Electronic Supplementary Material (ESI) for Analyst.

Supporting Information

A fluorescent probe for STED microscopy to study NIP-specific B cells

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1. Materials and General Methods for Chemical Synthesis

Reagents. Starting materials and reagents were of the highest grade available from commercial sources and were used as supplied. Fmoc-protected amino acids, Fmoc-NH-PEG(12)-COOH and coupling reagents were obtained from Iris Biotech (Marktredwitz, Germany) and Sieber amide resin was purchased from Novabiochem Merck (Darmstadt, Germany). Star635P-dyes were obtained from Abberior (Göttingen, Germany) and Atto647N dyes were received from Sigma Aldrich (now Merck, Schnelldorf, Germany). NIP-ε-aminocaproyl-OSu was purchased from LGC Biosearch Technologies (Steinach, Germany).

Solvents. Anhydrous solvents of extra dry or puriss. absolute grade (over molecular sieves) were obtained from Acros Organics (Geel, Belgium) and Sigma Aldrich (now Merck, Schnelldorf, Germany). Acetonitrile and methanol for HPLC (in respective grade and all other solvents of the grade puriss. p.a.) were purchased from commercially available sources and were used as supplied. Water for HPLC and buffer was purified using a purification system MilliQ Advantage A10 from Merck Millipore (Darmstadt, Germany). Phosphate buffered saline (PBS) buffer was degassed by stirring for 15 min under vacuum, which then was kept under Ar atmosphere. **Reactions.** All air- and water-sensitive reactions were conducted under inert atmosphere in non-heatable tubes (Eppendorf safe lock tubes) reaction applying a purge-and-refill technique. All reaction steps of solid phase (peptide) synthesis SP(P)S were conducted in a single vessel using an acid- and base-resistant Becton Dickinson (BD) Discardit II-syringe (Fraga, Spain) equipped with a polyethylene-frit for filtration. **Instruments.** Electrospray ionization (ESI) and high-resolution ESI mass spectra were recorded with a Bruker micrOTOF and a Bruker maXis spectrometer (Bremen, Germany). The values are given as *m/z* relation. Microwave-mediated SP(P)S was realized on a CEM Discover microwave instrument (Kamp-Lintfort, Germany). High-performance liquid chromatography (HPLC) was performed on a JASCO (Groß-Umstadt, Germany) with two pumps system PU-2080Plus, a multi wavelength detector MD-2010Plus with an analytical or preparative cell, 3-line degasser DG-2080-5S and interface LC-Net II/ADC using a Macherey-Nagel Nucleodur® RP C-18 analytical HPLC column (250 × 4.6 mm, 5 µm) and a semipreparative HPLC column (250 × 10 mm, 5 µm). The HPLC runs were carried out using a linear gradient of 0.1% aq. trifluoroacetic acid (TFA) (solvent A) and CH_3CN/0.1% TFA (solvent B) in 30 min. Flow rates were taken as 1 mL/min for the analytical, and 3 mL/min for the semi-preparative purpose. The UV absorbance of peptides could be detected at three different wavelengths simultaneously (215 nm, 254 nm and 280 nm). For compounds containing Star635P- or Atto647N fluorophores their respective major absorption wavelength was used for detection instead of 280 nm. Freeze-drying of compounds from aqueous solutions containing minimal amounts of methanol (MeOH) or CH_3CN was performed using a Christ-Alpha-2-4 lyophilizer attached to a high vacuum pump and a Christ RCV-2-18 ultracentrifuge (Osterode am Harz, Germany). UV/vis-Spectra for concentration determination of ε-4-hydroxy-3-iodo-5-nitrophenyl (ε-NIP) and fluorophore-labeled peptides were recorded with a Thermo Scientific Nanodrop 2000c (Waltham, Massachusetts, USA) and for detailed measurements on a JASCO V-750 spectrophotometer. Fluorescence measurements of the probes were performed on a JASCO FP-8500 spectrometer.

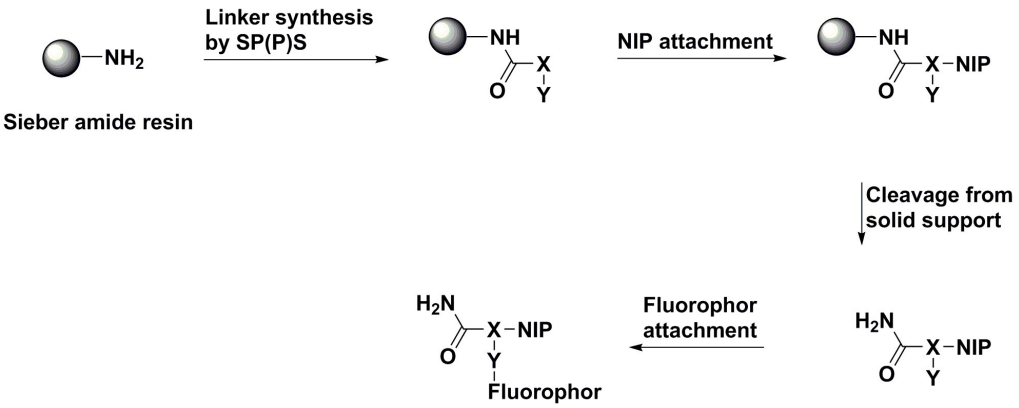
2. Materials and General Methods for Biochemical and Cellular Experiments

