Supporting Information

Site-specific Near-Infrared Fluorescent Labelling of Proteins on Cysteine Residues with \textit{meso}-Chloro-Substituted Heptamethine Cyanine Dyes

Coline Canovas, Pierre-Simon Bellaye, Mathieu Moreau, Anthony Romieu, Franck Denat, and Victor Goncalves*

\(^a\) Institut de Chimie Moléculaire de l’Université de Bourgogne, UMR6302, CNRS, Université Bourgogne Franche Comté, Dijon, France
\(^b\) Georges-François LECLERC Cancer Center - UNICANCER, Dijon, France
\(^c\) Institut Universitaire de France, 1, Rue Descartes, Bâtiment MONGÈ, 75231, Paris, France

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1. Abbreviations

BSA: Bovine serum albumin
DAD: Diode array detector
DIC: N,N'-diisopropylcarbodiimide
DIPEA: N,N-diisopropylethylamine
DMF: N,N-dimethylformamide
DTNB: 5,5-dithio-bis-(2-nitrobenzoic acid)
EDTA: Ethylenediaminetetraacetic acid
ESI: Electrospray ionisation
FA: Formic acid
FBS: Fetal bovine serum
FPLC: Fast protein liquid chromatography
GSH: Glutathione
HOBt: 1-Hydroxybenzotriazole
HPLC: High-performance liquid chromatography
HRMS: High-resolution mass spectrometry
IAM: Iodoacetamide
MS: Mass spectrometry
p.i.: Post-injection
PAGE: Polyacrylamide gel electrophoresis
PB: Phosphate buffer
PBS: Phosphate buffer saline
rpm: Round per minute
rt: Room temperature
RPMI: Roswell Park Memorial Institute medium
SD: Standard deviation
SDS: Sodium dodecyl sulfate
TCEP: Tris(2-carboxyethyl)phosphine
TFA: Trifluoroacetic acid
TIC: Total ion chromatogram
TIS: Triisopropylsilane
t<sub>R</sub>: Retention time
Tris: Tris(hydroxymethyl)aminomethane
2. Material and general procedures

All chemicals were purchased from Sigma-Aldrich, Acros Organics and Alfa Aesar and used without further purification. The cyanine-based fluorophores IR-783 (inner salt, sodium salt, dye content 90%) and DiSulfo-CYanine7 - Maleimide (K salt, FluorProbes®) were obtained from Sigma-Aldrich and Interchim respectively. Pertuzumab (Herceptin; Roche, U.K.) was obtained through the Georges-Francois LECLERC Cancer Center - UNICANCER (Dijon, France). The synthesis of the model peptide Ac-CKYWGRGDS-NH2 has been described previously.1 HPLC-gradient grade MeCN was obtained from Biosolve or Carlo Erba. LC-HRMS grade MeCN was obtained from Fisher Scientific. All aq. buffers used in this work and aq. mobile-phases for HPLC were prepared using water purified with a PURELAB Ultra system from ELGA (purified to 18.2 MΩ cm).

HRMS spectra were recorded on a mass spectrometer LTQ Orbitrap XL (Thermo Scientific) using an ESI source.

The peptide and related conjugate were characterized by HPLC analyses performed on an UltiMate 3000 system Dionex (Thermo Scientific) equipped with a DAD detector and coupled to a low-resolution mass spectrometry detector MSQ Plus (Thermo Scientific) equipped with an ESI source. Separation was achieved using an RP Kinetex™ column (Phenomenex) (2.6 µm, 100 Å, 50 × 2.1 mm) with ultrapure water and HPLC-grade MeCN: A: H2O 0.1% FA and B: MeCN 0.1% FA. Analyses were performed with the following gradient program: 5% to 100% of B in 5 min, 100% B for 1.5 min, 100% to 5% B in 0.1 min and 5% B for 1.9 min, at a flow rate of 0.5 mL/min.

The purity of peptide derivatives was determined from the integration of HPLC-MS chromatograms at 214 nm.

