

POLYFLUOROPHORE NANOREPORTERS FOR FLUOROGENIC IMAGING AND EARLY DIAGNOSIS

Field of Invention

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The current invention relates to polyfluorophore nanoporters and their application in fluorogenic imaging and early diagnosis.

Background

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15 Advances in nanotechnology have enabled extensive development of nanoparticle sensing agents with the impact on fundamental understanding of biology, close monitoring of therapeutic responses, and sensitive diagnosis of diseases. Due to the advantages of optical detection including high spatiotemporal resolution, high sensitivity and safe non-ionizing radiation, tremendous efforts have been devoted to development of optical nanoparticles for
20 sensing applications. With signal amplification and multiplexing capability, optical nanoparticles have found their unique position in *in vitro* diagnostics. Despite the excellent targeting ability, efficient accumulation and prolonged blood circulation time of nanoparticles for drug delivery, their *in vivo* sensing applications are mainly limited to preclinical settings, because their large sizes and optically active composition often result in slow clearance from
25 living body after systemic administration, raising long-term toxicity concerns. Moreover, the issue of light scattering and tissue reabsorption makes optical nanoparticles only effective for superficial imaging, further impeding them from clinical translation. Thus, unconventional sensing strategies are highly desired to invigorate *in vivo* detection capability of optical nanoparticles for early diagnosis.

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In vivo interconversion between small molecule and nanoparticle provides a precise way to exert their individual merits to compensate each other for advanced imaging and therapeutic applications. *In vivo* self-assembly of small-molecule fluorescence probes in response to disease biomarkers (such as pH, hydrolase, alkaline phosphatase, furin and caspase-3/7)
35 have proven to possess higher signal-to-background ratios than small molecule counterparts for cancer imaging. This is attributed to the combined pharmacokinetic advantages of both small molecules and nanoparticles: the small size of fluorescence probes ensures deep-tissue

penetration and broad biodistribution to major organs, while the biomarker-activated nanoparticle formation prolongs retention at disease site. Similarly, *in vivo* disassembly of nanoparticles into ultra-small nanoparticles or small-molecule fragments has shown to enhance the penetration of imaging components into the targeted tissues while facilitate the clearance in a shortened time frame. For instance, on-demand conversion of porphyrin microbubbles into nanoparticles bypassed the poor enhanced permeability and retention for microsized particles, leading to improved optical imaging of tumor. Moreover, in-situ cleavage and disassembly of dye-peptides conjugates from inorganic nanoparticles against biomarkers was applied for sensitive cancer diagnosis and enhanced targeting of therapeutics to tumor.

Despite the residence of residual inorganic nanoparticles at disease site, the cleaved dye-peptide conjugates could be renally cleared, providing a convenient way to detect cancer via fluorescence urine detection. Thus, these studies highlight the promise of molecule-nanoparticle interconversion to tackle the intrinsic challenges of optical nanoparticles for *in vivo* detection. However, synthetic approaches enabling complete in-situ conversion of nanoparticles into small molecules have yet to be reported for *in vivo* sensing.

Therefore, there exists a need to discover new nanoreporters for fluorogenic imaging and early diagnosis.

Drawings

FIG. 1 depicts the design of in-situ nanoparticle-to-molecule pharmacokinetic conversion sensing approach for real-time imaging and noninvasive longitudinal urinalysis of cancer and acute liver allograft rejection. (a) A schematic of early detection of cancer and acute liver allograft rejection via APNs-based optical urinalysis; (b) Molecular mechanisms of nanoparticle-to-molecule pharmacokinetic conversion and fluorescence turn-on of APNs for imaging and urinalysis; (c) Chemical structures of protease-reactive peptide brush (peptide sequence: Ac-FK and Ac-IEFD), renal clearance moiety (HP β CD) and targeting moiety (cRGD) for respective APNs; and (d) Chemical structures of APNs and their fluorogenic fragments CyCD or CyRGD in response to their respective proteases (cathepsin B (CatB) for APN_C, and granzyme B (GzmB) for APN_G) (R_1 = H or $(CH_3)_2NCO$, R_2 = HP β CD or cRGD, R_3 = H or $CH_2CHOHCH_3$).

FIG. 2 depicts *in vitro* sensing evaluation of APNs. (a,e) Fluorescence spectra of APNs (10 μ M) before and after incubation with their respective proteases (0.5 μ g, CatB or GzmB) at 37 °C. Fluorescence excitation at 650 nm. Inset: the corresponding fluorescence images acquired at 720 nm upon excitation at 675 nm. a.u., arbitrary units; (b,f) The near-infrared

fluorescence (NIRF) changes of APNs (10 μ M) at 720 nm after incubation with the indicated reactive oxygen species (90 μ M), enzyme and other analytes (90 μ M, excess) in PBS (10 mM, pH 7.4) at 37 °C. •OH, hydroxyl radical; H₂O₂, hydrogen peroxide; ONOO⁻, peroxynitrite; HClO, hypochlorous acid; GGT, gamma-glutamyl transferase; CE, carboxylesterases; AAP, alanine aminopeptidase; casp-3, caspase-3 (n = 3, mean $\pm$ s.d.); (c,g) Evaluation of enzyme-catalysed depolymerization of APNs through high-performance liquid chromatography (HPLC) analysis of APNs (10 μ M) after incubation with or without their respective proteases (0.5 μ g, CatB or GzmB) at 37 °C. HPLC traces of the pure compounds (CyCD, CyRGD, (4-aminophenyl)methanol (4-APM), 2,6-bis(hydroxymethyl)-p-cresol (BHMC), Ac-FK and Ac-IEFD) are also indicated for comparison; and (d,h) Representative transmission electron microscopy images of APNs before and after incubation with their respective proteases in buffer solution. The experiments in a, c–e, g and h were repeated independently three times with similar results.

FIG. 3 depicts *in vitro* characterization of APN_C and APN_G. (a&e) Average diameters of APN_C and APN_G determined by dynamic light scattering (DLS); (b&f) Average diameters of APN_C and APN_G as a function of time incubated with PBS and fetal bovine serum (FBS), respectively. Data are the mean $\pm$ SD. n=3 independent samples; (c&g) Absorption spectra of APN_C and APN_G (10 μ M) in the absence or presence of their respective proteases (0.5 μ g CatB and 0.5 μ g GzmB, respectively) in respective buffer; (d&h) Steady-state kinetics of the enzymatic reaction between APN_C (2.5, 5, 10, 20, 40 or 60 μ M) and CatB (0.5 μ g); and steady-state kinetics of the enzymatic reaction between APN_G (2.5, 5, 10, 20, 40 or 60 μ M) and GzmB (0.5 μ g). The initial velocity (v) was plotted against different concentrations of APN_C or APN_G. Data are the mean $\pm$ SD. n = 3 independent experiments; (i&j) near-infrared (NIR) fluorescence change fold of APN_C and APN_G in different pH buffer solution. Data are the mean $\pm$ SD. n = 3 independent experiments; (k&l) Fluorescence spectra of APNs (5 μ M) in FBS solution before and after incubation with their respective proteases (0.25 μ g) (CatB or GzmB) at 37 °C. Excitation at 650 nm; and (m) Zeta potentials of APN_C and APN_G in buffer with pH 7.4 and 6.8. Data are the mean $\pm$ SD. n = 3 independent experiments.

FIG. 4 depicts (a&d) cell viability of CT26 cells, LM3 cells and AML-12 cells after incubation with PBS, APN_C and APN_G at different concentrations for 24 h. Data are the mean $\pm$ SD. n = 3 independent experiments; (b) *in vitro* NIRF imaging of APN_C in CT26 cells or LM3 cells in the absence and presence of inhibitor CA-074. Blue fluorescence indicates cell nucleus stained with 4,6-diamidino-2-phenylindole (DAPI) and red fluorescence indicates the signals from APN_C; (c) mean near infra-red (NIR) fluorescence intensity in the panel b. Data are the mean $\pm$ SD. n = 3 independent cell experiments; (e) *in vitro* NIRF imaging of APN_G in CD8⁺ T

cells, RAW264.7 cells and AML-12 cells. Blue fluorescence indicates cell nucleus stained with DAPI and red fluorescence indicates the signals from APN_G; and (f) mean NIR fluorescence intensity in the panel (e). Data are the mean $\pm$ SD. $n = 3$ independent cell experiments. APN_C incubated CT26 cells (LM3 cells) showed a 26 (33)- or 6 (6)-fold higher NIRF intensity than that of PBS or CatB inhibitor (CA-074) treated CT26 cells (LM3 cells), respectively. Similarly, intense signal was observed in CD8⁺ T cells after treatment of APN_G, 6.5-fold higher than that of 4T1 cells and RAW264.7 cells. Thus, these results confirmed the biomarker specificity of APN_C and APN_G.

FIG. 5 depicts the biodistribution and clearance pathway of APNs and their activated fragments. (a) Schematic illustration of renal and hepatobiliary clearance pathways in living mice; (b) Chemical structures of CyCD (R = H or CH₂CHOHCH₃) and CyRGD and schematic illustration of APNs; (c) NIRF intensity of kidneys and liver from mice $t = 1$ h after injection of CyCD, CyRGD, APN_C or APN_G ($n = 3$, mean $\pm$ s.d.); (d) NIRF images of the abdominal cavity of mice at $t = 1$ h after injection of CyCD, CyRGD (2.5 $\mu\text{mol kg}^{-1}$ body weight), APN_C or APN_G (10 $\mu\text{mol kg}^{-1}$ body weight). Bladder (Bl), gallbladder (Gb), kidneys (Ki), liver (Li), muscle (Mu), spleen (Sp). NIRF images acquired at 720 nm upon excitation at 675 nm; (e) Accumulation of CyCD, CyRGD, APN_C or APN_G in liver at 1, 6 and 24 h post-injection ($n = 3$, mean $\pm$ s.d.); (f) Blood concentration (% ID g⁻¹, ID: injected doses) decay of CyCD, CyRGD, APN_C and APN_G after injection into living mice ($n = 3$, mean $\pm$ s.d.); and (g) The total renal clearance efficiencies and fecal excretion efficiencies of CyCD, CyRGD, APN_C and APN_G after 15 days injection ($n = 3$, mean $\pm$ s.d.).

FIG. 6 depicts (a&c) absorption (dark) and fluorescence (red) spectra of CyCD and CyRGD (30 μM) in PBS buffer (10 mM, pH 7.4) at 37 °C; (b&d) renal clearance efficiency as a function of time post-injection of CyCD and CyRGD (2.5 $\mu\text{mol kg}^{-1}$ body weight) in living mice, three lines represent the measurements in three independent mice; and (e) linear relationship between the fluorescence intensity of urine at 720 nm and injected dose of CyCD ($\mu\text{mol per mouse}$). Fluorescence excitation at 675 nm. The limit of detection (LOD) was estimated to be as low as 4 nM (S/N = 3). Data are the mean $\pm$ SD. $n = 3$ independent experiments.

FIG. 7 depicts the clearance studies for APN_C and APN_G. (a) A schematic illustration of the workflows for measurements of the clearance efficiencies and residual nanoreporters in healthy mice. Urine samples were collected at intervals of 3 days and faeces samples were collected at intervals of 6 days after i.v. injection of APN_C or APN_G (10 $\mu\text{mol kg}^{-1}$ body weight). Major organs were harvested at 15 days post-injection of APN_C or APN_G and were homogenized for extraction and quantification of residual nanoreporters. (b) NIRF imaging and

HPLC quantification of APN_C or APN_G in urine samples collected at each time points after i.v. injection. (c) NIRF imaging and HPLC quantification of APN_C or APN_G from faeces samples collected at each time points after i.v. injection. The corresponding fluorescence images acquired at 720 nm upon excitation at 675 nm with the IVIS spectrumCT system. (d)

5 Fluorescence quantification of residual nanoreporters in major organs from mice 15 days post-injection of PBS, APN_C or APN_G. Fluorescence intensities (720 nm) were recorded on a fluorescence spectrophotometer after homogenization of major organs in PBS buffer (10 mM, pH 7.4), centrifugation to remove insoluble components and then incubation with respective proteases. Fluorescence excitation at 675 nm. Data are the mean $\pm$ SD. n = 3 independent

10 mice. Residual nanoreporters in major organs from mice at 15 days post-injection of APN_C or APN_G had fluorescence intensities as low as those from the saline-injected mice, which further confirmed the complete excretion of APN_C or APN_G in mice after 15 days.

FIG. 8 depicts (a-c) representative NIRF images of living mice and resected organs at 1 h after

15 i.v injection of PBS (0.2 mL), CyCD (2.5 μ mol kg⁻¹ body weight) and CyRGD (2.5 μ mol kg⁻¹ body weight) respectively. The circles indicate the kidney; and (d) *ex vivo* NIRF quantification of major organs of mice at t = 1 h post-injection of PBS, CyCD or CyRGD. Data are the mean $\pm$ SD. n = 3 independent mice.

20 **FIG. 9** depicts *in vivo* biocompatibility studies using histological analysis. Haematoxylin and eosin (H&E) staining of major organs including heart, liver, spleen, lung, and kidney from mice after 14 days i.v injection of CyCD (2.5 μ mol kg⁻¹ body weight), CyRGD (2.5 μ mol kg⁻¹ body weight), APN_C and APN_G (10 μ mol kg⁻¹ body weight). (Scale bar: 200 μ m).

25 **FIG. 10** depicts *in vivo* stability studies of CyCD and CyRGD using optical characterization and MALDI-TOF mass spectrometry. Absorption and fluorescence spectra of the urine samples from living mice after i.v injection of (a) PBS, (b) CyCD, and (c) CyRGD. *In vivo* stability studies of CyCD and CyRGD through MALDI-TOF mass analysis of the urine samples after i.v injection; and (d-e) MALDI-TOF mass analysis of the pure compounds (CyCD and

30 CyRGD) in PBS are also indicated for comparison. Excreted CyCD and CyRGD in urine had the identical mass range compared to that of the pure compounds in PBS. Note that the MALDI-TOF mass spectra of CyCD and CyRGD were performed in the reflector mode.

FIG. 11 depicts real-time imaging and urinalysis of orthotopic liver cancer. (a) Schematic

35 illustration of mechanisms of APN_C for imaging and urinalysis of orthotopic liver tumour in living mice; (b) Representative NIRF images of living mice after injection of APN_C or APN_{CN} 15 days after subcutaneous tumour implantation; (c) Representative NIRF images of living mice after

injection of APN_C at different post-tumour-implantation times (0, 3 or 14 days). The circles indicate the (T) tumour, (L) liver and (K) kidney. NIRF images acquired at 720 nm upon excitation at 675 nm; (d) NIRF intensities of liver and kidney in living mice at t = 6 h injection of APN_C for different groups (n = 4, mean ± s.d.). Two-tailed Student's t-test; PBS versus tumour-implantation groups; (e) NIRF intensities of activated APN_C in the urine from living mice after injection of APN_C for different groups (n = 4, mean ± s.d.). Two-tailed Student's t-test; PBS versus tumour-implantation groups. NS, no statistically significant differences. Inset: the corresponding fluorescence images of urine samples acquired at 720 nm upon excitation at 675 nm; (f,g) The dynamic NIRF intensities of (f) liver, and (g) kidneys as a function of time after injection of APN_C in living mice (n = 4, mean ± s.d.). Two-tailed Student's t-test; PBS versus tumour-implantation groups. NS, no statistically significant differences; (h,i) Measurements of (h) liver function, and (i) kidney function in the mouse model of orthotopic liver cancer. Levels of Alanine Aminotranferase (ALT), Aspartate Aminotransferase (AST), serum creatinine (Cr) and blood urea nitrogen (BUN) from mice at different post-tumour-implantation times (n = 4, mean ± s.d.). Two-tailed Student's t-test; PBS versus tumour-implantation groups. NS, no statistically significant differences; (j) Linear regression analysis for studying correlation between percentage of activated APN_C in urine and tumour size (r² = 0.90; P < 0.0001).

FIG. 12 depicts *in vitro* sensing evaluation of APN_{CN}. (a&b) Absorption and fluorescence spectra of APN_{CN} (10 μM) before and after incubation with CatB (0.5 μg) at 37 °C. Fluorescence excitation at 650 nm. Inset: the corresponding fluorescence images acquired at 720 nm upon excitation at 675 nm; (c) The NIRF changes of APN_{CN} (10 μM) at 720 nm after incubation with indicated ROS (90 μM), enzymes, and other analytes (90 μM, excessive) in PBS buffer (10 mM, pH 7.4) at 37 °C. •OH, H₂O₂, ONOO⁻, HClO, GGT, CE, AAP, casp-3. (n=3 independent experiments, mean ± s.d.); (d) Steady-state kinetics of the enzymatic reaction between APN_{CN} (5, 10, 20, 40 or 60 μM) and CatB (0.5 μg); The initial velocity (v) was plotted against different concentrations of APN_{CN}. Data are the mean ± SD. n = 3 independent experiments; (e) Average diameters of APN_{CN} determined by DLS; (f) Representative transmission electron microscopy (TEM) images of APN_{CN} before and after incubation with catB in buffer solution; (g) The total renal clearance efficiencies and faecal excretion efficiencies of APN_{CN} after 15 days injection (n = 3 independent mice, mean ± s.d.); (h) Blood concentration (% ID g⁻¹) decay of APN_{CN} after injection into living mice (n = 3 independent mice, mean ± s.d.); and (i) H&E staining of major organs including heart, liver, spleen, lung, and kidney from mice after 14 days i.v injection of APN_{CN} (10 μmol kg⁻¹ body weight). (Scale bar: 200 μm). The experiments in a, b, f and i were repeated independently three times with similar results.

- FIG. 13** depicts real-time *in vivo* NIRF imaging of cancer using APN_C and APN_{CN} in living mice. (a) Schematic illustration of development of CT26 tumor-bearing mice and NIRF imaging at different post-injection timepoints after i.v. injection of APN_C or APN_{CN} (10 $\mu\text{mol kg}^{-1}$ body weight). CT26 cells was subcutaneously implanted into living mice. The control groups were implanted with saline (0.1 mL) or tumor-bearing mice treated with CatB inhibitor prior to APN_C or APN_{CN} administration. Real-time NIRF imaging was conducted at indicated timepoints after i.v injection of APN_C or APN_{CN}. (b) NIRF intensities of activated APN_C or APN_{CN} in the urine from living mice after injection of APN_C or APN_{CN} for different groups ($n = 4$, mean $\pm$ s.d.). Two-tailed Student's t-test; NS: no statistically significant differences. Inset: the corresponding fluorescence images of urine samples acquired at 720 nm upon excitation at 675 nm. (c) Representative NIRF images of living mice at different post-injection timepoints after injection of APN_C or APN_{CN}. APN_C or APN_{CN} had the highest signals at 6 h post-injection. The top and bottom circles indicate the kidney and tumor, respectively. NIRF images acquired at 720 nm upon excitation at 675 nm with the IVIS spectrumCT system. The non-inhibitor treated mice in APN_C group were imaged once removal and cleanup of hair with water. The dynamic NIRF intensities of (d) tumor, and (e) kidneys as a function of time post-injection of APN_C or APN_{CN} in living mice ($n = 4$ independent mice, mean $\pm$ s.d.).
- FIG. 14** depicts *ex vivo* NIRF imaging and urinalysis in tumor-bearing mice. (a) Representative NIRF images of the abdominal cavity of mice at $t = 6$ h i.v injection of APN_C or APN_{CN} (10 $\mu\text{mol kg}^{-1}$ body weight) after 15 days tumor implantation. The control groups were implanted with saline (0.1 mL) or tumor-bearing mice treated with CatB inhibitor prior to APN_C or APN_{CN} administration. Liver (Li), muscle (Mu), spleen (Sp), kidneys (Ki), bladder (Bl). (b&c) Signal quantification and *ex vivo* NIRF images of resected organs from mice at $t = 6$ h i.v injection of APN_C or APN_{CN} after 15 days tumor implantation. The liver from non-inhibitor treated mice showed higher fluorescence intensity than that of the control mice due to activation of APN_C at the tumor site to release CyRGD that cleared via the hepatobiliary clearance pathway, which was consistent with the previous imaging results in FIG. 8c. The NIRF images acquired at 720 nm upon excitation at 675 nm with the IVIS spectrumCT. Data are the mean $\pm$ SD. $n = 4$ independent mice; (d&e) Absorption and fluorescence spectra of excreted CyCD and CyRGD in the urine samples from tumor-bearing mice after injection of APN_C; and (f) MALDI-TOF mass analysis of CyCD and CyRGD in the urine samples from tumor-bearing mice after injection of APN_C. MALDI-TOF mass analysis of the pure compounds APN_C in PBS are also indicated for comparison. The mass spectra confirmed that APN_C was activated in tumor to release CyCD and CyRGD into urine. Note that the MALDI-TOF mass spectra of CyCD and CyRGD were performed in the reflector mode; the APN_C spectra were collected in linear mode

because it cannot be obtained using the reflector mode. The experiments in d-f were repeated independently three times with similar results.

FIG. 15 depicts real-time *in vivo* NIRF imaging of cancer in living mice. (a) Representative
 5 NIRF images of living mice after injection of APN_C at different post tumor implantation
 timepoints (2, 5, 10 or 15 days). The white circles indicate the tumor (T) and kidney (K). NIRF
 images acquired at 720 nm upon excitation at 675 nm with the IVIS spectrum imaging system;
 The dynamic NIRF intensities of (b) tumor, and (c) kidneys as a function of time post-injection
 of APN_C in living mice (n = 4, mean ± s.d.). For panel b, two-tailed Student's t-test; inhibitor-
 10 treated group versus non-inhibitor-treated groups. For panel c, twotailed Student's t-test; PBS
 versus tumor-implantation groups; (d) NIRF intensity of activated APN_C in the urine from living
 mice after injection of APN_C for different groups (n = 4, mean ± s.d.). Two-tailed Student's t-
 test; PBS versus tumor-implantation groups. NS: no statistically significant differences. Inset:
 the corresponding fluorescence images of urine samples acquired at 720 nm upon excitation
 15 at 675 nm with the IVIS spectrum imaging system; (e) Tumor volumes and percentages of
 activated APN_C in urine from living mice for different groups. Data are the mean ± SD. n = 4
 independent mice; and (f) Linear regression analysis for studying correlation between
 percentage of activated APN_C in urine and tumor size ($r^2 = 0.90$; $p < 0.0001$).

FIG. 16 depicts *ex vivo* NIRF imaging of tumor-bearing mice at different tumor implantation
 20 timepoints. (a) Schematic illustration of development of CT26 tumor-bearing mice and NIRF
 imaging at different tumor implantation timepoints. Real-time NIRF imaging was conducted at
 2-, 5-, 10-days post tumor implantation time after i.v injection of APN_C (10 $\mu\text{mol kg}^{-1}$ body
 weight); (b) Representative NIRF images of the abdominal cavity of mice at t = 6 h i.v injection
 25 of APN_C (10 $\mu\text{mol kg}^{-1}$ body weight) after 2-, 5-, or 10-days tumor implantation. Liver (Li),
 muscle (Mu), spleen (Sp), kidneys (Ki), bladder (Bl); (c-d) Signal quantification and *ex vivo*
 NIRF images of resected organs from mice at t = 6 h i.v injection of APN_C after 2-, 5-, or 10-
 days tumor implantation. The NIRF images acquired at 720 nm upon excitation at 675 nm with
 the IVIS spectrumCT system. Data are the mean ± SD. n = 4 independent mice. An increased
 30 NIRF signal was observed in both the liver and kidney with the tumor implantation time due to
 the increased hepatobiliary clearance of CyRGD and renal clearance of CyCD upon activation
 of APN_C in tumor; and (e) Volumes of urine samples collected from each mouse used for
 urinalysis experiments. NS: no statistically significant differences. Data are the mean ± SD. n
 = 4 independent mice.

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FIG. 17 depicts real-time *in vivo* NIRF imaging of orthotopic liver cancer in living mice. (a)
 Schematic illustration of development of HCC LM-3 tumor-bearing mice and NIRF imaging at

different post-injection timepoints after i.v. injection of APN_C (10 μ mol kg⁻¹ body weight). LM3 cells were orthotopically implanted into living mice. The control groups were implanted with PBS (0.1 mL) or tumorbearing mice treated with CatB inhibitor prior to APN_C administration. Real-time NIRF imaging was conducted at indicated timepoints after i.v injection of APN_C; (b)

5 Representative NIRF images of living mice at different post-injection timepoints after injection of APN_C. APN_C had the highest signals at 6 h post injection. The top and bottom circles indicate the kidney and tumor, respectively. NIRF images acquired at 720 nm upon excitation at 675 nm with the IVIS Lumina III system; and (c) Growth curves of tumors at different timepoints post tumor implantation (Data are the mean $\pm$ SD. n = 4 independent mice).

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FIG. 18 depicts real-time imaging and urinalysis of cancer. (a) Schematic illustration of mechanisms of APN_C for imaging and urinalysis of tumors in living mice; (b) Representative NIRF images of living mice after injection of APN_C (10 μ mol kg⁻¹ body weight) at 15 days post tumor implantation. The control group implanted with PBS or tumor-bearing mice treated with

15 a CatB inhibitor (CA-074) intratumorally twice for 2 days prior to APN_C administration; (c) Representative NIRF images of living mice after injection of APN_C at different post tumor implantation timepoints (2, 5 or 10 days). The white circles indicate the tumor and kidney. NIRF images acquired at 720 nm upon excitation at 675 nm; (d) NIRF intensities of tumor and kidney in living mice at t = 6 h injection of APN_C for different groups (n = 4, mean $\pm$ s.d.). The

20 dynamic NIRF intensities of (e) tumor, and (f) kidneys as a function of time post-injection of APN_C in living mice (n=4, mean $\pm$ s.d.); (g) NIRF intensities of activated APN_C in the urine from living mice after injection of APN_C for different groups (n = 4, mean $\pm$ s.d.). Statistically significant differences in the NIRF intensities between control and tumor implantation groups (*p<0.05, **p<0.01, ***p<0.001). n.s: no statistically significant differences. Inset: the

25 corresponding fluorescence images of urine samples acquired at 720 nm upon excitation at 675 nm. (h) Tumor volumes and percentages of activated APN_C in urine from living mice for different groups; and (i) Linear regression analysis for studying correlation between percentage of activated APN_C in urine and tumor size ($r^2 = 0.90$; $p < 0.0001$).

30 **FIG. 19** depicts *ex vivo* NIRF imaging and histological studies in orthotopic liver tumor mice. (a&b) *Ex vivo* NIRF images and signal quantification of resected organs from mice at t = 6 h i.v injection of APN_C at different post tumor implantation timepoints (3, 8 or 14 days). The white arrows indicate the tumor. The NIRF images acquired at 720 nm upon excitation at 675 nm with the IVIS Lumina III system. Data are the mean $\pm$ SD. n = 4 independent mice; and (c)

35 Photomicrographs of H&E staining in paraffinembedded major organs sections from control mice and liver tumor implanted mice (14 days). Histological studies showed no histological change in heart, spleen, lung and kidney, but tumor was observed in the liver, tumors were

indicated by the blue dashed curve (Scale bar: 200 μ m). The experiments in c were repeated independently three times with similar results.

FIG. 20 depicts real-time imaging and longitudinal urinalysis of acute immune-mediated hepatitis. (a) Schematic illustration of APN_G sensing mechanism in concanavalin A (Con-A)-induced acute immune-mediated hepatitis; (b) Representative NIRF images of living mice and urine samples with injection of APN_G (10 μ mol per kg body weight) after treatment with Con-A (12.5 mg per kg body weight) for 2, 5, 8 or 12 h. The control groups were treated with PBS or cyclosporine A (CsA, 10 or 50 mg kg⁻¹, termed low dose (LD) and high dose (HD), respectively). SCID mice were treated with Con-A and Balb/c mice were treated with lipopolysaccharide (LPS) intradermally (0.2 mg kg⁻¹). NIRF images were acquired at 720 nm upon excitation at 675 nm; (c,d) NIRF intensities of (c) liver, and (d) kidney in living mice at t = 1 h after intravenous injection of APN_G for different groups (n = 3, mean $\pm$ s.d.). Two-tailed Student's t-test; PBS versus Con-A- or LPS-treated groups. NS, no statistically significant differences; (e) Representative flow cytometry plots of the GzmB level of lymphocytes in the liver from mice at different post-treatment times; (f) Fluorescence enhancement in the urine from living mice after injection of APN_G at different treatment times (n = 3, mean $\pm$ s.d.); enhancement of the GzmB level as a percentage of CD44⁺ leucocytes in the liver from mice at different treatment times (n = 6, mean $\pm$ s.d.). Dashed frames indicate the first statistically significant times; (g) Enhancement of ALT, AST and cytokines at different times after treatment with Con-A (n = 6, mean $\pm$ s.d.). Dashed frames indicate the first statistically significant times. Two-tailed Student's t-test; PBS versus Con-A- or LPS-treated groups.

FIG. 21 depicts real-time *in vivo* NIRF imaging of immune-mediated hepatitis in living mice. (a) Schematic illustration of development of immune-mediated hepatitis mouse model and imaging at different post-treatment timepoints. Con-A was intravenously administered into living mice at 12.5 mg kg⁻¹ followed by i.v injection of APN_G (10 μ mol kg⁻¹ body weight) at different timepoints post-treatment of Con-A (1, 4, 7 or 11 h). The control groups were treated with PBS or CsA (10 or 50 mg kg⁻¹). SCID mice were treated with Con-A and Blab/c mice were treated with LPS intradermally. Real-time NIRF imaging was conducted at indicated timepoints after i.v injection of APN_G; and (b-c) Representative NIRF images of living mice after i.v injection of APN_G at different timepoints post-treatment of Con-A.

FIG. 22 depicts (a) representative photomicrographs of H&E staining in paraffin embedded liver and kidney sections from mice after various treatment. Histological studies showed no histological change in the kidneys after Con-A or LPS challenge. However, massive hepatic necrosis of the livers was only observed 12 h after Con-A treatment (Scale bar: 200 μ m). The

experiments were repeated independently three times with similar results; (b) confocal fluorescence microscopy images of liver slices from mice after various treatment. The blue and green signals come from DAPI and GzmB antibody staining, respectively; and the red channel indicates the signal from activated APN_G (Scale bar: 20 μ m). The experiments were repeated independently three times with similar results; and (c) mean NIRF intensity of activated APN_G in the panel b. Data are the mean $\pm$ SD. n = 3 independent experiments. Two-tailed Student's t-test; PBS versus Con-A or LPS-treated groups. NS: no statistically significant differences.

FIG. 23 depicts quantification of NIRF signals of APN_G in the liver, kidney, and bladder. (a-c) The dynamic NIRF intensities of liver, kidney and bladder as a function of time post-injection of APN_G in living mice at different timepoints post-treatment of Con-A (1, 4, 7 or 11 h). The control groups were treated with PBS or CsA. SCID mice were treated with Con-A and Blab/c mice were treated with LPS intradermally. Data are the mean $\pm$ SD. n = 3 independent mice.

FIG. 24 depicts *ex vivo* NIRF signal analysis of APN_G in the mouse model of immune-mediated hepatitis. (a) Representative NIRF images of the abdominal cavity of mice after 1 h i.v injection of APN_G (10 μ mol kg⁻¹ body weight) at different timepoints post-treatment of Con-A (1, 4, 7 or 11 h). The control groups were treated with PBS or CsA. SCID mice were treated with Con-A and Blab/c mice were treated with LPS intradermally. Liver (Li), muscle (Mu), spleen (Sp), kidneys (Ki), bladder (Bl). The experiments were repeated independently three times with similar results; and (b-c) *Ex vivo* NIRF images and signal quantification of resected organs from mice with i.v injection of APN_G after treatment of Con-A. The NIRF images acquired at 720 nm upon excitation at 675 nm with the IVIS spectrumCT system. Data are the mean $\pm$ SD. n = 3 independent mice.

FIG. 25 depicts the urinalysis of activated APN_G in the mouse model of immune-mediated hepatitis. (a) Percentage of activated APN_G in urine from living mice for different groups. Urine was collected for 12 h after injection of APN_G. Data are the mean $\pm$ SD. n = 3 independent mice. Two-tailed student's t-test. PBS versus treated groups, NS: no statistically significant differences; (b-c) Absorption and fluorescence spectra of excreted CyCD in the urine samples from living mice with i.v injection of APN_G; (d) MALDI-TOF mass analysis of CyCD in the urine samples after i.v injection of APN_G. MALDI-TOF mass analysis of the pure compounds APN_G in PBS are also indicated for comparison. The mass spectra confirmed that APN_G was activated in the liver to release CyCD into urine. Note that the MALDI-TOF mass spectra of CyCD were performed in the reflector mode; the APN_G spectra were collected in linear mode because it cannot be obtained using the reflector mode; and (e) Volumes of urine samples

collected from each mouse used for urinalysis experiments. Data are the mean $\pm$ SD. $n = 3$ independent mice. Two-tailed Student's t-test; PBS versus Con-A or LPS-treated groups. NS: no statistically significant differences.

- 5 **FIG. 26** depicts the flow cytometric analysis of GzmB expression in leukocytes after Con-A treatment. Quantification plots showing populations of GzmB⁺CD44⁺ cells among leukocytes in the (a) liver, (b) spleen, (c) lymph node, and (d) blood from mice after various treatments ($n = 6$, mean $\pm$ s.d.). Two-tailed Student's t-test; PBS versus Con-A-treated groups. NS: no statistically significant differences; and (e) Gating strategy for flow cytometry analysis of
- 10 GzmB⁺CD44⁺ leukocytes.

FIG. 27 depicts the representative flow cytometry plots of GzmB⁺CD44⁺ leukocytes in the liver, spleen, lymph node and blood from mice after various treatments.

- 15 **FIG. 28** depicts the measurements of liver function and cytokine levels in the mouse model of immune-mediated hepatitis. (a) The levels of ALT and AST from mice after various treatments. Two-tailed Student's t-test; healthy group versus grafted or LPS treated groups. NS: no statistically significant differences; and (b) Cytokine levels of tumor necrosis factor α (TNF- α), interferon γ (IFN- γ), Interleukin-2 (IL-2) and Interleukin-6 (IL-6) in serum after various
- 20 treatments ($n = 6$, mean $\pm$ s.d.). Two-tailed Student's t-test; healthy group versus grafted or LPS treated groups. NS: no statistically significant differences.

- FIG. 29** depicts the longitudinal optical urinalysis of acute liver allograft rejection in living rats. (a) Schematic illustration of the surgical procedure of liver transplantation, mechanisms of liver
- 25 allograft rejection, and activation of APN_G in grafted liver for optical urinalysis in living rats. After surgery, dendritic cells (DC) migrate from the graft to recipient lymphoid tissue, where they present alloantigen on major histocompatibility complex class I and II molecules and interact with naive alloreactive T cells, resulting in proliferation of alloreactive cytotoxic T cells (CTL). Such effector cells are recruited to the liver and attack donor cells, resulting in the
- 30 clinical manifestations of T-cell-mediated rejection. LSEC, liver sinusoidal endothelial cells; (b) NIRF intensities of excreted CyCD in the urine from living rats 8 h after injection of APN_G (10 μ mol per kg body weight) for different groups ($n = 4$, mean $\pm$ s.d.). Two-tailed Student's t-test; healthy group versus grafted or LPS-treated groups. Inset: the corresponding fluorescence images of urine samples acquired at 720 nm upon excitation at 675 nm; (c,d) *Ex vivo* NIRF
- 35 signal quantification of resected (c) liver and (d) kidneys from rats 8 h after injection of APN_G at different post-transplantation times ($n = 4$, mean $\pm$ s.d.). Two-tailed Student's t-test; healthy group versus grafted or LPS-treated groups. NS, no statistically significant differences; (e)

receiver operating characteristic (ROC) analysis of the diagnostic specificity and sensitivity for urinalysis, flow cytometry, ALT, AST and Alkaline Phosphatase (ALP) in differentiating between healthy rats (n = 12) and allografts (n = 8) 6 days after transplantation; (f) ROC analysis of the diagnostic specificity and sensitivity for cytokines including TNF- α , IFN- γ , IL-2, Interleukin-4 (IL-4) and IL-6 in differentiating between healthy rats (n = 21) and allografts (n = 14) 6 days after transplantation; (g) Detection timeline and area under the curve (AUC) comparing urinalysis with the clinical methods (ALT, AST and ALP) and cytokines for detection of liver allograft rejection; and (h) Representative confocal fluorescence microscopy images of regional liver slices and photomicrographs of haematoxylin and eosin staining in paraffin-embedded liver sections from rats 3 and 6 days after transplantation or isograft rats. The blue, green and red signals originate from 4,6-diamidino-2-phenylindole (DAPI), GzmB antibody staining and APN_G, respectively (scale bar, 200 μ m). The triangles and arrowheads indicate GzmB antibody and APN_G staining, respectively. The experiments in h were repeated independently three times with similar results.