Materials. Chemicals, buffers, and other reagents were purchased from Sigma-Aldrich (now Merck, Seelze, Germany) unless otherwise specified. Cell culture medium RPMI-1640 and fetal calf serum (FCS) were purchased from Gibco/Life Technologies (Carlsbad, USA) and Biochrom (Berlin, Germany), respectively. Penicillin/streptomycin 10.000 U/ml each, L-Glutamine 200 mM and Trypsin-EDTA solution were from Lonza (Basel, Switzerland). **Cells.** Human Ramos Burkitt lymphoma cells lacking expression of endogenous immunoglobulin heavy and light chains were kindly provided by Prof. Michael Reth (University of Freiburg, Germany). These cells were reconstituted with a murine NP-specific λ1 light chain and a membrane-bound murine immunoglobulin δ heavy chain. The NP-specific variable regions of the cDNAs of both Ig chains originated from B1.8 hybridoma cells. The murine light chain was expressed using the retroviral vector pMIGRII, which contains an IRES-EGFP cassette. For expression of the murine δ heavy chain the retroviral vector pMSCV (Clontech) was used. Retroviral transduction was performed using the retroviral packaging cell line Platinum-E. Two days after transfection, the virus-containing supernatant was used to infect the Ramos target cells. Transduced cells were selected for high expression of the NP-specific B cell receptor. To that

end, cells were stained with NIP-BSA-biotin (Santa Cruz) and streptavidin-conjugated APC (BD) and were subjected to FACS sorting. All cells were grown in RPMI 1640 medium supplemented with 10% FBS, 4 mM L-glutamine and 100 U/mL each penicillin and streptomycin at 37 °C in a humid atmosphere with 5% CO_2. **Instruments.** Fluorescent microscopy images shown in Fig. 3a and in Supporting Fig. 1 and 2 were acquired using a Nikon Ti Eclipse inverted microscope equipped with 0.75 NA/60 × oil objective and Nikon DS-Qi2 camera (Nikon GmbH, Dusseldorf, Germany). Confocal and STED images (shown in Fig. 3b) were obtained using a multicolor confocal and STED microscope (Abberior Instruments, Göttingen, Germany) based on an IX83 inverted microscope (Olympus, Hamburg, Germany) equipped with a UPLSAPO 100 × 1.4 NA oil immersion objective (Olympus, Hamburg, Germany). Confocal imaging was performed using a 640 nm excitation laser and signal detection between 650 and 720 nm. STED imaging was performed using a 640 nm excitation laser (for Star635P) and a 775 nm depletion laser (set to 30% of the max. power of 1.2 W), with 10 µs pixel dwell time and 20 nm pixel size. Flow cytometry was done with an LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). **Software for Data Analysis and Statistics.** Flow cytometry data were analyzed using FlowJo (FlowJo LLC, Ashland, OR, USA) and Microsoft Excel. Image processing was done using FiJi/ImageJ[1] (NIH, Bethesda, USA). Graphs and statistical analysis were carried out with Sigma Plot (Systat Software, San Jose, CA, USA) or GraphPad Prism 5.0 (San Diego, CA, USA). All values are given as mean ± standard error of the mean (SEM).

3. Synthesis of Fluorescent Marker

3.1 General Synthetic Scheme



Supporting Scheme S1: Probe synthesis started on a solid support (Sieber amide resin) applying solid phase (peptide) synthesis SP(P)S with standard Fmoc protocol, where X is for different backbones (PEG or peptides) and Y stands for the amino acids cysteine or lysine. The affinity tag NIP was introduced right before cleavage. In the final step fluorophores were attached either using the maleimide-thiol or the NHS ester-lysine conjugation reaction.

3.2 General Synthetic Procedure

General synthetic procedure (GSP) 1: Solid Phase (Peptide) Synthesis

Fmoc-based SP(P)S consisted of a cycle with the steps in the following order: deprotection, washing, coupling, washing, (coupling, washing). The deprotection and coupling reactions were microwave-assisted. As solid support a Sieber Amide resin with a loading density of 0.65 mmol/g was applied for all syntheses. The batch size for each experiment was stated correspondingly. The resin was swelled in dichloromethane (DCM)/*N*,*N*-dimethylformamide (DMF) (1:1, *v*/*v*) for 1 h prior to any coupling reaction.

Deprotection of the Fmoc-group was achieved by applying 20% piperidine in DMF (*v*/*v*, 2 ×, 30 sec, 50 W, 50 °C and 3 min, 50 °C, 50 W). For Asp containing sequences 0.1 M 1-hydroxybenzotriazole (HOBt) was added to the piperidine solution.

Loading of the first amino acid was performed by adding an activated solution consisting of amino acid (5.0 eq), HOBt (5.0 eq) and *N*,*N´*-diisopropylcarbodiimide (DIC) (5.0 eq) in DMF to the resin followed by microwave irradiation (20 W, 40 °C, 10 min). After first loading, the **coupling of amino acids** were realized by adding amino acid (4.0 eq/5.0 eq), 1-hydroxy-7-azabenzotriazole (HOAt) (4.0 eq/5.0 eq) and *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N´*,*N´*-tetramethyluronium hexafluorophosphate (HATU) (3.6 eq/4.5 eq), DIEA (10 eq) in DMF (450 µL per 10 µM resin) to the resin followed by microwave irradiation (25 W, 50 °C, 10 min). For each amino acid a double-coupling was performed, while Fmoc-PEG(12)-COOH was single-coupled. Fmoc-PEG(12)-COOH (5.0 eq), HOBt (5.0 eq) and DIC (5.0 eq) in DMF (450 µL per 10 µM resin) was added to the resin and the coupling was carried out at 20 W, 40 °C, 10 min. In between all steps the reactive solution was removed after reaction by successive washing with DMF (3 ×), DCM (3 ×) and DMF (3 ×). The cycle was repeated until complete linker was achieved. After final deprotection, the resin was washed with DMF, DCM, DMF, MeOH and DCM (3 × , respectively). The resin was dried under vacuum and stored at –21 °C until been further processed.