Proteins and related conjugates were characterised by HPLC analyses performed on a HPLC UltiMate 3000 system (Thermo Scientific) equipped with a DAD detector and coupled to a high-resolution mass spectrometer LTQ Orbitrap XL (Thermo Scientific) equipped with an ESI source. The separation was achieved on a Thermo Scientific™ MAbPac™ column (4 µm, 50 × 2.1 mm) with the following eluents: A: H2O, 0.1% FA + 0.02% TFA and B: MeCN/H2O (90/10), 0.1% FA + 0.02% TFA, and a linear gradient program: 5% to 100% of B in 15 min, 100% B for 3 min, 100% to 5% B in 0.1 min and 5% B for 6 min, at a flow rate of 0.3 mL/min, at 80 ºC. Multicharged mass spectra were deconvoluted with the Protein Deconvolution 2.0 software (Thermo Scientific).

SDS-PAGE analyses were performed on Mini-PROTEAN® TGX™ 4 to 20 % Precast gel (BioRad) in non-reductive conditions. 5 µL of sample solution at a concentration of about 1 mg/mL was mixed with 5 µL of loading buffer and denaturated at 90 ºC during 5 min. The loading buffer was constituted of 0.125 M Tris HCl, 20% glycerol, 4% SDS and 0.004% bromophenol blue. After loading, the electrophoresis was started (160 V, 45 min) in a running buffer constituted of 0.3% Tris HCl, 1.92 M glycine, 0.1% SDS and 0.05% sodium azide. The protein ladder used as reference was a PageRuler™ Plus Prestained (Thermo Scientific). For visualisation of IR-783 conjugates, a fluorescence image of the gel was recorded on an IVIS Lumina III In Vivo Imaging System (Perkin Elmer) in pair filter mode (740/790 nm). Then, the gel was stained with Coomassie brilliant blue during 1 h.

UV-visible spectra were obtained on an Agilent Cary 5000 UV-VIS-NIR (double beam) spectrophotometer by using a rectangular quartz cell (Hellma, 100-QS, 45 × 12.5 × 12.5 mm, pathlength: 10 mm, chamber volume: 3.5 mL), at 25 ºC. Fluorescence spectroscopic studies were performed with a HORiba Jobin Yvon Fluorolog spectrofluorometer (software FluorEssence) at 25 ºC, with a standard fluorometer cell (Labbox, LB Q, light path: 10 mm, width: 10 mm, chamber volume: 3.5 mL). The absorption spectra of cyanine dye IR-783 and related conjugate were recorded (220-900 nm) in PBS with concentrations in the micromolar range. Emission spectra were recorded in the range 725-900 nm after excitation at 710 nm (shutter: Auto Open, Ex. slit = 5 nm and Em. slit = 5 nm). Excitation spectra were recorded in the range 240-830 nm with emission measurement at 840 nm (shutter: Auto Open, Ex. slit = 12 nm and Em. slit = 5 nm). All fluorescence spectra were corrected until 850 nm. Fluorescence quantum yields were measured at 25 ºC by a relative method using cyanine dye IR-783 (ΦF = 6.5% in PBS pH 7.4) as standard (dilution by a factor of 3 between absorption and fluorescence measurements). The following equation was used:

\[
Φ_F(x) = (A_o/A_x)(F_o/F_x)(n_o/n_x)^2Φ_F(s)
\]
where A is the absorbance (in the range of 0.01-0.1 A.U.), F is the area under the emission curve, n is the refractive index of the solvents (at 25 °C) used in measurements, and the subscripts S and X represent standard and unknown, respectively. The following refractive index value was 1.337 for PBS.

3. Validation of the labelling strategy on model peptides

The synthesis of the model peptide Ac-CKYWGRGDS-NH$_2$ has been described previously.$^1$ The other peptides were synthesized according to the same methodology on a Liberty Blue™ Automated Microwave Peptide Synthesizer (CEM, USA).

