the stomach, or laceration of a mesenteric vessel. 1.7 mg/kg FBG800 or 15 µCi/kg of ¹²⁵I-FBG was injected intravenously into mice, rabbits, or pigs 30 min before or after injury. Sites of active clot were quantified using gamma counting, and were also imaged using invisible NIR light intraoperatively, for up to 3 hrs post-injection.

Results—After an injection of either ¹²⁵I-FBG or FBG800, sites of prior bleeding could be identified in the absence of active bleeding. Blood clearance was such that a SBR of 2.0 or greater could be achieved within 20 min after injection. A similarly labeled human serum albumin did not accumulate at any site, with an SBR of 1.0 or less.

Conclusions—Both radiolabeled (pre-operative gamma scanning) and NIR fluorescent (intraoperative real-time imaging) FBG can be used in experimental situations to identify the location of prior bleeding in the absence of active bleeding. Taken together, these contrast agents create a system for the identification and control of obscure GI bleeding.

Keywords

Fibrinogen; Gastrointestinal Bleeding; Pre-Operative Imaging; Intraoperative Imaging; Near-Infrared Fluorescence

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BACKGROUND

Gastrointestinal (GI) bleeding is a common disorder and a significant source of morbidity and mortality. Determining the site of bleeding is often difficult, and the inability to identify the source of bleeding results in prolonged hospitalization and compromises treatment.

Colonoscopy, endoscopy, nuclear scans, and angiography often fail to identify a source of bleeding because the bleeding from a particular site, though severe, may be intermittent. Radiolabeled red blood cell scans require active bleeding at a rate of 0.1–0.4 mL/min,¹ while angiography requires bleeding at a rate in excess of 0.5 mL/min.² If bleeding recurs after a negative test, total colectomy, a major and morbid procedure, may be required.^{3,4} Pre-operative or intraoperative localization would result in a much smaller and less morbid operation.

At all sites of bleeding, injury to the blood vessel wall triggers blood coagulation.^{5,6} Platelets adhere to endothelial proteins in the injury bed, followed by aggregation and fibrin formation. Fibrinogen (FBG), a circulating glycoprotein, plays an important role in the blood clotting process.⁷⁻⁹ Radiolabeled FBG has been used for the imaging of vascular thrombus, however, the use of FBG for the detection of bleeding sites has not been reported.^{10,11}

Attaching labels to FBG that would allow it to be detected either via radioactive gamma-ray emission or light-based fluorescence emission might permit detection of bleeding sites long after obvious bleeding has stopped, since a fresh clot remains enzymatically active with respect to the clotting cascade. Our laboratory has previously developed intraoperative near-infrared (NIR) fluorescence imaging systems that permit anatomy (using color video) and function (using NIR fluorescence) to be acquired simultaneously and in real-time.¹² Because of high photon penetration and low autofluorescence in the 700 to 900 nm wavelength range, invisible NIR light is favorable for intraoperative imaging,¹³⁻¹⁵ and most importantly, does not alter the look of the surgical field. In this report, we describe the detection of active clot, in the absence of active bleeding using both radioactive and optical contrast agents, in three different animal model systems.

METHODS

Reagents

The N-hydroxysuccinimide (NHS) ester (CW800-NHS) and carboxylic acid (CW800-CA) forms of IRDyeTM 800CW NIR dye were provided as dry powders from LI-COR (Lincoln, NE). They were resuspended at 30 mM in dimethylsulfoxide (DMSO; Sigma, St. Louis, MO) under reduced light conditions and stored at –80°C. Bovine FBG was purchased from MP Biomedicals (Irvine, CA), dissolved at 20 mg/mL in phosphate-buffered saline (PBS, pH 7.8) and stored at –20°C. Human thrombin (2000 NIH units/mg protein) was purchased from Sigma, and dissolved at 50 U/mL and stored at –80°C. Radiolabeled fibrinogen (¹²⁵I-FBG) and human serum albumin (¹²⁵I-HSA) were purchased from Amersham Biosciences (Piscataway, NJ).