GSP 2: Attachment of NIP

Synthesized linkers, peptides or PEG derivatives loaded on resin (5 µmol, 1.0 eq), were transferred to a reaction tube and swelled in anhydrous DMF (150 µL) under Ar atmosphere. *ε*-NIP-OSu (13.3 mg, 25 µmol, 5.0 eq) was dissolved in anhydrous DMF (150 µL) containing DIEA (8.44 µL, 6.26 mg, 50 µmol, 10 eq), and the mixture was transferred to the swollen resin. The resin was incubated overnight at room temperature. Reaction solution was filtered followed by washing of the resin with DMF, DCM, DMF, MeOH, and DCM (3 × 1.5 mL, respectively). The swollen resin in DCM was directly processed as described in GSP 3.

GSP 3: Cleavage from solid support

For the cleavage from resin, a concentrated solution of TFA, triisopropylsilane (TIS) and H_2O (95/2.5/2.5, *v*/*v*/*v*) was prepared. In case of Cys containing sequences, the concentrated solution consisted of TFA, TIS, H_2O and 1,2-ethanedithiol (EDT) (94/2.5/2.5/1, *v*/*v*/*v*/*v*). A cleavage solution, 20% of the concentrated mixture in DCM (1 mL, *v*/*v*) was transferred to the resin from GSP 2. The solution was filtered after 20 min shaking at room temperature. This cleavage procedure was repeated twice. The combined filtrates were mixed with concentrated cleavage solution and incubated for 1 h at room temperature to reach full deprotection of protecting groups. After cleavage the excess of TFA was removed by a gentle Ar stream. The product was precipitated in Et_2O (3 × 8 mL) and centrifuged for 20 min at 9000 rpm and –4 °C. The crude peptide was dissolved in aqueous CH_3CN and purified by HPLC followed by freeze-drying of the purified product.

GSP 4: Fluorophor labeling through NHS ester-lysine conjugation reaction

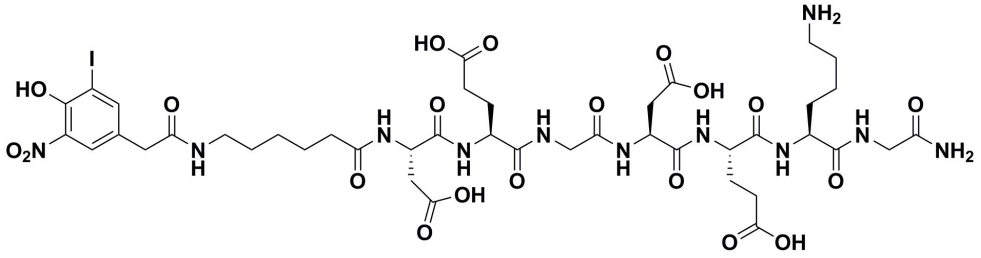
Under Ar atmosphere *ε*-NIP-labeled compound was dissolved in anhydrous DMF (PEG12 1.0 eq, and peptides 2.0 eq with a final concentration of 0.5–1 mM) followed by addition of DIEA (60 eq). To this solution the dye dissolved in anhydrous dimethyl sulfoxide (DMSO) or DMF (1.0 eq, 1 mg/mL) was added and the mixture was shaken overnight at room temperature excluding light. The reaction mixture was diluted with aqueous CH_3CN and purified by HPLC followed by freeze-drying of the purified product.

GSP 5: Fluorophor labeling through maleimide-cysteine conjugation reaction

ε-NIP-labeled peptide (2.0 eq) was dissolved in degassed PBS (50 mM+ 100 mM NaCl, pH 7.2, 100 µL) followed by addition of an aqueous TCEP solution (1.0 eq, 5 mg/mL) under Ar atmosphere. After 15 min, fluorophore in anhydrous DMSO or DMF (1.0 eq, 1 mg/mL) was added and the reaction solution was shaken for 2 h at room temperature excluding light. Afterwards, the solution was stored at +4 °C overnight. The reaction mixture was diluted in aqueous CH_3CN, purified by HPLC followed by freeze-drying of purified product.

3.3 Synthesis and Characterization

ε-NIP-DEGDEKG-NH_2 (1)