Peptide couplings were performed by using predefined Fmoc chemistry protocols on 208 mg (0.1 mmol) of Fmoc-Rink Amide aminomethyl-polystyrene resin (Iris Biotech - loading 0.48 mmol/g). DIC/HOBt in DMF were used as the activating reagents and a solution of piperidine 20% in DMF was used for Fmoc deprotections. Where necessary, the capping of the N-terminal amino acid was performed by reaction at room temperature, during 30 minutes, with acetic anhydride (472 µL, 5 mmol, 50 eq) and DIPEA (850 µL, 5 mmol, 50 eq) in DMF (5 mL). Peptidyl-resins were treated with a cleavage solution formed of TFA/TIS/DODT/H$_2$O, 92.5/2.5/2.5/2.5 (v/v) (5 mL) for 2.5 h. The resin was removed by filtration and the filtrate was concentrated under a flow of nitrogen. The peptides were precipitated in diethyl ether and recovered by centrifugation at 4000 rpm for 10 minutes. The crude peptides were purified by a semi-preparative HPLC on a BetaBasic-18 column (eluents: H$_2$O 0.1% TFA, MeCN 0.1% TFA) using a linear gradient solvent (10-50% MeCN in H$_2$O in 40 minutes) and the appropriate fractions were lyophilized to afford target peptides.

**Peptide CKYWGRGDS-NH$_2$·3TFA.** White fluffy powder, 55 mg, 39%, purity > 99%, MS: m/z calculated for C$_{46}$H$_{67}$N$_{15}$O$_{13}$S [M+2H]$^{2+}$ 535.7, found 535.8.

**Peptide Ac-KCYWGRGDS-NH$_2$·2TFA.** White fluffy powder, 98 mg, 73%, purity 98%. MS: m/z calculated for C$_{48}$H$_{69}$N$_{15}$O$_{14}$S [M+2H]$^{2+}$ 556.7, found 556.9.

**Peptide Ac-KYWGRGDSC-NH$_2$·2TFA.** White fluffy powder, 62 mg, 46%, purity 97%. MS: m/z calculated for C$_{48}$H$_{69}$N$_{15}$O$_{14}$S [M+2H]$^{2+}$ 556.7, found 556.8.

**Peptide Ac-KYWGRGDS-NH$_2$·2TFA.** White fluffy powder, 49 mg, 37%, purity 98%. MS: m/z calculated for C$_{48}$H$_{69}$N$_{15}$O$_{14}$S [M+2H]$^{2+}$ 556.7, found 556.8.

Monitoring of the reaction between model peptides and IR-783

10 µL of IR-783 solution at 20 mM in DMF (200 nmol, 25 equiv.) were added to 400 µL of a peptide solution at 0.02 mM in PB (0.66 M, pH 7.1-7.4). The resulting solution was stirred at rt, in dark, and monitored by HPLC-MS analysis after 1 h. No changes were observed after 15 h

**Figure S1.** For each tested peptide: (Top panel) RP-HPLC-MS analyses of the crude labelling reaction (elution profile at 214 nm). Bottom (left panel) UV-Vis absorbance spectrum of the main product; (right panel) ESI LRMS (positive mode).
Synthesis and spectral properties of the fluorescent conjugate 1, Ac-C(IR-783)KYWGGRGDS-NH₂

Doubly TFA salt of peptide Ac-CKYWGRGDS-NH₂ (11.0 mg, 8.2 µmol) and IR-783 (10.3 mg, 12.0 µmol, 1.5 equiv.) were dissolved in 3 mL of PB (0.66 M, pH 7.1). The resulting solution was stirred at rt, in dark, for 50 min. The crude peptide was purified on an UltiMate 3000 semi-preparative HPLC system Dionex (Thermo Scientific) equipped with a UV-Vis detector. Separation was achieved using dC18 SiliaChrom column from SiliCycle (10 µm, 100 Å, 250 × 20 mm) at a flow rate of 20 mL/min, with ultrapure water and HPLC-grade MeCN: A: H₂O 0.1% TFA and B: MeCN 0.1% TFA. Fractions were analyzed by analytical RP-HPLC-MS.