Conjugation Reactions for NIR Fluorescent FBG

All steps were performed under reduced light conditions. Reactions contained 15 mg/mL FBG and various molar ratios of CW800-NHS in PBS with reaction volumes ranging from 100 µL (analytical) to 5 mL (preparative). Conjugation was initiated by adding CW800-NHS, and constant agitation (without frothing) was continued for 2 hrs at RT. Quenching of unreactive NHS esters was not necessary given the purification system used (see below).

Gel Filtration Chromatography

The gel-filtration chromatography system consisted of an ÄKTA prime pump with fraction collector (Amersham Biosciences, Piscataway, NJ) and Econo-Pac P6 chromatographic cartridge with a cut-off of 6000 Da (Bio-Rad, Hercules, CA). Gel-filtration and on-line absorbance and fluorescence spectrometry was performed as described in detail previously.¹⁶ After conjugation, the sample was loaded into the injector and run at a flow rate of 1 mL/min using PBS as mobile phase. Full spectrum absorbance and fluorescence data were recorded every 10 sec. Desired products were collected by the fraction collector, pooled, and stored at 4°C in the dark without preservatives until use. Average yield was 85% or greater. The labeling ratio and the concentration of the conjugated protein (FBG800) were estimated using the extinction coefficients of FBG ($\epsilon_{280\text{nm}} = 330,000 \text{ M}^{-1}\text{cm}^{-1}$) and CW800-CA ($\epsilon_{777\text{nm}} = 242,000 \text{ M}^{-1}\text{cm}^{-1}$) in PBS, with correction for the 6.5% of measured absorbance at 280 nm due to CW800-CA:

$$\text{Labeling Ratio} = (\text{Abs}_{777\text{nm}} / \epsilon_{777\text{nm}}) / ((\text{Abs}_{280\text{nm}} - 0.065 * \text{Abs}_{777\text{nm}}) / \epsilon_{280\text{nm}})$$

ES-TOF Mass Spectroscopic Site Mapping

230 μL of 8 M urea/400 mM ammonium bicarbonate solution was added to 1 mg FBG or FBG800 in 20 μL of PBS. 5 μL of 45 mM dithiothreitol (DTT) was added and incubated for 15 min at 50°C. 5 μL of 100 mM iodoacetamide was added and incubated for 15 min at RT. The mixture was diluted with 750 μL of buffer (80 mg ammonium bicarbonate and 6 mg CaCl_2 in 10 mL H_2O), 20 μg of TPCK-trypsin (L-1-tosylamide-2-phenylethyl ketone-treated, Sigma) in water was added, and the solution was incubated for 24 hrs at 37°C. 20 μL of this peptide digest was used for analysis on a Waters (Milford, MA) LCT ES-TOF LC/MS equipped with dual wavelength absorbance detector (Waters), multi-wavelength fluorescence detector (Waters), a Sedex Model 75 evaporative light scatter detector (ELSD; Richards Scientific, Novato, CA), and a lock-spray. The absorbance detector was set to 254 and 700 nm (the maximum permitted wavelength), and fluorescence detector was set to excite at 770 nm and detect emission at 800 nm. Leucine enkephalin (0.5 ng/ μL) was used as a mass reference. Buffer A was 10 mM triethylammonium acetate, pH 7 (Glen Research, Sterling, VA) and buffer B was acetonitrile. Peptides were resolved on a $2.1 \times 150 \text{ mm}$ Symmetry C_{18} column (Waters) at a flow rate of 0.3 mL/min, using a gradient of 5% to 40% B over 35 min. Mass was measured in ES+ mode. Data were analyzed with MassLynx (Waters) software, and expected peptide masses calculated from the mass obtained from Peptidemass (<http://au.expasy.org/tools/peptide-mass.html>) with the addition of the mass of CW800-CA (1003.24) and subtraction of the mass of H_2O (18.02). 3-D protein structure was visualized on a Macintosh iMac computer running RasMac Molecular Graphics version 2.6-ucb1.0 (University of California, Berkeley, CA).