15

FIG. 30 depicts the development of a rat model of acute liver allograft rejection and measurements of liver function and kidney function. (a) Schematic illustration of development of a rat model of acute liver allograft rejection. Orthotopic liver transplantation was performed by using Dark Agouti (DA) and Lewis rats as donors and recipients, respectively. The control groups were healthy rats or treated with an immunosuppressive drug Tacrolimus (Tac) after surgery. Iso-graft, sham-operation and intradermally injection of LPS were also conducted for comparison; (b) The levels of ALT, AST and ALP from rats after liver transplantation (n = 4, mean $\pm$ s.d.); and (c) The levels of serum creatinine, BUN and uric acid from rats after liver transplantation (n = 4, mean $\pm$ s.d.). Two-tailed student's t-test. Healthy versus transplantation groups. NS: no statistically significant differences.

25

FIG. 31 depicts (a) representative confocal fluorescence microscopy images of regional liver slices, and (b) photomicrographs of H&E staining in paraffin-embedded kidney and liver sections from rats after liver transplantation. Histological studies showed no histological change in the kidneys after liver transplantation, but dilated sinusoids and hepatocyte debris of the liver was only observed at 6 days post allogeneic transplantation (Scale bar: 200 μ m). The experiments in a and b were repeated independently three times with similar results; and (c) Mean fluorescence intensity of anti-GzmB and activated APN_G in the panel a. Data are the mean $\pm$ SD. n = 3 independent experiments. Two-tailed Student's t-test; healthy group versus grafted or LPS treated groups. NS: no statistically significant differences.

35

FIG. 32 depicts the clearance efficiency, biodistribution and biocompatibility of CyCD and APN_G in healthy rats. (a) Renal clearance efficiency and fecal excretion efficiency of CyCD and APN_G in healthy rats after 15 days i.v. injection ($n = 3$, mean $\pm$ s.d.); (b) Accumulative APN_G in the liver as a function time of post-injection of APN_G ($n = 3$, mean $\pm$ s.d.); (c) *Ex vivo* NIRF quantification of resected organs of rats at $t = 6$ h post-injection of CyCD and APN_G. Data are the mean $\pm$ SD. $n = 3$ independent mice; (d) Representative NIRF images of resected organs from rats after 6 h i.v injection of CyCD ($2.5 \mu\text{mol kg}^{-1}$ body weight) and APN_G ($10 \mu\text{mol kg}^{-1}$ body weight); and (e) H&E staining of major organs including heart, liver, spleen, lung, and kidney from rats after 14 days i.v injection of CyCD or APN_G (Scale bar: $200 \mu\text{m}$). The experiments in e were repeated independently three times with similar results.

FIG. 33 depicts the *ex vivo* NIRF imaging after liver transplantation. Representative *ex vivo* NIRF images of resected organs from different rats groups. The NIRF images acquired at 720 nm upon excitation at 675 nm with the IVIS Lumina III system.

FIG. 34 depicts (a) percentage of activated APN_C in urine from living rats for different groups. Data are the mean $\pm$ SD. $n = 4$ independent mice. Two-tailed student's t-test. Healthy versus transplantation groups, NS: no statistically significant differences; and (b-c) absorption and fluorescence spectra of excreted CyCD in the urine samples from living rats with injection of APN_G.

FIG. 35 depicts flow cytometric analysis of GzmB expression in CD8⁺ T cells after liver transplantation. Quantification plots showing populations of GzmB⁺ cells among CD8⁺ T cells in (a) liver, (b) spleen, (c) lymph node, and (d) blood from rats ($n = 4$, mean $\pm$ s.d.). Two-tailed student's t-test. Healthy versus transplantation groups, NS: no statistically significant differences; and (e) Gating strategy for flow cytometry analysis of GzmB expression in CD8⁺ T cells in the liver.

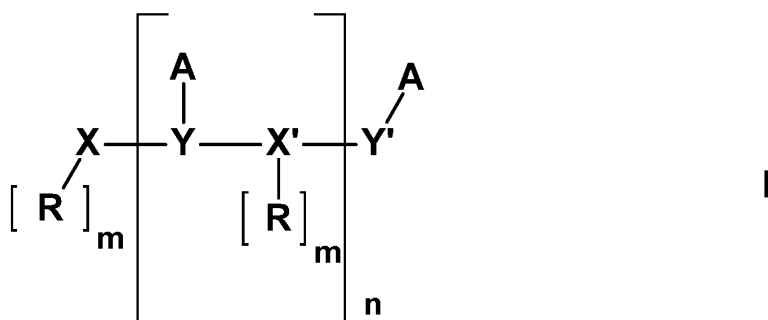
FIG. 36 depicts the representative flow cytometry plots of GzmB expression in CD8⁺ T cells in the liver, spleen, lymph node and blood from rats after liver transplantation. The experiments were repeated independently four times with similar results.

FIG. 37 depicts the measurements of cytokines levels after transplantation. Cytokines levels of (a) TNF- α , (b) IFN- γ , (c) IL-2, (d) IL-4, and (e) IL-6 in serum after liver transplantation ($n = 7$, mean $\pm$ s.d.). Two-tailed student's t-test. Healthy versus transplantation groups. NS: no statistically significant differences.

Description

It has been surprisingly found that molecules that overcome some or all of the problems identified above may be manufactured. Thus, in a first aspect of the invention, there is provided

- 5 a polymeric nanoreporter compound according to formula I:

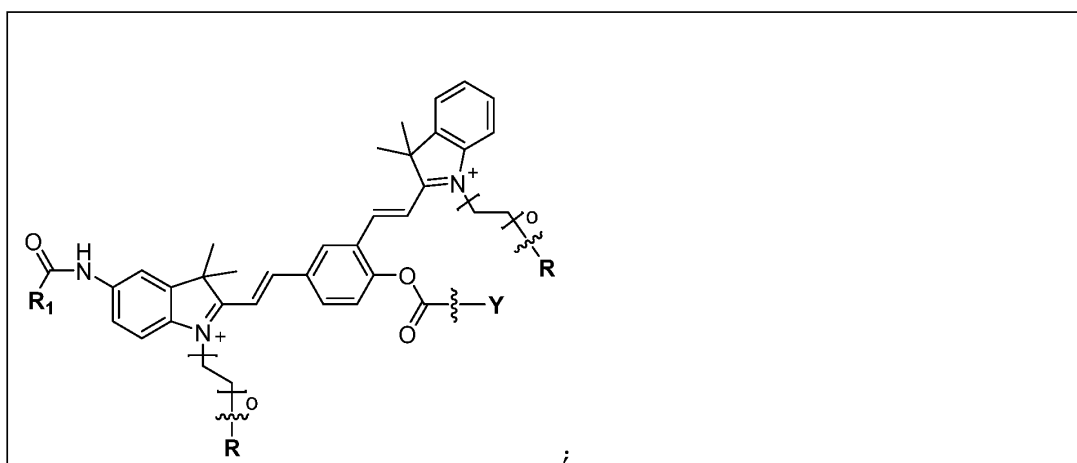


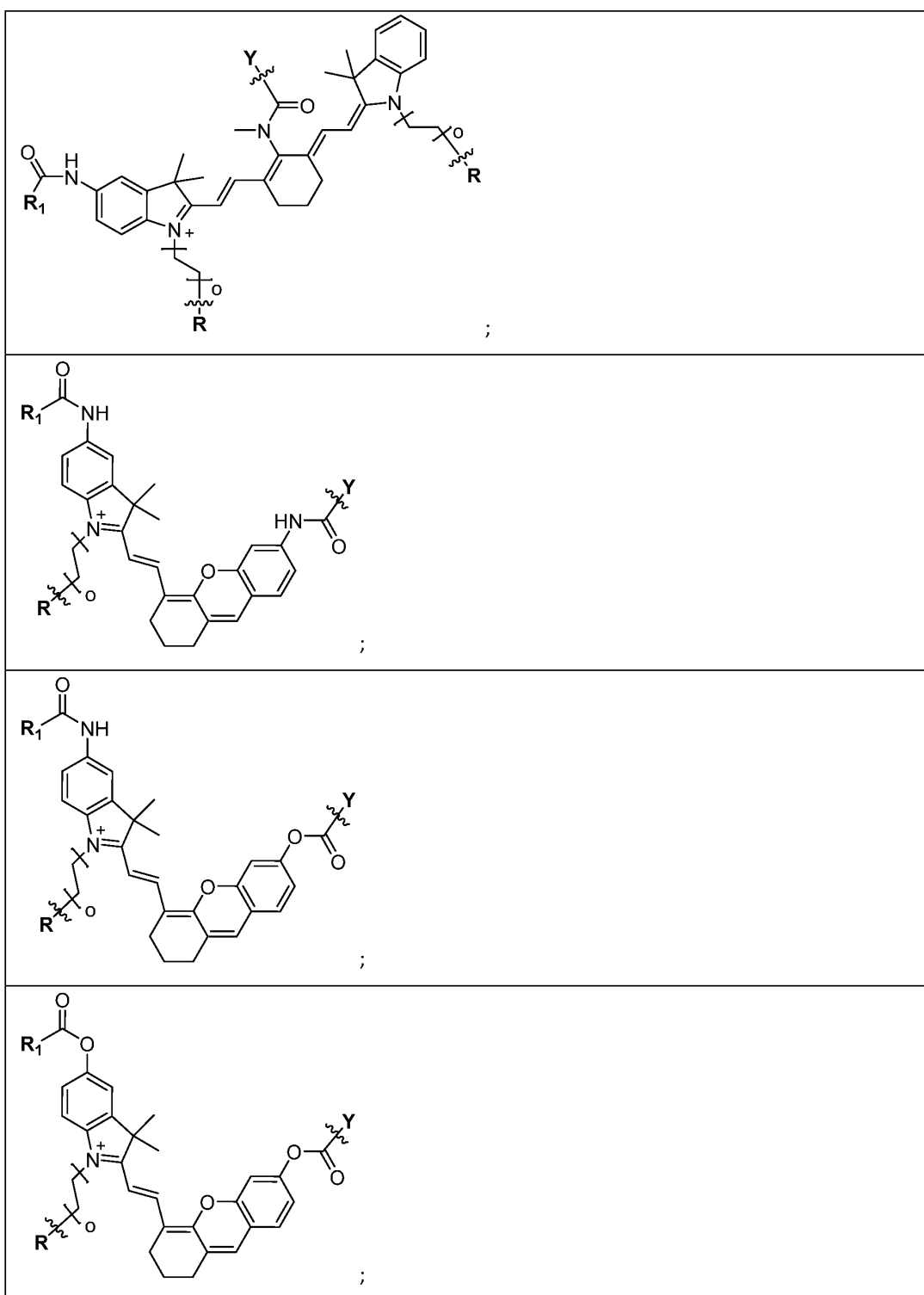
where:

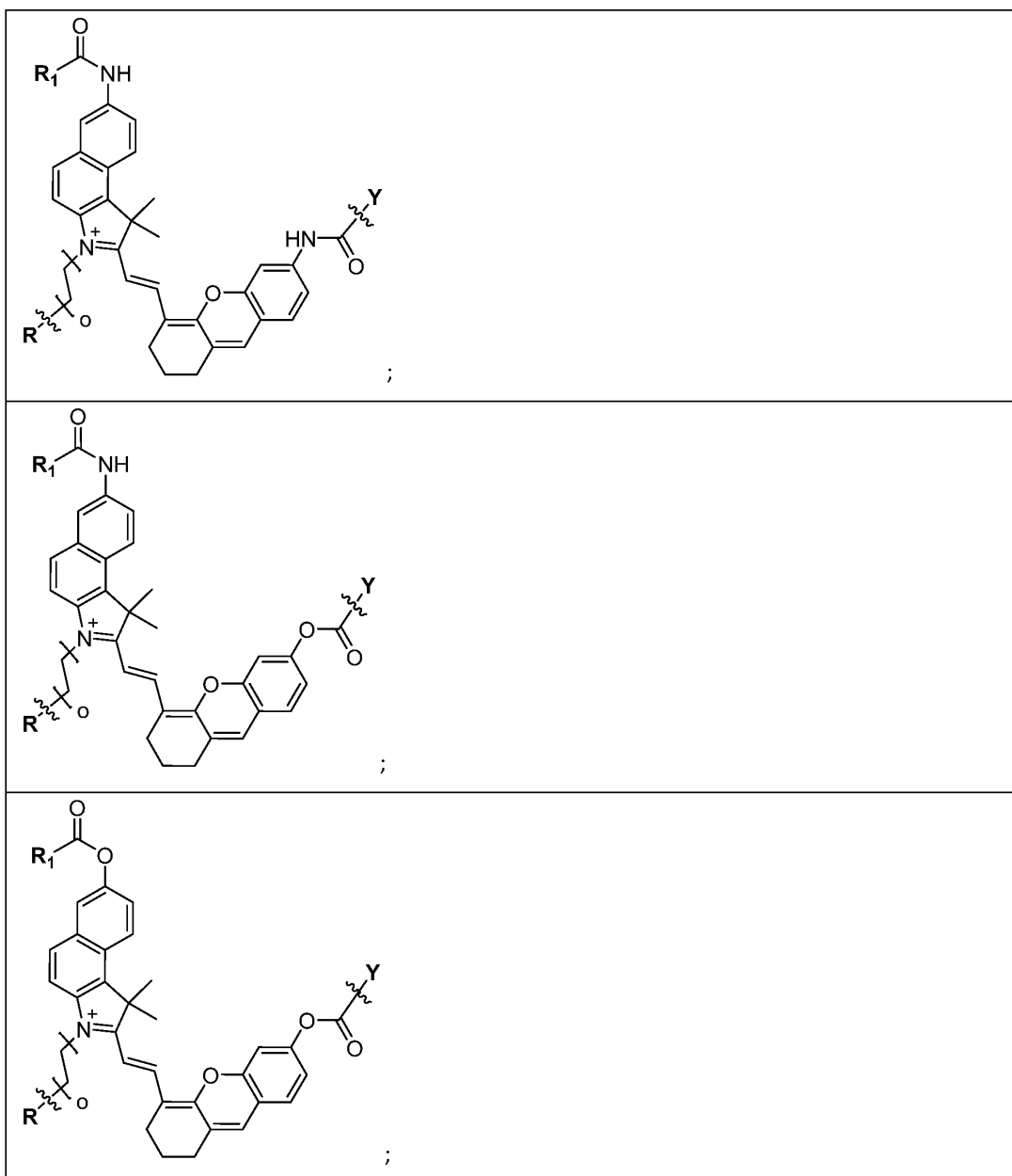
n is from 1 to 20 (e.g. from 1 to 5);

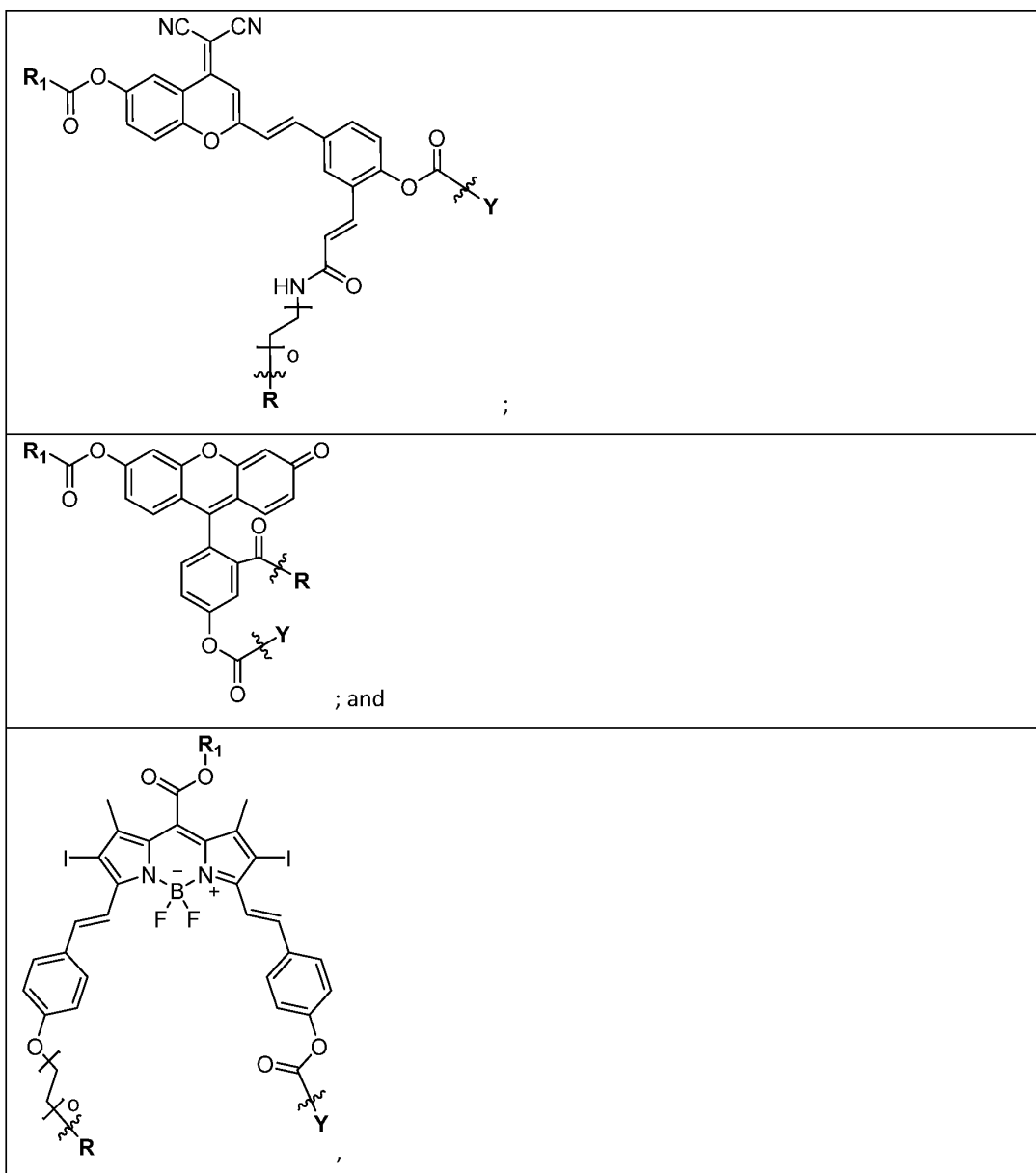
- 10 m represents 0 or 1, as determined by X or X';

X represents a caged fluorescent moiety selected from the group consisting of:



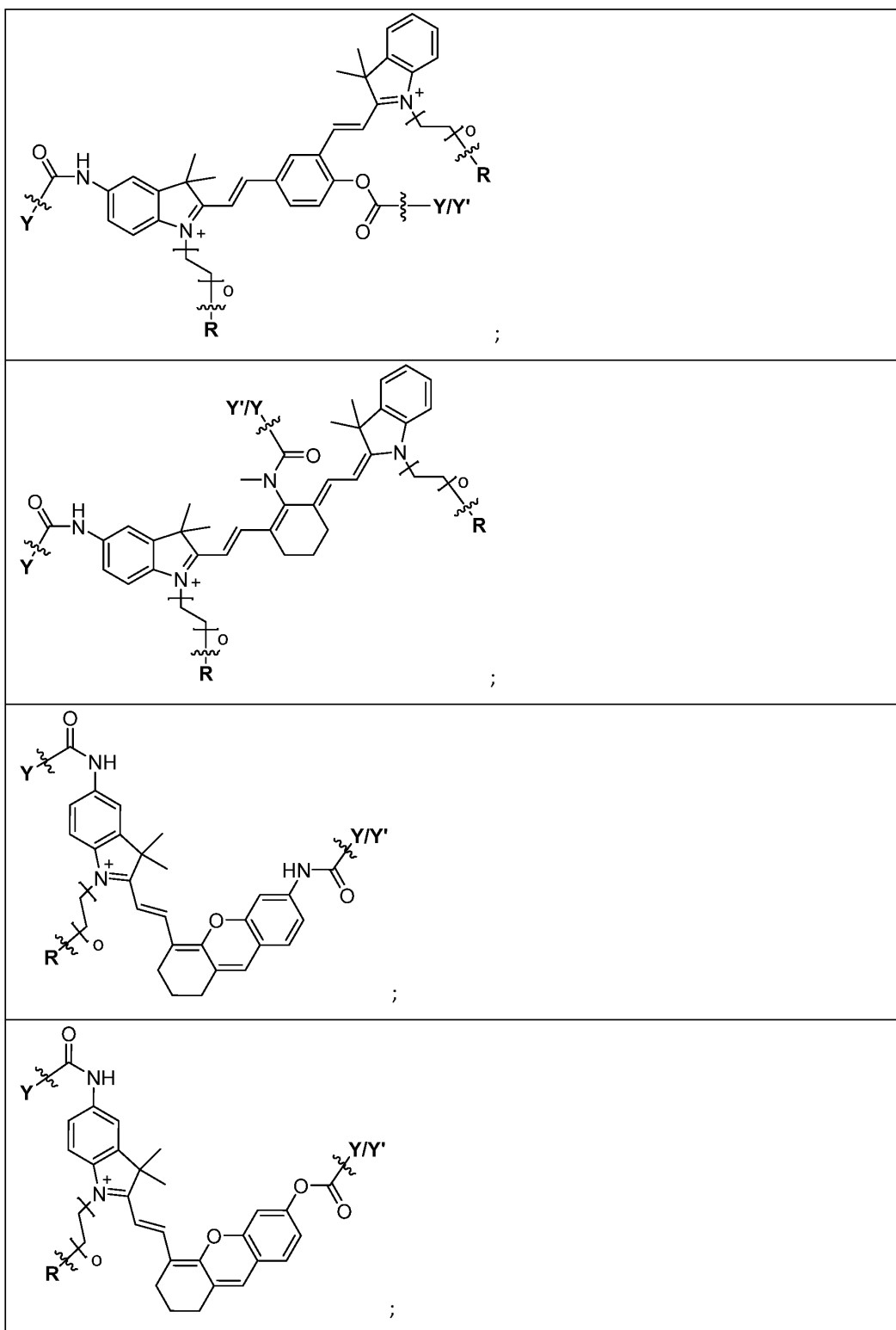


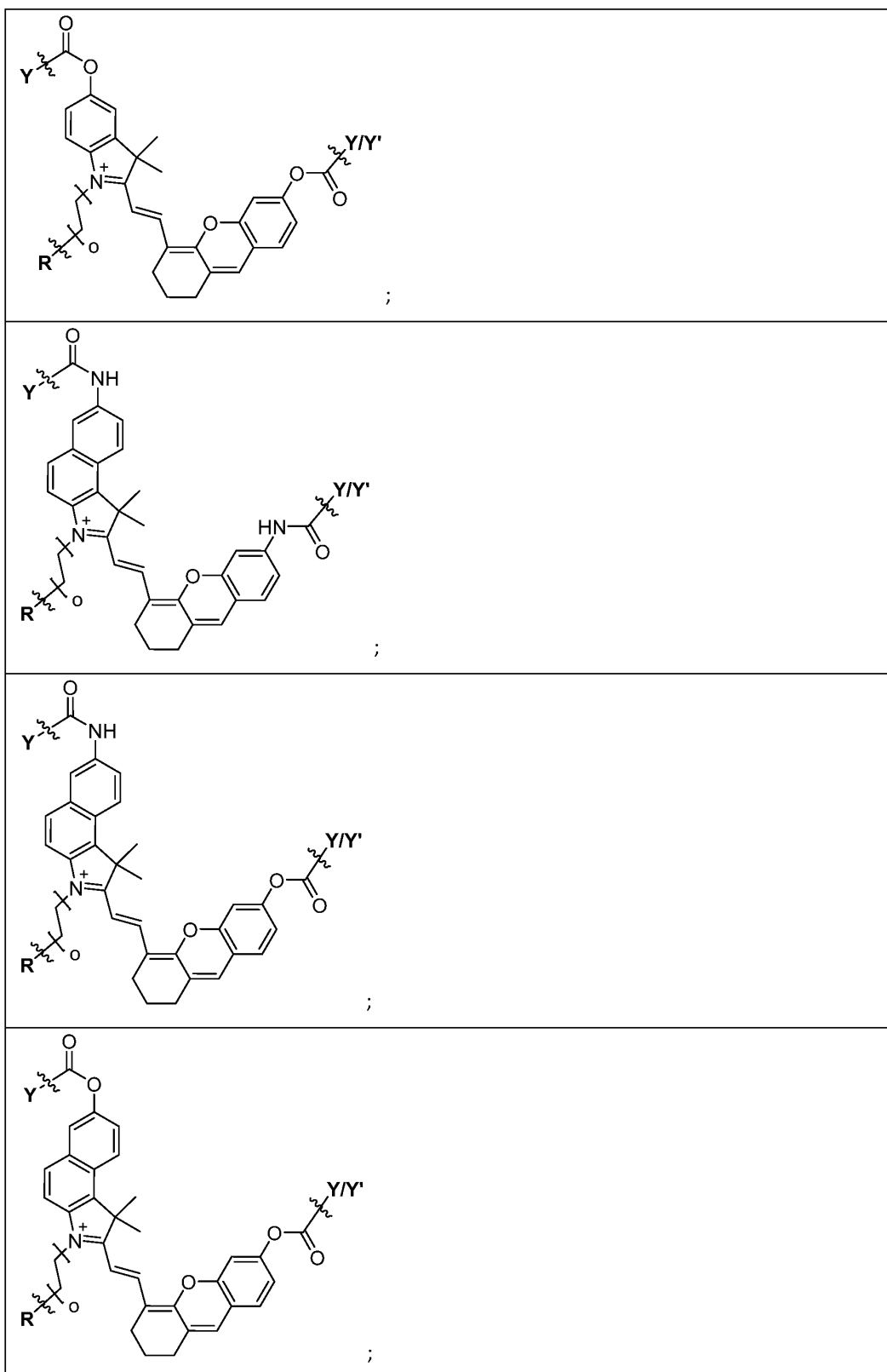


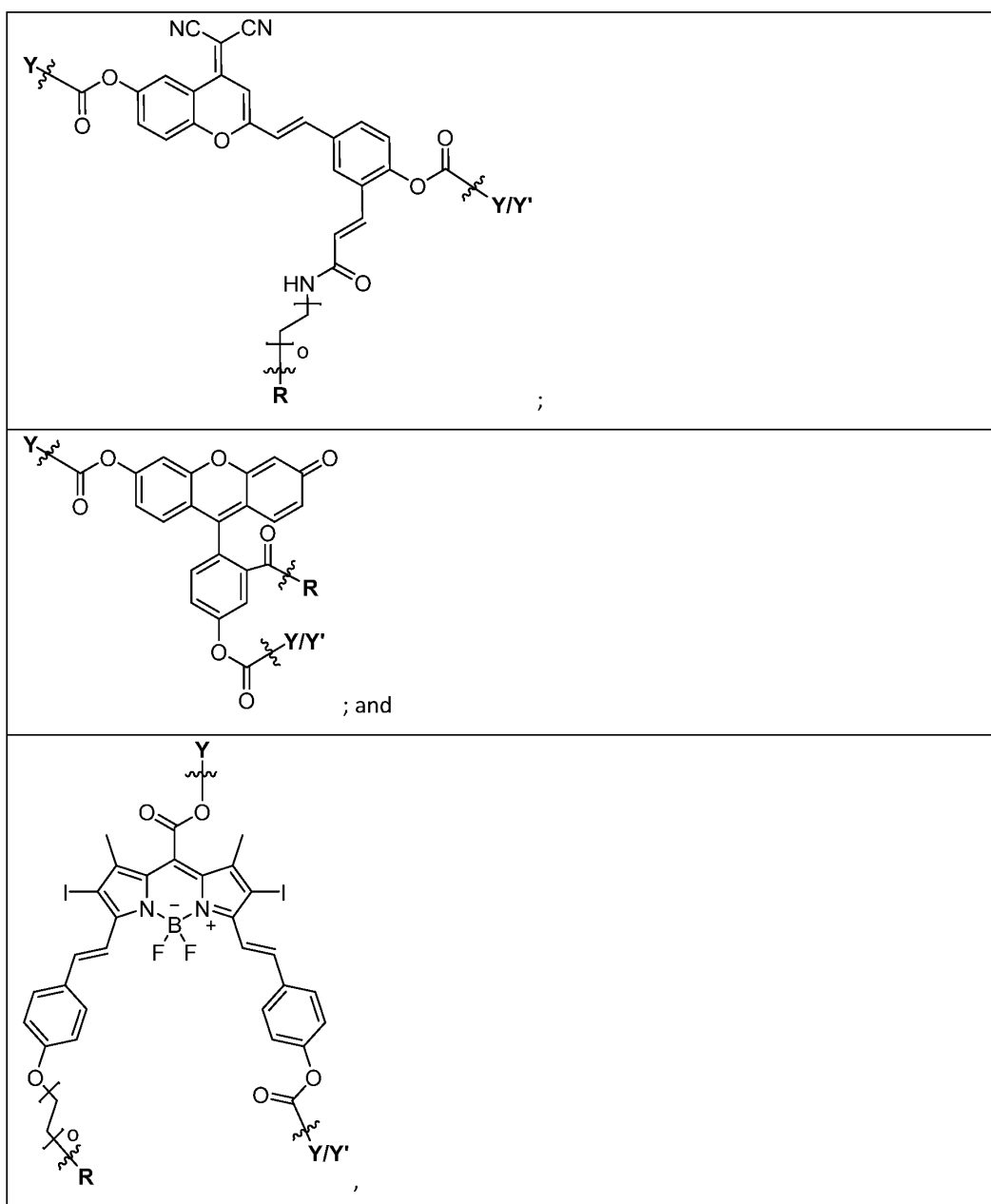


5

X' represents a caged fluorescent moiety selected from the group consisting of:



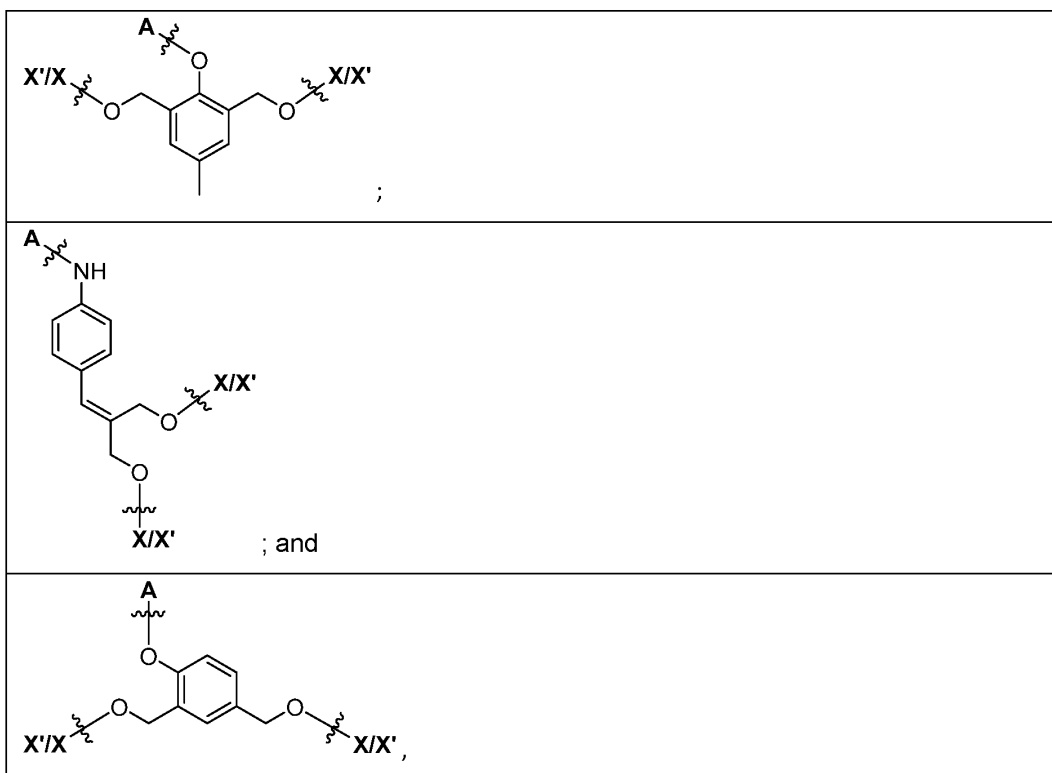




where the wiggly lines represent the point of attachment of X to the moieties R and Y or Y', respectively, where the moiety Y/Y' is Y' if the X' group is the terminal X' group and is Y if the X' group is not the terminal X' group;

- 5 each o independently represents 1 to 3;

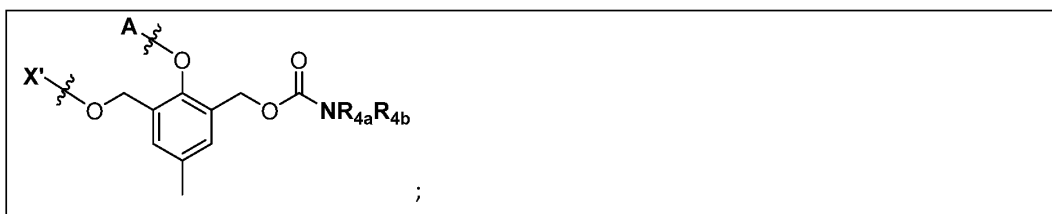
Y represents a self-immolative linker selected from:

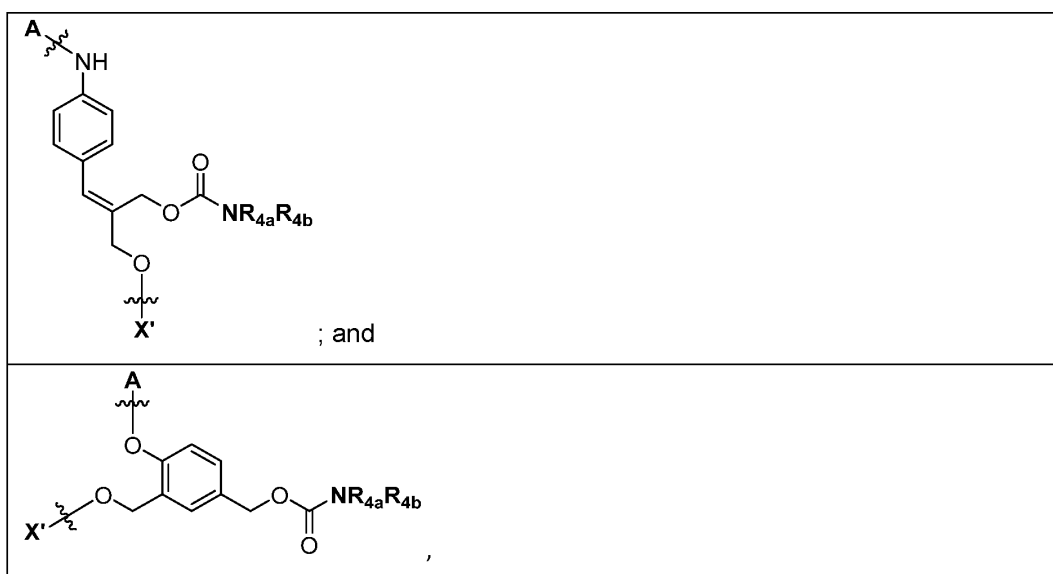


where the wiggly lines represent the point of attachment of Y to the moieties A, X and X', where the moiety X/X' is X' if the Y group is attached to the first X group of the polymeric nanoreporter compound chain and is otherwise attached to an X' group;

5

Y' represents a self-immolative linker selected from:

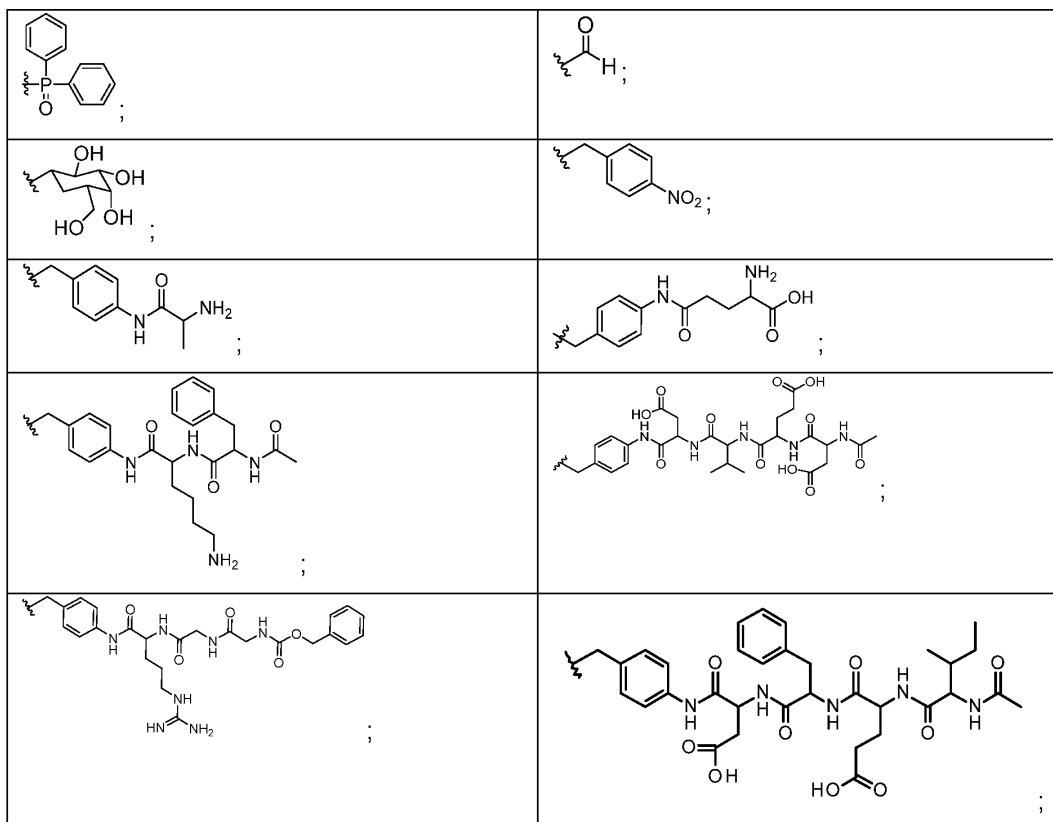


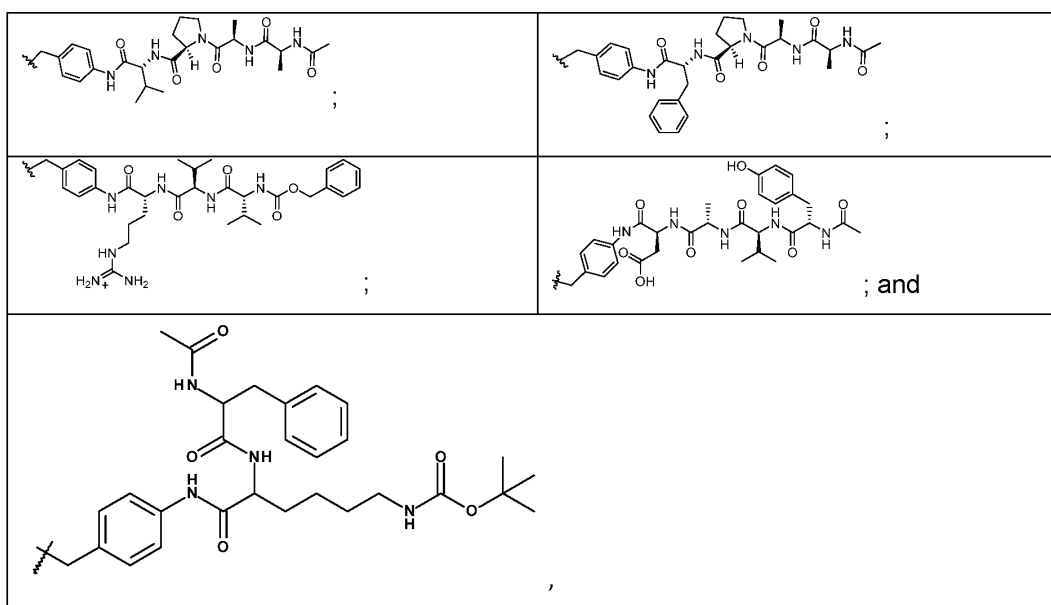


where the wiggly lines represent the point of attachment of Y to the moieties A and X';

where each R_{4a} and R_{4b} independently represents a C₁ to C₆ alkyl group,

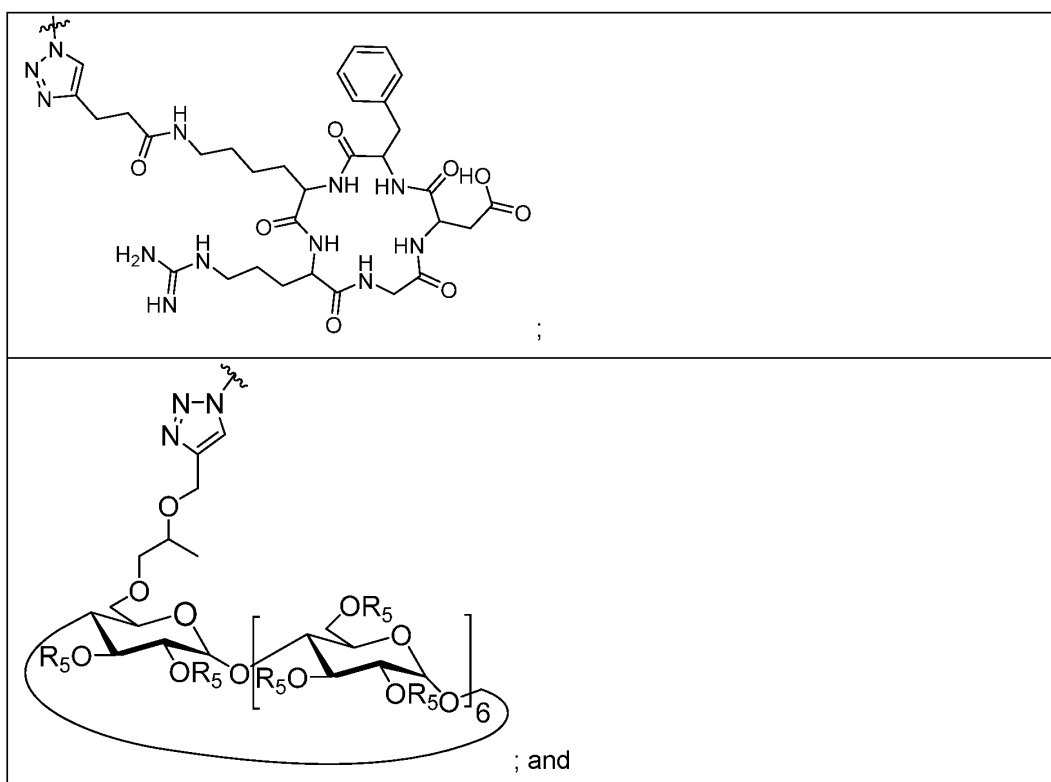
- 5 A represents a biomarker responsive moiety, selected from:

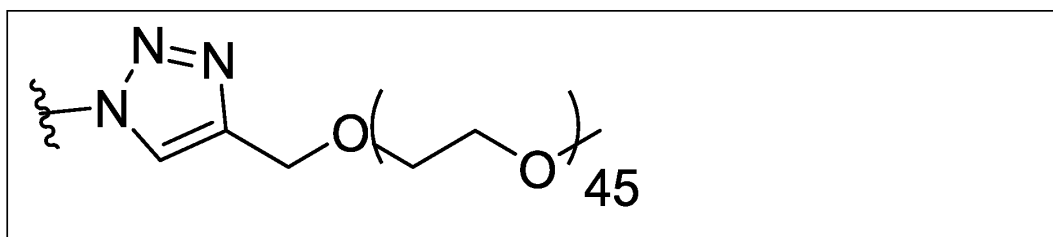




where the wiggly line represents the point of attachment to the rest of the molecule;

R represents a solubility enhancement moiety selected from one or more of:





where each R_5 is independently selected from H or $-\text{CH}_2\text{CH}(\text{OH})\text{CH}_3$, and the wiggly line represents the point of attachment to the rest of the molecule, or a pharmaceutically acceptable salt or solvate thereof.

- 5 In embodiments herein, the word “comprising” may be interpreted as requiring the features mentioned, but not limiting the presence of other features. Alternatively, the word “comprising” may also relate to the situation where only the components/features listed are intended to be present (e.g. the word “comprising” may be replaced by the phrases “consists of” or “consists essentially of”). It is explicitly contemplated that both the broader and narrower interpretations
- 10 can be applied to all aspects and embodiments of the present invention. In other words, the word “comprising” and synonyms thereof may be replaced by the phrase “consisting of” or the phrase “consists essentially of” or synonyms thereof and *vice versa*.

- The phrase, “consists essentially of” and its pseudonyms may be interpreted herein to refer to
- 15 a material where minor impurities may be present. For example, the material may be greater than or equal to 90% pure, such as greater than 95% pure, such as greater than 97% pure, such as greater than 99% pure, such as greater than 99.9% pure, such as greater than 99.99% pure, such as greater than 99.999% pure, such as 100% pure.

- 20 As used herein, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a composition” includes mixtures of two or more such compositions and the like.

- References herein (in any aspect or embodiment of the invention) to compounds of formula I
- 25 includes references to such compounds *per se*, to tautomers of such compounds, as well as to pharmaceutically acceptable salts or solvates, or pharmaceutically functional derivatives of such compounds.

- Pharmaceutically acceptable salts that may be mentioned include acid addition salts and base
- 30 addition salts. Such salts may be formed by conventional means, for example by reaction of a free acid or a free base form of a compound of formula I with one or more equivalents of an

appropriate acid or base, optionally in a solvent, or in a medium in which the salt is insoluble, followed by removal of said solvent, or said medium, using standard techniques (e.g. *in vacuo*, by freeze-drying or by filtration). Salts may also be prepared by exchanging a counter-ion of a compound of formula I in the form of a salt with another counter-ion, for example using a suitable ion exchange resin.

Examples of pharmaceutically acceptable salts include acid addition salts derived from mineral acids and organic acids, and salts derived from metals such as sodium, magnesium, or preferably, potassium and calcium.

Examples of acid addition salts include acid addition salts formed with acetic, 2,2-dichloroacetic, adipic, alginic, aryl sulphonic acids (e.g. benzenesulphonic, naphthalene-2-sulphonic, naphthalene-1,5-disulphonic and *p*-toluenesulphonic), ascorbic (e.g. L-ascorbic), L-aspartic, benzoic, 4-acetamidobenzoic, butanoic, (+) camphoric, camphor-sulphonic, (+)-(1S)-camphor-10-sulphonic, capric, caproic, caprylic, cinnamic, citric, cyclamic, dodecylsulphuric, ethane-1,2-disulphonic, ethanesulphonic, 2-hydroxyethanesulphonic, formic, fumaric, galactaric, gentisic, glucoheptonic, gluconic (e.g. D-gluconic), glucuronic (e.g. D-glucuronic), glutamic (e.g. L-glutamic), α -oxoglutaric, glycolic, hippuric, hydrobromic, hydrochloric, hydriodic, isethionic, lactic (e.g. (+)-L-lactic and ($\pm$)-DL-lactic), lactobionic, maleic, malic (e.g. (-)-L-malic), malonic, ($\pm$)-DL-mandelic, metaphosphoric, methanesulphonic, 1-hydroxy-2-naphthoic, nicotinic, nitric, oleic, orotic, oxalic, palmitic, pamoic, phosphoric, propionic, L-pyroglutamic, salicylic, 4-amino-salicylic, sebacic, stearic, succinic, sulphuric, tannic, tartaric (e.g. (+)-L-tartaric), thiocyanic, undecylenic and valeric acids.

Particular examples of salts are salts derived from mineral acids such as hydrochloric, hydrobromic, phosphoric, metaphosphoric, nitric and sulphuric acids; from organic acids, such as tartaric, acetic, citric, malic, lactic, fumaric, benzoic, glycolic, gluconic, succinic, arylsulphonic acids; and from metals such as sodium, magnesium, or preferably, potassium and calcium.

As mentioned above, also encompassed by formula I are any solvates of the compounds and their salts. Preferred solvates are solvates formed by the incorporation into the solid state structure (e.g. crystal structure) of the compounds of the invention of molecules of a non-toxic pharmaceutically acceptable solvent (referred to below as the solvating solvent). Examples of such solvents include water, alcohols (such as ethanol, isopropanol and butanol) and dimethylsulphoxide. Solvates can be prepared by recrystallising the compounds of the invention with a solvent or mixture of solvents containing the solvating solvent. Whether or

not a solvate has been formed in any given instance can be determined by subjecting crystals of the compound to analysis using well known and standard techniques such as thermogravimetric analysis (TGA), differential scanning calorimetry (DSC) and X-ray crystallography.

5

The solvates can be stoichiometric or non-stoichiometric solvates. Particularly preferred solvates are hydrates, and examples of hydrates include hemihydrates, monohydrates and dihydrates.

- 10 For a more detailed discussion of solvates and the methods used to make and characterise them, see Bryn *et al.*, *Solid-State Chemistry of Drugs*, Second Edition, published by SSCI, Inc of West Lafayette, IN, USA, 1999, ISBN 0-967-06710-3.

- 15 Compounds of formula I, as well as pharmaceutically acceptable salts, solvates and pharmaceutically functional derivatives of such compounds are, for the sake of brevity, hereinafter referred to together as the "compounds of formula I".

- 20 Compounds of formula I may contain double bonds and may thus exist as *E* (*entgegen*) and *Z* (*zusammen*) geometric isomers about each individual double bond. All such isomers and mixtures thereof are included within the scope of the invention.

Compounds of formula I may exist as regioisomers and may also exhibit tautomerism. All tautomeric forms and mixtures thereof are included within the scope of the invention.

- 25 Compounds of formula I may contain one or more asymmetric carbon atoms and may therefore exhibit optical and/or diastereoisomerism. Diastereoisomers may be separated using conventional techniques, e.g. chromatography or fractional crystallisation. The various stereoisomers may be isolated by separation of a racemic or other mixture of the compounds using conventional, e.g. fractional crystallisation or HPLC, techniques. Alternatively the
30 desired optical isomers may be made by reaction of the appropriate optically active starting materials under conditions which will not cause racemisation or epimerisation (i.e. a 'chiral pool' method), by reaction of the appropriate starting material with a 'chiral auxiliary' which can subsequently be removed at a suitable stage, by derivatisation (i.e. a resolution, including a dynamic resolution), for example with a homochiral acid followed by separation of the
35 diastereomeric derivatives by conventional means such as chromatography, or by reaction with an appropriate chiral reagent or chiral catalyst all under conditions known to the skilled person. All stereoisomers and mixtures thereof are included within the scope of the invention.

Unless otherwise stated, the term "alkyl" refers to an unbranched or branched, acyclic or cyclic, saturated or unsaturated (so forming, for example, an alkenyl or alkynyl) hydrocarbyl radical, which may be substituted or unsubstituted (with, for example, one or more halo atoms). Where the term "alkyl" refers to an acyclic group, it is preferably C₁₋₆ alkyl (such as ethyl, propyl, (e.g. n-propyl or isopropyl), butyl (e.g. branched or unbranched butyl), pentyl or, more preferably, methyl). Where the term "alkyl" is a cyclic group (which may be where the group "cycloalkyl" is specified), it is preferably C₃₋₆ cycloalkyl and, more preferably, C₅₋₆ cycloalkyl. In particular, embodiments of the invention, an alkyl group referred to herein may be acyclic.

Further embodiments of the invention that may be mentioned include those in which the compound of formula I is isotopically labelled. However, other, particular embodiments of the invention that may be mentioned include those in which the compound of formula I is not isotopically labelled.

The term "isotopically labelled", when used herein includes references to compounds of formula I in which there is a non-natural isotope (or a non-natural distribution of isotopes) at one or more positions in the compound. References herein to "one or more positions in the compound" will be understood by those skilled in the art to refer to one or more of the atoms of the compound of formula I. Thus, the term "isotopically labelled" includes references to compounds of formula I that are isotopically enriched at one or more positions in the compound.

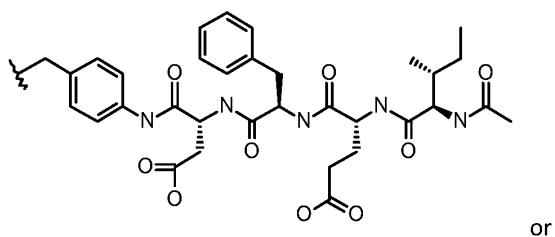
The isotopic labelling or enrichment of the compound of formula I may be with a radioactive or non-radioactive isotope of any of hydrogen, carbon, nitrogen, oxygen, sulfur, fluorine, chlorine, bromine and/or iodine. Particular isotopes that may be mentioned in this respect include ²H, ³H, ¹¹C, ¹³C, ¹⁴C, ¹³N, ¹⁵N, ¹⁵O, ¹⁷O, ¹⁸O, ³⁵S, ¹⁸F, ³⁷Cl, ⁷⁷Br, ⁸²Br and ¹²⁵I).

When the compound of formula I is labelled or enriched with a radioactive or nonradioactive isotope, compounds of formula I that may be mentioned include those in which at least one atom in the compound displays an isotopic distribution in which a radioactive or non-radioactive isotope of the atom in question is present in levels at least 10% (e.g. from 10% to 5000%, particularly from 50% to 1000% and more particularly from 100% to 500%) above the natural level of that radioactive or non-radioactive isotope.

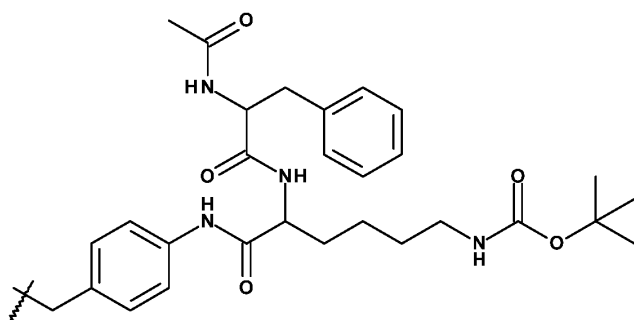
In embodiments of the invention that may be mentioned herein, one or more of the following may apply:

n may be 2;

A may represent:



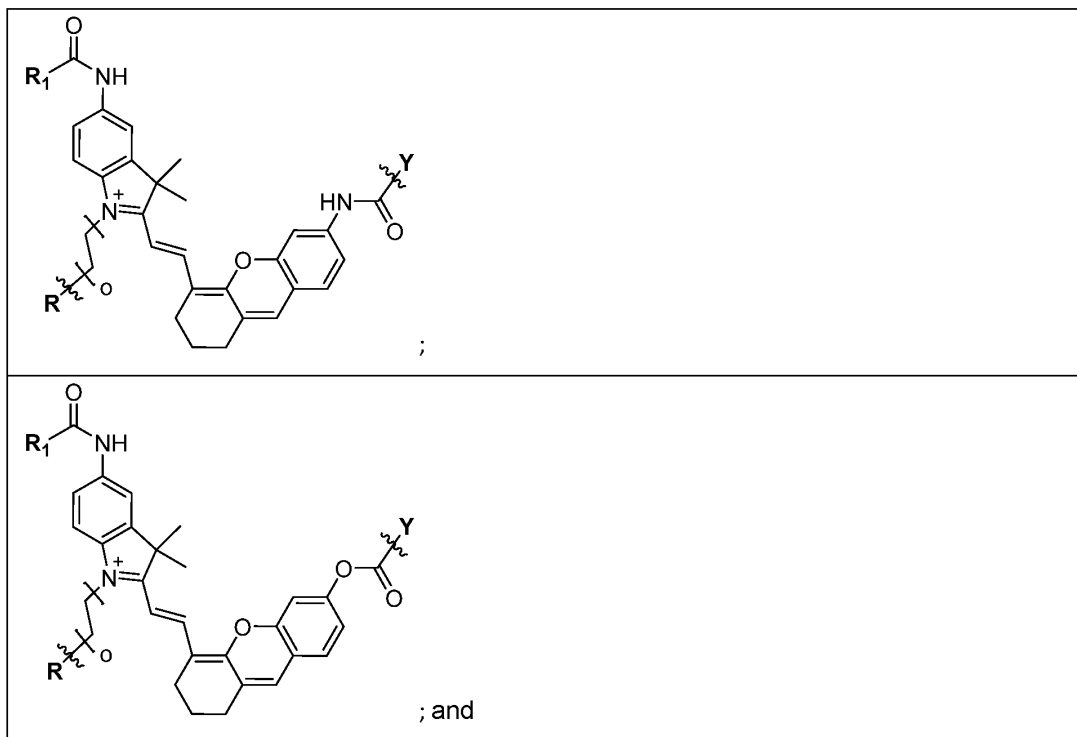
5

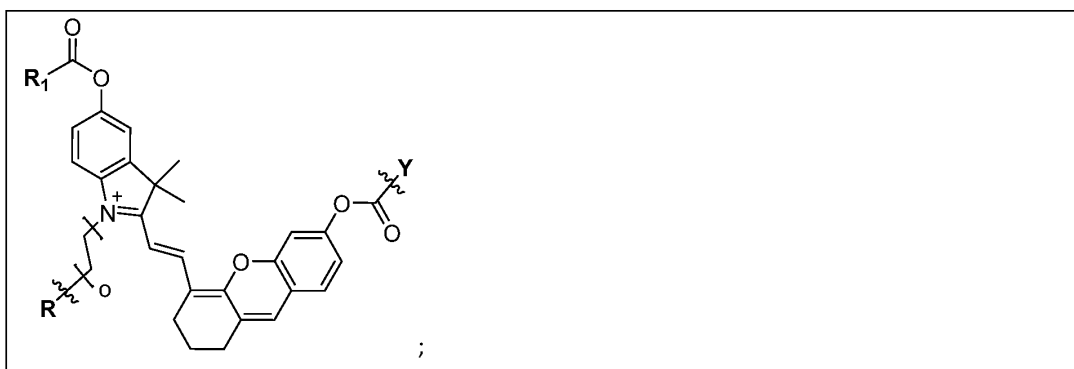


, where the wiggly line represents

the point of attachment to the rest of the molecule;

X may be selected from:

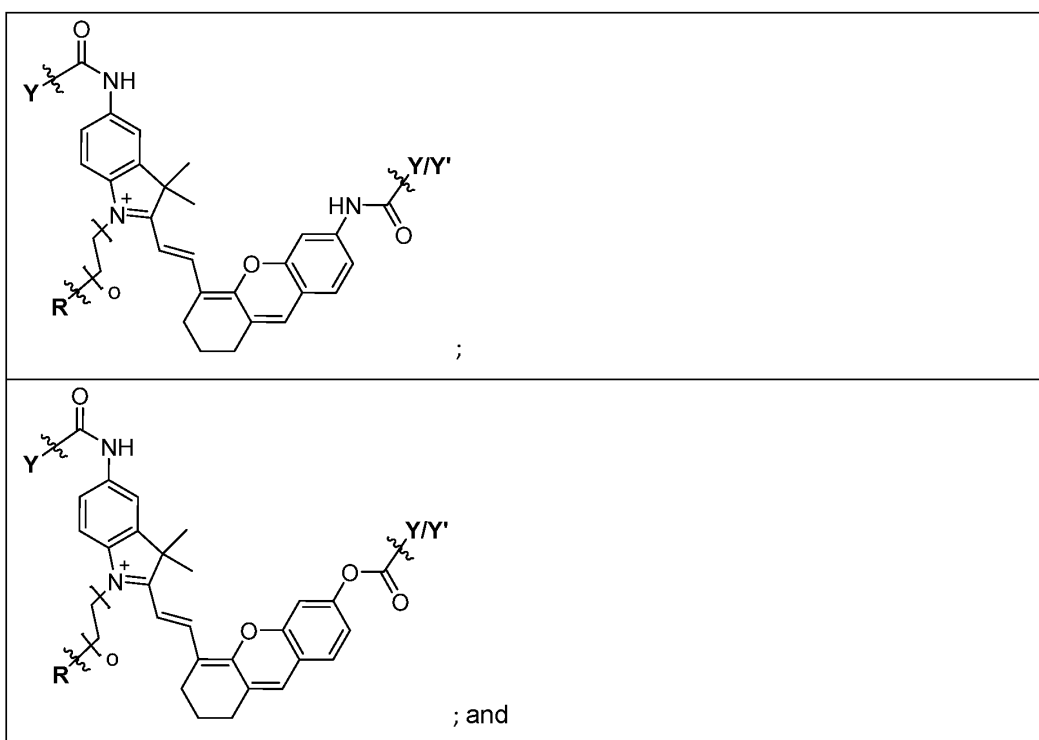


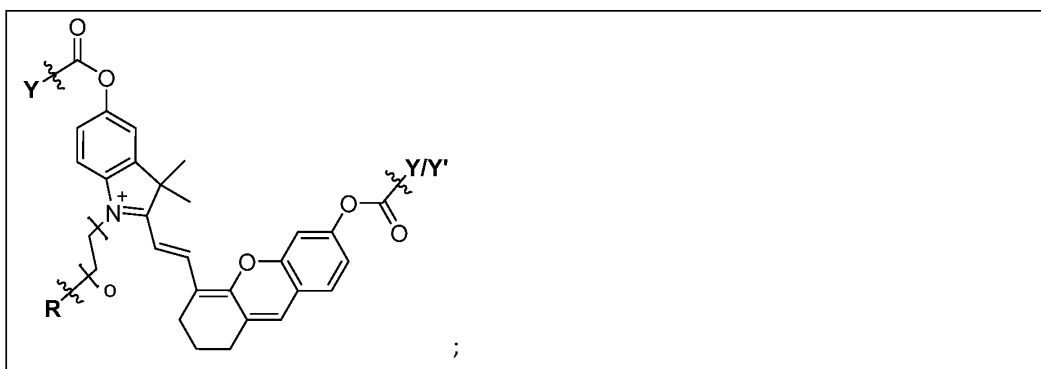


where the wiggly lines represent the point of attachment of X to the moieties R and Y, respectively;

R_1 represents $R_{2a}R_{2b}N$ and R_{2a} and R_{2b} independently represent a methyl group;

X' may be selected from:





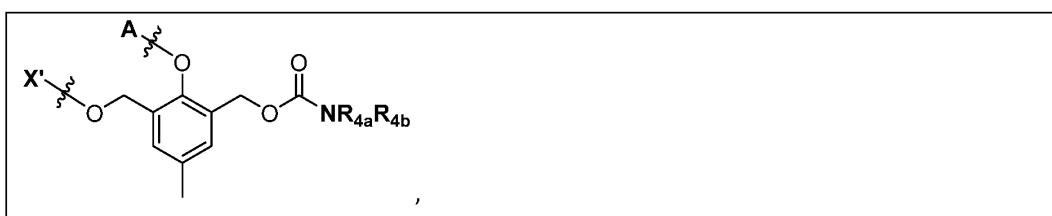
where the wiggly lines represent the point of attachment of X to the moieties R and Y or Y', respectively, where the moiety Y/Y' is Y' if the X' group is the terminal X' group and is Y if the X' group is not the terminal X' group;

Y may be:



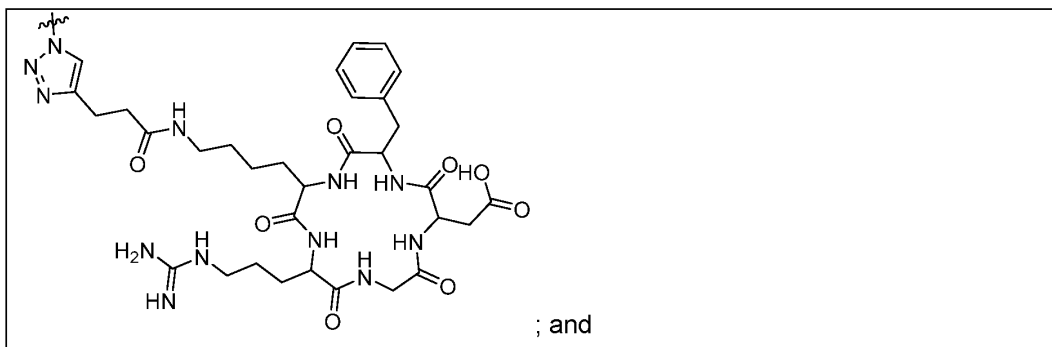
- 5 where the wiggly lines represent the point of attachment of Y to the moieties A, X and X', where the moiety X/X' is X' if the Y group is attached to the first X group of the polymeric nanoreporter compound chain and is otherwise attached to an X' group;

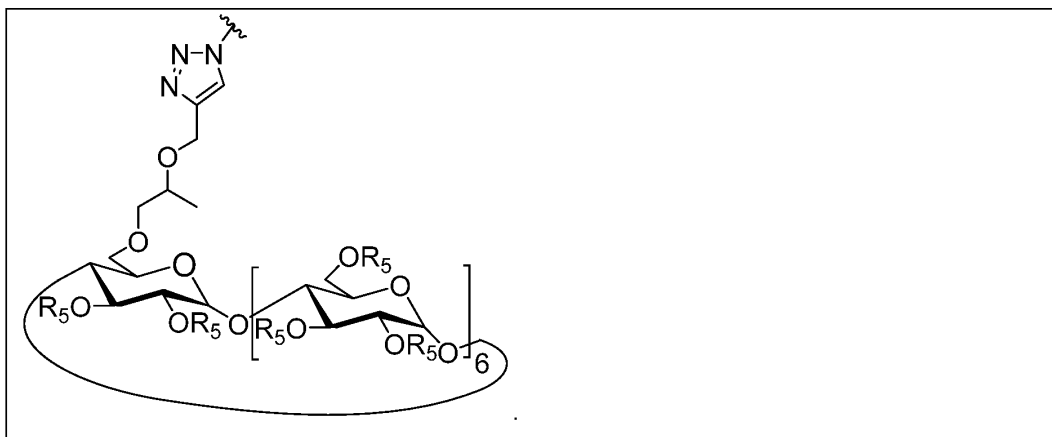
Y' may be:



where the wiggly lines represent the point of attachment of Y to the moieties A and X';

- 10 each R_{4a} and R_{4b} may independently represent a methyl group;
each R represents a solubility enhancement moiety selected from one or both of:

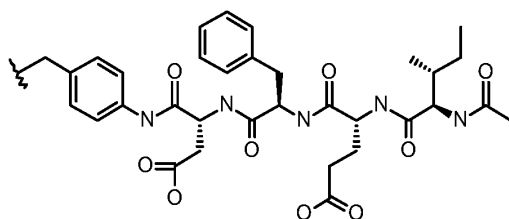




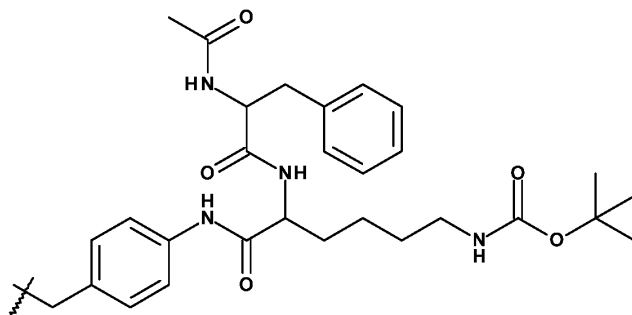
In embodiments of the invention that may be mentioned herein, one or more of the following may apply:

n may be 2;

5 A may represent:



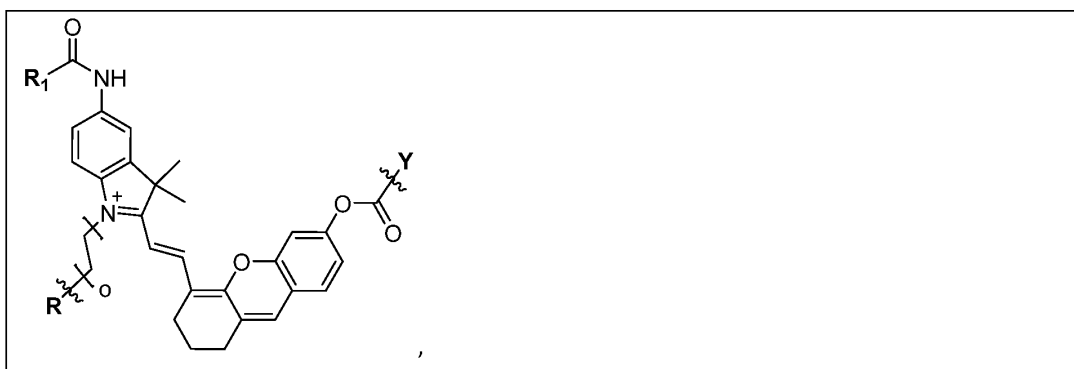
or



, where the wiggly line represents

the point of attachment to the rest of the molecule;

10 X may be:

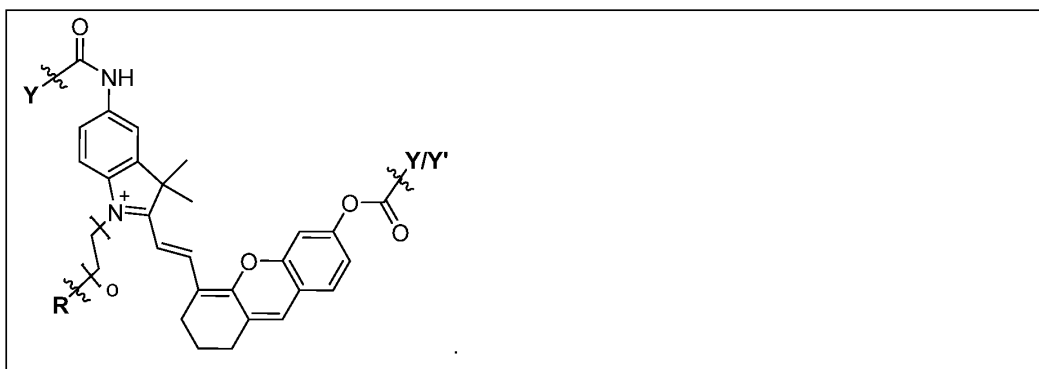


where the wiggly line represents the point of attachment of X to the moieties R and Y, respectively;

R_1 represents $R_{2a}R_{2b}N$ and R_{2a} and R_{2b} independently represent a methyl group;

X' may be:

5



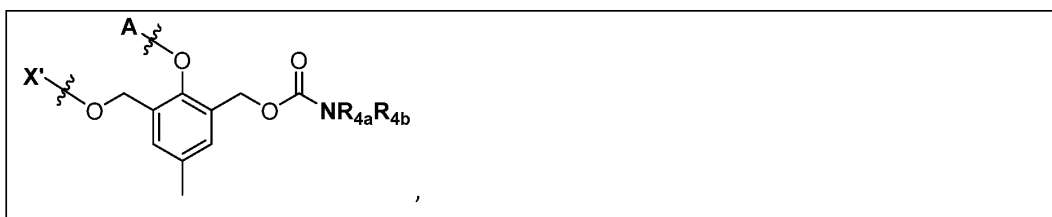
where the wiggly lines represent the point of attachment of X to the moieties R and Y or Y' , respectively, where the moiety Y/Y' is Y' if the X' group is the terminal X' group and is Y if the X' group is not the terminal X' group;

Y may be:



10 where the wiggly lines represent the point of attachment of Y to the moieties A, X and X' , where the moiety X/X' is X' if the Y group is attached to the first X group of the polymeric nanoreporter compound chain and is otherwise attached to an X' group;

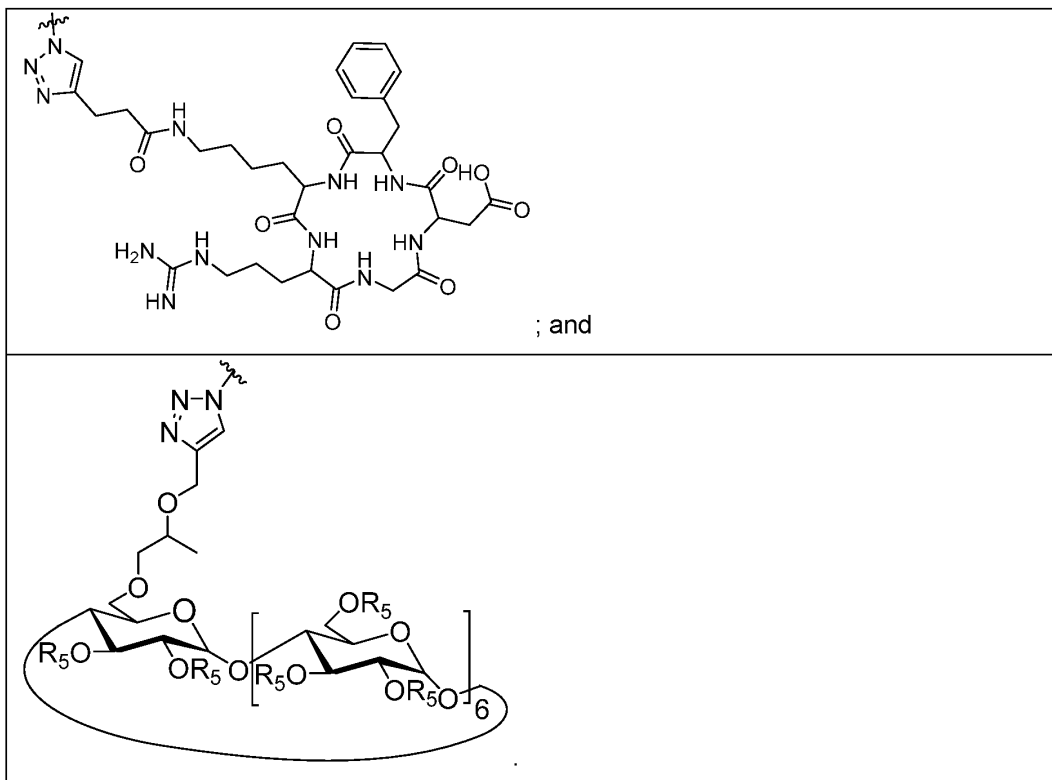
Y' may be:



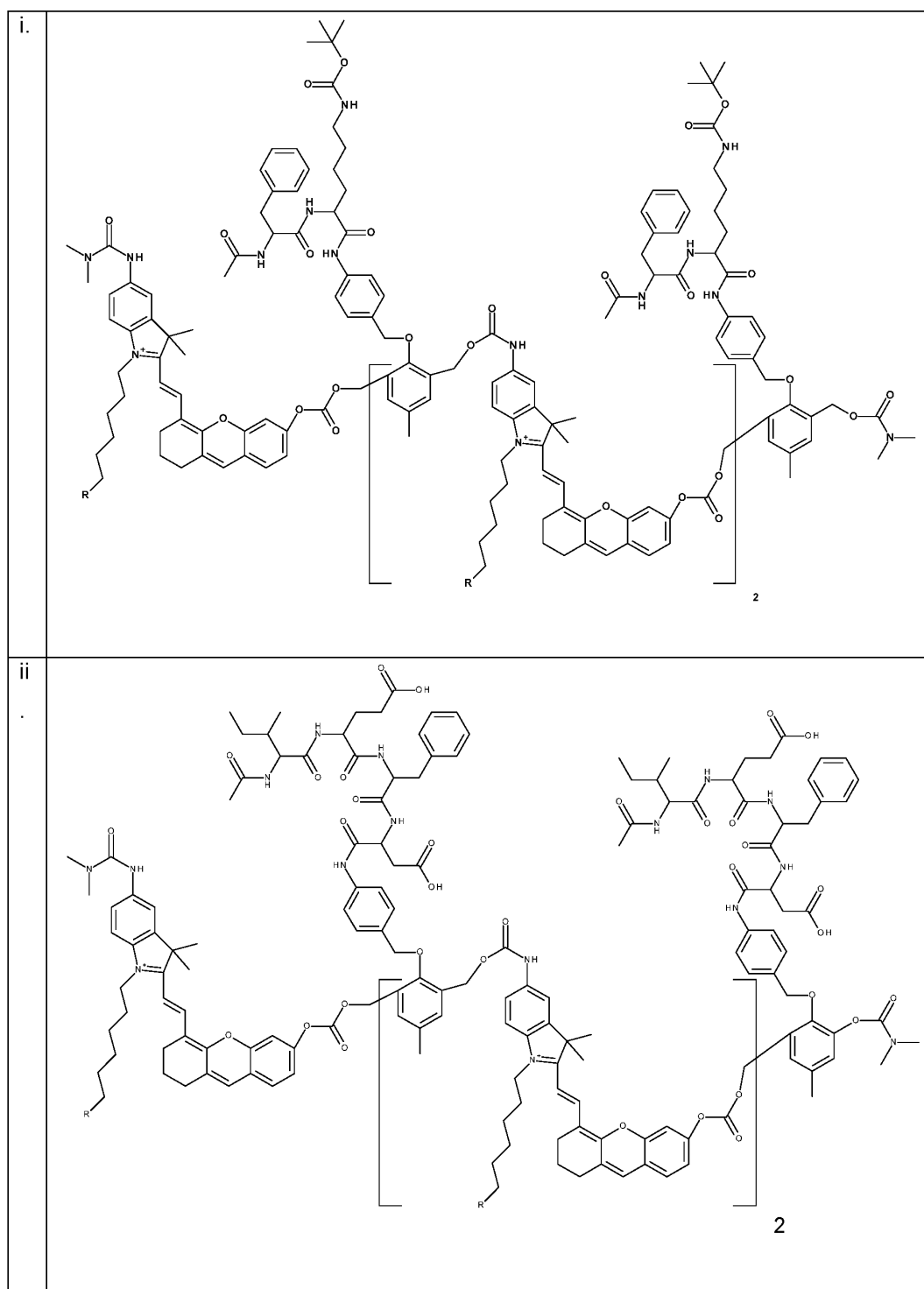
where the wiggly lines represent the point of attachment of Y to the moieties A and X';

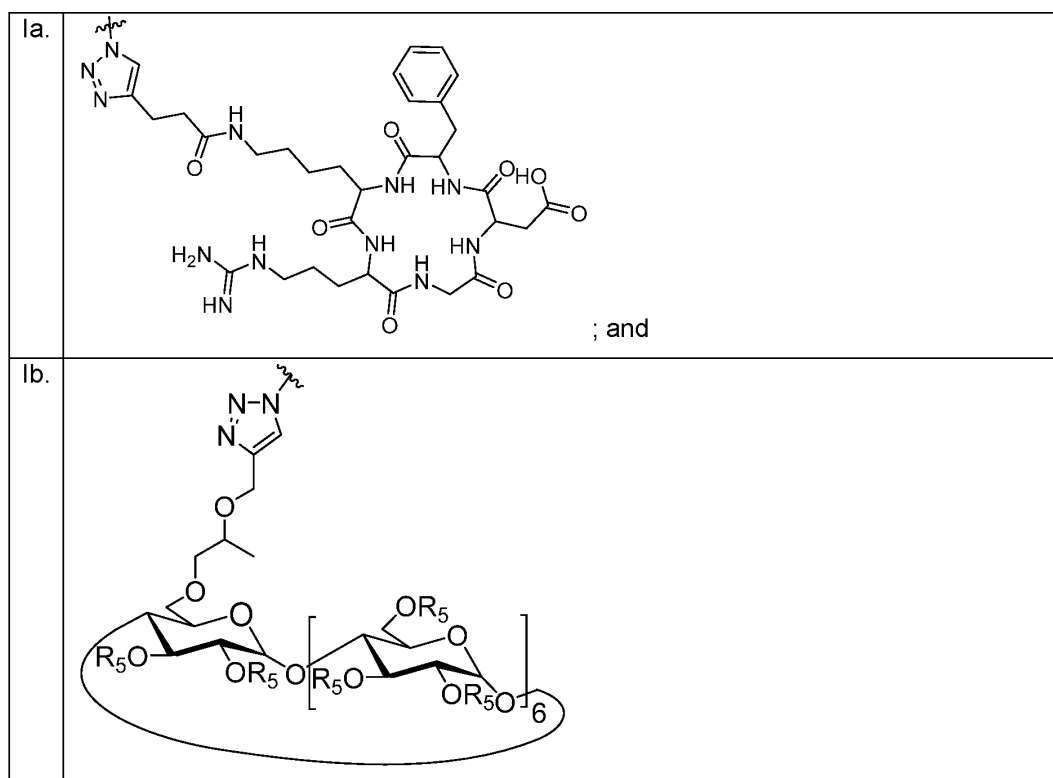
each R_{4a} and R_{4b} may independently represent a methyl group;

each R represents a solubility enhancement moiety selected from one or both of:



- 5 In particular embodiments of the invention that may be mentioned herein, the polymeric nanoreporter compound, or salt or solvate thereof, may be selected from:



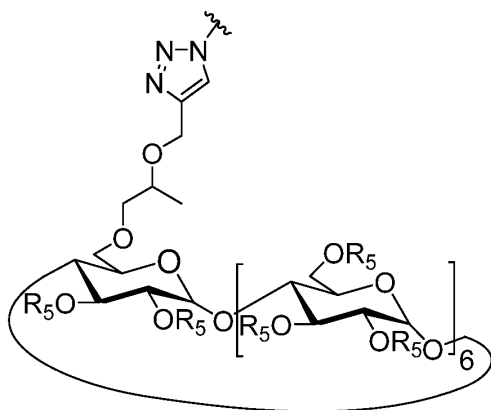


; and

where the ratio of Ia to Ib is 1:9; and

where R in (ii) is:

5



The molecules disclosed herein may be particularly useful in the detection of a pathological condition in a subject. Thus, in further aspects of the invention there is provided:

(A) a method of detecting a pathological condition in a subject, the method comprising the steps of:

(a) administering a polymeric nanoreporter compound according to formula I, or a pharmaceutically acceptable salt or solvate thereof, as described hereinbefore that targets the selected pathological condition to a subject; and

(b) detecting any fluorescence in one or both of urine from the subject and an organ or a tissue in the subject that is targeted by the polymeric nanoreporter compound according to formula I, wherein the presence of the pathological condition in the subject is indicated by fluorescence in the urine, the tissue or organ, or both the urine and the tissue or organ;

(B) use of a polymeric nanoreporter compound according to formula I, or a pharmaceutically acceptable salt or solvate thereof, as described hereinbefore in the manufacture of a medicament for the diagnosis of a pathological condition;

(C) a polymeric nanoreporter compound according to formula I, or a pharmaceutically acceptable salt or solvate thereof, as described hereinbefore for use in the diagnosis of a pathological condition.

The terms "*patient*" and "*patients*" include references to mammalian (e.g. human) patients. As used herein the terms "subject" or "patient" are well-recognized in the art, and, are used interchangeably herein to refer to a mammal, including dog, cat, rat, mouse, monkey, cow, horse, goat, sheep, pig, camel, and, most preferably, a human. In some embodiments, the subject is a subject in need of treatment or a subject with a disease or disorder. However, in other embodiments, the subject can be a normal subject. The term does not denote a particular age or sex. Thus, adult and newborn subjects, whether male or female, are intended to be covered.

In embodiments of the above aspects, the pathological condition may be a cancer, an organ allograft rejection, and immune-mediated hepatitis.

As will be appreciated, if an underlying pathological condition is found, then the subject may then be treated for this condition by the administration of a pharmaceutically effective amount of one or more therapeutic agents useful for treating said condition. Alternatively or additionally, the subject may be treated by one or more of radiotherapy and surgery.

The term "effective amount" refers to an amount of a compound, which confers a therapeutic effect on the treated patient (e.g. sufficient to treat or prevent the disease). The effect may be

objective (i.e. measurable by some test or marker) or subjective (i.e. the subject gives an indication of or feels an effect).

Compounds of formula I may be administered by any suitable route, but may particularly be administered orally, intravenously, intramuscularly, cutaneously, subcutaneously, transmucosally (e.g. sublingually or buccally), rectally, transdermally, nasally, pulmonarily (e.g. tracheally or bronchially), topically, by any other parenteral route, in the form of a pharmaceutical preparation comprising the compound in a pharmaceutically acceptable dosage form. Particular modes of administration that may be mentioned include oral, intravenous, cutaneous, subcutaneous, nasal, intramuscular or intraperitoneal administration.

Compounds of formula I will generally be administered as a pharmaceutical formulation in admixture with a pharmaceutically acceptable adjuvant, diluent or carrier, which may be selected with due regard to the intended route of administration and standard pharmaceutical practice. Such pharmaceutically acceptable carriers may be chemically inert to the active compounds and may have no detrimental side effects or toxicity under the conditions of use. Suitable pharmaceutical formulations may be found in, for example, Remington *The Science and Practice of Pharmacy*, 19th ed., Mack Printing Company, Easton, Pennsylvania (1995). For parenteral administration, a parenterally acceptable aqueous solution may be employed, which is pyrogen free and has requisite pH, isotonicity, and stability. Suitable solutions will be well known to the skilled person, with numerous methods being described in the literature. A brief review of methods of drug delivery may also be found in e.g. Langer, *Science* (1990) 249, 1527.

Otherwise, the preparation of suitable formulations may be achieved routinely by the skilled person using routine techniques and/or in accordance with standard and/or accepted pharmaceutical practice.

The amount of compound of formula I in any pharmaceutical formulation used in accordance with the present invention will depend on various factors, such as the severity of the condition to be treated, the particular patient to be treated, as well as the compound(s) which is/are employed. In any event, the amount of compound of formula I in the formulation may be determined routinely by the skilled person.

For example, a solid oral composition such as a tablet or capsule may contain from 1 to 99 % (w/w) active ingredient; from 0 to 99% (w/w) diluent or filler; from 0 to 20% (w/w) of a disintegrant; from 0 to 5% (w/w) of a lubricant; from 0 to 5% (w/w) of a flow aid; from 0 to 50%

(w/w) of a granulating agent or binder; from 0 to 5% (w/w) of an antioxidant; and from 0 to 5% (w/w) of a pigment. A controlled release tablet may in addition contain from 0 to 90 % (w/w) of a release-controlling polymer.

- 5 A parenteral formulation (such as a solution or suspension for injection or a solution for infusion) may contain from 1 to 50 % (w/w) active ingredient; and from 50% (w/w) to 99% (w/w) of a liquid or semisolid carrier or vehicle (e.g. a solvent such as water); and 0-20% (w/w) of one or more other excipients such as buffering agents, antioxidants, suspension stabilisers, tonicity adjusting agents and preservatives.

10

Depending on the disorder, and the patient, to be treated, as well as the route of administration, compounds of formula I may be administered at varying diagnostically effective doses to a patient in need thereof.

- 15 However, the dose administered to a mammal, particularly a human, in the context of the present invention should be sufficient to effect a therapeutic response in the mammal over a reasonable timeframe. One skilled in the art will recognize that the selection of the exact dose and composition and the most appropriate delivery regimen will also be influenced by *inter alia* the pharmacological properties of the formulation, the nature and severity of the condition
20 being treated, and the physical condition and mental acuity of the recipient, as well as the potency of the specific compound, the age, condition, body weight, sex and response of the patient to be treated, and the stage/severity of the disease.

- Administration may be continuous or intermittent (e.g. by bolus injection). The dosage may
25 also be determined by the timing and frequency of administration. In the case of oral or parenteral administration the dosage can vary from about 0.01 mg to about 1000 mg per day of a compound of formula I.

- In any event, the medical practitioner, or other skilled person, will be able to determine
30 routinely the actual dosage, which will be most suitable for an individual patient. The above-mentioned dosages are exemplary of the average case; there can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

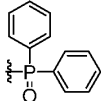
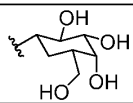
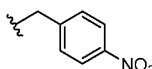
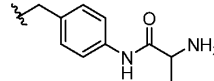
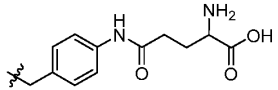
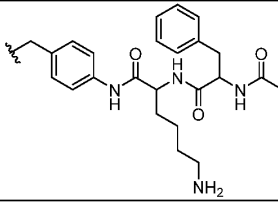
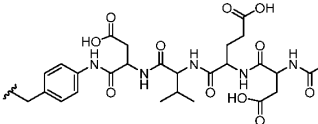
- 35 The aspects of the invention described herein (e.g. the above-mentioned compounds, combinations, methods and uses) may have the advantage that, in the treatment of the conditions described herein, they may be more convenient for the physician and/or patient

than, be more efficacious than, be less toxic than, have better selectivity over, have a broader range of activity than, be more potent than, produce fewer side effects than, or may have other useful pharmacological properties over, similar compounds, combinations, methods (treatments) or uses known in the prior art for use in the treatment of those conditions or otherwise.

As will be appreciated, the aspects of the invention described herein (e.g. the above-mentioned compounds, combinations, methods and uses) may have potential for close monitoring of graft conditions in a patient-friendly yet sensitive, specific and dynamic way.

Further, the aspects of the invention described herein (e.g. the above-mentioned compounds, combinations, methods and uses) may outperform existing blood and urine tests, allowing for ultrasensitive detection of cancer and early diagnosis of liver allograft rejection with allograft specificity unattainable by existing non-invasive methods. Thus, the aspects of the invention described herein (e.g. the above-mentioned compounds, combinations, methods and uses) may not only translate optical nanoparticles for *in vivo* sensing, but also provide ultrasensitive urine tests for early diagnosis of a pathological condition in a subject.

As will be appreciated, the biomarker responsive moieties may be selective for specific conditions. The conditions associated with particular biomarker responsive moieties are set out in the table below.

i) Reactive oxygen species		
Superoxide anion	Peroxynitrite	
		
ii) Cancer-related biomarkers		
β -galactosidase	Nitroreductase	Alanine aminopeptidase
		
γ -glutamyl transpeptidase	Cathepsin B	Caspase-3
		
Urokinase		

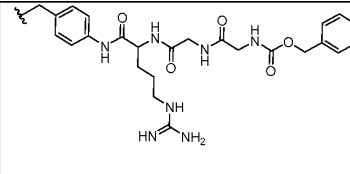
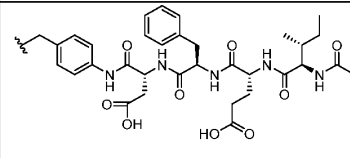
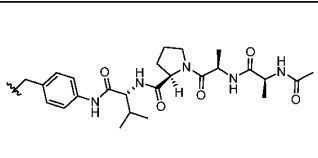
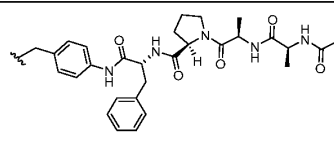
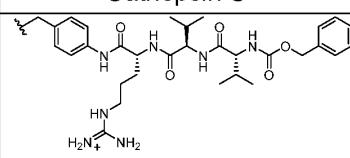
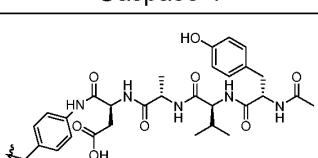
		
iii) Immune-related biomarkers		
Granzyme B	Neutrophil Elastase	Cathepsin G
		
Cathepsin S	Caspase-1	
		

Table AA

Further aspects and embodiments of the invention will now be discussed by reference to the following non-limiting examples.

5

Examples

Materials

All chemicals were purchased from Sigma-Aldrich or Tokyo Chemical Industry (TCI) unless otherwise stated. Con-A, CsA, CA-074, Tac, LPSs, gamma-glutamyl transferase, carboxylesterases, bicinchoninic acid protein assay kit, ALT activity assay, AST activity assay kit, ALP activity assay kit, creatinine assay kit and urea assay kit were purchased from Sigma-Aldrich. Alanine aminopeptidase and recombinant caspase-3 were purchased from R&D Systems. Granzyme B (GzmB) was purchased from Novoprotein Scientific Inc. Mouse TNF- α (430904), IFN- γ (430804) and IL-6 (431304) enzyme-linked immunosorbent assay (ELISA) kits were purchased from BioLegend. Mouse IL-2 (ab223588) was purchased from Abcam. Rat TNF- α (ab236712), IFN- γ (ab46107), IL-2 (ab100769), IL-4 (ab100770) and IL-6 (ab234570) ELISA kits were purchased from Abcam. FITC (fluorescein isothiocyanate) anti-mouse CD3 (catalogue number 100204, dilution 1:50), phycoerythrin (PE) anti-mouse CD8 (catalogue number 100708, dilution 1:80), purified anti-mouse CD16/32 (catalogue number 156604, dilution 1:200), Alexa Fluor 700 anti-mouse CD45 (catalogue number 103128, dilution 1:200), peridinin-chlorophyll-protein anti-mouse CD4 (catalogue number 100538, dilution

1:80), allophycocyanin (APC) anti-human/mouse GzmB (catalogue number 372204, dilution 1:20) and BV510 anti-mouse/human CD44 (catalogue number 103043, dilution 1:40) were purchased from BioLegend. Live/Dead Fixable Blue Dead Cell Stain was purchased from Thermo Fisher Scientific (catalogue number L23105, dilution 1:1000). Mouse GzmB
 5 monoclonal antibody (catalogue number sc-8022, dilution 1:200) was purchased from Santa Cruz Biotechnology. Secondary antibody Alexa Fluor 488-conjugated goat anti-mouse IgG was purchased from Thermo Fisher Scientific (catalogue number A28175, dilution 1:1000). Anti-rat CD16/32 (catalogue number 550270, dilution 1:200), BV510 fixable viability stain (catalogue number 564406, dilution 1:1000), BV786 anti-rat CD45 (catalogue number 740914,
 10 dilution 1:100), FITC anti-rat CD3 (catalogue number 559975, dilution 1:200), APC anti-rat CD8a (catalogue number 200610, dilution 1:200), BV421 anti-rat CD4 (catalogue number 100563, dilution 1:400) and BV650 anti-rat CD44 (catalogue number 740455, dilution 1:100) were purchased from BD Biosciences. PE anti-rat GzmB (catalogue number 130-116-486, dilution 1:50) was purchased from Miltenyi Biotec. Rat GzmB monoclonal antibody (catalogue
 15 number Sc-8022) was purchased from Santa Cruz Biotechnology. Secondary antibody Alexa Fluor 488-conjugated goat anti-rat IgG was purchased from Thermo Fisher Scientific (catalogue number A11006, dilution 1:1000). Ultrapure water was supplied by a Milli-Q Plus System (Millipore). Dulbecco's modified Eagle medium (DMEM) and FBS were purchased from GIBCO. RBC Lysis Buffer was purchased from Invitrogen.

20

Mouse colon carcinoma line CT26, mouse macrophage cell line RAW 264.7 cells and mouse hepatocyte cell line AML-12 were purchased from the American Type Culture Collection (ATCC).

25 Analytical techniques

Column chromatography

Silica gel (Silicycle, 230-400 mesh) was used for column chromatography.

Thin layer chromatography (TLC)

30 Thin layer chromatography (TLC) was carried out on Merck Silica gel 60 F-254 Glass plates.

Ultraviolet-visible (UV-vis) spectroscopy

UV-vis spectra were recorded on a Shimadzu UV-2450 spectrophotometer using quartz cuvettes (1 cm path length) or Thermo Scientific NanoDrop 2000.

35

Fluorescence spectroscopy

Fluorescence spectra were acquired with a Fluorolog 3 time-correlated single photon counting spectrofluorometer (Horiba Jobin Yvon) using quartz cuvettes (1 cm path length) or Thermo Scientific NanoDrop 2000.

5 *Fluorescence imaging*

Fluorescence imaging was performed on the IVIS spectrumCT (PerkinElmer, Inc, USA) for mice and IVIS Lumina III (PerkinElmer, Inc, USA) for rats.

High performance liquid chromatography (HPLC)

- 10 HPLC analyses and purification were performed on an Agilent 1260 system, using acetonitrile (CH₃CN) with 0.1% trifluoroacetic acid (TFA)/water (H₂O) with 0.1% TFA as the eluent.

Nuclear magnetic resonance (NMR) spectroscopy

- Proton-nuclear magnetic resonance (¹H NMR) spectra were conducted with a Bruker 300 MHz
15 NMR instrument. Chemical shifts are reported in ppm relative to residual protic solvent resonances. Mestre Nova LITE v5.2.5-4119 software (Mestre lab Research S.L.) was used to analyze the NMR spectra. Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet) or m (multiplet). Coupling constants are reported as a J value in hertz (Hz). The number of protons (n) for a given resonance is indicated nH based on the spectral integration values.

20

Electrospray ionization-mass spectrometry (ESI-MS)

ESI-MS spectra were obtained on a Thermo Finnigan Polaris Q quadrupole ion trap mass spectrometer (ThermoFisher Corporation) equipped with a standard ESI source.

- 25 *Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry*

MALDI-TOF analyses were conducted on a Bruker ultraflex TOF/TOF instrument.

pH measurement

- The pH values were tested using a digital pH-meter (SevenCompact S220, Zurich,
30 Switzerland).

Gel-permeation chromatography (GPC)

GPC analyses were performed on an Agilent 1260 system, using tetrahydrofuran as the eluent.

- 35 *DLS*

DLS and zeta potential were measured on a Malvern Nano-ZS particle sizer.

TEM

TEM images were captured using a JEM 1400 transmission electron microscope (JEOL, Tokyo, Japan).

5 General procedure for cytokine detection

Blood was sampled from living mice or rats at indicated timepoints in heparinized capillary tubes and serum was isolated by centrifugation for analysis. Levels of TNF- α , IFN- γ , IL-2, IL-4, and IL-6 were measured by ELISA kits according to the manufacturer's protocol.

10 General procedure for tissue imaging

Tissues were cut into sections using a cryostat (Leica, Germany). The tissue sections were examined using a Nikon ECLIPSE 80i microscope (Nikon Instruments Inc, USA). Confocal fluorescence microscopy images of tissue sections were acquired on a LSM800 confocal laser scanning microscope (Carl Zeiss, Germany).

15

General procedure for collection of blood samples

Blood samples were collected using heparinized capillary tubes (Paul Marienfeld, Germany).

General procedure for collection of urine samples

20 Urine samples were collected with metabolic cages (Lab Products Inc, USA).

General procedure for histological studies

All tissues were fixed with 4% paraformaldehyde (PFA), dehydrated in a series of ethanol solution, embedded in paraffin and cut into sections with a thickness of 10 μ m for H&E staining.

25 The sections were washed with xylene and ethanol and then immersed in hematoxylin working solution for 5 min and eosin working solution for 3 min, followed by washing with distilled water. The stained sections were examined using a Nikon ECLIPSE 80i microscope. For immunofluorescence staining, liver and kidney tissues were fixed with 4% PFA, dehydrated using 30% sucrose solution, embedded in frozen optimal cutting temperature (O.C.T.) medium, and then cut into sections with a thickness of 10 μ m. The sections were dried at room temperature for 60 min, washed three times using PBS containing 0.1% Triton X-100, and incubation with 3% bovine serum albumin (BSA) solution at room temperature for additional 60 min, followed by washing with PBS. The sections were then incubated with anti-GzmB antibody for 60 min at 37 °C. After being washed three times with PBS to remove unbound antibody, the sections were counterstained with respective secondary antibody for 60 min at 35 room temperature. Next, the cell nuclei were stained with DAPI. The stained sections were imaged using a LSM800 confocal laser scanning microscope.

General procedure for flow cytometry analysis

For the analysis of GzmB level in leukocytes in mice, liver, spleen, blood and lymph nodes were harvested from mice at different times after treatment with con-A and prepared as single-cell suspensions. Briefly, liver was minced, gently grounded in ice-cold 1× PBS and filtered through a 70 µm strainer. Red blood cells in collected cell suspension were removed using eBioscience 1× RBC Lysis Buffer. The immune cells in liver were then isolated by density gradient centrifugation of a single-cell suspension with 40% and 70% Percoll (850 g, 20 min), followed by washing with ice-cold 1× PBS. Spleen and lymph nodes were gently ground in ice-cold 1× PBS with a syringe plunger and filtered through a 70 µm cell strainer to afford a single-cell suspension. Red blood cells in a single-cell suspension of spleen were removed with eBioscience 1× RBC Lysis Buffer. Immune cells in blood were isolated by density gradient centrifugation with Histopaque 1077 (400 g, 30 min). After single-cell suspension, the collected cells from liver, spleen, blood and lymph nodes were blocked with anti-mouse CD16/32 and stained with Alexa Fluor 700 anti-mouse CD45, Live/Dead Fixable Blue Dead Cell Stain, FITC anti-mouse CD3, PE anti-mouse CD8, peridinin-chlorophyll-protein anti-mouse CD4, APC anti-human/mouse GzmB and BV510 anti-mouse/human CD44 according to the vendors' protocols. For the analysis of GzmB expression in CD8⁺ T cells in rats, liver, spleen, blood and lymph nodes were harvested from rats at different times after transplantation and subjected to single-cell suspension as described above. Thereafter, the cells collected from these organs were blocked with anti-rat CD16/32 and stained with BV786 anti-rat CD45, BV510 fixable viability stain, FITC anti-rat CD3, APC anti-rat CD8a, BV421 anti-rat CD4, BV650 anti-rat CD44 and PE anti-rat GzmB. The stained cells were analysed using flow cytometry.

General procedure for statistical analysis

The *in vitro*, *in vivo* and *ex vivo* fluorescence signals were quantified with region of interest analysis using Living Image 4.3 software. Results are expressed as the mean ± s.d. unless stated otherwise. Investigators were blinded to group allocation during experiments. Statistical comparisons were determined between two groups by t-test and among three groups or more by one-way analysis of variance. For all tests, P values less than 0.05 were considered statistically significant (*P < 0.05, **P < 0.01 and ***P < 0.001). All statistical calculations were performed using GraphPad Prism 8.0, including assumptions of tests used (GraphPad Software).

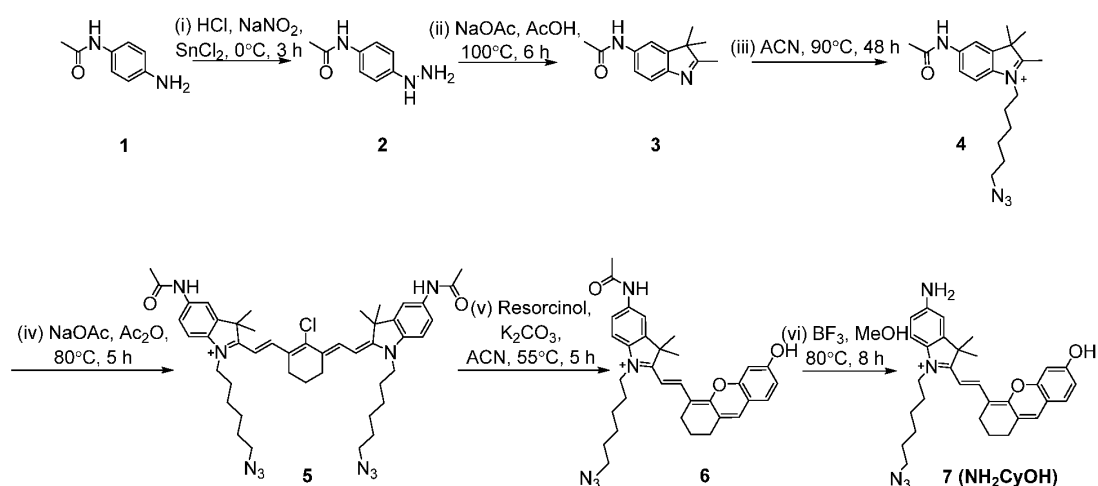
Example 1. Development of activatable polymeric nanoreporters (APNs) that possess biomarker-triggered renal clearance and fluorogenic response for noninvasive near-infrared fluorescence (NIRF) *in vivo* imaging and urinalysis of diseases

APNs comprise three key building blocks (FIG. 1b): a protease-reactive peptide brush, a cascaded self-immolative linker, a caged fluorophore unit with a renal clearance moiety and/or a targeting moiety. The fluorophore units are connected with the self-immolative linkers to form the polymer backbone which is grafted by the peptide brushes also via the self-immolative linkers. APNs are thus amphiphilic and spontaneously assembled into nanoparticles in aqueous solution. At intrinsic state, APNs are nonfluorescent because the phenol group of the fluorophore unit is caged to inhibit its electron-donating ability. At activated state, the disease-overexpressed protease cleaves the peptide brushes and induces cascade self-elimination to depolymerize the backbone of APNs, releasing the renal-clearable fluorophore fragments (FIG. 1b,d) for real-time NIRF imaging and urinalysis (FIG. 1a,b). Such protease-initiated fragmentation not only converts APNs from nanoparticles to small-molecule fragments, but also uncage the phenol group of the renal-clearable fluorophores, leading to a sensitive fluorogenic turn-on response. Therefore, APNs bypass the slow clearance of nanoparticles and shallow tissue-penetration of light, implementing noninvasive *in vivo* optical detection for early diagnosis. Further, owing to catalytic signal amplification of enzymatic depolymerization and high signal-to-background ratio of fluorescence turn-on detection, APNs is sensitive for early diagnosis.

Example 2. Syntheses of APNs

APNs were constructed via polycondensation of the fluorophore unit (NH_2CyOH) with an azide group on the alkyl chain and the cascaded self-immolative linker (4-aminophenyl conjugated phenylene dimethanol) with an aniline group (Fig. 1d). The two proteases CatB and GzmB respectively associated with tumor progression and lymphocyte activation in allograft rejection were chosen as the biomarker for construction of APNs. The aniline group of self-immolative linker was caged with a dipeptide Acetyl-Phe-Lys (Ac-FK) or a tetrapeptide Acetyl-Ile-Glu-Phe-Asp (Ac-IEFD) (FIG. 1c), which are the specific substrates of CatB (Verdoes, M. *et al.*, *J. Am. Chem. Soc.* **2013**, 135, 14726-14730) and GzmB (He, S. *et al.*, *J. Am. Chem. Soc.* **2020**, 142, 7075-7082), respectively. These peptide-conjugated self-immolative monomers were respectively polymerized with NH_2CyOH to form polymer C and G. The azide group on the side chain of polymer C/G was further conjugated with the alkyne-functionalized HP β CD or alkyne-functionalized cyclic arginine-glycine-aspartate motif (cRGDfK) via a click reaction to yield APN_C and APN_G, respectively.

Synthesis of fluorophore unit (NH_2CyOH)



Compound 2

- 5 A mixture of N-(4-aminophenyl) acetamide (compound 1, 3000 mg, 20 mmol) and HCl (12 M, 10 mL) was stirred in ice bath. Sodium nitrite (1380 mg, 20 mmol) dissolved in HCl (10 mL) was added drop wise and stirred for 1 h. Then, tin chloride (14460 mg, 80 mmol) in HCl (10 mL) was added to the above mixture and stirred for another 2 h in the ice bath. The precipitated yellow solid was filtered and dried to obtain compound 2 (3200 mg, 80% yield), which was
- 10 used in the next step without further purification.

¹H NMR (300 MHz, CD₃OD): δ 2.14 (s, 3H), 7.00 (d, *J* = 6 Hz, 2H), 7.54 (d, *J* = 6 Hz, 2H). ESI-MS (*m/z*): calcd: 165.09, found: 165.75.

Compound 3

- 15 A mixture of compound 2 (3200 mg, 16 mmol), 3-methylbutan-2-one (1720 mg, 20 mmol), glacial acetic acid (30 mL) and sodium acetate (1600 g, 19 mmol) was refluxed under 100 °C for 6 h. Then, the hot solution was cooled to room temperature and concentrated. The residues were purified by silica gel column chromatography to afford compound 3 (2940 mg, 85% yield).

20

¹H NMR (300 MHz, CD₃OD): δ 1.32 (s, 6H), 2.13 (s, 3H), 2.28 (s, 3H), 7.36 (s, 2H), 7.71 (s, 1H). ESI-MS (*m/z*): calcd: 216.13, found: 216.85.

Compound 4

A mixture of compound 3 (2940 mg, 13.6 mmol) and 1-azido-6-bromohexane (3090 mg, 15 mmol) was stirred at 90 °C in acetonitrile in a 50 ml round-bottom flask. After 48 h, the residue was purified by HPLC to give compound 4 (3256 g, 70%).

- 5 ¹H NMR (300 MHz, CDCl₃): δ 1.52 (m, 12H), 1.91 (m, 2H), 2.22 (s, 3H), 2.74 (s, 3H), 3.27 (t, 2H), 4.39 (t, 2H), 7.46 (d, *J* = 9 Hz, 1H), 7.77 (d, *J* = 9 Hz, 1H), 8.14 (s, 1H), 9.61 (s, 1H). ESI-MS (*m/z*): calcd: 342.23, found: 342.64.

Compound 5

- 10 A mixture of compound 4 (3078 mg, 9.0 mmol), (*E*)-2-chloro-3-(hydroxymethylene) cyclohex-1-ene-1-carbaldehyde (765 mg, 4.5 mmol) and anhydrous sodium acetate (1170 mg, 13.5 mmol) in acetic anhydride (30 ml) was refluxed at 80 °C for 5 h. The reaction mixture was cooled and concentrated under reduced pressure. The residue was purified using silica gel column chromatography to afford compound 5 as a green solid (4800 mg, 65% yield).

- 15 ¹H NMR (300 MHz, CDCl₃): δ 1.45 (s, 2H), 1.63 (s, 8H), 1.69 (s, 12H), 1.81 (m, 8H), 2.32 (m, 6H), 2.61 (s, 4H), 3.28 (t, 4H), 3.98 (s, 4H), 5.98 (d, *J* = 15 Hz, 2H), 6.96 (d, *J* = 9 Hz, 2H), 7.96 (d, *J* = 9 Hz, 2H), 8.11 (s, 2H), 8.28 (d, *J* = 12 Hz, 2H), 10.76 (s, 2H). ESI-MS (*m/z*): calcd: 819.46, found: 819.76.

20

Compound 6

- A mixture of potassium carbonate (840 mg, 6.0 mmol) and resorcinol (330 mg, 3.0 mmol) in acetonitrile (15 ml) was stirred at 55 °C for 30 min. Then, a solution of compound 5 (1640 mg, 2 mmol) in acetonitrile (15 ml) was added dropwise to the above mixture. The reaction mixture
25 was stirred at 55 °C for an additional 5 h. After the solvent was evaporated under reduced pressure, the crude product was purified by silica gel column chromatography to afford compound 6 (773 mg, 70% yield).

- 30 ¹H NMR (300 MHz, CDCl₃): δ 1.45 (m, 6H), 1.86 (s, 6H), 1.92 (s, 4H), 2.27 (s, 3H), 2.58 (t, 2H), 2.73 (t, 2H), 3.28 (t, 2H), 4.07 (t, 2H), 6.05 (d, *J* = 15 Hz, 1H), 6.96 (d, *J* = 6 Hz, 1H), 7.07 (d, *J* = 9 Hz, 2H), 7.34 (m, 2H), 7.44 (d, *J* = 9 Hz, 1H), 7.75 (s, 1H), 8.14 (d, 1H), 8.60 (d, *J* = 15 Hz, 1H), 9.71 (s, 1H). ESI-MS (*m/z*): calcd: 552.30, found: 552.56.