The doubly TFA salt of Ac-C(IR-783)KYWGGRGDS-NH₂ (1) was isolated as a green amorphous powder (14.4 mg, yield 86%, TFA salt). RP-HPLC-MS: tᵦ = 4.29 min, purity = 95%. HRMS: C₈₆H₁₁₇N₁₇O₂₀S₃⁺ ([M + 2H]⁺) m/z calcd: 901.89060; found: 901.89222; C₈₆H₁₁₆N₁₇O₂₀S₃Na⁺ ([M + H + Na]⁺) m/z calcd: 912.88157; found: 912.88194; C₈₆H₁₁₅N₁₇O₂₀S₃Na₂⁺ ([M + 2Na]²⁺) m/z calcd: 923.87254; found: 923.86964.
Figure S2. RP-HPLC-MS analysis of the fluorescent conjugate 1 (left: elution profile at 214 nm, top right: ESI LRMS recorded in the positive mode, bottom right: UV-Vis absorbance spectrum recorded during the HPLC analysis in the range 200-800 nm).

Figure S3. ESI HRMS recorded in the positive mode.
Figure S4. Left: Overlaid absorbance, emission and excitation spectra of the fluorescent conjugate 1 in PBS (pH 7.4). The emission spectrum was recorded in the range 725-900 nm upon excitation at 710 nm (slit 5 nm), and the excitation spectrum was recorded in the range 240-830 nm upon emission at 840 nm (slit 12 nm and 5 nm respectively). \( \lambda_{\text{Abs, max}} = 792 \text{ nm}, \lambda_{\text{Em, max}} = 825 \text{ nm} \); Right: Overlaid excitation and emission spectra of free IR-783 and fluorescent conjugate 1 showing a bathochromic displacement of 20 nm for the excitation and of 33 nm for the emission after conjugation to peptide. Please note: emission spectra cannot be corrected in the range 850-900 nm, which explains the artefact observed at 850 nm.

Stability studies of the conjugate 1 in FBS/RPMI (25 : 75, v/v)

6.67 µL of a 30 mM stock solution of fluorescent conjugate 1 in DMF (200 nmol) were stirred in a thermomixer (1000 rpm, 37 °C, dark) with 145 µL of FBS/RPMI (25 : 75, v/v). Final concentration in conjugate 1 was 1 mM. After 0 min, 10 min, 20 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h, 55 h, 70 h and 148 h, a sample of 15 µL of solution was collected and diluted with 60 µL of absolute EtOH. Samples were vortexed and centrifuged (11700 rpm, 15 min) to remove the precipitated proteins. The supernatant was analyzed by RP-HPLC-MS (UV detection at 214 nm).

Stability studies of the fluorescent conjugate 1 in the presence of GSH

10.0 µL of a 5 mM stock solution of fluorescent conjugate 1 in ultrapure water containing 6.7% of DMF (50 nmol) were stirred in a thermomixer (1000 rpm, 25 °C, dark) with 0.3 mM solution of GSH in PBS (480 µL). Final concentration in conjugate 1 was 0.1 mM. After 10 min, 1.3 h, 2.6 h, 5.1 h, 8.3 h, 21.4 h, 2 days, 5 days and 6 days the reaction was analyzed by RP-HPLC-MS (UV detection at 214 nm).

Figure S5. Time-dependent changes in the amount of intact fluorescent conjugate 1 upon incubation in FBS (25% in RPMI) (red curve) or in the presence of GSH (0.3 mM) (blue curve).
4. Evaluation of the strategy by the labelling of pertuzumab fragment Fab’

Synthesis of pertuzumab fragment F(ab’)2

Pertuzumab (10 mg) was digested by the pepsin protease from porcine gastric mucosa (Sigma Aldrich) in sodium acetate buffer (0.1 M, pH 4.0). Antibody concentration was 1 mg/mL and pepsin concentration, 0.05 mg/mL. After 15 h at 37 °C, the enzymatic digestion was stopped by adding Tris buffer (1.0 M, pH 9.0, 2 mL). The buffer was exchange from NaOAc to PB and the reaction was concentrated on an Amicon Ultra Ultracel-30 kDa (Merck Millipore). The fragment F(ab’)2 was purified by FPLC (Äkta Pure 25 M chromatography system, GE Healthcare Life Sciences) on an Hitrap Protein L column (Capto L resin, 7 × 25 mm, 1 mL) with AcOH buffer (0.05 M, pH 3.0) as eluent, and then by size-exclusion separation on a Superdex™ 200 10/300 GL column (cross-linked agarose and dextran, 13 µm, 10 × 300-310 mm, 24 mL), at 0.8 mL/min, with PB (20 mM, pH 7.3) as eluent. The fragment was then concentrated by ultrafiltration on an Amicon Ultra Ultracel-30 kDa to obtain a solution of F(ab’)2 at 4.98 mg/mL* in PB (0.02 M, pH 7.3). The pure F(ab’)2 was characterised by RP-HPLC-MS (orbitrap). (protein recovery = 69%, HPLC purity at 280 nm = 95%).