Thrombin-Catalyzed Fibrin Polymerization

Polymerization was followed by turbidity at 350 nm using a HR2000 fiber optic spectrometer connected to a DH-2000-BAL light source and CUV-ALL-UV 4-way cuvette holder (Ocean Optics, Dunedin, FL). FBG800 (497 μL of 0.5 mg/mL) of various labeling ratios in PBS was mixed with human thrombin (3 μL of 50 U/mL) and placed into the cuvette immediately. Turbidity was monitored at RT for 30 min. Maximum slope was obtained from the turbidity curves, and relative slope was calculated from the slope of FBG alone, which was set to 1.0.

Animal Models of Bleeding

Animals were housed in an AAALAC-certified facility and were studied under the supervision of an approved institutional protocol. 30 g CD-1 mice were from Charles River Laboratories (Wilmington, MA). 3 kg adult New Zealand White rabbits were from Robinson Services (Mocksville, NC). 30 kg Yorkshire pigs were from E.M. Parsons and Sons (Hadley, MA). All

animals acclimated to the animal facility for at least 48 hrs prior to experimentation, and were euthanized after experimentation using pentobarbital (mice) or rapid intravenous injection of Fatal-Plus (Vortech Pharmaceuticals, Dearborn, MI; rabbits and pigs).

Anesthesia was induced with intraperitoneal injection of pentobarbital (40 mg/kg) to mice and intramuscular injection of ketamine (35 mg/kg) and xylazine (5 mg/kg) to rabbits. Pig anesthesia was induced with 4.4 mg/kg intramuscular Telazol (Fort Dodge Labs, Fort Dodge, IA) and anesthesia was maintained through a 7 mm endotracheal tube with 1.5% isoflurane/98.5% O₂ at 5 L/min. After anesthesia, a laparotomy was made in the midline; the stomach and a loop of small intestine were externalized. A mesenteric vessel of the mice and pigs was lacerated then permitted to clot, resulting in intra-mesenteric thrombus formation. For rabbits and pigs, a gastrotomy was made and the posterior wall mucosa was pulled through. A 5-mm mucosectomy was performed with a scalpel. The injured area was allowed to clot and marked with a 4-0 silk suture for identification.

Contrast Agent Administration

1.7 mg/kg FBG800 protein (10 nmol/kg of fluorophore) was injected intravenously in mice (N = 4) and pigs (N = 3). 15 μ Ci/kg ¹²⁵I-FBG was injected intravenously in rabbits (N = 3). This dose is approximately 100 ng and is much less than the total fibrinogen in the rabbit. Injections occurred 30 min before or 30 min after injury as described below. For controls, the same dose of CW800-labeled human serum albumin (HSA800, N = 3)¹⁶ or radiolabeled albumin (¹²⁵I-HSA, N = 3) was used.

Intraoperative Imaging System

The intraoperative NIR fluorescence imaging system optimized for animal surgery has been described previously in detail.¹² Briefly, it is composed of two wavelength-isolated excitation sources, one generating 0.5 mW/cm² 400–700 nm “white” light, and the other generating 5 mW/cm² 725–775 nm light over a 15 cm diameter field of view. Simultaneous photon collection of color video and NIR fluorescence images is achieved with custom-designed optics that maintains separation of the white light and NIR fluorescence (>795 nm) channels. Spatial resolution at a field-of-view of 20 x 15 cm is 625 μ m, and at a field-of-view of 4 x 3 cm is 125 μ m. After computer-controlled (LabVIEW) camera acquisition via custom LabVIEW (National Instruments, Austin, TX) software, anatomic (white light) and functional (NIR fluorescence light) images can be displayed separately and merged. All images are refreshed up to 15 times per sec. The entire apparatus is suspended on an articulated arm over the surgical field, thus permitting non-invasive and non-intrusive imaging.