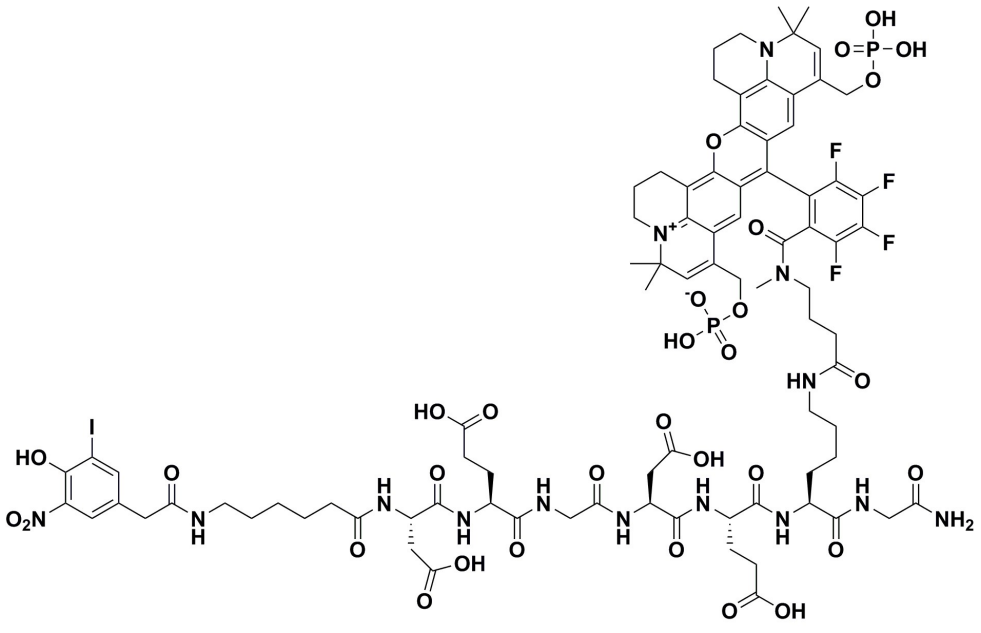


$C_{42}H_{60}IN_{11}O_{20}$
1165.90 g/mol

H-DEGDEKG-NH_2 (50 µmol) was synthesized on a Sieber amide resin by manual SPPS according to GSP 1 using the orthogonal protected amino acids Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH, Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Asp(O^{t}Bu). Then, ε-NIP attachment was performed using ε-NIP-OSu according to GSP 2. Afterwards, the product **1** was cleaved from the support as described in GSP 3. The crude was purified by HPLC and the product **1** was obtained as a light-yellow solid after lyophilisation.

Analytical data: HPLC (analytical, gradient 5 → 90% B in 30 min): t_R = 14.5 min. *m/z* (ESI) = 627.6 [M − 2H + 4Na]$^{2+}$, 638.6 [M − 3H + 5Na]$^{2+}$, 1188.3 [M + Na]$^+$, 581.6 [M − 3H]$^{2-}$, 1164.3 [M − H]$^-$. *m/z* (HR-ESI-MS) = calculated: 627.6242 [M − 2H + 4Na]$^{2+}$, 638.6152 [M − 3H + 5Na]$^{2+}$, 1188.2953 [M + Na]$^+$, 581.6458 [M − 3H]$^{2-}$, 1164.2988 [M − H]$^-$, found: 627.6227 [M − 2H + 4Na]$^{2+}$, 638.6145 [M − 3H + 5Na]$^{2+}$, 1188.2916 [M + Na]$^+$, 581.6458 [M − 3H]$^{2-}$, 1164.3043 [M − H]$^-$.

KM1: ε-NIP-DEGDEK(Star635P)G-NH_2 (2)



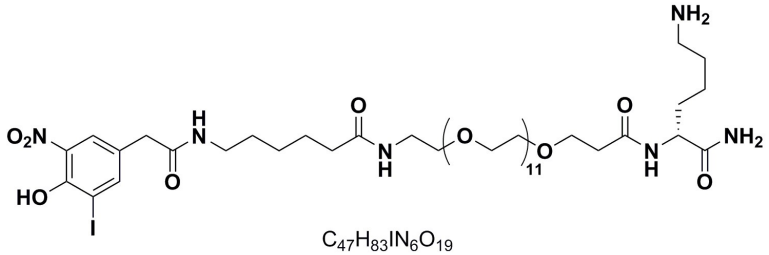
$C_{85}H_{103}F_4IN_{14}O_{31}P_2$
2081.67 g/mol

ε-NIP-DEGDEKG-NH_2 (**1**, 900 µg, 388 nmol, 2.0 eq) was labeled with the fluorophore Star635P in anhydrous DMF (20.0 µL, 200 µg, 194 nmol, 1.0 eq) according to GSP 4. The product KM 1 (2, 287 µg, 138 nmol, 71%) was isolated as a blue solid.

Analytical data: HPLC (analytical, gradient 10 → 90% B in 30 min): t_R = 16-5–17.8 min. *m/z* (ESI) = 1085.7 [M − 2H + 4Na]$^{2+}$, 1096.2 [M − 2H + 5Na]$^{2+}$, 1039.8 [M − 2H]$^{2-}$, 1050.3 [M − 3H + Na]$^{2-}$. *m/z* (HR-ESI-MS) = calculated: 692.5050 [M − 3H]$^{3-}$,

699.8324 [M − 4H + Na]$^{3-}$, 1039.2612 [M − 2H]$^{2-}$, 1050.2522 [M − 3H + Na]$^{2-}$, found: 692.5059 [M − 3H]$^{3-}$, 699.8321 [M − 4H + Na]$^{3-}$, 1039.2612 [M − 2H]$^{2-}$, 1050.2522 [M − 3H + Na]$^{2-}$.