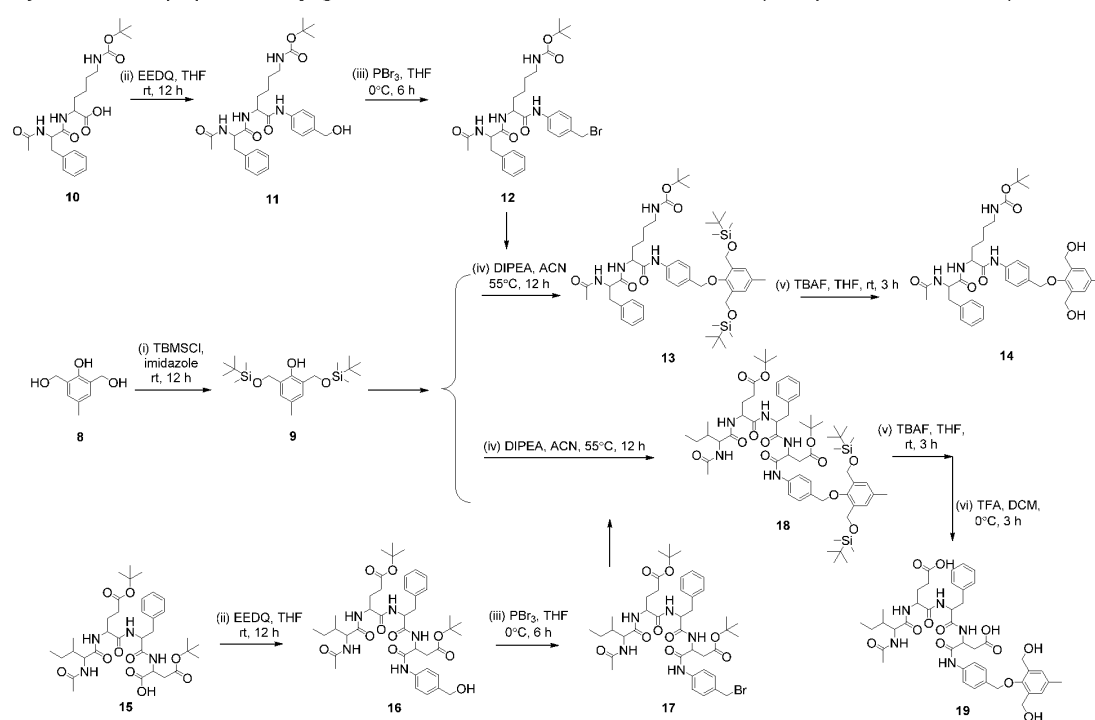
Compound 7

- 35 A mixture of compound 6 (773 mg, 1.4 mmol) and boron trifluoride methanol (4400 μL, 28 mmol) was stirred at 80 °C under nitrogen atmosphere for 8 h. After the solvent was

evaporated under reduced pressure, the crude product was purified using HPLC to give compound 7 (510 mg, 71%).

^1H NMR (300 MHz, CD_3OD): δ 1.58 (m, 8H), 1.79 (s, 6H), 1.91 (m, 4H), 2.74 (m, 4H), 4.30 (t, 2H), 6.40 (d, J = 15 Hz, 1H), 6.80 (s, 2H), 7.06 (s, 1H), 7.17 (s, 1H), 7.40 (m, 3H), 8.62 (d, 1H).
ESI-MS (m/z): calcd: 510.29, found $[\text{M}+\text{H}^+]$: 511.56.

Syntheses of peptide-conjugated cascaded self-immolative linker (compound 14 and 19)



10

Compound 9

A mixture of 2,6-bis(hydroxymethyl)-p-cresol (compound 8, 2000 mg, 12 mmol) and imidazole (1620 mg, 24 mmol) was dissolved in dimethylformamide (DMF, 15 mL) and stirred in ice bath.
After 30 min, *tert*-butyldimethylsilyl chloride (3650 mg, 24 mmol) was added to the reaction mixture and stirred at room temperature for 12 h. The mixture was diluted with diethyl ether and washed with saturated ammonium chloride solution. Then, the organic phases were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography to afford compound 9 (3960 mg, 83%).

20

¹H NMR (300 MHz, CDCl₃): δ 0.12 (s, 12H), 0.94 (s, 18H), 2.26 (s, 3H), 4.82 (s, 4H), 6.90 (s, 2H), 8.03 (s, 1H). ESI-MS (*m/z*): calcd: 396.25, found: 396.29.

Compound 10

- 5 Peptide 10 (Ac-FK, 2176 mg, 5.0 mmol) was prepared by solid phase peptide synthesis.

¹H NMR (300 MHz, CDCl₃): δ 1.20-1.44 (m, 12H), 1.65-1.97 (m, 5H), 3.03 (m, 4H), 4.44 (m, 2H), 7.16 (m, 4H), 7.42 (m, 1H), 11.09 (s, 2H). ESI-MS (*m/z*): calcd: 435.24, found [M+H⁺]: 436.36.

10

Compound 11

- To a solution of compound 10 (435 mg, 1.0 mmol) in tetrahydrofuran (THF, 20 mL) were added (4-aminophenyl) methanol (490 mg, 4.0 mmol) and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (960 mg, 4.0 mmol). The reaction mixture was stirred for 12 h at room temperature before it was concentrated under reduced pressure. The residue was washed with distilled water and extracted with dichloromethane (DCM). The organic layer was further washed with brine, dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The residue was purified using HPLC to give compound 11 (405 mg, 75% yield).

- 20 ¹H NMR (300 MHz, CD₃OD): δ 1.29 (m, 2H), 1.41 (s, 9H), 1.50 (m, 2H), 1.75 (m, 2H), 1.92 (s, 3H), 3.01 (m, 4H), 4.42 (m, 1H), 4.57 (s, 2H), 4.64 (t, 1H), 7.20 (m, 5H), 7.30 (d, *J* = 9 Hz, 2H), 11.09 (d, *J* = 9 Hz, 2H). ESI-MS (*m/z*): calcd: 540.20, found: 540.41.

Compound 14

- 25 To a solution of compound 11 (405 mg, 0.75 mmol) in dry THF (10 mL), phosphorus tribromide (540 mg, 2.0 mmol) was added. The reaction mixture was stirred for 6 h in an ice bath and then it was quenched with distilled water followed by extraction with DCM. The organic layer was washed with brine, dried over anhydrous magnesium sulfate and concentrated under reduced pressure to afford compound 12 (360 mg, 80% yield), which was used in the next step without further purification. To a solution of compound 12 (360 mg, 0.6 mmol) in acetonitrile (8 ml) were added compound 9 (396 mg, 1.0 mmol) and *N,N*-diisopropylethylamine (160 µl, 1.22 mmol). After the reaction mixture was stirred at 55 °C for 12 h, it was poured into distilled water, extracted by DCM and concentrated under a vacuum to yield compound 13, which was used in the next step without further purification. Compound 13 (459 mg, 0.5 mmol)
- 35 and tetrabutylammonium fluoride (1.0 M in THF, 1.5 ml, 1.5 mmol) were dissolved in anhydrous THF (6 ml) and stirred for 3 h at room temperature. The reaction mixture was poured into diluted hydrogen chloride (HCl) aqueous solution, extracted with ethyl acetate and

concentrated under a vacuum. Purification of the residue by silica gel column chromatography afforded compound 14 (138 mg, 40% yield).

¹H NMR (300 MHz, CDCl₃): δ 1.28 (s, 9H), 1.71 (m, 6H), 2.00 (s, 3H), 2.23 (s, 3H), 2.82 (m, 4H), 4.10 (m, 2H), 4.55 (m, 2H), 4.77 (s, 4H), 6.87 (s, 2H), 7.10 (m, 1H), 7.52 (m, 4H), 7.69 (m, 4H). ESI-MS (*m/z*): calcd: 690.36, found: 690.25.

Compound 15

Peptide 15 (Ac-IEFD, 3382 mg, 5.0 mmol) was prepared by solid phase peptide synthesis according to the inventors' previous study (Kwon, E. J., Dudani, J. S. & Bhatia, S. N., *Nat. Biomed. Eng.* **2017**, 1, 0054).

¹H NMR (300 MHz, CDCl₃): δ 0.90 (m, 6H), 1.22 (m, 4H), 1.44 (s, 18H), 1.75-2.00 (m, 4H), 2.09 (s, 3H), 2.85 (s, 2H), 3.33 (m, 2H), 4.41 (m, 2H), 4.76 (m, 1H), 7.18 (m, 5H). ESI-MS (*m/z*): calcd: 676.37, found [M+H⁺]: 677.31.42.

Compound 16

To a solution of compound 15 (676 mg, 1.0 mmol) in THF (20 mL) were added (4-aminophenyl) methanol (490 mg, 4.0 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (960 mg, 4.0 mmol). The reaction mixture was stirred for 12 h at room temperature before it was concentrated under reduced pressure. The residue was washed with distilled water and extracted with DCM. The organic layer was further washed with brine, dried over anhydrous magnesium sulphate and concentrated under reduced pressure. The residue was purified using HPLC to give compound 16 (473 mg, 70% yield).

¹H NMR (300 MHz, CD₃OD): δ 0.90 (m, 6H), 1.80 (s, 18H), 1.84 (m, 2H), 2.04 (s, 3H), 2.19 (m, 2H), 2.65-3.25 (m, 6H), 4.14 (m, 3H), 4.57 (s, 3H), 7.23 (m, 5H), 7.30 (d, *J* = 9 Hz, 2H), 7.59 (d, *J* = 9 Hz, 2H). ESI-MS (*m/z*): calcd: 781.43, found: 781.74.

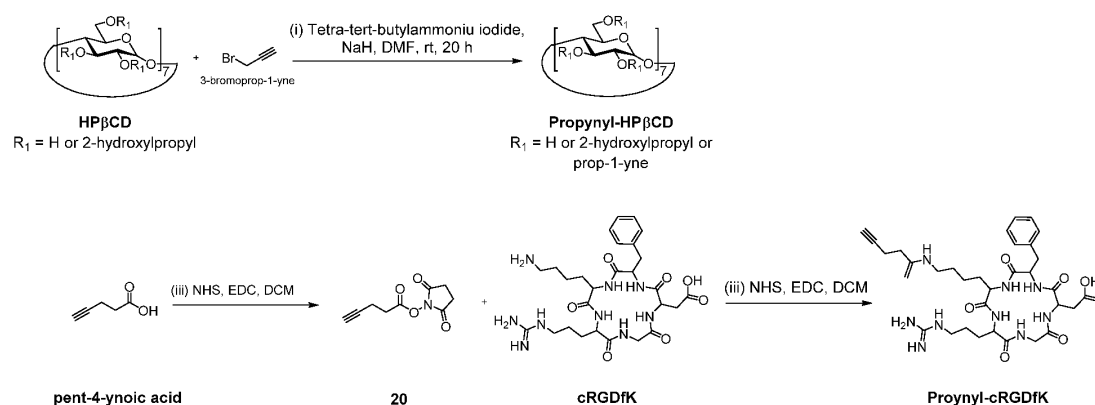
Compound 19

To a solution of compound 16 (390 mg, 0.5 mmol) in dry THF (10 mL) was added phosphorus tribromide (540 mg, 2.0 mmol). The reaction mixture was stirred for 6 h in ice bath and then it was quenched with distilled water followed by extraction with DCM. The organic layer was washed with brine, dried over anhydrous magnesium sulphate and concentrated under reduced pressure to afford compound 17 (590 mg, 70% yield), which was used in the next step without further purification. To a solution of compound 17 (422 mg, 0.5 mmol) in acetonitrile (8 mL) were added compound 9 (396 mg, 1.0 mmol) and *N,N*-diisopropylethylamine

(160 μ l, 1.22 mmol). After the reaction mixture was stirred at 55 °C for 12 h, it was poured into distilled water, extracted by DCM and concentrated under a vacuum to yield compound 18, which was used in the next step without further purification. Compound 18 (580 mg, 0.5 mmol) and tetrabutylammonium fluoride (1.0 M in THF, 1.5 ml, 1.5 mmol) were dissolved in anhydrous THF (6 ml) and stirred for 3 h at room temperature. The reaction mixture was poured into diluted HCl aqueous solution, extracted with ethyl acetate and concentrated under vacuum. The residue and trifluoroacetic acid (5 mL) in DCM (5 mL) were stirred in an ice bath under nitrogen atmosphere for 3 h. After the solvent was evaporated under reduced pressure, the crude product was purified using HPLC to give compound 19 (123 mg, 30% yield).

^1H NMR (300 MHz, CDCl_3): δ 0.89 (m, 6H), 2.02 (m, 3H), 2.16 (s, 3H), 2.32 (m, 2H), 2.37 (s, 3H), 2.75-3.25 (m, 6H), 3.93 (m, 2H), 4.12 (m, 3H), 4.22 (m, 3H), 4.56 (s, 2H), 7.23 (m, 7H), 7.56 (m, 3H), 7.74 (s, 1H), 10.26 (s, 2H). ESI-MS (m/z): calcd: 819.37, found: 820.47.

15 Syntheses of renal clearance moiety propynyl-HP β CD and tumor targeting moiety propynyl-cRGD



20 Propynyl-HP β CD

Propynyl-HP β CD was synthesized according to the inventors' previous study (Huang, J. *et al.*, *Nat. Mater.* **2019**, *18*, 1133-1143).

Propynyl-cRGD

25 Pent-4-ynoic acid (98 mg, 1.0 mmol), N-hydroxysuccinimide (138 mg, 1.2 mmol), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (186 mg, 1.2 mmol) were dissolved in anhydrous DCM (6 mL) and stirred for 6 h at room temperature. The reaction mixture was poured into water, extracted with ethyl acetate and concentrated under a vacuum. Purification of the

residue by silica gel column chromatography afforded compound 20. Compound 20 (24 mg, 0.12 mmol) and cRGD (60 mg, 0.1 mmol) were dissolved in anhydrous THF (6 mL) and stirred for 6 h at room temperature. The reaction mixture was purified using HPLC to give the compound propynyl-cRGD (55 mg, 80% yield).

5

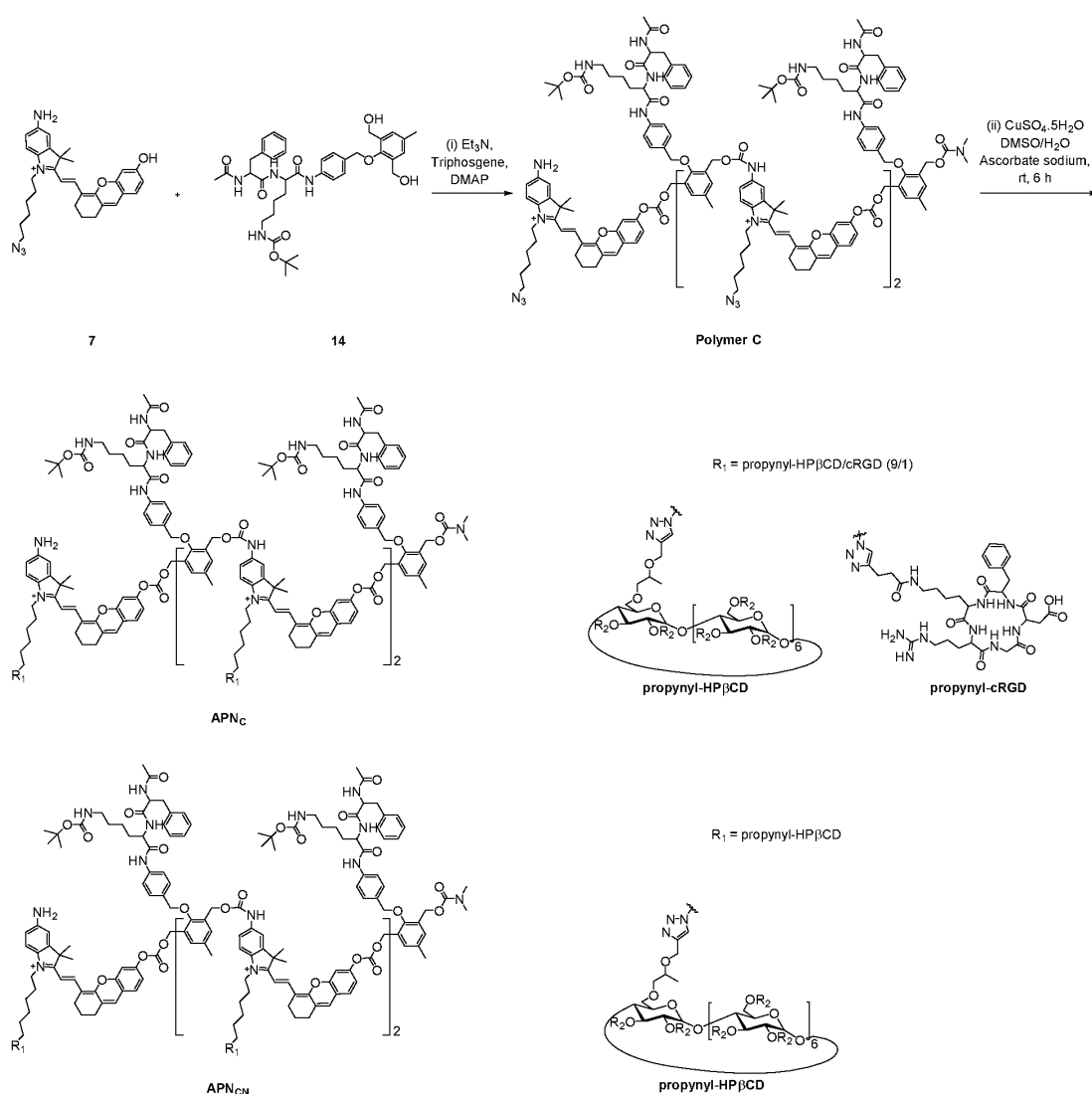
^1H NMR (300 MHz, CD_3OD): δ 1.01 (m, 2H), 1.34 (m, 5H), 1.65 (m, 2H), 1.85 (m, 1H), 2.26 (t, 1H), 2.36 (m, 2H), 2.43 (m, 2H), 2.58 (m, 1H), 2.79 (m, 2H), 2.96 (m, 3H), 3.10 (m, 4H), 3.94 (m, 1H), 4.25 (m, 2H), 4.51 (m, 1H), 4.74 (m, 1H), 7.27 (m, 5H), 7.80-8.50 (m, 4H). ESI-MS (m/z): calcd: 683.34, found $[\text{M}+\text{H}^+]$: 684.30.

10

General procedure for synthesis of polymer APN_C , APN_G and APN_CN

Compound 7 (16 mg, 0.03 mmol) and triethylamine (25 mg, 0.25 mmol) were added dropwise to a solution of triphosgene (75 mg, 0.25 mmol) in anhydrous DCM in an ice bath under argon atmosphere. After 30 min, the reaction mixture was concentrated under vacuum to remove residual triphosgene, followed by adding dropwise a solution of 4-dimethylaminopyridine (61 mg, 0.5 mmol) and compound 14 or 19 (0.05 mmol). After 10 min, dimethylamine (2 μL in 100 μL anhydrous DCM) was added and the reaction mixture was stirred for an additional 10 min, then poured into H_2O , extracted using ethyl acetate and concentrated under a vacuum to yield polymer C or G. The polymer C or G (7.6 mg or 8.4 mg) and propynyl-substrate (propynyl-HP β CD/propynyl-cRGD = 0.9/0.1 mole ratio for preparing APN_C and propynyl-HP β CD for preparing APN_CN and APN_G) was dissolved in dimethylsulfoxide/water (4/1, v/v), followed by addition of a solution of sodium ascorbate (2.0 mg, 0.01 mmol) and cupric sulfate (2.5 mg, 0.01 mmol) in distilled water. After the mixture was stirred at room temperature under a nitrogen atmosphere in the dark for 6 h, the reaction mixture was purified by dialysis in distilled water and freeze dried to afford the corresponding compound as a blue solid.

25



The MALDI-TOF mass spectra of **APN_C** and **APN_{CN}** was performed in the linear mode because they cannot be obtained under the reflector mode. The mass ranges of **APN_C** are identical to the sum of molecular weight of 3 of NH_2CyOH , 3 of compound 14 and 3 of propynyl-HP β CD/propynylcRGD (APN_C or $\text{APN}_{CN} = 3 \times (\text{NH}_2\text{CyOH} + \text{compound 14} + \text{HP}\beta\text{CD/cRGD})$). The experiments were repeated independently three times with similar results.

The considerations of introducing amine-protected lysine in the Ac-FK ligand are two-fold. Firstly, it can reduce the synthetic challenge. To prepare the **APN_C** or **APN_{CN}**, the synthetic routes have been rational designed to protect the amine of lysine in order to yield compound 13, followed by polymerization with NH_2CyOH to form polymer C. Unprotected lysine amine is reactive and can yield by-products on above two steps, and thus the amine-protected lysine

5

10

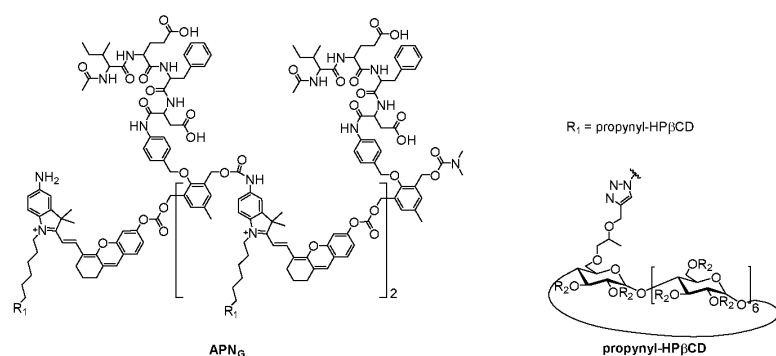
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19

Polymer G

(i) Et_3N ,
Triphosgene,
DMAP

(ii) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$,
DMSO/ H_2O ,
Ascorbate sodium,
rt, 6 h



15

55

1H), 7.15-7.30 (m, 11H), 7.35 (m, 3H), 7.61 (m, 3H), 8.34 (m, 1H). MALDI-TOF MS found: 7,500-10,000.

The structures of intermediates and APNs were affirmed by NMR spectroscopy, gel-permeation chromatography (GPC) and MALDI-TOF mass spectrometry.

Example 3. Characterization, stability and selectivity of APNs

APNs prepared in Example 2 were characterized and taken for stability and selectivity studies.

10

In vitro stability and selectivity studies

APN_C, APN_{CN} and APN_G solutions (10 μ M) were incubated in different buffer solutions with a pH range from 5.0 to 9.0 at 37 °C for 4 h, or FBS for 3 weeks, or the indicated reactive oxygen species (90 μ M), metal ions (90 μ M) and enzymes including caspase-3 (0.5 μ g) in PBS buffer (10 mM, 50 mM NaCl, 0.1% CHAPS, 10 mM ethylenediaminetetraacetic acid (EDTA), 5% glycerol, 1 mM dithiothreitol (DTT), pH 7.4), alanine aminopeptidase (1.0 U) in HEPES buffer (10 mM, pH 7.4), GGT (10 μ M) in PBS (10 mM, pH 7.4) and carboxylesterases (0.5 μ g) in PBS (10 mM, pH 7.4). Fluorescence enhancement of APNs was measured on a fluorescence spectrophotometer after incubation. Unit definition: 1 U of enzyme will hydrolyse 1 μ mol of the corresponding substrate per minute in optimized conditions. The PBS used for these experiments was purged with nitrogen gas for 30 min before the measurement.

20

Results and discussion

The hydrophobic fluorophore backbone and the hydrophilic brushes of APNs made them amphiphilic and thus spontaneously assemble into nanoparticles in aqueous solution with a spherical morphology (as shown by TEM, FIG. 2d,h), with average hydrodynamic diameters of 180 and 170 nm for APN_C and APN_G, respectively (FIG. 3a,b,e,f). No precipitation and obvious change in size were detected for APNs during the storage in PBS or FBS for 3 weeks at least (FIG. 3b,f).

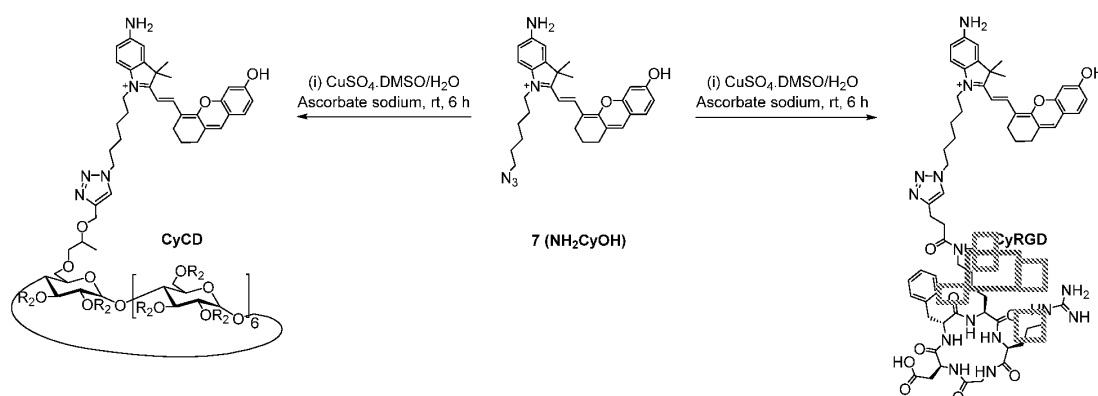
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Example 4. Synthesis of CyCD and CyRGD

The depolymerized fluorescent fragments of the APNs in Example 2 including HP β CD-substituted NH₂CyOH (CyCD) and cRGD-substituted NH₂CyOH (CyRGD) were also synthesized.

35

General procedure for the synthesis of CyCD and CyRGD



- 5 A mixture of compound 7 (10 mg, 0.02 mmol) and propynyl-substrate (propynyl-HPβCD or propynyl-cRGD, 0.024 mmol) was dissolved in dimethylsulfoxide (DMSO)/water (3mL/3mL), followed by addition of a solution of sodium ascorbate (2.0 mg, 0.01 mmol) and cupric sulfate (2.5 mg, 0.01 mmol) in distilled water (0.1mL). After the mixture was stirred at room temperature under a nitrogen atmosphere in dark for 6 h. The reaction mixture was purified by HPLC to afford the corresponding compound as a blue solid.

CyCD: ¹H NMR (300 MHz, D₂O): δ 1.26 (m, 17H), 1.61 (m, 4H), 1.74 (m, 6H), 2.57 (m, 2H), 2.70 (m, 2H), 3.01 (m, 2H), 3.50-4.30 (m, 70H), 4.36 (m, 2H), 4.54 (m, 2H), 5.18-5.34 (m, 10H), 6.93 (m, 4H), 7.16 (m, 4H), 8.54 (d, *J* = 15 Hz, 1H). MALDI-TOF MS found: 1,600-2,200.

15

CyRGD: ¹H NMR (300 MHz, CD₃OD): δ 0.89 (m, 4H), 0.91 (m, 2H), 1.34 (m, 6H), 1.57 (m, 6H), 1.64 (m, 1H), 1.75-2.70 (m, 8H), 2.76 (s, 6H), 2.80-3.30 (m, 9H), 3.97 (m, 1H), 4.32 (m, 2H), 4.42 (m, 1H), 4.57 (t, 1H), 4.86 (m, 1H), 5.36 (t, 1H), 6.49 (d, 1H), 6.79 (m, 2H), 7.25 (m, 5H), 7.44 (m, 1H), 7.97 (m, 1H), 8.20-8.70 (m, 3H). ESI-MS (*m/z*): calcd: 1,193.63, found: 1,193.74.

20

Example 5. Optical measurement of APNs, CyCD and CyRGD

- The optical profiles of APNs (prepared in Example 2), and CyCD and CyRGD (prepared in Example 4) were determined.

25

Preparation of stock solutions

APNs, CyCD and CyRGD were dissolved in ultrapure water to obtain stock solutions. H₂O₂, HOCl, and O₂^{•−} stock solutions were prepared by directly diluting commercially available H₂O₂, NaOCl, and KO₂, respectively. •OH was generated by Fenton reaction between H₂O₂ and Fe(ClO₄)₂. ONOO[−] was generated from 3-morpholiniosydnonimine hydrochloride. Stock solutions of carboxylesterases, gamma-glutamyl transferase, alanine aminopeptidase, caspase-3, MgSO₄, CuSO₄, and FeSO₄ were prepared with distilled water.

Optical measurement

APN_C and APN_{CN} solutions (10 μM) was incubated with CatB (0.5 μg) in buffer solution (25 mM MES, 5 mM DTT, pH 5.5; assay buffer: 25 mM MES, pH 6.0) at 37 °C. APN_G solution (10 μM) were incubated with GzmB (0.5 μg) in buffer solution (50 mM MES, 50 mM NaCl, 5 mM DTT, pH 5.5; assay buffer: 50 mM Tris, pH 7.5) at 37 °C. UV-vis and fluorescence spectra of the solutions were measured on UV-Vis and fluorescence spectrophotometer after 4 h incubation. Fluorescence images were acquired using an IVIS spectrumCT system with excitation at 675 ± 10 nm and emission at 720 ± 10 nm and an acquisition time of 1 s. The sensing capability of APNs was analyzed through HPLC. UV-Vis and fluorescence spectra of the CyCD and CyRGD solution (30 μM) in PBS (10 mM, pH 7.4) were recorded on UV-Vis and fluorescence spectrophotometers. Fluorescence quantum yields were measured using indocyanine green as a standard (Φ = 13% in DMSO, Benson, R. C. & Kues, H. A., *J. Chem. Eng. Data* **1977**, 22, 379–383).

Enzyme kinetic assay

Various concentrations of APN_C, APN_{CN} and APN_G (2.5, 5, 10, 20, 40 or 60 μM) were incubated with CatB (0.5 μg) and GzmB (0.5 μg), respectively, at 37 °C for 15 min. After incubation, the mixtures were analyzed using HPLC for quantification. The initial reaction velocity (nM min^{−1}) was calculated, plotted against the concentration of APN_C, APN_{CN} or APN_G, and fitted to a Michaelis-Menten curve. The kinetic parameters were calculated by use of the Michaelis-Menten equation (Gu, K. *et al.*, *J. Am. Chem. Soc.* **2016**, 138, 5334-5340):

$$V = V_{\max}[S]/(K_m + [S]) \quad (1)$$

where V is the initial velocity, and [S] is substrate concentration.

Measurement of fluorescence quantum yields

ICG was used as a standard with a known fluorescence quantum yield (Φ) value of 13% in DMSO (Benson, R. C. & Kues, H. A., *J. Chem. Eng. Data* **1977**, 22, 379-383). Fluorescence quantum yields were calculated using the following equation: $\Phi_s/\Phi_f = (A_s/A_f) \times (Abs_s/Abs_f) \times (\eta_s^2/\eta_f^2)$, Where Φ_s and Φ_f are the fluorescence quantum yields of the standard and the samples, respectively; A_s and A_f are the emission areas of the standard and the samples,

respectively; Abs_s and Abs_f are the absorbance of the standard and the samples at the wavelength of excitation; n_s and n_f are the refractive indices of the standard and the samples, respectively.

5 Calculation of partition coefficients

In silico calculation of the partition coefficients (Log D at pH 7.4) was conducted using Marvin and JChem calculator plug-ins (ChemAxon, Hungary).

Results and discussion

10 The same fluorophore backbone endowed APN_C and APN_G with nearly identical optical profiles with an absorption maximum at 600 nm and were initially non-fluorescence with a low fluorescent quantum yield of 0.07% in PBS (FIG. 2a,e and 3c,g). This is because the fluorophore NH₂CyOH were in a 'caged' state wherein the electron-donating ability of the aromatic hydroxyl group was diminished. In response to their respective protease, the
15 absorption spectra of APNs changed, with the appearance of a new peak at 700 nm (FIG. 3c,g); meanwhile, the fluorescence at 720 nm increased by ~11-fold for APN_C and APN_G (FIG. 2a,e and 3k,l and Table 1), with the fluorescence quantum yield increased to 2.1% (7-fold higher than 0.3% of ICG in water). These optical features were identical to those of CyCD having a strong electron-donating phenolate group on the fluorophore ('uncaged' state). This
20 proved the depolymerization of APNs to form CyCD or CyRGD due to enzymatic cleavage of the amide linkage between the peptide substrate and the self-immolative linker, followed by a 1,6-elimination and 1,4-elimination. The cleavage of peptide substrate was further confirmed by detection of new HPLC peaks (FIG. 2c,g). TEM images showed their sizes decreased dramatically to less than 3 nm after protease-induced depolymerization (FIG. 2d,h). APNs
25 were negatively charged (FIG. 3m). The catalytic efficiencies (K_{cat}/K_m) of CatB towards APN_C, and GzmB towards APN_G were calculated to be 7 and 0.4 M⁻¹ s⁻¹, respectively (FIG. 3d,h). Additionally, both APNs barely changed their NIR fluorescence over a pH range from 5.0 to 9.0 or in the presence of other interfering analytes including reactive oxygen species, other enzymes and metal ions, proving their high stability and specificity (FIG. 2b,f and 3i,j).

30

Table 1. Photophysical properties of APNs and their activated fragments (CyCD and CyRGD).

Fluorophores/Nanoreporters		λ_{ab} (nm)	λ_{em} (nm)	Stokes Shift (nm)	Φ	Log D (pH = 7.4)
Activated fragments	CyCD	600	720	120	2.1%	-10.90
	CyRGD	600	720	120	2.2%	-2.40

	APN _C	600	720	120	0.07%	Not applicable
APNs	APN _{CN}	600	720	120	0.07%	Not applicable
	APN _G	600	720	120	0.08%	Not applicable

Note: λ_{ab} and λ_{em} : wavelength of maximum absorbance and emission, respectively; Φ : fluorescence quantum yield; Log D value: distribution coefficient.

Therefore, although the amine of lysine is protected by tert-butyloxycarbonyl group, Ac-FK of APN_C can be effectively cleaved, resulting in the fluorescence enhancement (FIG. 2a). This demonstrated the protection did not affect the sensing ability towards Cat B.

Example 6. *In vitro* cytotoxicity and cell imaging studies of APN_C and APN_G

The APNs prepared in Example 2 were taken for cytotoxicity studies. Further, their ability to detect CatB and GzmB in cells were investigated.

In vitro cytotoxicity and cell imaging studies

Murine colorectal carcinoma cells (CT26), murine macrophage cells (RAW 264.7) and murine hepatocytes (AML-12) were cultured in DMEM with 10% FBS in a humidified environment at 37 °C, which contains 5% CO₂ and 95% air. CD8⁺ T cells were isolated from spleen of Balb/c mouse by using a Dynabeads® Untouched™ Mouse CD8 Cells kit and cultured in RPMI 1640 culture medium in a humidified environment at 37 °C which contains 5% CO₂ and 95% air.

For cytotoxicity studies, the cells were seeded in 96-well plates at a density of 8,000 cells per well and cultured for 24 h. Then, the cell culture medium was replaced by 200 μ L fresh DMEM cell culture medium containing APNs at different concentrations followed by incubation for 24 h. After that, the cell culture medium was removed, and the cells were carefully washed with PBS. Then, 120 μ L of fresh DMEM cell culture medium containing 20 μ L 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium, inner salt (MTS) was added to each well and the cells were incubated for another 4 h. The absorbance of each well at 490 nm was measured using a SpectraMax M5 microplate reader to calculate the cell viability.

For cell fluorescence imaging, CT26, human hepatocellular carcinoma cells (LM3), CD8⁺ T cells, RAW 264.7 and AML-12 (1×10^5 cells per well in 1 ml of DMEM cell culture medium or RPMI 1640 culture medium) were seeded into confocal cell culture dishes (diameter 35 mm) and cultured overnight. The cells were incubated with APNs (10 μ M) for 2 h. For cell imaging with inhibitor, CT26 or LM3 cells were pre-treated with CA-074 (60 μ M), followed by incubation with APNs (10 μ M). Then, the medium was removed, and the cells were washed with PBS buffer for three times. The cells were fixed with 4% paraformaldehyde solution and stained with DAPI. Fluorescence microscopy images of cells were captured using a Laser Scanning Microscope LSM800 (Zeiss). The excitation and emission wavelengths were 640 and 655-710 nm for APNs, and 405 and 410-470 nm for DAPI. Cellular fluorescence intensities were quantified using ImageJ software.

Results and discussion

After confirming their minimal cytotoxicity (FIG. 4a,d), the ability of APN_C and APN_G to detect CatB and GzmB in cells were investigated in murine colorectal carcinoma CT26 cell line (human hepatocellular carcinoma LM3 cell) and granzyme B-positive cytotoxic T cells (CD8⁺ T cells), respectively (FIG. 4b,c,e,f). APN_C incubated CT26 cells showed a 26- or 6-fold higher NIRF intensity than that of PBS or CatB inhibitor (CA-074) treated CT26 cells, respectively (FIG. 4c). Similarly, intense NIRF signal was observed in CD8⁺ T cells after treatment of APN_G, which was 6.5-fold higher than that of GzmB negative 4T1 cells and RAW264.7 cells (FIG. 4f). Thus, these results confirmed that APN_C and APN_G could be specifically activated by CatB and GzmB in cells, respectively.

Example 7. Pharmacokinetic studies of APNs, CyCD and CyRGD

APNs (prepared in Example 2), and CyCD and CyRGD (prepared in Example 4) were taken for pharmacokinetic studies.