Signal Intensity Measurements

After injection of FBG800, signal to background ratio (SBR) was measured by using as background the signal from an identically sized and shaped region of interest (ROI) over adjacent mesenteric vessels or GI mucosa. After injection of ¹²⁵I-FBG, a standard Geiger counter was swept over the areas of injury to determine whether the site of injury could be recognized simply on the basis of meter readings. The entire stomach was then removed and opened. A portion of stomach away from both the gastrotomy site and the site area of mucosal injury was resected and used for background measurements. Next, the gastrotomy site was resected. Finally, the area of mucosal injury was recognized and resected. All tissues were weighed, placed in scintillation vials, and counted in a standard scintillation counter. Statistical comparisons were made with a Student's t-test.

RESULTS

Synthesis of FBG800 and its Optical and Biological Properties

After reaction with fluorophore, FBG800 was successfully separated from free NHS ester and free CW800-CA with a purity of >95% by gel-filtration (data not shown). As expected, the labeling ratio increased as the mixing ratio increased (Figure 1A). However, as the labeling ratio increased, the relative per-fluorophore fluorescence decreased (Figure 1B) and the per-molecule fluorescence plateaued (Figure 1C). Due to this phenomenon, it was concluded that maximal per-molecule fluorescence yield occurs at a labeling ratio of 6–8 (Figure 1C).

However, increasing fluorophore substitution resulted in marked changes in biopotency. Even at a labeling ratio of 1.0, FBG800 bioactivity was 50% of native FBG (Figure 1C). Above a labeling ratio of 2.0, FBG polymerization was inhibited significantly (Figure 1C). FBG at a labeling ratio of 2.0 was therefore used for all further studies. Peak absorption of CW800-CA and FBG800 was 775 nm and 785 nm, respectively (Figure 1D). Peak fluorescence emission of CW800-CA and FBG800 was 794 nm and 800 nm, respectively (Figure 1E), showing the characteristic bathochromic shift after conjugation.¹⁶

Identification of Fluorophore Location(s) on FBG800

In order to determine the conjugation site(s) of CW800, we performed tryptic mass-spec fingerprinting. The total ion chromatogram demonstrated that peptides from both FBG and FBG800 were well separated, with a small number of peaks of 700 nm absorbance (the highest available wavelength on our detector) and 800 nm fluorescence were observed from FBG800, but not from FBG (data not shown). By measuring the mass of the peptides with 700 nm absorbance and 800 nm fluorescence, we obtained two conjugation sites, at amino acids Lys399 and Lys185 of the B β chain. Fortunately, neither conjugation site (Figure 1F) is located in the fibrinopeptide A or B,⁹ which are released by thrombin cleavage and are not included in the fibrin clot.⁸

Intraoperative Thrombus Detection with ¹²⁵I-FBG

Radiolabeled FBG has the potential to be used with a gamma camera to confirm occult GI bleeding and to partially localize the bleeding site. For proof of principle, the low energy gamma emitter ¹²⁵I was used. In the first group, rabbits injected 30 min prior to gastrotomy were analyzed. Radioactivity was counted 3 hrs after the injury, and the counts at the sites of injury were divided by the counts at the uninjured area to evaluate the fold increase over background. The ratio of the counts at the site of injury using ¹²⁵I-FBG as compared to background, uninjured mucosa was 4.8 ± 1.0 (N = 3). The ratio of the counts at the site of the gastrotomy as compared to the uninjured mucosa was 6.9 ± 1.8 (N = 3). Importantly, the gastrotomy site and the injury site were readily identified *in situ* by a standard handheld Geiger counter. To evaluate whether the increased counts were due to a non-specific effect of protein accumulation at the sites, ¹²⁵I-HSA was used as a control. In these animals, the ratio of the counts at the site of mucosal injury as compared to the uninjured mucosa was 1.7 ± 1.0 (N = 3), and at the gastrotomy as compared to the uninjured mucosa was 1.0 ± 0.7 (N = 3). There was a statistically significant difference between the ratios in the injured tissue with fibrinogen as compared to the ¹²⁵I-HSA control ($p < 0.05$).