*note: the concentration of the F(ab’)2 in mg/mL was calculated by UV spectrophotometry using the extinction coefficient of the F(ab’)2 at 280 nm (1.45 L.g⁻¹.cm⁻¹, value from ExPASy).

Figure S6. RP-HPLC elution profiles (TIC and UV detection at 280 nm), and deconvoluted ESI mass spectrum of F(ab’)2.
Figure S7. Coomassie brilliant blue staining of the SDS-PAGE analysis of the full antibody pertuzumab (1) and the purified F(ab')\(_2\) (2).

Synthesis of Fab'

F(ab')\(_2\) (400 µg) was reduced with TCEP (HCl salt, 2.5 equiv.) in ammonium acetate buffer (0.1 M, pH 5.8). F(ab')\(_2\) concentration was 1 mg/mL. The reaction was monitored by RP-HPLC-MS (orbitrap). After 2 h of incubation at 25 °C, the fragment Fab' was purified by FPLC (Äkta Pure 25 M chromatography system, GE Healthcare Life Sciences) by size exclusion chromatography on a Superdex\textsuperscript{TM} 75 10/300 GL column (cross-linked agarose and dextran, 13 µm, 10 × 300-310 mm, 24 mL), at 0.8 mL/min, with PB (20 mM, EDTA 5 mM, pH 7.3) as eluent. The fragment was then concentrated by ultrafiltration on an Amicon Ultra UltraCel-30 kDa (Merck Millipore) to obtain a solution of Fab' at 1.26 mg/mL\textsuperscript{*} in PB (20 mM, EDTA 5 mM, pH 7.3) (protein recovery = 68%, HPLC purity at 280 nm = 77%). The Fab' fragment is relatively stable at -18 °C but should be kept at a concentration <2 mg/mL in PB with EDTA (5 mM) to avoid spontaneous cysteine oxidation.

\textsuperscript{*} note: the concentration of the Fab' in mg/mL was calculated by UV spectrophotometry using the extinction coefficient of the Fab' at 280 nm (1.45 L.g\textsuperscript{-1}.cm\textsuperscript{-1}, value from ExPASy).
Figure S8. RP-HPLC elution profiles (detection at 280 nm) of the Fab' fragment before (top) and after (bottom) purification. The product and impurities are identified.

Figure S9. Deconvoluted ESI mass spectrum of the pertuzumab fragment Fab'.

Preparation of conjugate 2

2.5 μL of 5 mM stock solution of IR-783 in DMF (12.5 nmol, 25 equiv.) were added to a 1 mg/mL solution of Fab’ (25.0 μg, 0.5 nmol) in PB (20 mM, EDTA 5 mM, pH 7.3). The solution was stirred in a thermomixer (1000 rpm, 37 °C). The reaction was monitored by RP-HPLC-MS (orbitrap).
Figure S10. RP-HPLC elution profiles (TIC and dual UV-Vis detection at 280 nm and 650 nm) and deconvoluted ESI mass spectrum of the Fab' labelling reaction after 26 h.