Pharmacokinetic studies

Female Balb/c mice were anaesthetized by intraperitoneal injection of ketamine/xylazine (50 mg per kg body weight ketamine and 5 mg per kg body weight xylazine) for the entire duration of the experiment. The end of the tail was cut for blood extraction. Blood was sampled in heparinized capillary tubes as a reference before injection. Mice were intravenously injected with CyCD and CyRGD (2.5 μ mol per kg body weight) and blood was sampled 1, 4, 9, 16, 25, 35, 55, 75, 95, 120, 240, 720 and 1,440 min after injection. For pharmacokinetic studies of APNs, blood was sampled from living mice 1, 9, 35, 95, 120, 240, 720 and 1,440 min after injection of APNs (10 μ mol per kg body weight). Collected blood samples were stored in an

ice box to prevent clotting before centrifugation at 1,096 g for 20 min. CyCD and CyRGD were directly quantified using HPLC. APNs were extracted and degraded to fragments for HPLC quantification. Quantification results were presented as a biexponential decay curve to estimate blood $t_{1/2\beta}$ values.

5

In vivo stability and biocompatibility studies

The collected urine in PBS buffer (10 mM, pH 7.4) was measured using UV-vis, fluorescence spectrophotometer and MALDI-TOF mass spectrometry. Heart, liver, spleen, lung and kidneys were collected from Balb/c mice 14 days after injection of APNs (10 μ mol per kg body weight),
10 CyCD or CyRGD (2.5 μ mol per kg body weight), or from SD rats 14 days after injection of APN_G (10 μ mol per kg body weight) or CyCD (2.5 μ mol per kg body weight), and placed into 4% PFA for histological examination.

Renal clearance efficiency and fecal excretion efficiency studies

15 For clearance studies in mice, female Balb/c mice were intravenously injected with APNs (10 μ mol per kg body weight), CyCD or CyRGD (2.5 μ mol per kg body weight) and placed in metabolic cages. Urine and faeces were collected for 15 d after injection. Collected urine was centrifuged at 1,585 g for 8 min and filtered using a 0.22 μ m syringe filter. Excretion of CyCD and CyRGD in the urine was imaged with the IVIS SpectrumCT system and quantified using
20 HPLC. Excreted APNs were incubated with respective proteases and degraded to fragments for imaging with the IVIS SpectrumCT system and HPLC quantification. Mice were killed to image resected organs after urine and faeces collection. Fluorescence intensities for resected organs were analysed with region of interest analysis using Living Image 4.3 software. Major organs were collected, homogenized in PBS buffer and centrifuged at 1,585 g for 15 min to
25 remove insoluble components. The final supernatants were incubated with respective proteases and measured on a fluorescence spectrophotometer. For clearance studies in rats, SD rats were intravenously injected with APN_G (10 μ mol per kg body weight) or CyCD (2.5 μ mol per kg body weight) and placed in metabolic cages. Urine and faeces were collected for 7 days after injection. Excreted CyCD was quantified with HPLC. APN_G was extracted and
30 incubated with GzmB to degrade into fragments for HPLC quantification.

Results and discussion

The pharmacokinetics of intravenously injected APNs and their fragments including CyCD and CyRGD were investigated by HPLC tracking of their blood concentrations (FIG. 5f). The blood
35 concentrations of CyCD and CyRGD decreased close to 0% ID g⁻¹ at 2 h and at 4 h post-injection, respectively, but it was prolonged to 24 h post-injection for APNs. The elimination half-lives ($t_{1/2\beta}$) of CyCD (22.9 min) and CyRGD (29.7 min) were much shorter than for APN_C

(2.1 h) and APN_G (2.0 h). The renal clearance efficiencies versus faecal excretion efficiencies of CyCD (CyRGD) were determined to be $91 \pm 3.0\%$ vs $1.9 \pm 1.0\%$ ID ($16 \pm 3.1\%$ vs $68.7 \pm 4.7\%$ ID) 24 h after injection, respectively (FIG. 5g and 6b,d). The efficient renal clearance of CyCD is attributed to its high hydrophilicity and molecular weight far below the glomerular filtration cutoff (<50 kDa). The renal clearance efficiencies of APN_C and APN_G were only $3.4 \pm 0.8\%$ and $2.4 \pm 0.8\%$ ID after 12 days after injection (FIG. 5g and 7), showing minimal activation by basal levels of proteases in healthy mice. Excretion of APN_C and APN_G in faeces reached $70 \pm 4.7\%$ and $65 \pm 5.3\%$ ID, respectively (FIG. 5g and Table 2), and the APNs were completely undetectable in all major organs from 12 days after injection, suggesting their complete clearance from the body (FIG. 7d).

Table 2. Elimination half-life, renal clearance efficiency and faecal excretion efficiency of APNs and their activated fragments (CyCD and CyRGD) in living mice.

Fluorophores/Nanoreporters		$t_{1/2\beta}$	Renal clearance efficiency (%)	Faecal excretion efficiency (%)
Activated fragments	CyCD	23 min	$91 \pm 3.0\%$	$1.9 \pm 1.0\%$
	CyRGD	30 min	$16 \pm 3.1\%$	$68.7 \pm 4.7\%$
APNs	APN _C	2.1 h	$3.4 \pm 0.8\%$	$70 \pm 4.7\%$
	APN _G	2.0 h	$2.4 \pm 0.8\%$	$65 \pm 5.3\%$

Note: $t_{1/2\beta}$: elimination blood half-life values; Data are the mean $\pm$ SD. n = 3 independent mice.

Example 8. Biodistribution of APNs, CyCD and CyRGD

APNs (prepared in Example 2), and CyCD and CyRGD (prepared in Example 4) were taken for biodistribution studies.

In vivo biodistribution studies

All mouse studies were performed in compliance with the guidelines set by the Institutional Animal Care and Use Committee, Sing Health. Female NCr mice, female Balb/c mice and male Balb/c mice (6 weeks old) and NSG mice with a SCID phenotype (6 weeks old) were obtained from InVivos. All rat studies were conducted in accordance with the National Institute Guide for the Care and Use of Laboratory Animals. Specific pathogen-free male Sprague Dawley (SD) rats, Lewis rats, Dark Agouti (DA) rats (12–15 weeks old and 250–300 g body weight) and Balb/c nude mice (4–5 weeks old) were purchased from the Zhejiang Academy

of Medical Sciences and Beijing Vital River, respectively. The experimental protocols were approved by the animal ethics review committees of the First Affiliated Hospital, Zhejiang University School of Medicine. The maximal tumour size/burden of 1,100 mm³ permitted by these ethics committees and the maximal tumour size/burden in this study was not exceeded.

- 5 Female NCr mice were intravenously injected with 0.2 ml saline (control), CyCD, CyRGD (2.5 µmol per kg body weight) or APNs (10 µmol per kg body weight) and imaged 60 min after injection. The abdominal cavity and resected organs from mice were imaged once they were killed 60 min after injection. Fluorescence images were acquired using the IVIS SpectrumCT system with excitation at 675 ± 10 nm and emission at 720 ± 10 nm. SD rats were
- 10 intravenously injected with CyCD (2.5 µmol per kg body weight) or APN_G (10 µmol per kg body weight) and imaged 6 h after injection. The resected organs from rats were imaged once they were killed 6 h after injection. Fluorescence images were acquired using the IVIS Lumina III system with excitation at 675 ± 10 nm and emission at 720 ± 10 nm. Fluorescence intensities for each organ were analysed with region of interest analysis using Living Image 4.3 software.
- 15 Mice and rats were killed, and major organs were collected, homogenized in PBS buffer and centrifuged at 1,585 g for 15 min to remove insoluble components. The supernatant containing extracted APNs, CyCD or CyRGD was taken for HPLC analysis.

Results and discussion

- 20 The biodistribution of APNs was studied and compared with that of CyCD and CyRGD (FIG. 5b). Fluorescence signals were detected for CyCD in the kidneys at 1 h after injection but was minimal in other organs (FIG. 5d and 8b,d). In contrast, CyRGD mainly accumulated in the liver, gallbladder and intestine (FIG. 5d and 8c,d), because it has higher distribution coefficients (Log *D* = -2.40) and thus higher hydrophobicity than CyCD (Log *D* = -10.90). The
- 25 large size of APNs (~170 nm > renal filtration threshold ~5 nm) resulted in their accumulation in the liver, showing minimal fluorescence as they are intrinsically non-fluorescent (FIG. 5c,d). HPLC quantification (FIG. 5e) revealed that liver accumulations of CyRGD, APN_C and APN_G is ~4.2, 4.7 and 4.8% ID g⁻¹ at 1 h after injection, respectively, which further increased at 6 h and then dropped at 24 h, respectively. Furthermore, histological staining revealed that all
- 30 APNs and their activated fragments had high biosafety (FIG. 9).

Example 9. *In vivo* stability of CyCD

- To determine *in vivo* stability of CyCD (prepared in Example 4), the optical and chemical
- 35 profiles of CyCD excreted renally from living mice were measured by following the protocol in Example 5, and compared with pure compounds.

Measurement of LOD

A dilution series of CyCD (0.00625, 0.0125, 0.025, 0.05 and 0.1 $\mu\text{mol kg}^{-1}$) was injected into living mice, followed by fluorescence measurement of collected urine samples. The LOD was calculated using the equation (Hu, J. J. *et al.*, *J. Am. Chem. Soc.* **2015**, 137, 6837-6843):

$$\text{LOD} = 3\sigma/k \quad (2)$$

where σ is the standard deviation of blank, and k is the slope of the plot of emission intensities against the ID of CyCD.

Results and discussion

- CyCD had no change on its optical spectra (FIG. 10b,c) or chemical structures (MALDI, FIG. 10d,e) after circulation in living mice, confirming minimal *in vivo* metabolism of CyCD in mice. To assess the sensitivity of CyCD for urinalysis, CyCD was injected into living mice at different concentrations, followed by fluorescence readout of collected urine samples. The LOD for injected CyCD was calculated to be 4 nmol per mouse (FIG. 6e). These data confirmed the high renal clearance efficiency and minimal *in vivo* metabolism of CyCD in living mice, making it a stable and sensitive tracer for optical urinalysis.

Example 10. Real-time imaging and longitudinal urinalysis of cancer

- Early diagnosis of cancer is crucial for curative treatment and increased survival rate. However, it remains challenging to detect ultrasmall tumors (< 2 mm micrometastatic level) owing to the low sensitivity and specificity of current methods (Pashayan, N. & Pharoah, P. D., *Science* **2020**, 368, 589-590). The tumour-targeting ability of APN_C was first studied by comparing with the control nanosensor APN_{CN} in a subcutaneous CT26 tumour-bearing mouse model.

Mouse model of CT26 colorectal cancer

- Mice were randomly selected and subcutaneously implanted with CT26 cancer cells in DMEM at a density of 1×10^6 cells/mouse on the right side of the back. The control groups were implanted with saline (0.1 mL) or tumour-bearing mice treated with CA-074 (10 mg kg^{-1}) intratumorally twice for 2 days prior to APN_C administration (Ryu, J. H. *et al.*, *J. Mater. Chem.* **2011**, 21, 17631-17634). Imaging was conducted at different timepoints (2, 5, 10, and 15 days) post tumor implantation. Body weights of all the mice were recorded during experiments. At the end, mice were euthanized, and major organs were placed into 4% PFA for histological examination.

Urinalysis in a mouse model of cancer

For the orthotopic liver tumour model, urine was collected from mice at $t = 9$ h after injection of APN_C (10 μ mol per kg body weight) 3, 8 and 14 days after tumour implantation, or injection of CA-074 before APN_C administration 14 days after tumour implantation, or from control mice after saline (0.1 ml) injection. For the subcutaneous tumour model, urine was collected from

5 living mice at $t = 9$ h after injection of APN_C or APN_{CN} (10 μ mol per kg body weight) 2, 5, 10 or 15 days after tumour implantation, or intratumoral injection of CA-074 before APN_C or APN_{CN} administration 15 days after tumour implantation, or from control mice after saline (0.1 ml) implantation. Fluorescence images were acquired using the IVIS SpectrumCT or IVIS Lumina III system with excitation at 675 ± 10 nm and emission at 720 ± 10 nm. To study the percentage

10 amount of activated APN_C in urine, urine was collected from living mice for 72 h after intravenous injection of APN_C 2, 5, 10 and 15 days after tumour implantation, or intratumoral injection of CA-074 before APN_C administration 15 days after tumour implantation time, or from control mice with saline implantation. The collected urine samples were centrifuged at 1,585 g for 8 min, filtered with a 0.22 μ m syringe filter, measured on a spectrophotometer and

15 analysed using HPLC as well as MALDI-TOF MS.

Real-time in vivo NIRF imaging of cancer in living mice

For the orthotopic liver tumour model, 14 days after tumour implantation, real-time NIRF imaging was conducted at $t = 2, 6, 24, 48$ and 72 h after intravenous injection of APN_C (10 μ mol per kg body weight) or intraperitoneal injection of CA-074 (10 mg per kg body weight) before APN_C administration. Imaging was also conducted at different times (3 and 8 days) after tumour implantation. For the subcutaneous tumour model, 15 days after tumour implantation, real-time NIRF imaging was conducted at $t = 2, 4, 6, 10, 24, 48, 72$ and 96 h after intravenous injection of APN_C or APN_{CN} (10 μ mol per kg body weight) or intratumoral

20 injection of CA-074 (10 mg per kg body weight) before APN_C administration. Imaging was also conducted at different times (2, 5 and 10 days) after tumour implantation. Fluorescence images were acquired using the IVIS SpectrumCT or IVIS Lumina III system with excitation at 675 ± 10 nm and emission at 720 ± 10 nm and the acquisition time of 1 s. Mice were killed 6 h after injection of APN_C at different times after tumour implantation. The abdominal cavity and

25 resected organs from mice were imaged after they were killed.

30

Determination of liver function and kidney function

To study liver function and kidney function in mice, blood was collected from the tail vein of mice under isoflurane anaesthesia at $t = 2, 5, 8,$ or 12 h after treatment with Con-A or

35 intravenous injection of CsA before Con-A administration, SCID mice, LPS-treated mice or mice with orthotopic liver tumours. The collected blood samples were centrifuged for 20 min at 1,096 g. Serum ALT, AST, creatinine and BUN were determined using commercial kits. To

study liver function and kidney function in rats, the blood samples were collected from the abdominal aorta 2, 3, 4 and 6 days after transplantation, intravenous injection of Tac daily after liver transplantation, or sham operation with only trauma on the belly, or from isograft rats or LPS-treated rats. The blood samples were centrifuged for 20 min at 1,096 g. Serum
5 ALT, AST, ALP, creatinine, BUN and uric acid were determined using commercial kits according to the manufacturer's protocol.

Subcutaneous tumour and orthotopic liver tumour mouse models

Balb/c mice were randomly selected and subcutaneously implanted with CT26 cancer cells in
10 DMEM at a density of 1×10^6 cells per mouse on the right-hand side of the back. The control groups were implanted with saline (0.1 ml) and the tumour-bearing mice treated with CA-074 (10 mg kg^{-1}) intratumorally twice for 2 days before APN_C administration (Ryu, J. H. *et al.*, *J. Mater. Chem.* **2011**, *21*, 17631–17634). Imaging was conducted at different times (2, 5, 10 and 15 days) after tumour implantation. Balb/c nude mice were randomly selected and injected
15 with HCC LM3 cells in a suspension containing PBS plus Matrigel into the left lateral lobe of the liver (Ding, Y. *et al.*, *Theranostics* **2020**, *10*, 5195–5208). Imaging was conducted at different times (3, 8 and 14 days) after tumour implantation. Body weights of all the mice were recorded during experiments.

20 Results and discussion

The maximum tumour-to-background ratio and kidney-to-background ratio (KBR) were observed 6 h after injection of APN_C (9.6 and 7.8 times higher than control mice, respectively, FIG. 11b and 12-16), and were nearly twice as high as those for APN_{CN} (5.0- and 4.0-fold increases). This demonstrated that the tumour-targeting moiety (cRGDfK) on APN_C enhanced
25 tumour accumulation.

The feasibility of APN_C for real-time imaging and urinalysis of tumours was further evaluated in an orthotopic liver tumour model (FIG. 11a). Kidney function was first evaluated at different post-implantation times. Serum creatinine and BUN had no increment in all groups of mice
30 (FIG. 11i), ensuring intact kidney function for identical renal clearance efficiencies of CyCD during the detection period. After 14 days from LM3 cell implantation, longitudinal imaging was conducted after intravenous injection of APN_C (FIG. 11c and 17).

After 15 days CT26 cells implantation, longitudinal NIRF imaging were conducted after
35 intravenous injection of APN_C (FIG. 13a). Due to the targeting ability of APN_C, the tumor was easily delineated by NIRF signal of APN_C; meanwhile, the signal was detected in the kidneys (FIG. 18b and 13c). The maximum tumor to background ratio (TBR) and kidney to background

ratio (KBR) were observed 6 h post-injection of APN_C (FIG. 18d,e; 9.6- and 7.8-fold higher than control mice, respectively), and then they slowly decreased at later imaging timepoints due to the rapid renal clearance of CyCD. In contrast, the signal of APN_C for mice pre-treated with a CatB inhibitor (CA-074) was close to the baseline in control mice (FIG. 18d-f). These data confirmed that the overexpressed CatB in the tumor catalyzed the depolymerization of APN_C, leading to the production of fluorescent and renal-clearable CyCD (FIG. 14f).

The tumour was delineated by APN_C; meanwhile, the signal was detected in the kidneys (FIG. 11c). The liver-to-background ratio (LBR) and KBR reached maxima 6 h after injection (FIG. 11f,g; 5.6 and 6.9 times higher than those of control mice, respectively), which slowly decreased at later times due to the renal clearance of CyCD. In contrast, the signal of APN_C for mice pretreated with a CatB inhibitor (CA-074) was close to the baseline in control mice (FIG. 11f,g).

To reveal the sensitivity of APN_C in cancer detection, NIRF imaging were conducted at different tumor implantation days (2, 5 and 10 days) (FIG. 16a and 18c). At 2 days post tumor implantation when the average tumor volume only reached ~2.1 mm³ (~1.6 mm in diameter), the maximum TBR and KBR increased to 1.4-fold and 1.5-fold relative to the control mice (FIG. 18c,d). Moreover, similar NIRF signal evaluation as a function of imaging time was observed for the mice groups at 5- and 10 days post tumor implantation (FIG. 18e,f); however, the maximum TBR (and KBR) increased to 2.5-fold (and 3.6-fold) and 6.3-fold (and 5.7-fold), respectively. *Ex vivo* fluorescence imaging of the resected tumors and kidneys showed similar trend in signal changes (FIG. 14 and 16). To reveal the sensitivity of APN_C in orthotopic cancer detection, imaging was also conducted early after tumour implantation (3 and 8 days, FIG. 17 and 11c). At 3 days after tumour implantation when the tumour volume was ~3.4 mm³ (~1.9 mm in diameter), the maximum LBR and KBR increased 2.0- and 2.4-fold relative to the control mice (FIG. 11d). Moreover, similar signal evolution as a function of imaging time was observed for the mouse groups 8 days after tumour implantation (FIG. 11f,g); however, the maximum LBR and KBR increased to 4.7- and 6.2-fold, respectively. *Ex vivo* imaging of the tumours and kidneys showed a similar trend in signal changes (FIG. 19). These data indicated the signal of APN_C was closely correlated with the increasing level of CatB in growing tumor.

By virtue of the high renal clearance efficiency of CyCD, the CatB levels in tumors were detected directly through NIRF measurement of urinary CyCD from tumor-bearing living mice after injection of APN_C. The NIRF signals increased to 1.6- 1.8-, 4.9- and 8.0-fold for 2-, 5-, 10- and 15 days post tumor implantation relative to the control mice, respectively (FIG. 18g). The urinary signal evolution coincided with the real-time NIRF imaging, as the excreted CyCD

was released from APN_C by activation of intramural CatB. The percentages of activated APN_C in urine were quantified and found that the values had a close correlation to the tumor sizes (FIG. 18h,i). Tumors were readily detected by APN_C injection and urinalysis when the tumor diameter was only ~1.6 mm (FIG. 18i). The intratumoral CatB levels were detected through measurement of urinary CyCD after injection of APN_C. The signals increased 1.8-, 3.8- and 7.8-fold for 3, 8 and 14 days after tumour implantation relative to the control mice, respectively (FIG. 11e). The percentages of activated APN_C in urine had a close correlation with the tumour sizes (FIG. 11j). APN_C-based urinalysis enabled ultrasensitive detection of an orthotopic liver tumour with diameter down to ~1.9 mm (FIG. 11j), close to the detection limit (~1.6 mm) in the subcutaneous CT26 tumour model (FIG. 15-16). Clinical liver function assays showed lower sensitivity, as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) had no notable increase even 14 days after tumour implantation when the diameter of the tumour was ~6.4 mm (FIG. 11h).

Therefore, these results showed that APN_C-based urinalysis has the potential for early diagnosis of cancer.

Example 11. Real-time imaging and urinalysis of acute immune-mediated hepatitis

Real-time non-invasive monitoring of the presence and activation of T lymphocytes is critical for diagnosing immune-mediated diseases and solid-organ allografts rejection, which however remains challenging. The ability of APN_C to monitor T-lymphocyte activation was tested in the murine model of acute immune-mediated hepatitis induced by lectin (Con-A) (Tiegs, G., Hentschel, J. & Wendel, A. A, *J. Clin. Invest.* **1992**, *90*, 196-203). The pathology involves specificity binding of Con-A to mannose receptors on liver sinusoidal endothelial cells and Kupffer cells, followed by the activation and recruitment of T-cells into the liver (FIG. 20a).

Mouse model of acute immune-mediated hepatitis

Male Balb/c mice were randomly selected and treated with Con-A (12.5 mg kg⁻¹, intravenous injection). The control groups were treated with saline (0.2 ml) or CsA (10 or 50 mg kg⁻¹, termed low dose or high dose, intravenous injection) 15 h and 1 h before Con-A administration. For specificity studies, male SCID mice and Balb/c mice were treated with Con-A (12.5 mg kg⁻¹, intravenous injection) and LPS (0.2 mg kg⁻¹, intradermal injection) on the right thigh, respectively. After drug administration, the weights of mice and signs of discomfort were monitored daily during the entire experiments. Imaging, blood and urine sampling were conducted at different times after treatment with Con-A or LPS. The control groups were

treated with saline, or an immunosuppressive drug (CsA) before Con-A administration (Tiegs, G., Hentschel, J. & Wendel, A., *J. Clin. Invest.* **1992**, 90, 196–203), or Con-A in SCID mice.

Urinalysis in a mouse model of immune-mediated hepatitis

- 5 Urine was collected from living mice at $t = 3$ h after injection of APN_G (10 μ mol per kg body weight) 1, 4, 7 and 11 h after treatment with Con-A, or intravenous injection of CsA before Con-A administration, or 7 h after treatment with Con-A for SCID mice, or 3 h after treatment with LPS for Balb/c mice. Fluorescence images were acquired using the IVIS SpectrumCT system with excitation at 675 ± 10 nm and emission at 720 ± 10 nm. To study the percentage
10 amount of activated APN_G in urine, urine was collected from living mice for 12 h after injection of APN_G 1, 4, 7 and 11 h after treatment with Con-A, or intravenous injection of CsA before Con-A administration, or 7 h after treatment with Con-A for SCID mice, or 3 h after treatment with LPS for Balb/c mice. The collected urine samples were centrifuged at 1,585 g for 8 min, filtered with a 0.22 μ m syringe filter, measured on a spectrophotometer and analysed using
15 HPLC as well as MALDI-TOF MS.

Real-time in vivo NIRF imaging of acute immune-mediated hepatitis in living mice

- Real-time NIRF imaging was conducted in Balb/c mice at $t = 0.5, 1, 2, 3, 6, 12, 24$ and 48 h after intravenous injection of APN_G (10 μ mol per kg body weight) 1, 4, 7 and 11 h after
20 treatment with Con-A, or intravenous injection of CsA (10 or 50 mg per kg body weight) before Con-A administration. Imaging was also conducted in SCID mice. Fluorescence images were acquired using the IVIS SpectrumCT system with excitation at 675 ± 10 nm and emission at 720 ± 10 nm and the acquisition time of 1 s. Mice were killed 1 h after injection of APN_G at different times after treatment with Con-A. The abdominal cavity and resected organs from
25 mice were imaged after they were killed. NIRF intensities were analyzed by the ROI analysis using the Living Image 4.3 Software. Major organs were placed into 4% PFA for histological examination.

Specificity studies in living mice with LPS-induced local skin oedema

- 30 Male Balb/c mice were intradermally injected with LPS (0.2 mg kg⁻¹) on the right thigh (Medicherla, S. *et al.*, *J. Inflamm. Res.* **2010**, 3, 9-16), followed by intravenous injection of APN_G (10 μ mol kg⁻¹ body weight) at 3 h post-treatment of LPS. Real-time NIRF imaging of living mice was conducted using the IVIS spectrumCT system. NIRF intensities were analyzed by the ROI analysis using the Living Image 4.3 Software. Major organs were placed into 4%
35 paraformaldehyde for histological examination. Note that such a low dosage of LPS does not induce organ injury. The LPS-induced local skin oedema on Balb/c mice was used as a control disease model to validate the tissue specificity of APN_G.

Results and discussion

Con-A was intravenously administered into living Balb/c mice at a dose of 12.5 mg/kg (Yamashita, J. *et al.*, *J. Immunol.* **2011**, *186*, 3410-3420), followed by intravenous injection of APN_G at different time points post-treatment with Con-A (1, 4, 7 and 11 h, FIG. 21a). The control groups were treated with saline, or an immunosuppressive drug (CsA: Cyclosporin A) prior to Con-A administration (Tiegs, G., Hentschel, J. & Wendel, A. A, *J. Clin. Invest.* **1992**, *90*, 196-203), or Con-A in severe combined immunodeficient (SCID) mice. Furthermore, the LPS-induced local skin edema on Balb/c mice was used as a control disease model to validate the tissue specificity of APN_G. No histological change in the kidneys after Con-A or LPS challenge (FIG. 22), and massive hepatic necrosis of the livers was only observed 12 h after Con-A treatment (FIG. 22). Such intact kidney function ensured the identical renal clearance efficiency of CyCD during the detection period. Longitudinal NIRF imaging showed that 2 h after treatment with Con-A, the NIRF signals of APN_G in the liver and kidney were close to those of the control mice (FIG. 20b,c). However, at 5 h after treatment with Con-A, the signals in the liver and kidneys increased to 2.4-fold and 1.9-fold, respectively (FIG. 20c,d), indicating that APN_G was activated in the liver to release CyCD that in turn filtrated into kidneys. Similar NIRF signal evolution was observed for the mice groups 7 and 11 h after treatment with Con-A (FIG. 21 and 23); however, the maximum signals of the liver (and kidneys) 8 and 12 h after treatment were 3.1 times (3.0 times) and 2.5 times (1.8 times) higher than that of control mice, respectively (FIG. 20c,d). By contrast, when the mice were protected with the immunosuppressant CsA for inhibiting T cells activation, the NIRF signal decreased 2.6-fold(1.6-fold) in the liver (kidneys) or close to the basal level of saline-treated control mice, depending on the CsA doses. Further, the NIRF signals had no increment in both the liver and kidneys on SCID mice or mice with LPS-induced local skin edema (FIG. 20c,d). *Ex vivo* NIRF imaging and biodistribution studies showed similar results (FIG. 24). These data indicated that APN_G was specifically activated by GzmB in liver but not in other tissues.

The NIRF signal of excreted CyCD in urine was measured after injection of APN_G into treated mice to validate its feasibility for optical urinalysis. The first statistically significant NIRF enhancement was observed 5 h (5.5-fold) after treatment; it continued increasing at 8 h (7.3-fold) and then decreased at 12 h (5.0-fold) after treatment (FIG. 20b,f). Consistent with imaging data, the urinary signals were close to the background level with administration of high-dose CsA, or in SCID mice as well as mice with LPS-induced local skin edema, further proving the tissue and GzmB specificity of APN_G (FIG. FIG. 20b,f and 25). Moreover, the signal intensity of APN_G was found to be correlated well with GzmB concentration, because flow cytometry analysis showed that the level of GzmB from lymphocytes in the liver increased by 2.7-, 5.7-

and 2.2-fold increase at 5, 8 and 12 h after treatment with Con-A relative to that of control mice (FIG. 20e,f and 26-27).

Clinical and preclinical assays were used to test the liver function (ALT and AST) and the level of proinflammatory cytokines including TNF- α , IFN- γ , IL-2 and IL-6 in sera of mice (FIG. 28). The ALT and AST had a statistically significant increase at 12 h (10.6- and 16.9-fold) after treatment. In contrast, the cytokines increased earlier at 2 h (13.1-, 2.5-, 3.0- and 4.2-fold for TNF- α , IFN- γ , IL-2 and IL-6, respectively) after con-A treatment because they are upstream activators for lymphocytes activation (FIG. 20g). Although the detection timepoint of cytokines (2 h) was ahead of APN_G-based urinalysis (5 h), the levels of these cytokines also increased in mice with LPS-induced local skin oedema, suggesting that cytokine measurements have low specificity. These results thus confirmed that APN_G enabled longitudinal, sensitive optical imaging and urinalysis of T cell infiltration in liver during acute immune-mediated hepatitis in a disease tissue and biomarker specific manner.

15

Example 12. Optical urinalysis of acute liver allograft rejection

Orthotopic liver transplantation is the only option for patients with end-stage liver disease; however, allograft rejection remains a major complication after transplantation (Lechler, R. I. *et al.*, *Nat. Med.* **2005**, *11*, 605-613). Invasive biopsy is the gold standard routinely used to diagnose allograft rejection and to monitor the efficacy of immunosuppressive drug therapy (Jones, K. D. & Ferrell, L. D., *Semin. Diagn. Pathol.* **1998**, *15*, 306-317). However, this procedure can cause secondary injury and provides only a static and focal pathological state (Portmann, B. *et al.*, *Verh. Dtsch Ges. Pathol.* **1995**, *79*, 277-290). Hence, early detection of allograft rejection is crucial to preserve the function of the transplant. Encouraged by the promising results of APN_G (prepared in Example 2) for monitoring T cell activation in the liver of living mice, we sought to evaluate its diagnostic/translational potential in the rat model of acute liver allograft rejection (FIG. 1a). APN_G was modulated to respond to GzmB that was overexpressed by T lymphocytes. In both immune-mediated hepatitis and acute liver allograft rejection models, APN_G-based fluorescence urinalysis is proven to be as sensitive as flow cytometry of tissue samples and biopsy, but obviously more advantageous as it is non-invasive and dynamic. Liver function and kidney function studies were performed by following the protocol in Example 10.

Rat model of acute liver allograft rejection

Dark Agouti (DA) rats and Lewis rats were used as donors and recipients, respectively, for orthotopic liver transplantation. The surgery was carried out according to "two cuff technique"

(Kamada, N. & Calne, R. Y., *Transplantation* **1979**, *28*, 47-50). Briefly, the donor DA rat was subjected to anesthesia and subsequent systemic heparinization. The donor liver from the DA rat was detached, immersed in 4 °C Ringer's balanced solution, and immediately transplanted orthotopically into the abdomen of the recipient Lewis rat. The anastomosis of the supra-
5 hepatic vena cava was continuously sutured. The cuff technique was applied to connect portal vein and infra-hepatic vena cava. After then, the bile duct was reconstructed by an end-to-end anastomosis over an indwelling stent. After orthotopic liver transplantation, standard rodent chow and sterilized water were available ad libitum. For treatment groups, the recipient Lewis rats were treated with tacrolimus (0.3 or 1.5 mg kg⁻¹, termed low dose (LD) or high dose (HD),
10 intramuscular injection) daily after liver transplantation (Guo, L. *et al.*, *Liver Transpl.* **2004**, *10*, 743-747). Sham-operation rats with only trauma on the belly and syngeneic liver transplantation between Lewis rats were conducted. In addition, healthy Lewis rats were hutreated with LPS (0.2 mg kg⁻¹, intradermally injection) (Hu, J. J. *et al.*, *J. Am. Chem. Soc.* **2015**, *137*, 6837-6843) was included as a control diseases model to assess the specificity of
15 APN_G. After surgery, the weight of rats and signs of discomfort were monitored daily during the entire period of experiments. *Ex vivo* imaging, blood and urine sampling were conducted at different time points post-operation. At the end, major organs were placed into 4% PFA for histological examination.

20 *Urinalysis in a rat model of acute liver allograft rejection*

Urine was collected from recipient rats at t = 8 h after intravenous injection of APN_G (10 μmol per kg body weight) 2, 3, 4 and 6 days after transplantation, or intravenous injection of Tac daily after liver transplantation, from rats 4 days after sham-operation with only trauma on the belly, from rats 4 days after syngeneic liver transplantation or from rats 3 h after treatment with
25 LPS. Fluorescence images were acquired using the IVIS Lumina III system with excitation at 675 ± 10 nm and emission at 720 ± 10 nm. The collected urine samples were centrifuged at 1,585 g for 8 min, filtered with a 0.22 μm syringe filter, measured on a spectrophotometer and analysed using HPLC. Urinary protein was determined by using a bicinchoninic acid protein assay kit.

30

Ex vivo NIRF imaging of acute liver allograft rejection in rats

Ex vivo NIRF imaging was conducted in recipient rats at 8 h post-injection of APN_G (10 μmol kg⁻¹ body weight) at 2-, 3-, 4-, and 6-days post-transplantation, or intravenously injection of Tac (0.3 or 1.5 mg kg⁻¹, intramuscular injection) daily after liver transplantation. *Ex vivo*
35 imaging was conducted in rats 4 days after sham-operation with only trauma on the belly or at 4 days after syngeneic liver transplantation. Fluorescence images were acquired using the IVIS Lumina III system with excitation at 675 ± 10 nm and emission at 720 ± 10 nm and the

acquisition time of 1 s. NIRF intensities were analyzed by the ROI analysis using the Living Image 4.3 Software. Major organs were placed into 4% PFA for histological examination.

Specificity studies in living mice with LPS-induced local skin oedema

- 5 Male Balb/c mice were intradermally injected with LPS (0.2 mg kg^{-1}) on the right thigh (Medicherla, S. *et al.*, *J. Inflamm. Res.* **2010**, 3, 9-16), followed by intravenous injection of APN_G ($10 \text{ } \mu\text{mol per kg body weight}$) 3 h after treatment with LPS. Real-time NIRF imaging of living mice was conducted using the IVIS SpectrumCT system. NIRF intensities were analyzed by the ROI analysis using the Living Image 4.3 Software. Major organs were placed into 4%
- 10 PFA for histological examination. This low dosage of LPS does not induce organ injury (Wang, W. *et al.*, *Am. J. Physiol. Ren. Physiol.* **2007**, 293, F1131–F1136). Lewis rats were intradermally injected with LPS (0.2 mg kg^{-1}) on the right thigh (Medicherla, S. *et al.*, *J. Inflamm. Res.* **2010**, 3, 9-16), followed by intravenous injection of APN_G ($10 \text{ } \mu\text{mol per kg body weight}$) 3 h after treatment with LPS. Urine was collected for 8 h after injection of APN_G, centrifuged
- 15 at $1,585 \text{ g}$ for 8 min and filtered with a $0.22 \text{ } \mu\text{m}$ syringe filter, followed by fluorescence imaging using the IVIS Lumina III system with excitation at $675 \pm 10 \text{ nm}$ and emission at $720 \pm 10 \text{ nm}$.

Results and discussion

- Allogeneic orthotopic liver transplantation (termed allograft) was performed by using DA and
- 20 Lewis rats as donors and recipients, respectively (FIG. 29a). The control groups included rats that were healthy or treated with an immunosuppressive drug, Tac, after surgery (FIG. 30a). The liver and kidney function were first evaluated at different post-operation timepoints. The ALT, AST and ALP had statistically significant increases (5.6-, 3.7- and 3.2-fold) at 4 days after transplantation (FIG. 30b), however, the serum creatinine, BUN and uric acid had no
 - 25 increment in any group (FIG. 30c). Moreover, histological studies showed normal tissue morphology at 3 days after allogeneic transplantation but dilated sinusoids and hepatocyte debris of the liver was observed at 6 days after allogeneic transplantation (FIG. 29h and 31b), while no histological change was detected in the kidneys in any rats (FIG. 31b). Such intact kidney function in rats ensured the same renal clearance efficacy of CyCD within 6 days post-
 - 30 transplantation.

- APN_G was intravenously injected into living rats at different post-transplantation time points (2, 3, 4 and 6 days, FIG. 30a) for longitudinal optical urinalysis of infiltration of T cells into transplants. Note that the biodistribution and clearance pathways of APN_G were similar in
- 35 healthy rats and mice (FIG. 32). The NIRF signal of urinary CyCD was measured and normalized by the fold of urinary protein to cancel out the individual difference in urine output. Using pre-graft urine signals from healthy mice as the baseline level, the first statistically

significant NIRF enhancement for excreted CyCD was observed at 3 days (3.2-fold) after transplantation (FIG. 29b), and respectively increased to 4.9- and 3.9-fold 4 days and 6 days after transplantation (FIG. 29b). Moreover, when the allografted rats were treated with Tac for maintenance therapy, the signal was significantly decreased to 1.8-fold at low dose and even
5 regressed to the baseline level at high dose.