In the second group, rabbits injected 30 min after gastrotomy with either ¹²⁵I-FBG or ¹²⁵I-HSA were analyzed. The ratio of the counts at the site of the injury using ¹²⁵I-FBG as compared to background, uninjured mucosa was 3.0 ± 0.5 (N = 3). The ratio of the counts at the site of the gastrotomy as compared to the uninjured mucosa was 2.4 ± 1.0 (N = 3). The differences were statistically significant ($p < 0.05$). Again, the gastrotomy site and the injury site were readily identified by a standard handheld Geiger counter. To evaluate whether the increased

counts were due to a non-specific effect of protein accumulation at the sites, ^{125}I -HSA was used as a control. In these animals, the ratio of the counts at the site of mucosal injury as compared to the uninjured mucosa was 1.2 ± 0.1 ($N = 3$), and at the gastrotomy as compared to the uninjured mucosa was 1.0 ± 0.4 ($N = 3$). There was a statistically significant difference between the ratios when ^{125}I -FBG was used as compared with ^{125}I -HSA ($p < 0.05$).

Real-Time Intraoperative Thrombus Detection with FBG800 using Invisible NIR Fluorescent Light

In the mice ($N = 4$), FBG800 was injected intravenously 30 min after mesenteric vessel injury, when no active bleeding was seen. As shown in Figure 2A, FBG800 rapidly accumulated at the site of thrombus formation, and within 20 min SBR was optimal. The control protein HSA800 showed no accumulation (Figure 2A, $N = 4$). The SBR of the clot was ≈ 2.0 , while that of the adjacent area remained < 1.0 . In order to evaluate the performance of FBG800 in animals approaching the size of humans, mesenteric vessel injury and mucosal resection of the stomach were performed in 30 kg Yorkshire pigs ($N = 3$). 30 min after these injuries, when no active bleeding was present, FBG800 was injected intravenously. It was found to accumulate rapidly at the sites of clot formation, with optimal SBR achieved by 30 min (Figure 2B). Intravenous injection of HSA800 resulted in no accumulation at any site of injury (data not shown).

DISCUSSION

Current methods for detecting GI bleeding all require active bleeding to localize the source. High false-negative rates of these tests are due to the fact that GI bleeding is often intermittent. There is a tremendous need for a test that can detect the site even if bleeding is not ongoing at the time of the test. In this study we described two novel contrast agents that do not require active bleeding to find the site of clot, and thus, previous hemorrhage. Although we focused this study on FBG-based contrast agents, one might imagine that other components of the clotting cascade, or markers on the endothelium itself, could be used in a similar fashion, but with different intensities, uptake, and decay characteristics.

As we have demonstrated in three different animal model systems, ^{125}I -FBG and FBG800 can be administered before, during, or after injury and still be used to identify the site of bleeding. Since the size of the FBG protein is rather large (330 kDa), the plasma half-life is expected to be several days.¹⁷ This may work to the surgeon's advantage because these agents could be given to patients at high risk for re-bleeding, as most of these patients are, and thus identify the bleeding source if, and when, re-bleeding occurs. Either radiolabeled or NIR fluorophore-labeled FBG might be administered to patients at presentation, in combination with methods that are currently being used, to detect bleeding or to increase the sensitivity of conventional tests. Although ^{125}I -FBG was used in this study for proof of principle, ^{131}I -FBG would provide the capability for full-body gamma-ray scanning of humans. In terms of clinical use, radiolabeled and NIR fluorescent FBG are complementary. Radiolabeled FBG does not require a laparotomy as does FBG800, and it provides full-body scanning. FBG800 needs to be used in an operative setting, but it doesn't expose patient and caregivers to radioactivity, and provides sensitive and high-resolution visualization of active clot.