Blocking of Fab' cysteine residues with iodoacetamide

30 µL of 100 mM stock solution of IAM in ultrapure water (3 µmol, 2000 eq) were added to a 0.83 mg/mL solution of Fab' (75.0 µg, 1.5 nmol) in PB (20 mM, EDTA 5 mM, pH 7.3). The resulting solution was stirred in a thermomixer 1000 rpm, at 37 °C and for 1 h. The blocked fragment Fab' was purified with FPLC (Äkta Pure 25 M chromatography system, GE Healthcare Life Sciences) by size-exclusion chromatography on an Hitrap desalting column (Sephadex G-25 Superfine resin, cross-linked dextran, bead size 15-70 µm, 16 × 25 mm, 5 mL) with PB 20 mM (pH 7.3) as eluent, at 2.5 mL/min. Concentration by ultrafiltration on an Amicon Ultra Ultracel-30 kDa gave a solution of IAM-blocked Fab' at 1.16 mg/mL* in PB 20 mM (pH 7.3) (protein recovery = 57%, HPLC purity at 280 nm = 67%).

*note: the concentration of the blocked Fab' in mg/mL was calculated by UV spectrophotometry using the extinction coefficient of the Fab' at 280 nm (1.45 L.g⁻¹.cm⁻¹, value from ExPASy).
Figure S11. RP-HPLC elution profiles (TIC and UV detection at 280 nm) and deconvoluted ESI mass spectrum of the blocked Fab’.

Control experiment on the blocked Fab’

2.5 µL of 5 mM stock solution of IR-783 in DMF (12.5 nmol, 25 equiv.) were added to a 1 mg/mL solution of Fab’ (25.0 µg, 0.5 nmol) in PB (20 mM, EDTA 5 mM, pH 7.3). The resulting solution was stirred in a thermomixer (1000 rpm, 37 °C). The reaction was monitored by RP-HPLC-MS (orbitrap).
Figure S12. RP-HPLC elution profiles (TIC and dual UV-Vis detection at 280 nm and 650 nm) and deconvoluted ESI mass spectrum of the blocked Fab’ after 22.5 h.

Monitoring of the progress of the labelling reaction

Figure S13. Evolution of the conversion for the labelling of Fab’ with free cysteines and with blocked cysteines. Conversion were calculated from peaks integration in the HPLC chromatograms at 280 nm.
Labeling of Fab’ with a commercially available DiSulfo-CY\textsubscript{avino}7 - Maleimide

1.5 µL of 5 mM stock solution of DiSulfo-CY\textsubscript{avino}7 - Maleimide derivative in DMF (7.5 nmol, 15 equiv.) were added to a 1 mg/mL solution of Fab’ (25.0 µg, 0.5 nmol) in PB (20 mM, EDTA 5 mM, pH 7.3). The resulting solution was stirred in a thermomixer (1000 rpm, 37 °C). The reaction was monitored by RP-HPLC-MS (orbitrap).

Figure S14. RP-HPLC elution profiles (TIC and dual UV-Vis detection at 280 nm and 650 nm) of the reaction mixture after 1 h.

Figure S15. Deconvoluted ESI mass spectrum of the unmodified Fab’ and of the reaction mixture after 1 h.
5. Labelling of BSA

Preparation of the conjugate

30.1 µL of a 5 mM stock solution of *IR-783* in DMF (150 nmol, 10.0 equiv.) was added to a 2 mg/mL solution of BSA (1 mg, 15 nmol) in PB (20 mM, pH 7.3). The solution was stirred in a thermomixer (800 rpm, 25 °C). The reaction was monitored by RP-HPLC-MS (orbitrap). After ca. 5 h, the product was purified by FPLC (Åkta Pure 25 M chromatography system, GE Healthcare Life Sciences) on an Hitrap desalting column (Sephadex G-25 Superfine resin, cross-linked dextran, bead size 15-70 µm, 16 × 25 mm, 5 mL) with PB (20 mM, pH 7.3) as eluent, at 2.5 mL/min, and then by size-exclusion separation on a Superdex™ 75 10/300 GL column (cross-linked agarose and dextran, 13 µm, 10 × 300-310 mm, 24 mL), at 0.8 mL/min, with PB (20 mM, pH 7.3) as eluent. The pure fluorescently labelled BSA was obtained as a 4.54 mg/mL* solution in PBS (protein recovery = 39%, HPLC purity at 280 nm > 90%).