To evaluate the specificity of APN_G towards allograft rejection, sham-operation rats with only trauma on the belly, syngeneic liver transplantation between Lewis rats (termed isograft), and a separate cohort of rats with LPS-induced local skin oedema were used. Notably, no
10 substantial increase of urine signals was observed from isografted rats, sham-operation rats, and rats with LPS-induced local skin oedema. The signal evolution behaviours of urinalysis coincided well with the *ex vivo* NIRF imaging data in the liver and kidneys (FIG. 29c,d and 33), as APN_G was specifically activated in grafted liver to produce CyCD that cleared through kidneys to urine (FIG. 34). These data confirmed that APN_G specifically detected and
15 distinguished allograft rejection from traumatism and local inflammation.

To validate whether the signal intensity of APN_G correlated with the infiltration extent of T cells into allograft, the levels of GzmB in cytotoxic T lymphocytes from the transplant liver at different post-operation times were analyzed by flow cytometry. The earliest timepoint to
20 detect GzmB upregulation in the liver was 3 days post-operation with a 4.8-fold increment relative to the healthy rats (FIG. 35-36), which was consistent with APN_G-based urinalysis assay. Similar evolution was observed in the spleen, lymph nodes and blood (FIG. 35-36). Moreover, no increment was observed in isografted rats in comparison with healthy rats. The immunofluorescence staining showed that the signal of CyCD in the liver was observed at 3
25 days after transplantation (FIG. 29h), but not for isografted rats, sham-operation rats, and rats with LPS-induced local skin oedema (FIG. 31a); furthermore, the signal enhancement increased to 13-fold, 37-fold and 81-fold at 3-, 4- and 6 days after transplantation, respectively (FIG. 31c).

30 ELISA assays on analysis of proinflammatory cytokines in sera of rats revealed that cytokines including TNF- α , IFN- γ , IL-2, IL-4 and IL-6 showed a statistically significant increase (1.3-, 2.0-, 1.7-, 1.4-, and 1.8-fold) 2 days post-operation (FIG. 37). However, the statistically significant increase for cytokines was also observed in isografted, sham-operation rats, and skin edema rats (FIG. 37), implying that serum cytokines had low disease specificity.

35

In both immune-mediated hepatitis and acute liver allograft rejection models, APN_G-based urinalysis is proven to be as sensitive as flow cytometry and biopsy, but with the advantage of

being non-invasive and dynamic. Moreover, APN_G detected the infiltration of T cells into liver allograft earlier than clinical imaging methods including ultrasonography (Dravid, V. *et al.*, *AJR Am. J. Roentgenol.* **1994**, 163, 585-589) and hepatobiliary scintigraphy (>24 h earlier) (Ogura, Y. *et al.*, *Radiology* **2000**, 214, 795-800; and Shah, A. N., Dodson, F. & Fung, J., *Semin. Nucl. Med.* **1995**, 25, 36-48), clinical assays (AST, ALT and ALP) (24 h earlier), and histological analysis (72 h earlier). It is worth highlighting that APN_G-based urinalysis distinguished acute allograft rejection from traumatism and local inflammation, which was not possible for blood tests. This is because APN_G-based urinalysis detected the excreted urinary CyCD which was specifically released after activation of APN_G by T lymphocytes localized in allograft liver. Such allograft specificity has been long desired, as transplant patients can have other background diseases that can contribute to elevated levels of proinflammatory cytokines in blood. With the predictive power superior to other tested methods, APN_G-based urinalysis holds potential for close monitoring of graft conditions in a patient-friendly yet sensitive, specific and dynamic way.

Thus, we demonstrated in-situ nanoparticle-to-molecule pharmacokinetic conversion as an effective *in vivo* sensing approach. This was exemplified by development of APNs as a universal polymeric scaffold that underwent biomarker-initiated catalytic depolymerization and subsequent release of renal-clearable fluorogenic fragments for optical urinalysis of cancer (see Example 10) or allograft rejection in rodent models. The design of APNs is distinct from that of dye-peptide conjugated inorganic nanosensors. First, APNs underwent a complete conversion from nanoparticles to small-molecule fragments upon interacting with protease, while the reported inorganic nanosensors left the inorganic core in tissues (Kwong, G. A. *et al.*, *Nat. Biotechnol.* **2013**, 31, 63-70). Second, the fluorogenic response of APNs was solely determined by the structural evolution of the fluorophore unit upon protease-catalyzed reaction, while the conventional electron-transfer-induced fluorescence quenching was the signaling mechanism of the reported inorganic nanosensors, which was distance-dependent and easily disturbed by biomolecular interactions (Kwon, E. J., Dudani, J. S. & Bhatia, S. N., *Nat. Biomed. Eng.* **2017**, 1, 0054; and Dudani, J. S. *et al.*, *Adv. Funct. Mater.* **2016**, 26, 2919-2928). Third, the released fluorophore fragment (CyCD) was specifically designed for high renal clearance (~91% ID at 24 h, see Example 7), while the dye-peptide conjugates have the moderate-to-low renal clearance highly dependent on the peptide sequence (Kwon, E. J., Dudani, J. S. & Bhatia, S. N., *Nat. Biomed. Eng.* **2017**, 1, 0054). Thus, APNs represent an unparalleled class of optical nanosensors with high clinical translation potential for imaging and diagnosis.

To assess sensitivity and specificity for all tested assays, ROC analysis was conducted (FIG. 29e,f). In contrast to the limited predictive power of cytokines for early detection ($0.69 < \text{AUC}$

< 0.91), APN_G-based urinalysis and flow cytometry were highly discriminatory, producing higher AUC of 0.98 and 0.96, respectively. Although ALT and AST had a comparable predictive power (AUC = 0.96 and 0.98), they were insensitive as their detection time was delayed to 4 days post-operation time (FIG. 29g). The comparison proved that the APN_G-based urinalysis had the highest sensitivity to diagnose acute liver allograft rejection, followed by flow cytometry, and then the clinical blood tests of ALT and AST and lastly cytokines. With the predictive power of AUC = 0.98 superior to other tested methods, APN_G-based fluorescence urinalysis holds great potential for close monitoring of graft conditions during maintenance immunosuppression therapy and early identification of allograft rejection in a patient-friendly yet sensitive, specific and dynamic way.

Comparative Example 1

Equipped with the peptide brushes cleavable by cancer-overexpressed CatB and the cancer-targeting moiety (cRGDfK), APN_C specifically homed to tumor and efficiently underwent in-site enzymatic fragmentation to liberate the fluorescence-on CyCD for real-time imaging and urinalysis (see Example 10). By virtue of in-situ signal amplification from catalytic reaction, ultrahigh renal clearance of CyCD, and high contrast fluorogenic turn-on response, APN_C-based longitudinal urinalysis enabled ultrasensitive detection of the diminutive tumor with diameter down to ~1.6 mm. This is significantly more sensitive than many clinical imaging technologies (single photon emission computed tomography (SPECT, Welt, S. *et al.*, *J. Clin. Oncol.* **1994**, 12, 1193-1203), positron emission tomography (PET, Erdi, Y. E., *Mol. Imaging Radionucl. Ther.* **2012**, 21, 23-28) and magnetic resonance imaging (MRI, Serres, S. *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **2012**, 109, 6674-6679), which are unlikely to detect tumors with the diameter smaller than 7 mm (Table 3); it is also more sensitive than assays detecting circulating tumor DNA and carcinoembryonic antigen which only become detectable in blood when tumor diameter reached 10 mm and 6.9 mm, respectively (Kwong, G. A. *et al.*, *Nat. Biotechnol.* **2013**, 31, 63-70; and Aalipour, A. *et al.*, *Nat. Biotechnol.* **2019**, 37, 531-539). Moreover, the sensitivity of APN_C is comparable to or potentially higher than the reported inorganic nanosensors with the limit of detection for the tumor down to ~1.8 mm in mouse models of lung adenocarcinoma cancer (Table 3) (Kirkpatrick, J. D. *et al.*, *Sci. Transl. Med.* **2020**, 12, eaaw0262). In view of the high probability for cancer treatment to reach a systemic cure when the tumor size is smaller than 5 mm (Kwong, G. A. *et al.*, *Nat. Biotechnol.* **2013**, 31, 63-70), APN_C can potentially serve as an ideal non-invasive urine test for early diagnosis and clinical decision-making during cancer therapy.

Table 3. Comparison of APN_C with other preclinical and clinical methods in terms of detectable size threshold of tumor.

Technologies	Agents	Detection threshold in diameter	Human trial or animal model	References
Optical	APN _C	~1.6 mm	CT26 tumor-bearing mice	This study
		~1.9 mm	Orthotopic LM3 tumor-bearing mice	
	Activity-based nanosensors	~ 2 mm	A mouse model of ovarian cancer	<i>Nat. Biomed. Eng.</i> 2017 , 1, 0054.
	ICG	20 mm	Patients with intraparenchymal brain metastatic tumor	<i>Neurosurg. Focus.</i> 2021 , 50, E11.
LC-MS	Activity-based nanosensors	5.1 mm and 1.78 mm	LS174T colorectal and lung adenocarcinoma cancer model	<i>Nat. Biotechnol.</i> 2013 , 31, 63-70; and <i>Sci. Transl. Med.</i> 2020 , 12, eaaw0262.
MRI	Gadobutrol	5 to 10 mm	Brain metastases in human	<i>Proc. Natl. Acad. Sci. USA.</i> 2012 , 109, 6674-6679.
PET	FDG-18	~7 mm	Human	<i>Mol. Imaging Radionucl. Ther.</i> 2012 , 21, 23-28.
SPECT	¹³¹ I-mAbF19	~10 mm	Patients with hepatic metastases from colorectal carcinoma	<i>J. Clin. Oncol.</i> 1994 , 12, 1193-1203.
X-ray	Not applicable	10 to 20 mm	Lung cancer	<i>Acta. Radiol.</i> 1980 , 362, 131-134.
CT	Not applicable	8 mm	Patients with pulmonary nodules	<i>J. Thorac. Dis.</i> 2018 , 10, S2611-S2627.
Circulating tumor DNA	Not applicable	10 mm	LS174T colorectal cancer xenograft model	<i>Nat. Biotechnol.</i> 2019 , 37, 531-539.

Carcinoem-bryonic antigen	Not applicable	6.9 mm	LS174T colorectal cancer xenograft model	<i>Nat. Biotechnol.</i> 2013 , 31, 63-70.
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The slow *in vivo* clearance and shallow tissue penetration of light are the major hurdles for clinical translation of optical nanoparticles as imaging agents for early diagnosis. To tackle these challenges, it is reported here the first kind of optical nanoparticles that specifically

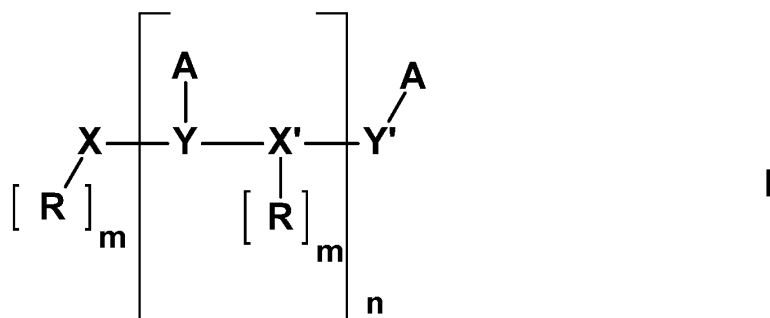
 5 activate nanoparticle-to-molecule pharmacokinetic conversion at the disease site, sensitively trigger fluorescence signal to report the level of biomarkers, and rapidly excrete from living body via safe renal clearance pathway for non-invasive urinalysis. The ultrahigh renal clearance efficacy and biomarker-specific release of the fluorogenic fragment (CyCD) coupled with in-situ amplification from catalytical fragmentation make APN-based fluorescence turn-

 10 on urinalysis outperform the traditional blood and urine tests, allowing for ultrasensitive detection of cancer and early diagnosis of acute liver allograft rejection with long-wanted allograft specificity unattainable by other non-invasive methods. The modular chemistry of APNs affords both structural and functional diversity for sensitive detection of diseases potentially beyond cancer and allograft rejection. Thus, the present invention not only provides

 15 new opportunities to translating optical nanoparticles for *in vivo* sensing but also highlights a universal nanoplatform to fill the gaps of lacking ultrasensitive urine tests for early diagnosis.

Claims

1. A polymeric nanoreporter compound according to formula I:

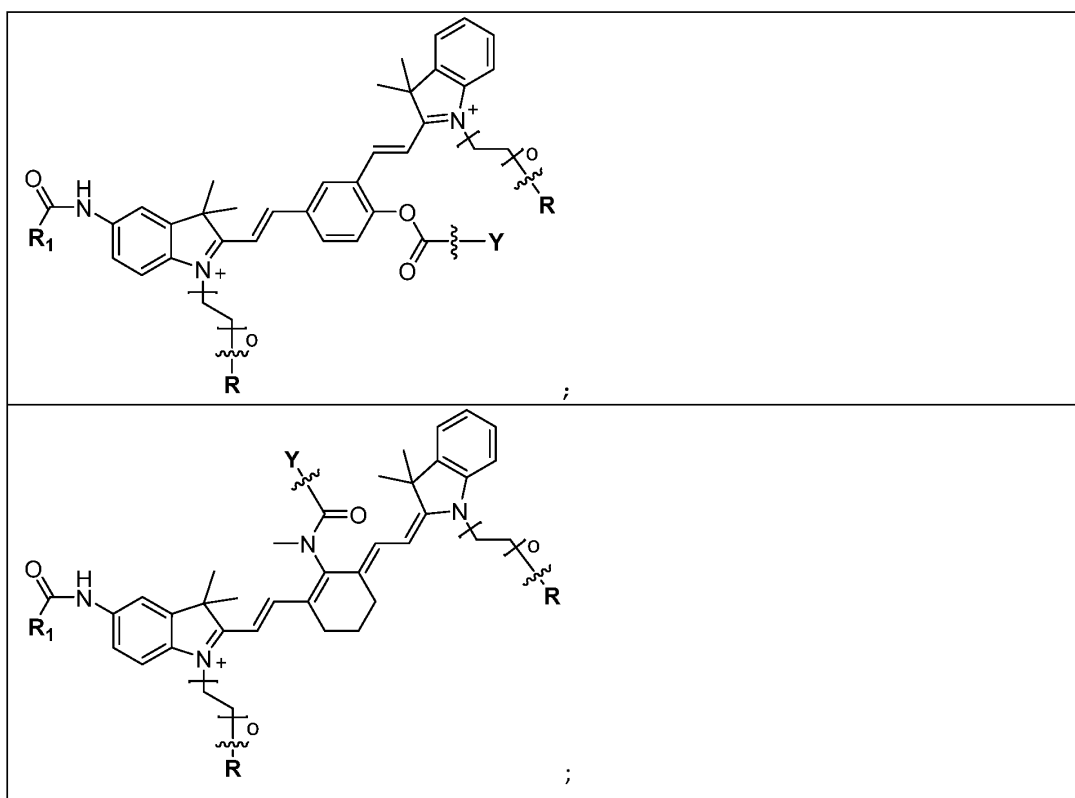


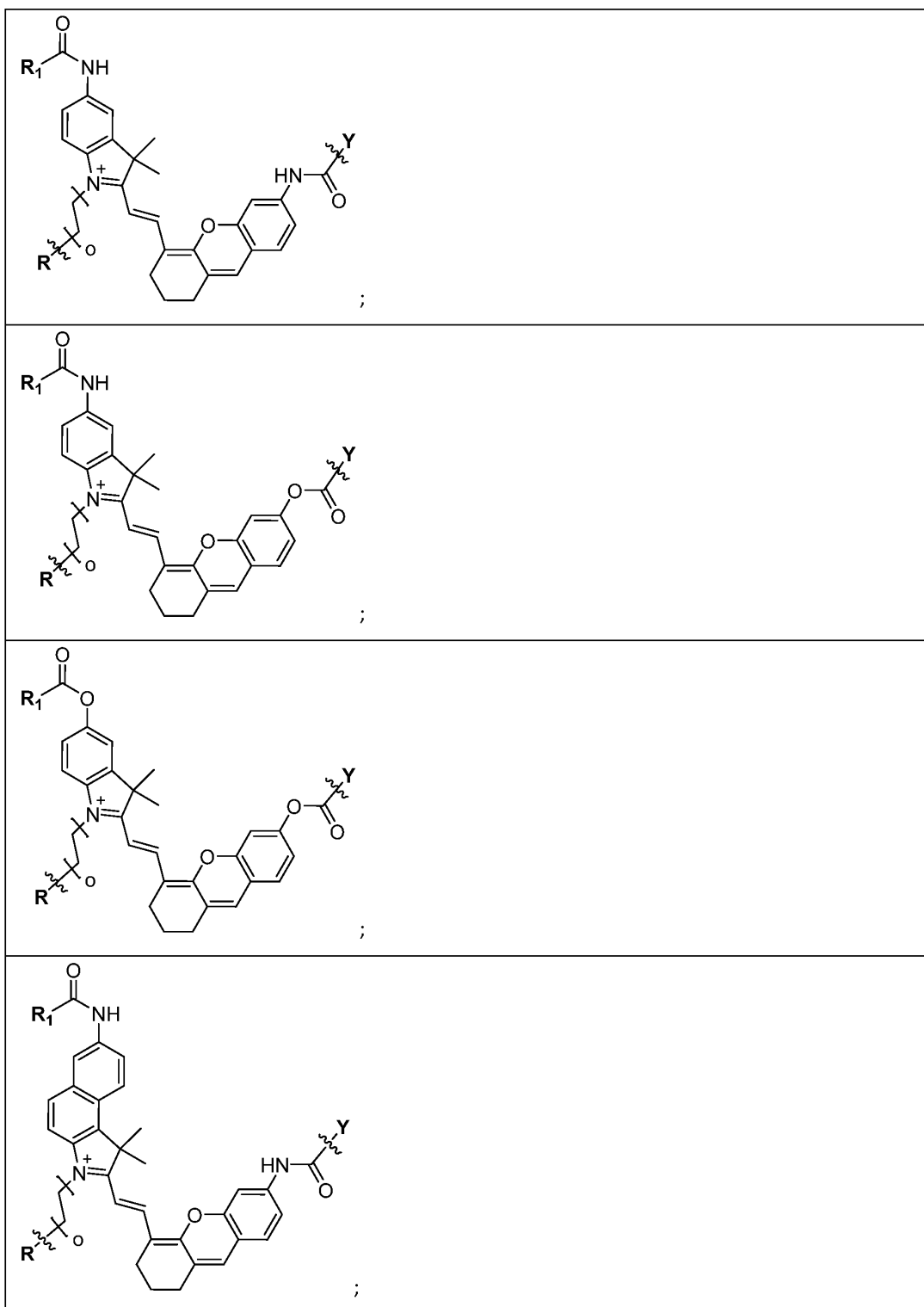
where:

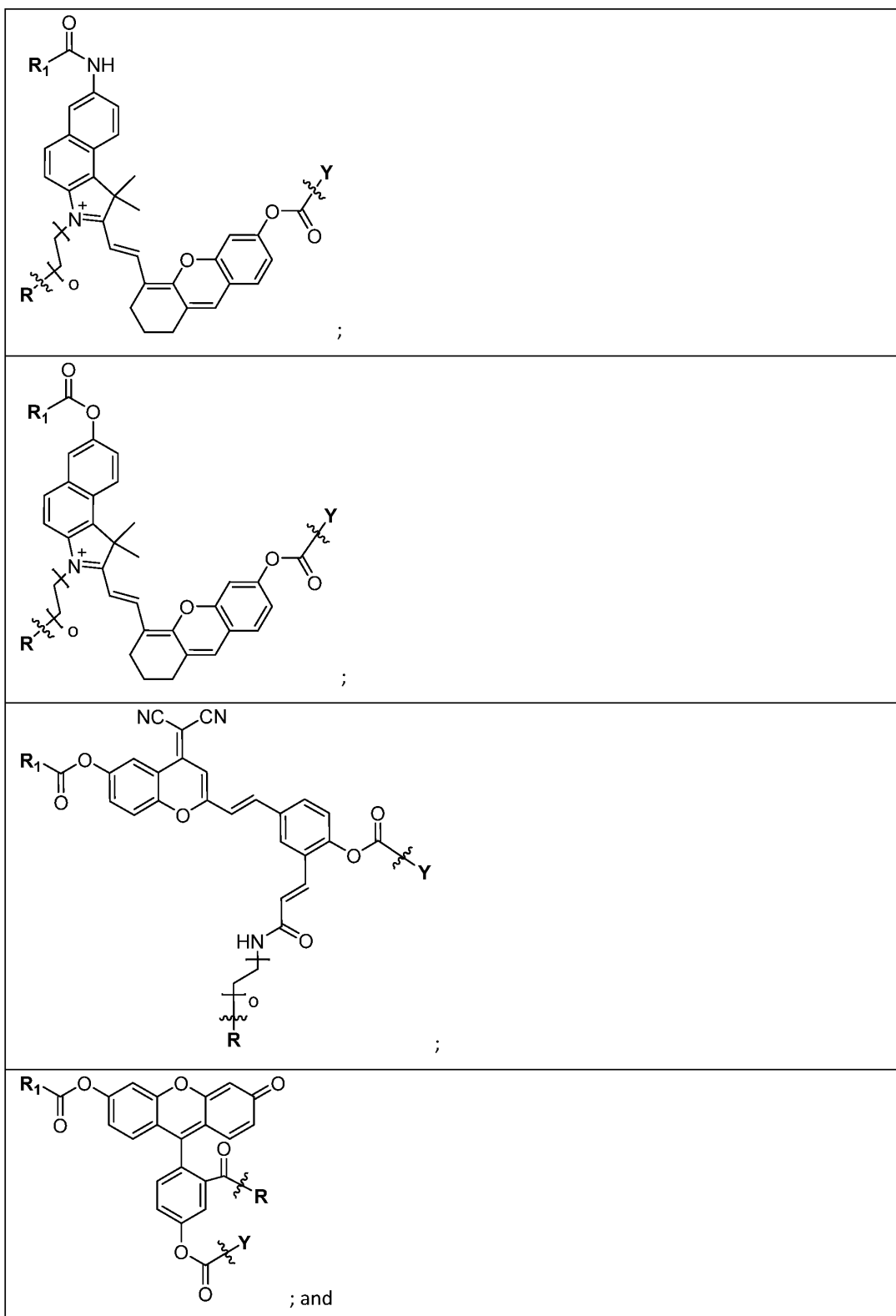
n is from 1 to 20;

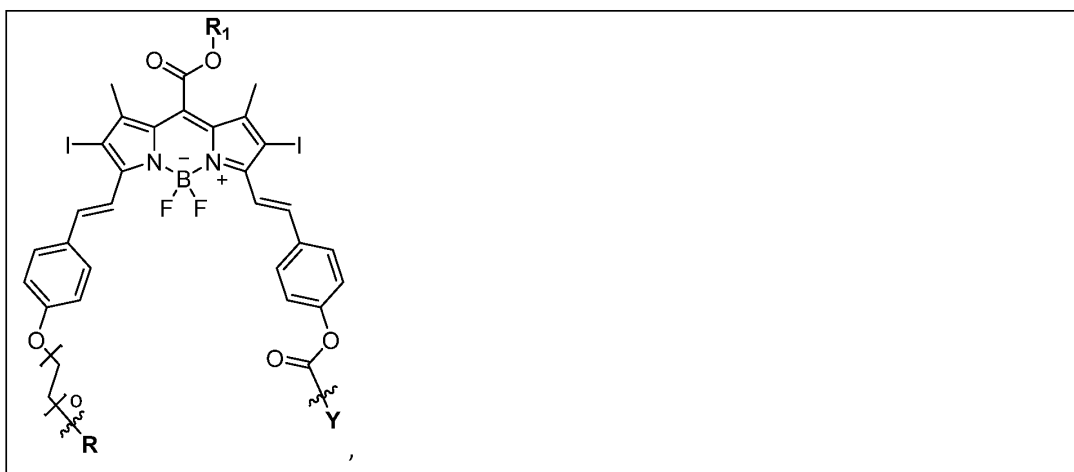
m represents 0 or 1, as determined by X or X';

X represents a caged fluorescent moiety selected from the group consisting of:







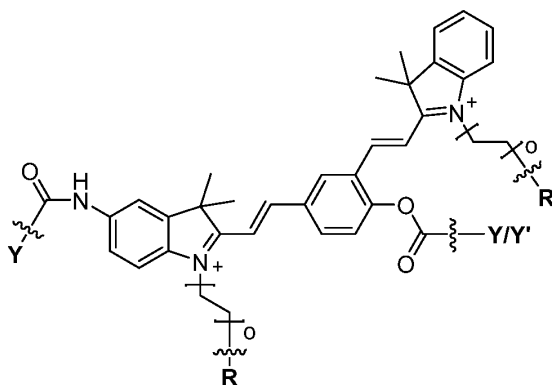


where the wiggly lines represent the point of attachment of X to the moieties R and Y, respectively;

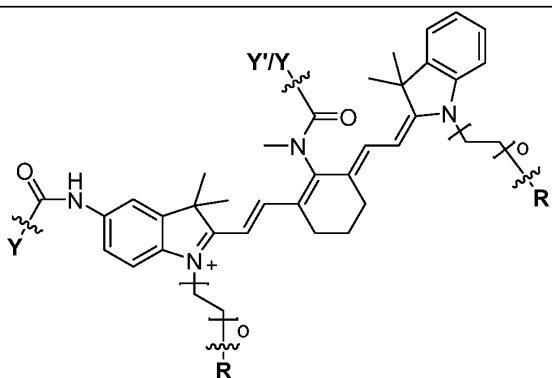
R_1 represents $R_{2a}R_{2b}N$;

R_{2a} and R_{2b} independently represent a C_1 to C_6 alkyl group;

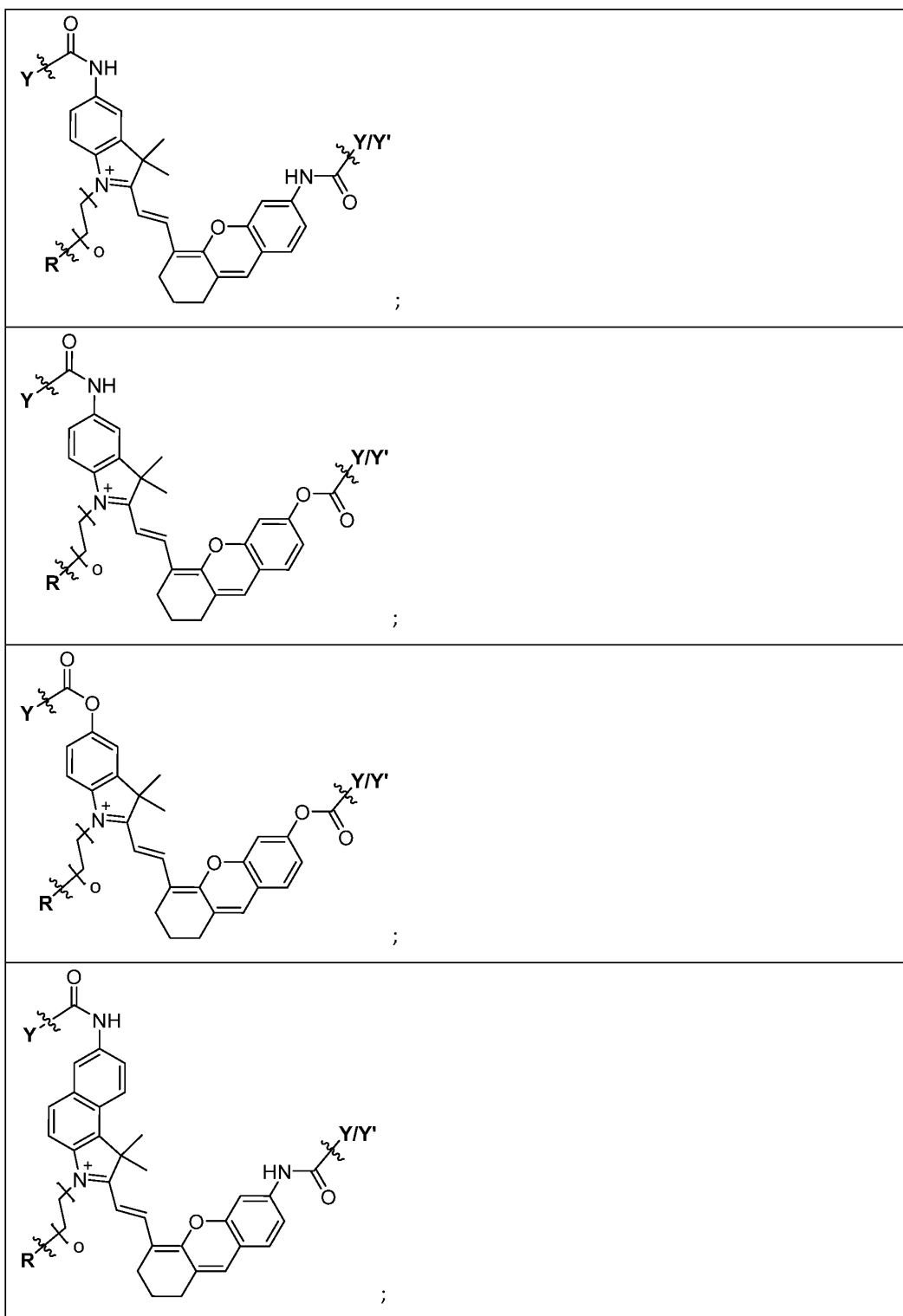
X' represents a caged fluorescent moiety selected from the group consisting of:

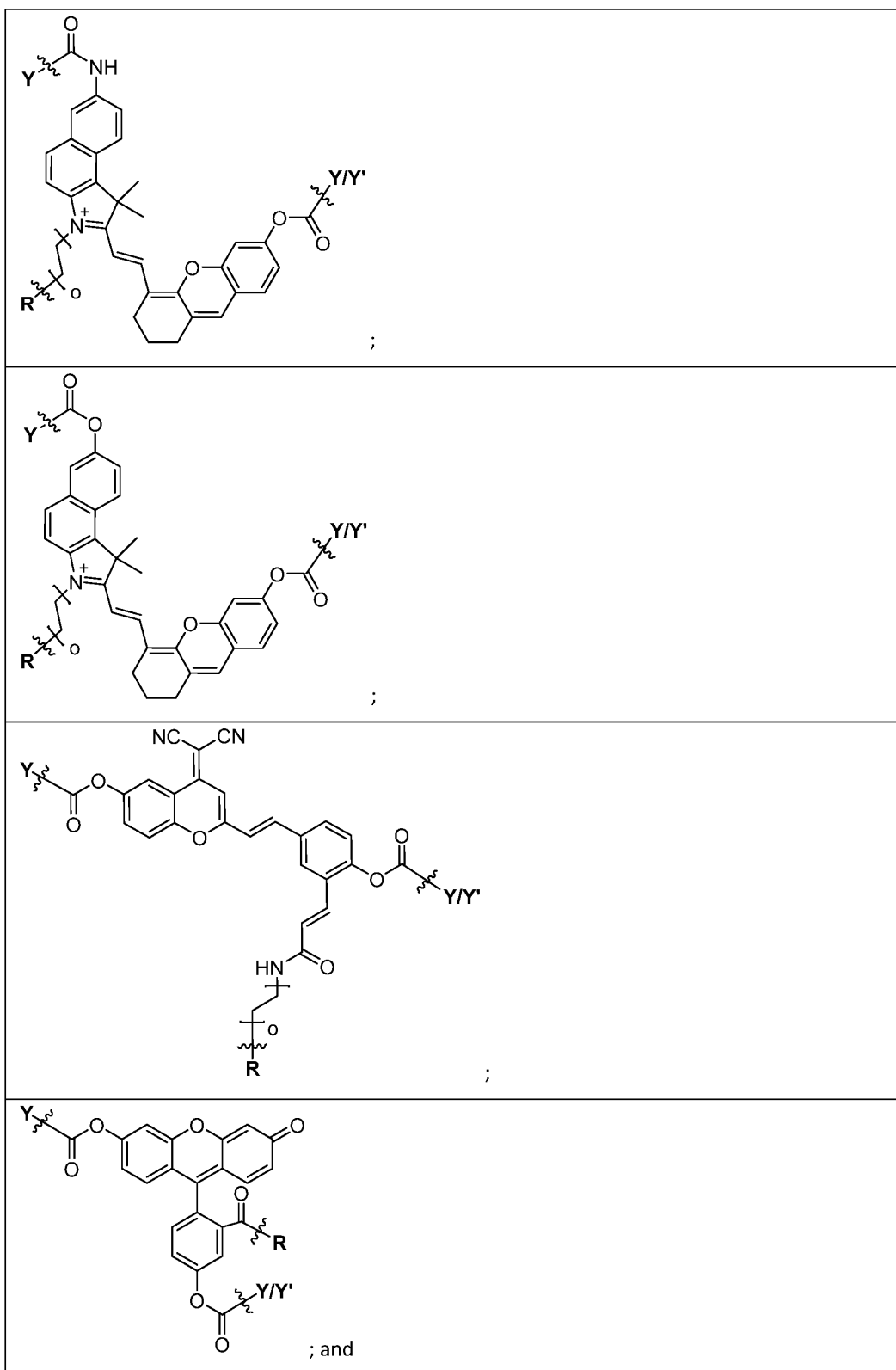


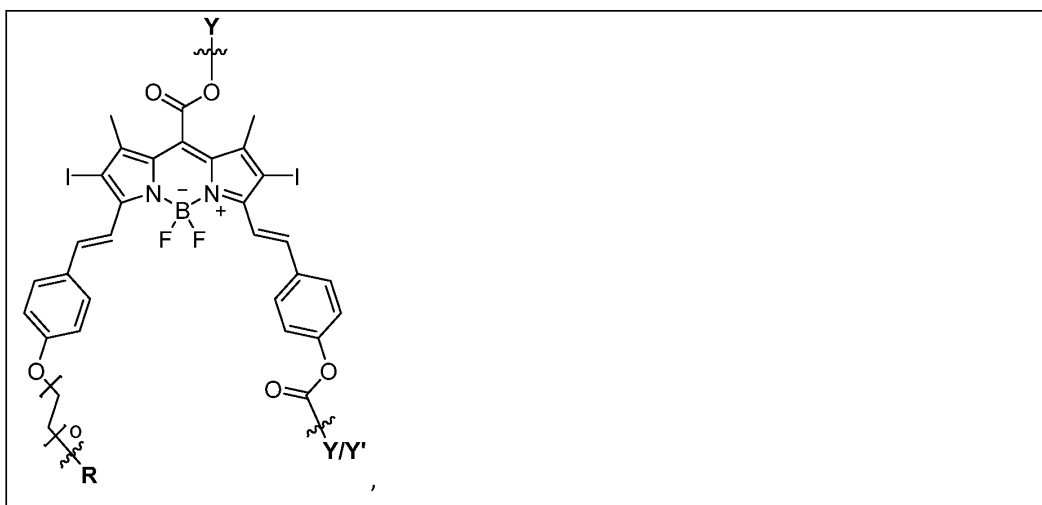
;



;



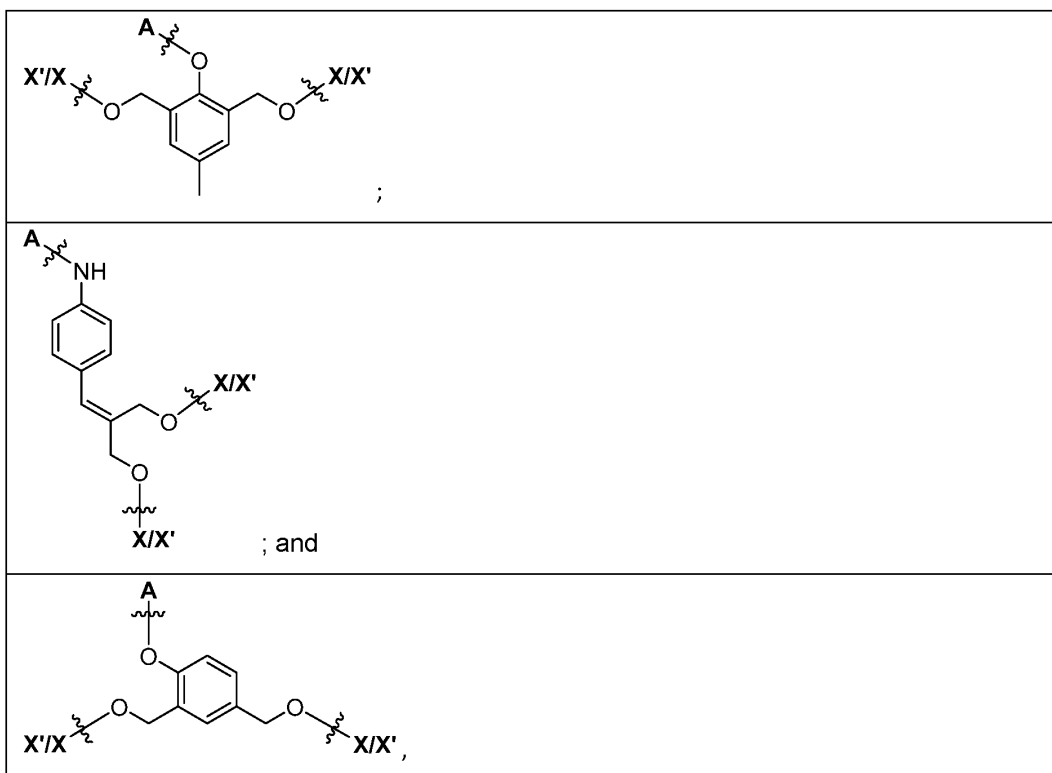




where the wiggly lines represent the point of attachment of X to the moieties R and Y or Y', respectively, where the moiety Y/Y' is Y' if the X' group is the terminal X' group and is Y if the X' group is not the terminal X' group;