ε-NIP-PEG(12)-K-NH_2 (3)

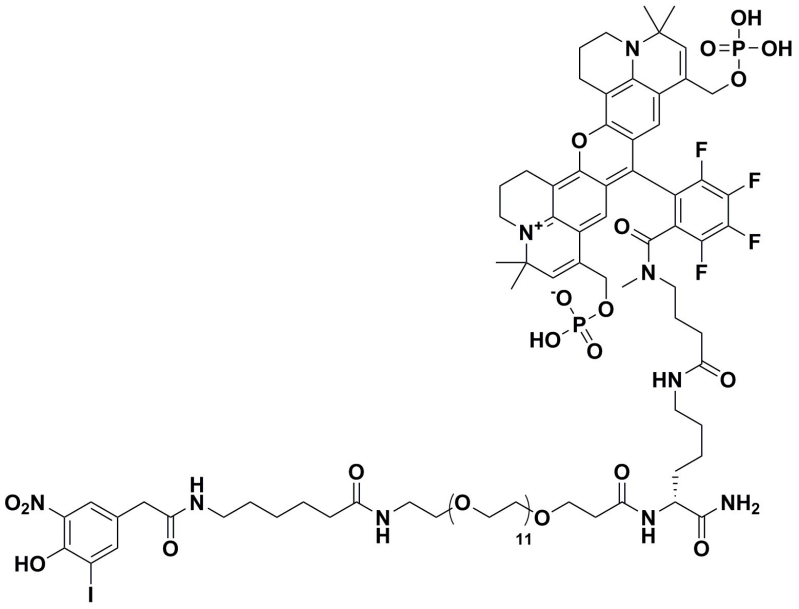


$C_{47}H_{83}IN_6O_{19}$
1163.11 g/mol

H-PEG(12)-K-NH_2 (10 µmol) was synthesized on a Sieber amide resin by manual SPPS according to GSP 1 using Fmoc-PEG(12)-COOH and the orthogonal protected amino acid Fmoc-Lys(Boc)-OH. Then, ε-NIP was *N*-terminally attached to the resin-bound peptide according to GSP 2. Afterwards, the product **1** was cleaved from the support as described in GSP 3. The crude was purified by HPLC and the product **3** was obtained as a light-yellow solid after lyophilisation.

Analytical data: HPLC (analytical, gradient 20 → 90% B in 30 min): t_R = 12.1 min. *m/z* (ESI) = 582.2 [M + 2H]$^{2+}$, 593.2 [M + H+ Na]$^{2+}$, 1163.5 [M + H]$^+$. *m/z* (HR-ESI-MS) = calculated: 582.2452 [M + 2H]$^{2+}$, 593.2361 [M + H + Na]$^{2+}$, 1163.4830 [M + H]$^+$, 1185.4638 [M + Na]$^+$, found: 582.2446 [M + 2H]$^+$, 593.2363 [M + H+ Na]$^{2+}$, 1163.4831 [M + H]$^+$, 1185.4650 [M + Na]$^+$.

KM4: ε-NIP-PEG(12)-K(Star635P)-NH_2 (4)



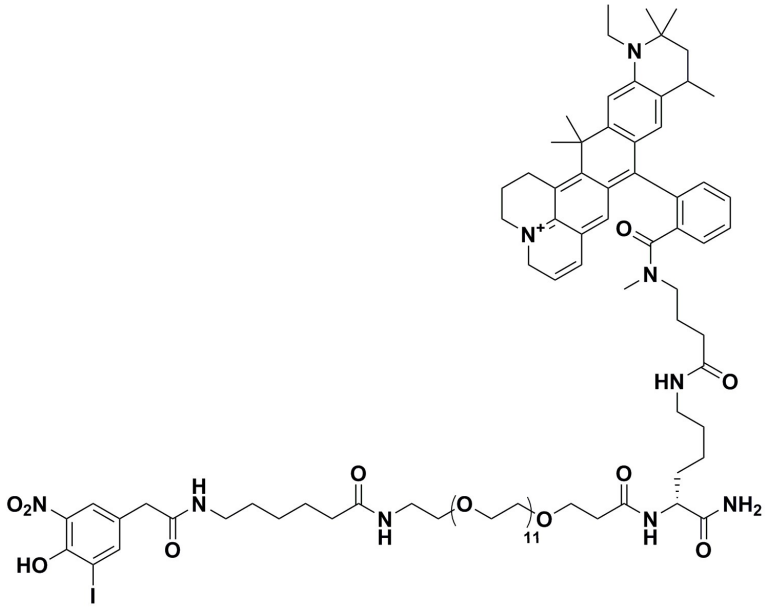
$C_{90}H_{126}N_9O_{30}P_2F_4I$
2078.88 g/mol

ε-NIP-PEG(12)-K-NH_2 (**3**, 170 µg, 146 nmol, 1.0 eq) was labeled with the fluorophore Star635P NHS ester in anhydrous DMSO (15 µL, 150 µg, 142 nmol, 1.0 eq) according to GSP 4. The product KM4 (**4**, 166 µg, 79.8 nmol, 56%) was isolated as a blue solid.

Analytical data: HPLC (analytical, gradient 20 → 90% B in 30 min): t_R = 15.1–16.3 min. *m/z* (ESI) = 730.6 [M − 2H + 5Na]$^{3+}$, 1084.4 [M − 2H + 4Na]$^{2+}$, 1038.4 [M − 2H]$^{2-}$, 2077.7 [M − H]$^-$. *m/z* (HR-ESI-MS) = calculated: 730.2127 [M − 2H + 5Na]$^{3+}$,

1083.8245 [M − 2H + 4Na]$^{2+}$, 1037.8461 [M − 2H]$^{2-}$, 2076.6994 [M − H]$^{-}$, found: 730.2137 [M − 2H + 5Na]$^{3+}$, 1083.8232 [M − 2H + 4Na]$^{2+}$, 1037.8491 [M − 2H]$^{2-}$, 2076.7044 [M − H]$^{-}$.