*note:* the concentration of the BSA in mg/mL was calculated by UV spectrophotometry using the extinction coefficient of the BSA at 280 nm (0.65 L.g⁻¹.cm⁻¹, value from ExPASy). A correction factor (0.04) was used for taking account for the contribution of the *IR-783* absorbance at 280 nm.

![Figure S16. RP-HPLC elution profiles (TIC and dual UV-Vis detection at 280 nm and 650 nm) and deconvoluted ESI mass spectrum of the fluorescent BSA conjugate 3.](image-url)
**Figure S17.** UV-Vis absorption spectrum of the fluorescent BSA conjugate 3 recorded in PBS pH 7.4 at 25 °C. Data were recorded on a CLARIOstar microplate reader (BMG LABTECH) with an Lvis plate (BMG LVis MicroDrop, wavelength step width: 1.0 nm, with a concentration in the micromolar range). $\lambda_{\text{Abs, max}} = 795$ nm.

*In vitro* stability of the fluorescent BSA conjugate 3 in human plasma

9.71 µL of a stock solution of 3 at 2.06 mg/mL in PBS pH 7.4 (20 µg) were stirred in a theromixer (800 rpm, 37 °C, dark) with 20 µL of human plasma. The final concentration in 3 was 0.5 mg/mL. After 1, 2, 4, 8, 24 or 48 h 5 µL of the mixture were sampled and frozen. Then, each sample were analysed by SDS-PAGE (Mini-PROTEAN® TGX™ 4 to 20% Precast gel, Biorad), in non-reductive conditions. The samples were not denaturated at 90 °C prior to the electrophoresis to avoid unsuitable degradation.

**Figure S18.** SDS-PAGE analysis of human plasma (1), conjugate 3 without incubation in human plasma (2), conjugate 3 after incubation in human plasma during respectively 0, 1, 2, 4, 8, 24 and 48 h (3 to 9) after Coomassie blue staining (left panel) and by fluorescence (right panel) with an excitation at 740 nm and emission at 845 nm (bandwidth of 20 and 40 nm respectively).
6. In vivo evaluation of the fluorescent BSA conjugate 3

Mouse model

A syngeneic mouse model of colorectal cancer was used. BALB/c mice (8 weeks old, n = 9) were randomized into 2 groups with 1/ mice xenografted with CT26 murine tumor cells (ATCC, CRL-2639, n = 5) and 2/ non-injected mice (healthy mice (n = 4)). Briefly, 1x10^6 CT26 cells were resuspended in non-supplemented RPMI culture medium and injected subcutaneously (100µl, 27G needle) on the right flank of the mice. Tumor volume was monitored daily form D8 post-injection. Imaging was performed at D14 with tumor ranging from 600 to 1400 mm^3 (Figure S19).

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Figure S19. Table of the tumors volume evolution in mm^3; tumor implantation at J0.

Biodistribution study by NIR fluorescence imaging

Compound 3 (30 µg, 100 µl in PBS) was administered by intravenous injection in the mouse tail vein under anaesthesia (Isoflurane 2%). Whole animal imaging was performed at 1 h, 4 h and 24 h post-injection by acquisition of the fluorescence on IVIS Lumina III In Vivo Imaging System (PerkinElmer) in pair filter mode (760/845 nm). After the last imaging (24 h post-injection) mice were euthanized and organs (tumor, liver, kidneys, lungs, blood, muscle, urine, spleen, heart, intestine) were collected for ex vivo fluorescence imaging.
**Figure S20.** Images of the biodistribution of the fluorescent BSA conjugate 3 by the measurement of fluorescence (filter mode Ex./Em. 760/845 nm) in living mice at 1 h, 4 h and 24 h p.i.. Fluorescence signal, displayed as radiant efficiency, is superimposed to white light images. Tumor position is shown by a white arrow.

**Figure S21.** Fluorescence imaging of organs (tumor, liver, kidneys, lungs, blood, muscle, urine, spleen, heart, intestine) after euthanasia of the mice at 24 h p.i. (filter pair mode Ex./Em. 760/845 nm).
Figure S22. Biodistribution values expressed as average radiant efficiency ([p/s/cm²/sr] / [µW/cm²]) for each collected organ (Prism 7 software - GraphPad).
7. References