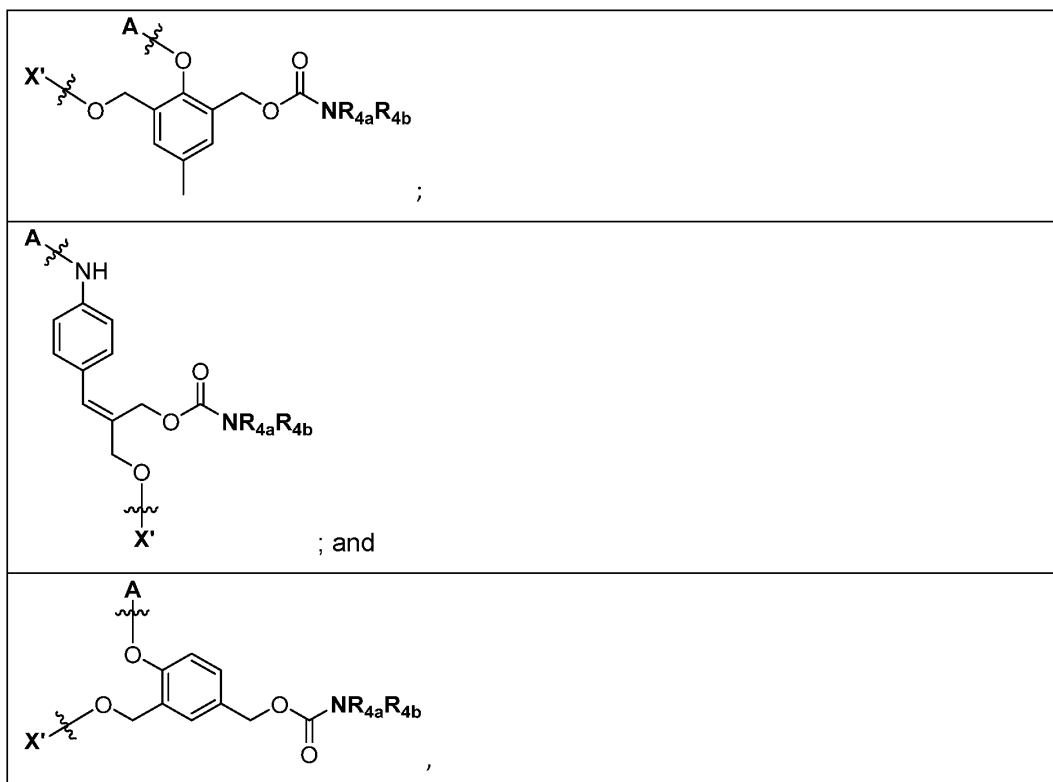
each o independently represents 1 to 3

Y represents a self-immolative linker selected from:



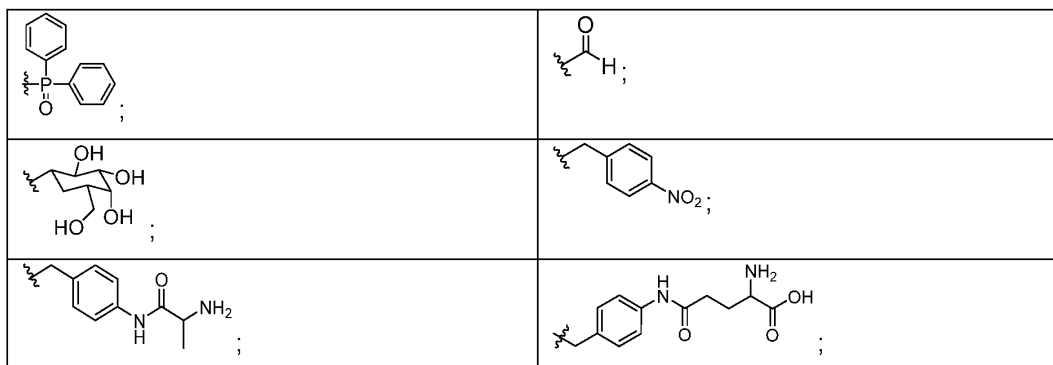
where the wiggly lines represent the point of attachment of Y to the moieties A, X and X', where the moiety X/X' is X' if the Y group is attached to the first X group of the polymeric nanoreporter compound chain and is otherwise attached to an X' group;

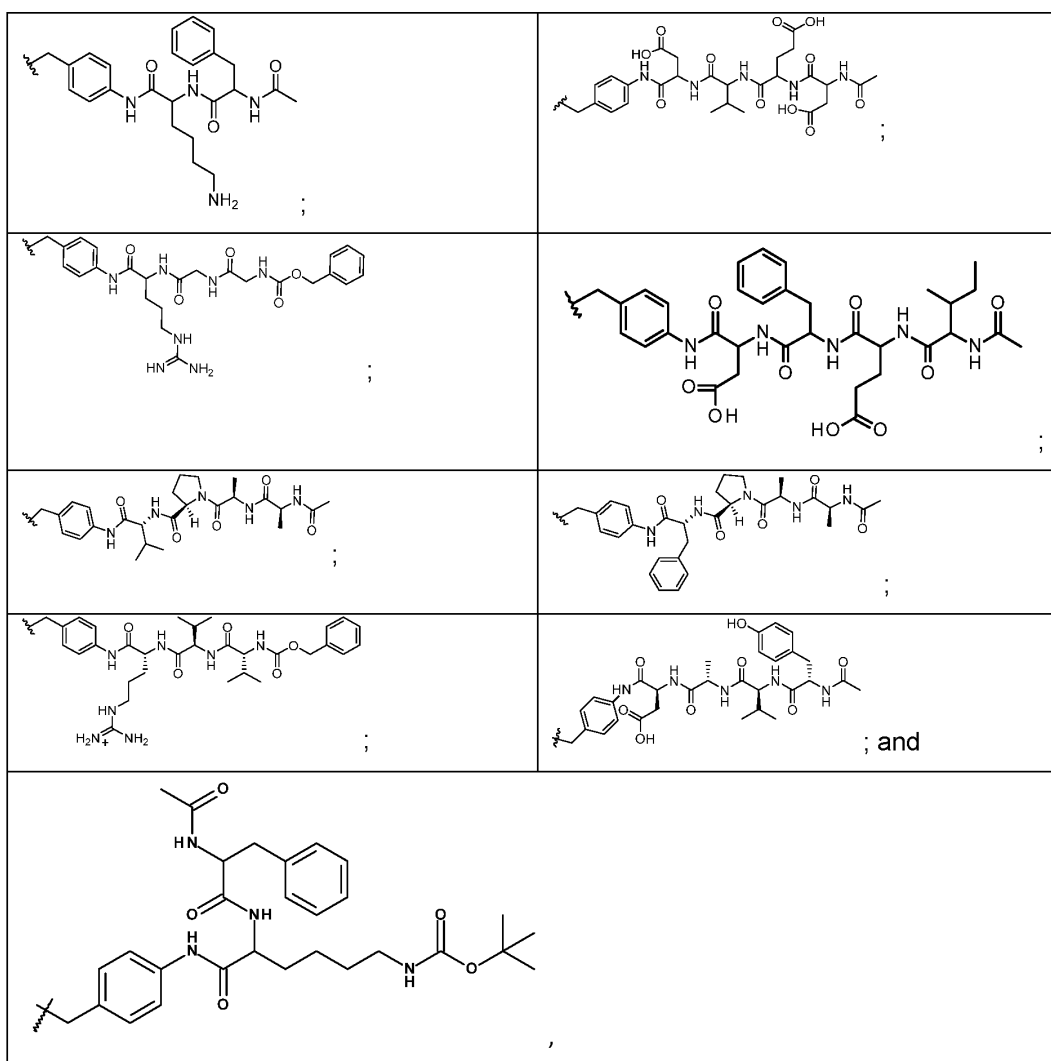
Y' represents a self-immolative linker selected from:



where the wiggly lines represent the point of attachment of Y to the moieties A and X';
where each R_{4a} and R_{4b} independently represents a C₁ to C₆ alkyl group,

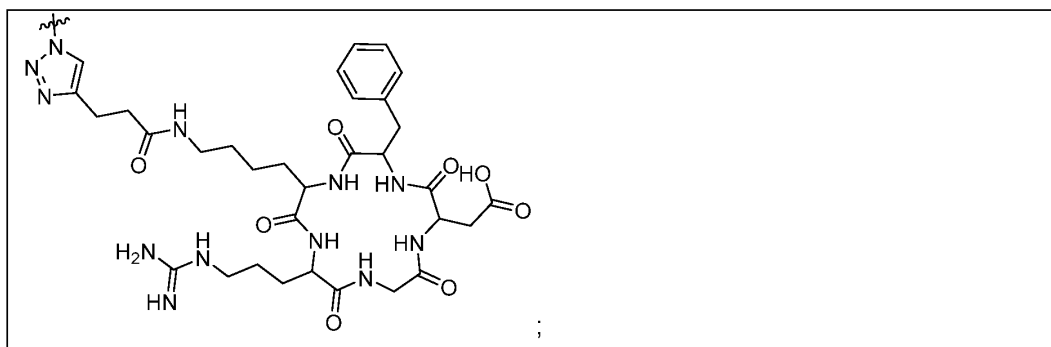
A represents a biomarker responsive moiety, selected from:

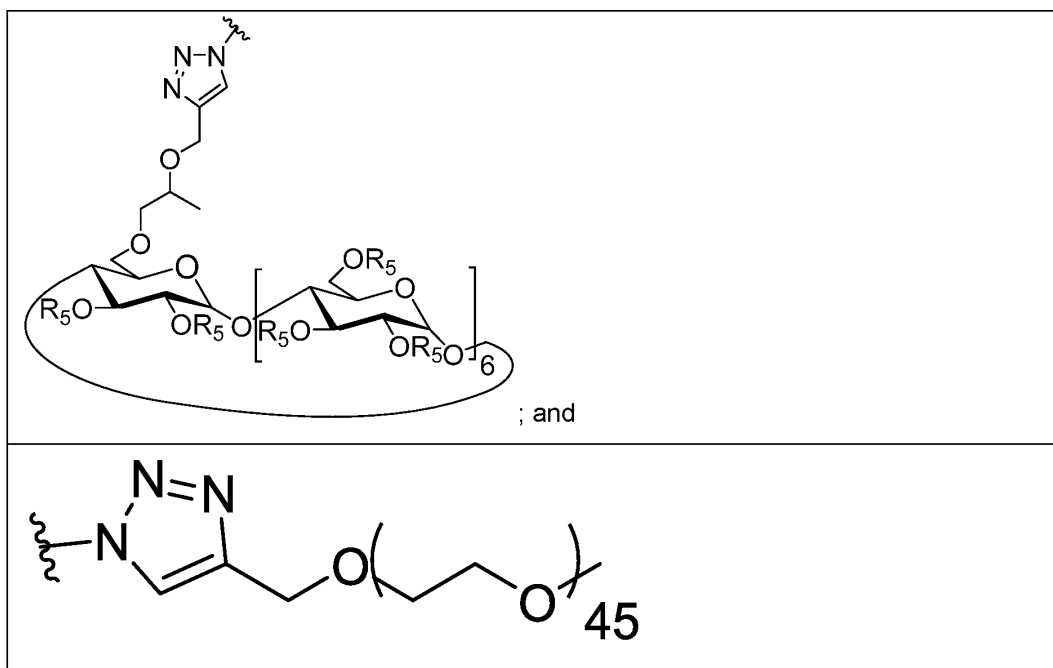




where the wiggly line represents the point of attachment to the rest of the molecule;

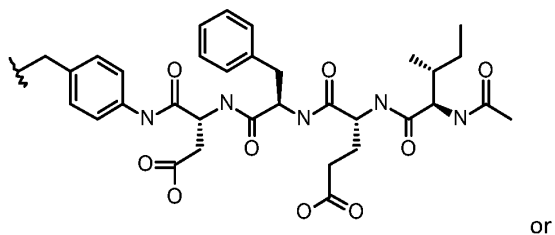
R represents a solubility enhancement moiety selected from one or more of:

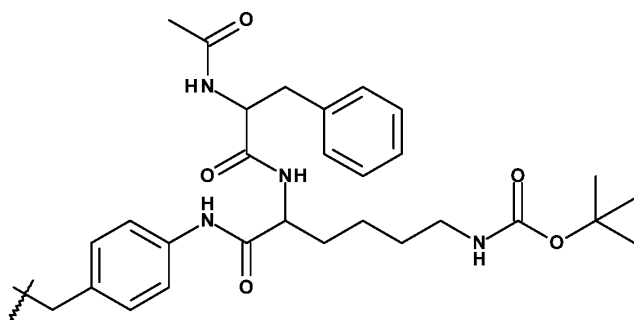




where each R_5 is independently selected from H or $-\text{CH}_2\text{CH}(\text{OH})\text{CH}_3$, and the wiggly line represents the point of attachment to the rest of the molecule, or a pharmaceutically acceptable salt or solvate thereof.

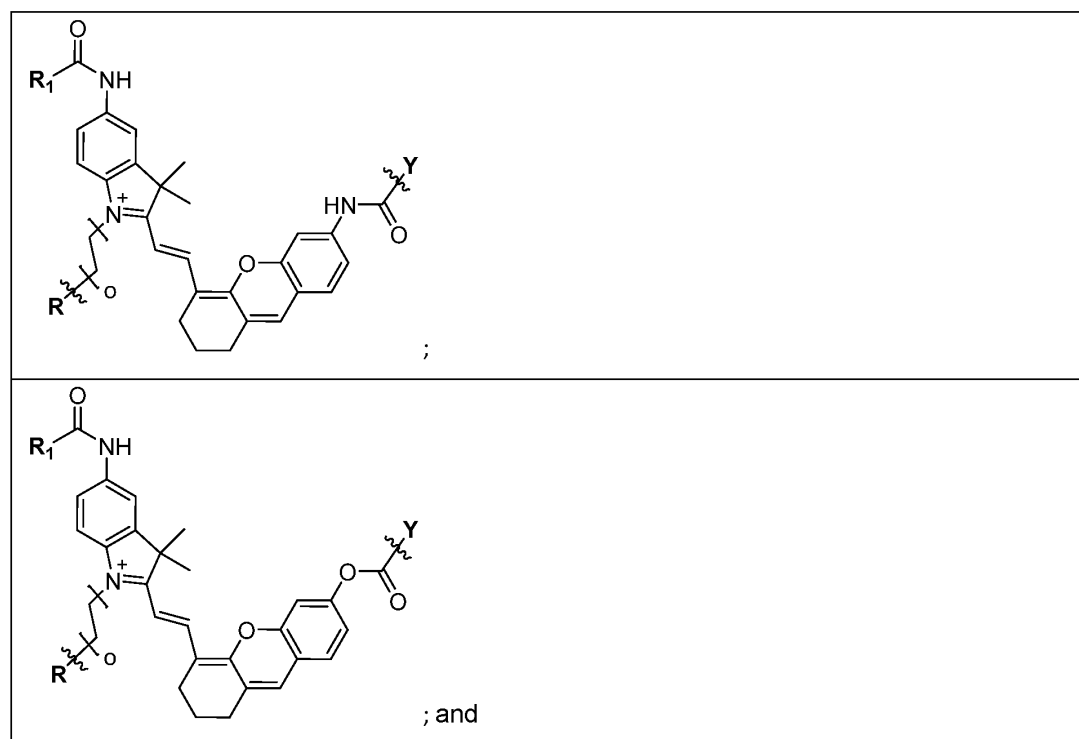
2. The polymeric nanoreporter compound, or salt or solvate thereof, according to Claim 1, wherein n is 2.
3. The polymeric nanoreporter compound, or salt or solvate thereof, according to Claim 1 or Claim 2, wherein A represents:

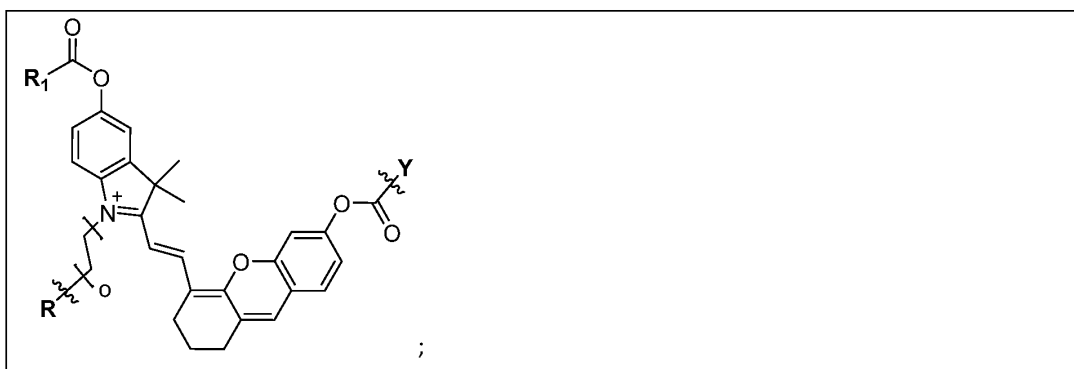




the point of attachment to the rest of the molecule.

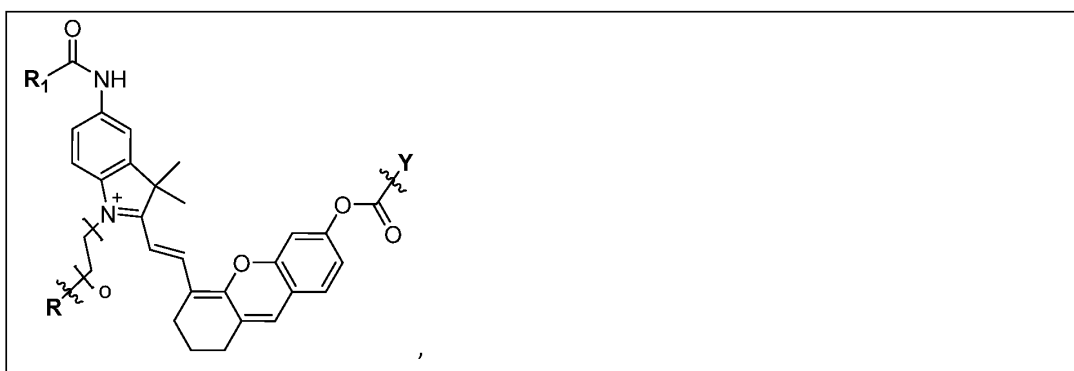
4. The polymeric nanoreporter compound, or salt or solvate thereof, according to any one of the preceding claims, wherein X is selected from:





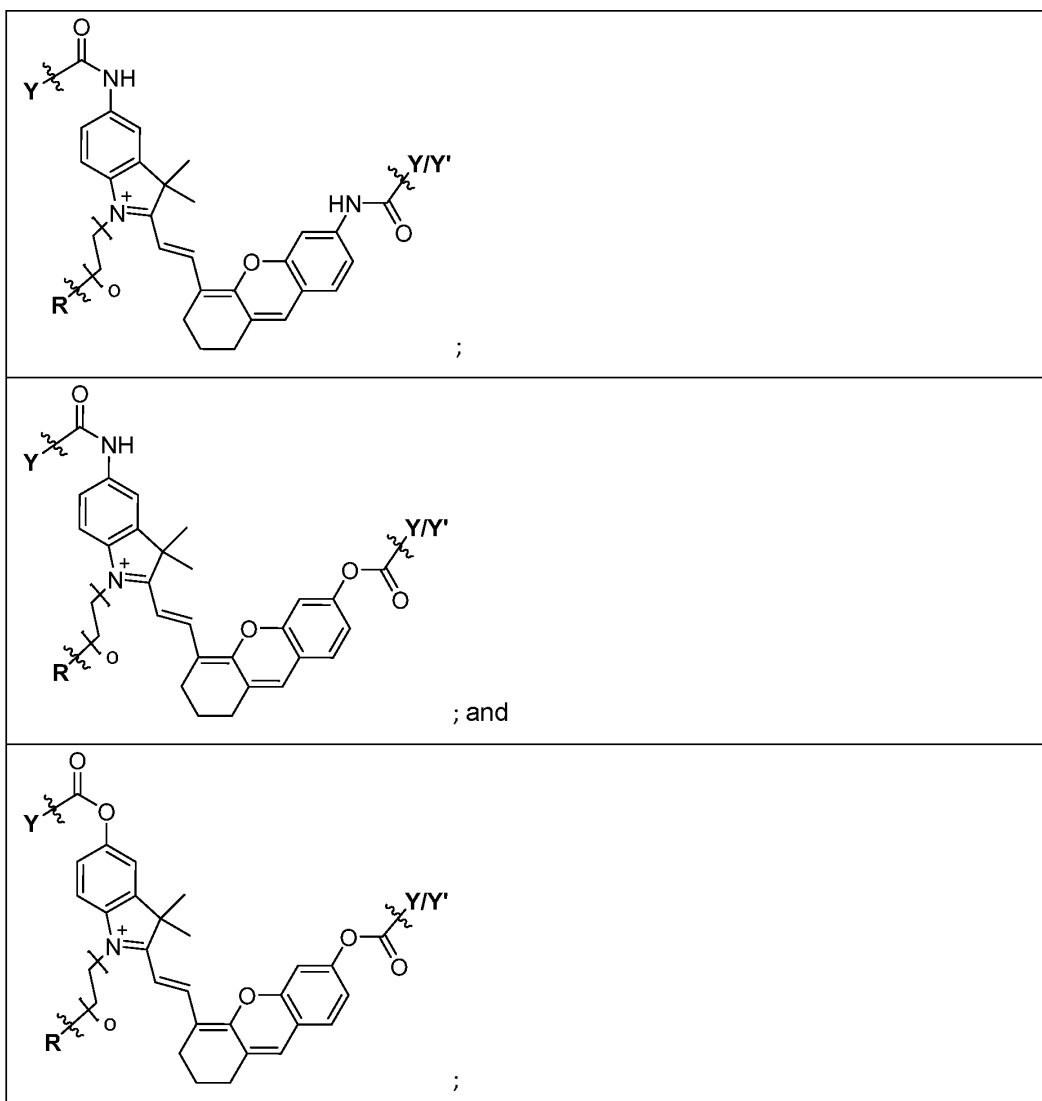
where the wiggly lines represent the point of attachment of X to the moieties R and Y, respectively.

5. The polymeric nanoreporter compound, or salt or solvate thereof, according to Claim 4, wherein X is:



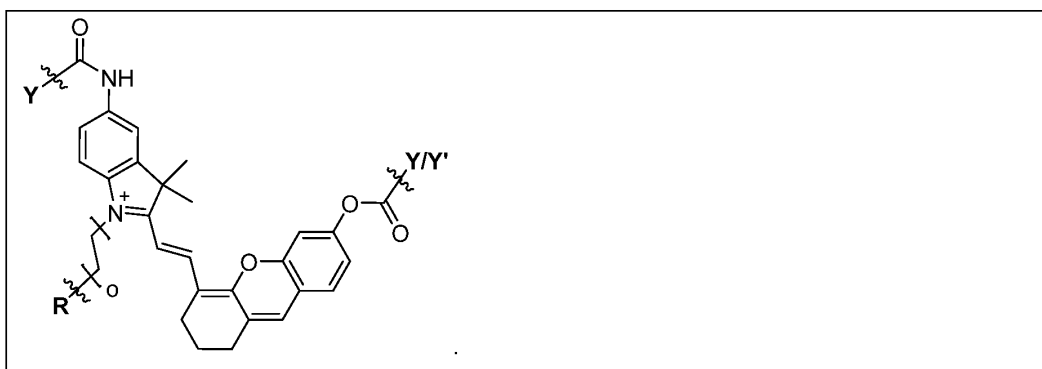
where the wiggly line represents the point of attachment of X to the moieties R and Y, respectively.

6. The polymeric nanoreporter compound, or salt or solvate thereof, according to any one of the preceding claims, wherein R_1 represents $R_{2a}R_{2b}N$ and R_{2a} and R_{2b} independently represent a methyl group.
7. The polymeric nanoreporter compound, or salt or solvate thereof, according to any one of the preceding claims, wherein X' is selected from:



where the wiggly lines represent the point of attachment of X to the moieties R and Y or Y', respectively, where the moiety Y/Y' is Y' if the X' group is the terminal X' group and is Y if the X' group is not the terminal X' group.

7. The polymeric nanoreporter compound, or salt or solvate thereof, according to Claim 6, wherein X' is:

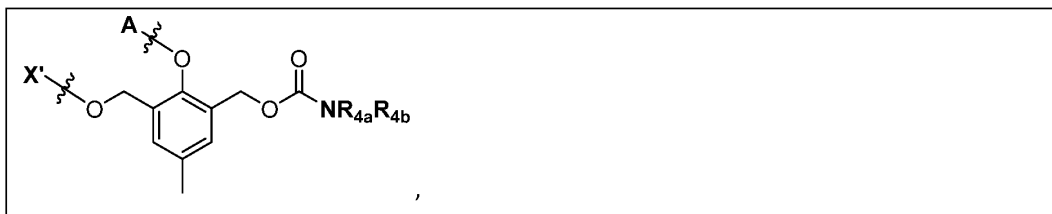


8. The polymeric nanoreporter compound, or salt or solvate thereof, according to any one of the preceding claims, wherein Y is:



where the wiggly lines represent the point of attachment of Y to the moieties A, X and X', where the moiety X/X' is X' if the Y group is attached to the first X group of the polymeric nanoreporter compound chain and is otherwise attached to an X' group.

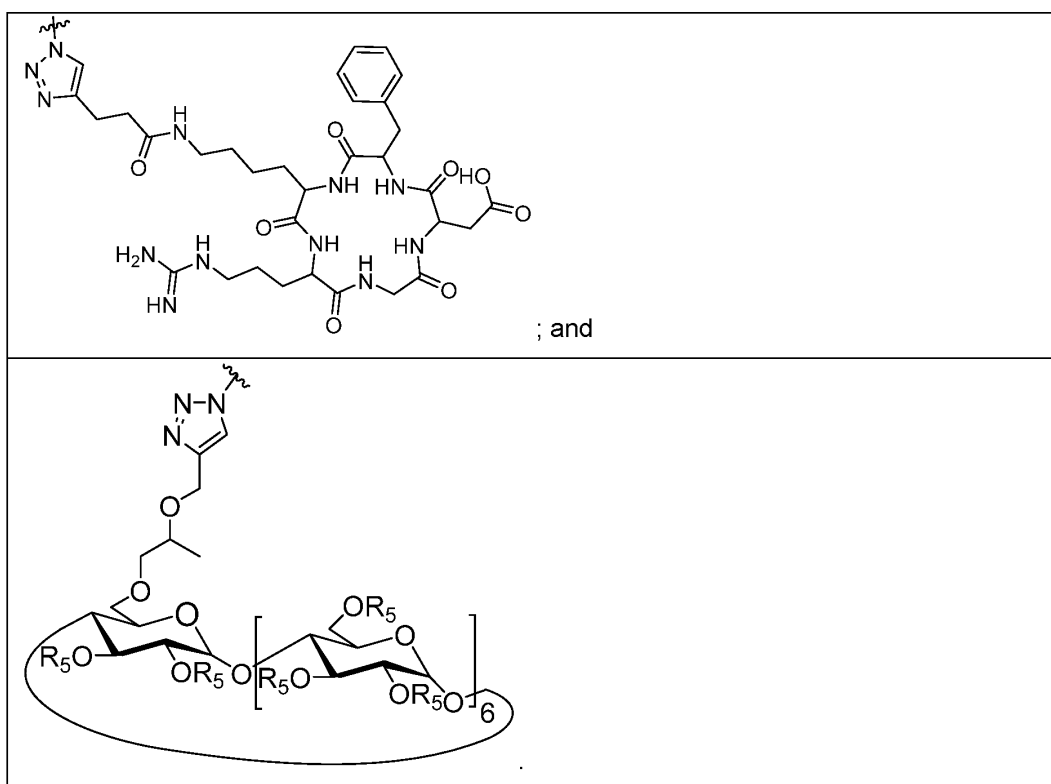
9. The polymeric nanoreporter compound, or salt or solvate thereof, according to any one of the preceding claims, wherein Y' is:



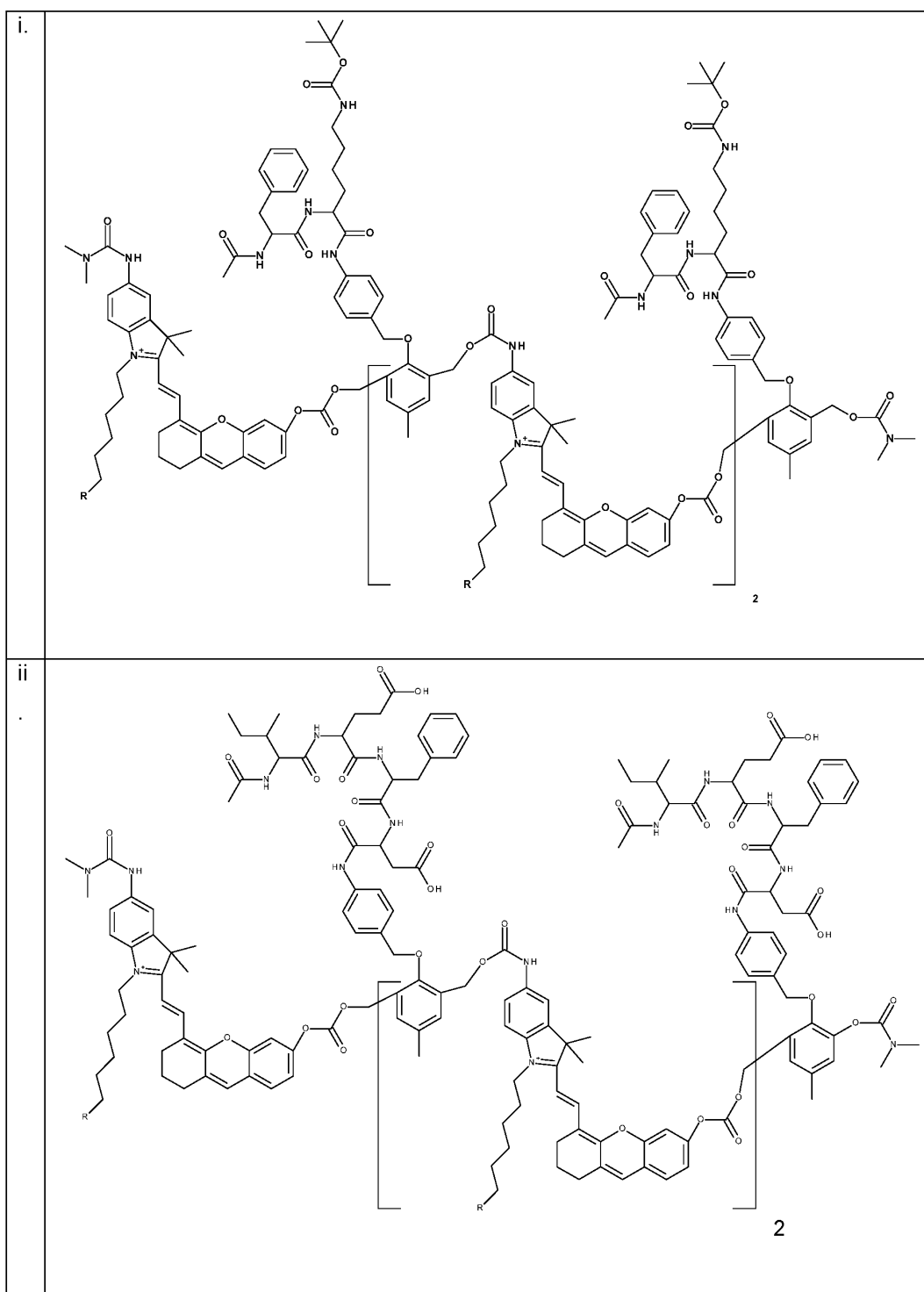
where the wiggly lines represent the point of attachment of Y to the moieties A and X'.

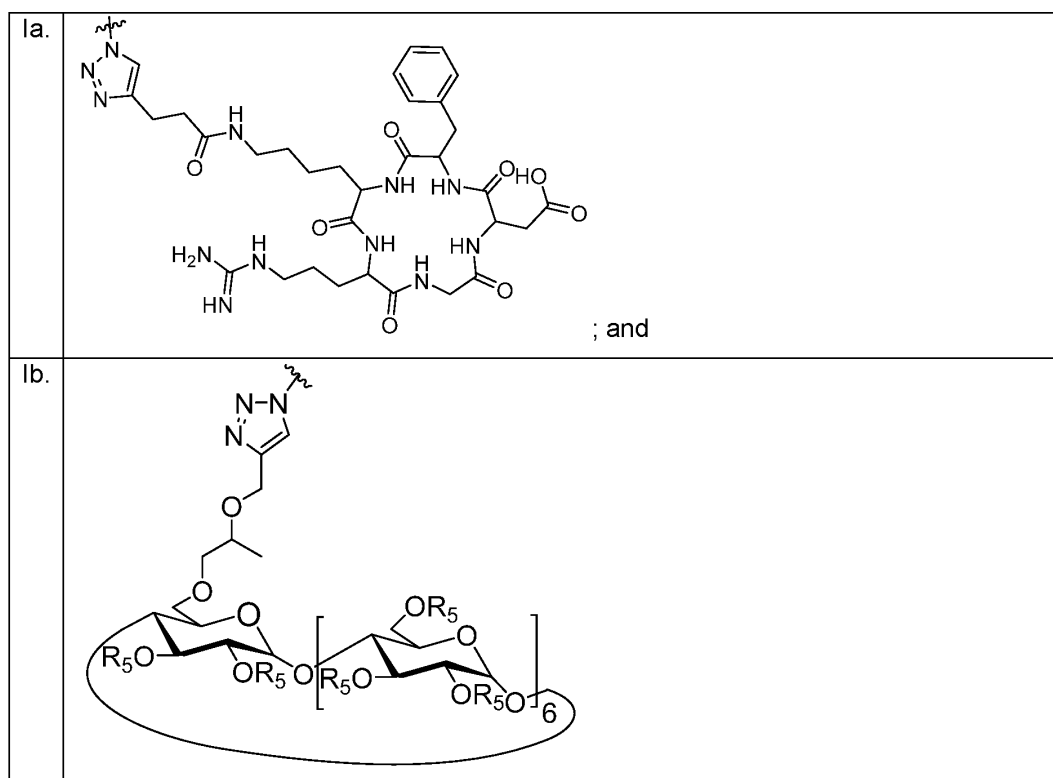
10. The polymeric nanoreporter compound, or salt or solvate thereof, according to any one of the preceding claims, wherein each R_{4a} and R_{4b} independently represents methyl group.

11. The polymeric nanoreporter compound, or salt or solvate thereof, according to any one of the preceding claims, wherein R represents a solubility enhancement moiety selected from one or both of:



12. The polymeric nanoreporter compound, or salt or solvate thereof, according to any one of the preceding claims, wherein the compound is selected from:

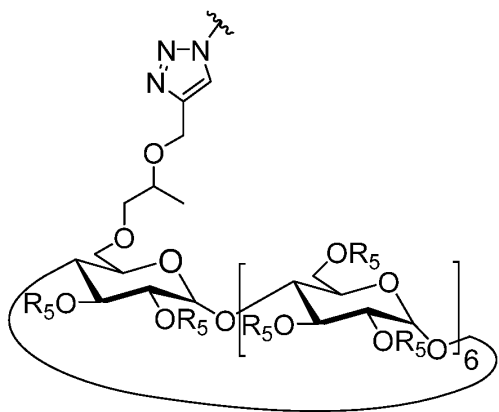




; and

where the ratio of 1a to 1b is 1:9; and

where R in (ii) is:



13. A method of detecting a pathological condition in a subject, the method comprising the steps of:

- (a) administering a polymeric nanoreporter compound according to formula I, or a pharmaceutically acceptable salt or solvate thereof, as described in any one of Claims 1 to 13 that targets the selected pathological condition to a subject; and
- (b) detecting any fluorescence in one or both of urine from the subject and an organ or a tissue in the subject that is targeted by the polymeric nanoreporter compound according to formula I, wherein the presence of the pathological condition in the subject is indicated by fluorescence in the urine, the tissue or organ, or both the urine and the tissue or organ.

15. Use of a polymeric nanoreporter compound according to formula I, or a pharmaceutically acceptable salt or solvate thereof, as described in any one of Claims 1 to 13 in the manufacture of a medicament for the diagnosis of a pathological condition.

16. A polymeric nanoreporter compound according to formula I, or a pharmaceutically acceptable salt or solvate thereof, as described in any one of Claims 1 to 13 for use in the diagnosis of a pathological condition.

17. The method according to Claim 13, the use according to Claim 14 and the polymeric nanoreporter compound for use according to Claim 16, wherein the pathological condition is a cancer, an organ allograft rejection, and immune-mediated hepatitis.