KM5: ε-NIP-PEG(12)-K(Atto647N)-NH_2 (5)

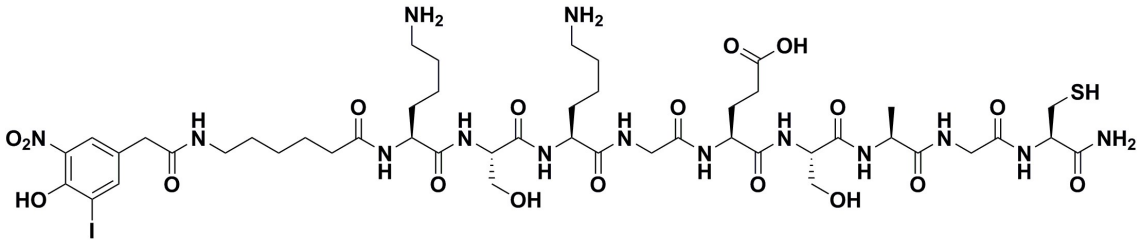


$C_{89}H_{132}IN_9O_{21}$
1791.99 g/mol

ε-NIP-PEG(12)-K-NH_2 (**3**, 170 µg, 146 nmol, 1.24 eq) was labeled with the fluorophore Atto647N NHS ester in anhydrous DMSO (11 µL, 110 µg, 130 nmol, 1.0 eq) according to GSP 4. The product KM5 (**5**, 229 µg, 128 nmol, 98%) was isolated as a blue solid.

Analytical data: HPLC (analytical, gradient 20 → 90% B in 30 min): t_R = 22.3, 22.7 min. *m/z* (ESI) = 612.3 [M + 3H]$^{3+}$, 907.4 [M + 2H]$^{2+}$, 1790.9 [M + H]$^{+}$. *m/z* (HR-ESI-MS) = calculated: 619.6086 [M + 3Na]$^{3+}$, 917.9183 [M + 2Na]$^{2+}$, 1790.8655 [M + H]$^{+}$, found: 619.6098 [M + 3Na]$^{3+}$, 917.9181 [M + 2Na]$^{2+}$, 1790.8640 [M + H]$^{+}$.

ε-NIP-KSKGESAGC-NH_2 (6)

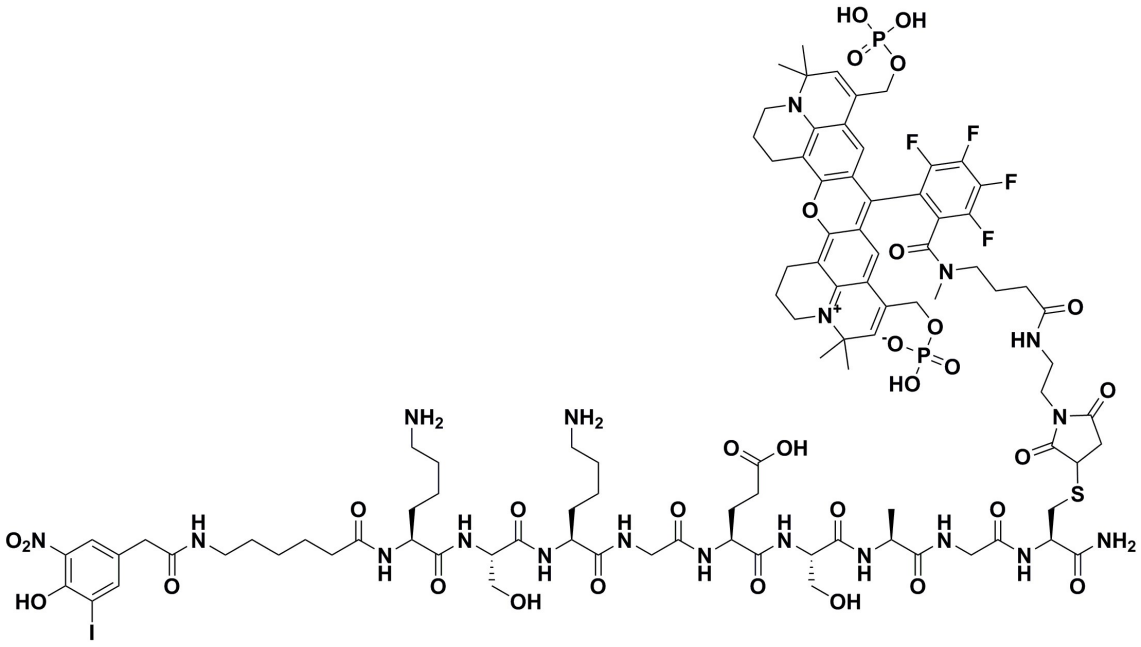


$C_{47}H_{75}IN_{14}O_{18}S$
1283.16 g/mol

H-KSKGESAGC-NH_2 (25 µmol) was synthesized on a Sieber amide resin by manual SPPS according to GSP 1 using the orthogonal protected amino acids Fmoc-Cys(Trt)-OH, Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Glu(^{t}Bu)-OH and Fmoc-Lys(Boc)-OH. Then, ε-NIP was *N*-terminally attached to the resin-bound peptide according to GSP 2. Afterwards, the product **1** was cleaved from the support as described in GSP 3. The crude was purified by HPLC and freeze-dried afterwards to yield the product **3** as a light-yellow solid.