There are a number of issues that need to be addressed in the further development of this technology. More work is required to determine which clotting factors are optimal. It is likely that depending on the time of hemorrhage that different proteins might be more useful. Although background clot did not have high radioactivity, in the setting of massive bleeding and ongoing transfusion, the radioactivity or fluorescence might be washed out and produce a high background that would make localization difficult. Irrigation of the bowel may be useful to overcome this problem but this has not been tested. Furthermore, interventions that might

cause mucosal damage at sites distant from the actual bleeding site, such as colonoscopy, might produce false positive results.

A critical step in the development of NIR fluorescent proteins such as FBG800 is the simultaneous optimization of total fluorescent yield and biopotency. Although it is usually possible to achieve high substitution ratios (Figure 1B), internal filter effects and dye quenching limit total fluorescent yield (Figure 1C). As we have shown previously, wide spacing of heptamethine indocyanines over the surface of a large protein can minimize, but not eliminate these effects.¹⁶ Fluorophore substitution can also alter the molecule's biopotency, which needs to be verified independently (Figure 1C).

In summary, we describe a radiolabeled ¹²⁵I-FBG and an 800-nm fluorescent FBG that in conjunction with the appropriate imaging system can be used to detect enzymatically active clot even after the cessation of bleeding in a well defined experimental setting. The fluorescence of FBG800 has been optimized for 800 nm, a region of the spectrum with low autofluorescence and scatter, and fluorophore substitution has been titrated carefully to simultaneously maximize photon yield and biopotency. ¹²⁵I-FBG and FBG800 are complementary in their use, creating a system for the diagnosis and treatment of obscure GI bleeding.

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ABBREVIATIONS

DMSO, dimethylsulfoxide
 ES-TOF, electrospray time-of-flight
 FBG, fibrinogen
 GI, gastrointestinal
 HSA, human serum albumin
 LC/MS, liquid chromatography/mass spectrometry
 NHS, N-hydroxysuccinimide
 NIR, near-infrared
 PBS, phosphate-buffered saline
 RT, room temperature
 SBR, signal to background ratio.

REFERENCES

1. Smith R, Copely DJ, Bolen FH. 99mTc RBC scintigraphy: correlation of gastrointestinal bleeding rates with scintigraphic findings. *AJR Am J Roentgenol* 1987;148:869–874. [PubMed: 3495120]
2. Baum S, Athanasoulis CA, Waltman AC, Ring EJ. Gastrointestinal hemorrhage. II. Angiographic diagnosis and control. *Adv Surg* 1973;7:149–198. [PubMed: 4544255]
3. Farner R, Lichliter W, Kuhn J, Fisher T. Total colectomy versus limited colonic resection for acute lower gastrointestinal bleeding. *Am J Surg* 1999;178:587–591. [PubMed: 10670878]