Analytical data: HPLC (analytical, gradient 20 → 80% B in 30 min): t_R = 8.78 min. *m/z* (ESI) = 642.2 [*M* + 2H]$^{2+}$, 1283.4 [*M* + H]$^{+}$. *m/z* (HR-ESI-MS) = calculated: 642.2147 [*M* + 2H]$^{2+}$, 664.1967 [*M* + 2Na]$^{2+}$, 1283.4222 [*M* + H]$^{+}$, found: 642.2149 [*M* + 2H]$^{2+}$, 664.1959 [*M* + 2Na]$^{2+}$, 1283.4216 [*M* + H]$^{+}$.

KM7: ε-NIP- KSKGESAGC(Star635P)-NH_2 (7)

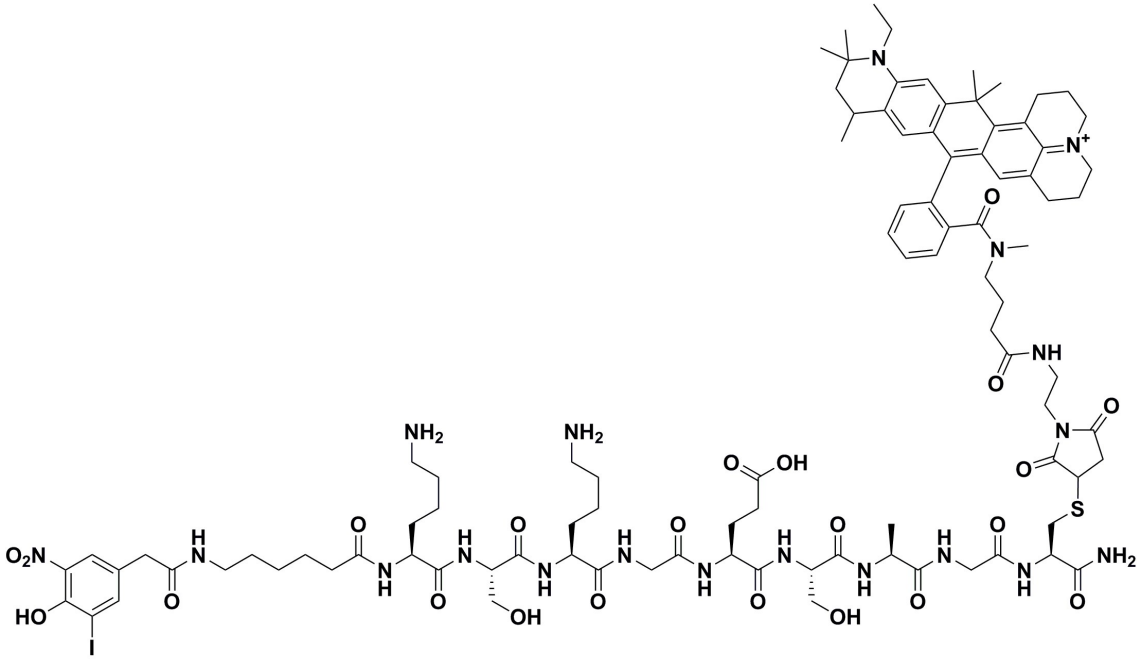


$C_{96}H_{126}F_4IN_{19}O_{31}P_2S$
2339.07 g/mol

ε-NIP-KSKGESAGC-NH_2 (**6**, 375 µg, 292 nmol, 1.16 eq) was labeled with the fluorophore Star635P maleimide in anhydrous DMSO (20 µL, 270 µg, 251 nmol, 1.0 eq) according to GSP 5. The product KM7 (**7**, 587 µg, 251 nmol, 98%.) was isolated as a blue solid.

Analytical data: HPLC (analytical, gradient 20 → 80% B in 30 min): t_R = 13.1–14.1 min. *m/z* (ESI) = 802.6 [*M* + 3Na]$^{3+}$. *m/z* (HR-ESI-MS) = calculated: 802.2240 [*M* + 3Na]$^{3+}$, 778.2275 [*M* − 3H]$^{3-}$, 1167.8449 [*M* − 2H]$^{2-}$, found: 802.2207 [*M* + 3Na]$^{3+}$, 778.2272 [*M* − 3H]$^{3-}$, 1167.8449 [*M* − 2H]$^{2-}$.

KM8: ε-NIP-KSKGESAGC(Atto647N)-NH_2 (8)



$C_{95}H_{133}IN_{19}O_{22}S^+$
2052.18 g/mol

ε-NIP-KSKGESAGC-NH_2 (**6**, 303 µg, 236 nmol, 2.0 eq) was labeled with the fluorophore Atto647N maleimide in anhydrous DMSO (10.2 µL, 102 µg, 117 nmol, 1.0 eq) according to GSP 5. The product KM8 (**8**, 146 µg, 71.1 nmol, 61%) was isolated as a blue solid.

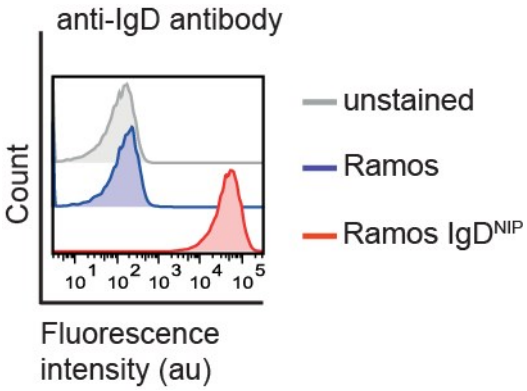
Analytical data: HPLC (analytical, gradient 20 → 80% B in 30 min): t_R = 18.7–19.0 min. *m/z* (ESI) = 684.6 $[M + 2H]^{3+}$, 1026.5 $[M + H]^{2+}$. *m/z* (HR-ESI-MS) = calculated: 513.4713 $[M + 3Na]^{4+}$, 684.2926 $[M + 2H]^{3+}$, 1025.9353 $[M + H]^{2+}$, found: 513.4710 $[M + 3Na]^{4+}$, 684.2926 $[M + 2H]^{3+}$, 1025.9357 $[M + H]^{2+}$.