4. Schuetz A, Jauch KW. Lower gastrointestinal bleeding: therapeutic strategies, surgical techniques and results. *Langenbecks Arch Surg* 2001;386:17–25. [PubMed: 11405084]
5. Dahlback B. Blood coagulation. *Lancet* 2000;355:1627–1632. [PubMed: 10821379]
6. Spronk HM, Govers-Riemsag JW, ten Cate H. The blood coagulation system as a molecular machine. *Bioessays* 2003;25:1220–1228. [PubMed: 14635257]
7. Doolittle RF, Everse SJ, Spraggon G. Human fibrinogen: anticipating a 3-dimensional structure. *Faseb J* 1996;10:1464–1470. [PubMed: 8940292]
8. Fuss C, Palmaz JC, Sprague EA. Fibrinogen: structure, function, and surface interactions. *J Vasc Interv Radiol* 2001;12:677–682. [PubMed: 11389218]
9. Herrick S, Blanc-Brude O, Gray A, Laurent G. Fibrinogen. *Int J Biochem Cell Biol* 1999;31:741–746. [PubMed: 10467729]
10. Haines ST, Bussey HI. Diagnosis of deep vein thrombosis. *Am J Health Syst Pharm* 1997;54:66–74. [PubMed: 9117794]
11. Knight LC. Scintigraphic methods for detecting vascular thrombus. *J Nucl Med* 1993;34:554–561. [PubMed: 8441057]
12. De Grand AM, Frangioni JV. An operational near-infrared fluorescence imaging system prototype for large animal surgery. *Technol Cancer Res Treat* 2003;2:553–562. [PubMed: 14640766]
13. Frangioni JV. In vivo near-infrared fluorescence imaging. *Curr Opin Chem Biol* 2003;7:626–634. [PubMed: 14580568]
14. Ntziachristos V, Bremer C, Weissleder R. Fluorescence imaging with near-infrared light: new technological advances that enable in vivo molecular imaging. *Eur Radiol* 2003;13:195–208. [PubMed: 12541130]
15. Sevick-Muraca EM, Houston JP, Gurfinkel M. Fluorescence-enhanced, near infrared diagnostic imaging with contrast agents. *Curr Opin Chem Biol* 2002;6:642–650. [PubMed: 12413549]
16. Ohnishi S, Lomnes SJ, Laurence RG, Gogbashian A, Mariani G, Frangioni JV. Organic alternatives to quantum dots for intraoperative near-infrared fluorescent sentinel lymph node mapping. *Mol Imaging* 2005;4:172–181. [PubMed: 16194449]
17. Coyne CP, Hornof WJ, Kelly AB, O'Brien TR, DeNardo SJ. Extraction, radioiodination, and in vivo catabolism of equine fibrinogen. *Am J Vet Res* 1985;46:2572–2577. [PubMed: 4083596]
18. Brown JH, Volkmann N, Jun G, Henschen-Edman AH, Cohen C. The crystal structure of modified bovine fibrinogen. *Proc Natl Acad Sci U S A* 2000;97:85–90. [PubMed: 10618375]

A.

B.

C.

D.

E.

F.

Figure 1. Synthesis and Optical Properties of FBG800

- A) The labeling ratio of FBG800 as a function of the protein to fluorophore mixing ratio.
 B) Relative per fluorophore fluorescence, as a function of labeling ratio, compared to free CW800-CA (1 μ M) in PBS.
 C) Per molecule total fluorescence yield calculated from the labeling ratio and per fluorophore fluorescence, and compared to the same dye concentration (1 μ M) of free CW800-CA in PBS (closed circles). The relative ratio of bioactivity of FBG800 to native FBG measured using a fibrin polymerization assay (open circles).
 D) Absorbance spectra of CW800-CA and FBG800 (labeling ratio=1.2) in PBS. Peak absorption of CW800-CA and FBG800 was 775 nm and 785 nm, respectively.

E) Emission spectra of CW800-CA and FBG800 (labeling ratio=1.2). Peak emission of CW800-CA and FBG800 was 794 nm and 800 nm, respectively.

F) The conjugation sites of CW800 to FBG (white) were established using trypsin digestion and ES-TOF LC/MS peptide analysis, and are shown overlaid with the crystal structure¹⁸ of the dimer. The amino acid substitution sites are: Lys399 (red) and Lys185 (green) of the B β chain.

A.

B.

Figure 2. Real-Time Intraoperative Thrombus Detection using Invisible Light

A) FBG800 (left) or HSA800 (right) was injected into the mouse 30 min after injury of a mesenteric vessel (arrows). Shown are color video (left of each set) and NIR fluorescence (right of each set). All NIR fluorescence images have identical exposure times and normalizations. Results are representative of N = 4 animals.

B) FBG800 was injected into the pig 30 min after injury (arrows) of a mesenteric vessel (top) or mucosal resection of the stomach (bottom). Shown are color video (left), NIR fluorescence (right), and a pseudo-colored (lime green) merge of the two (right). Results are representative of N = 3 animals.