4. Biochemical and Cellular Experiments

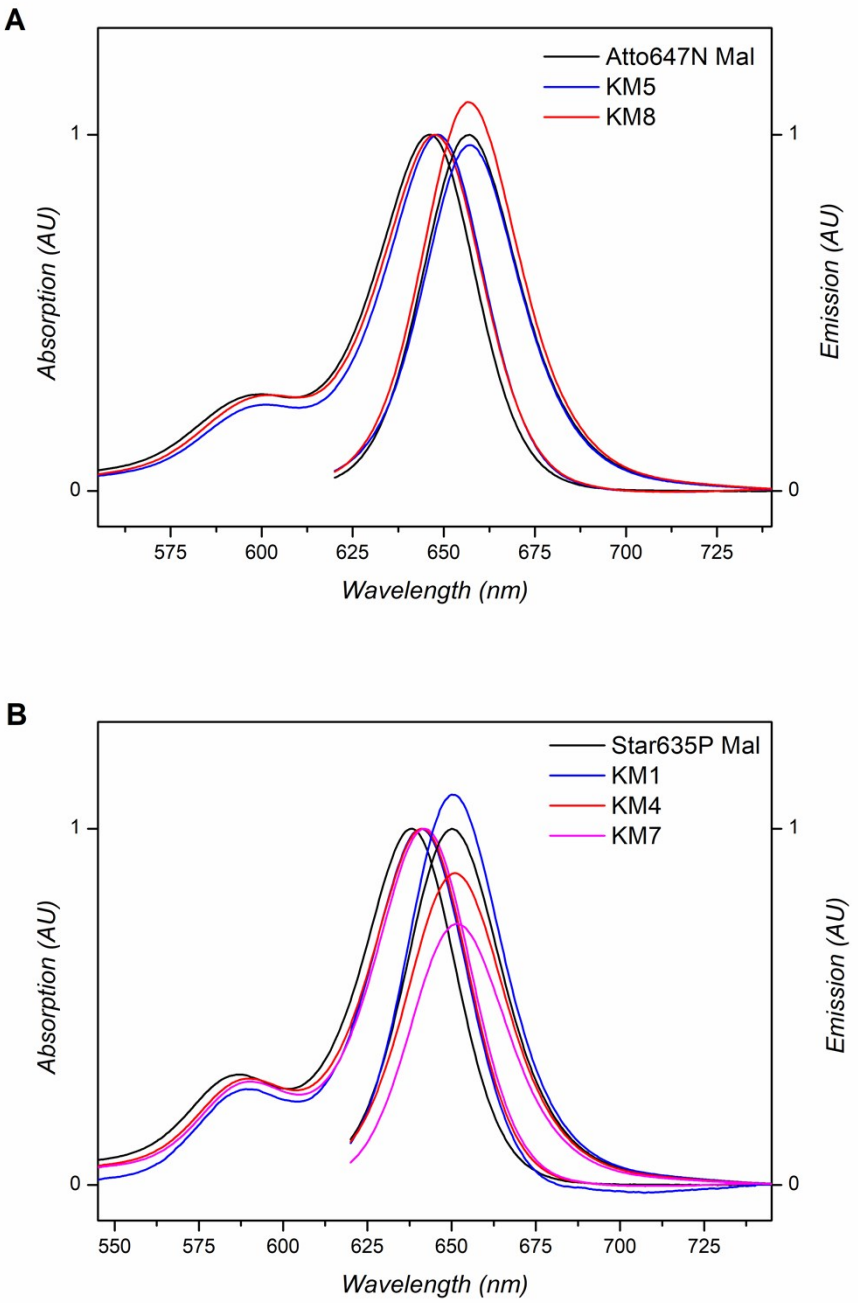
4.1 Staining of B cells with fluorescent probes

Ramos and Ramos IgD^{NIP} cells (~200,000/sample) were centrifuged in 1.5 mL tubes for 4 min at 300 × g and cell pellets were resuspended with 50 µL ice-cold complete medium supplemented with fluorescently labeled NIP at the different concentrations (250 nM, 500 nM and 1 µM) and incubated on ice for 30 min. After incubation with the probes, cells were washed 2 × in 1 mL ice-cold Dulbecco´s PBS (DPBS). After the last washing step pellets were resuspended in 1 mL of ice-cold DPBS. For non-fixed cells imaging experiments (shown in Fig. S2), resuspended cells were transferred to a 24 well glass bottom plate (Greiner Bio-One GmbH, Austria) and centrifuged at ~50 × g for 5 min at 4 °C to allow the cells to adhere to the glass and directly imaged. For fixed cells experiments (shown in Fig. 3a-b and b and Fig. S3), resuspended cells were transferred to a 12 well plate containing PLL treated coverslips and plate centrifuged at ~50 × g for 5 min at 4 °C to allow the cells to adhere to the coverslips. After centrifugation the supernatant was discarded and 1 mL fixation solution composed of 4% paraformaldehyde and 0.1% glutaraldehyde in DPBS was added to each well. Plates were centrifuged at ~50 × g for 10 min at 4 °C and then incubated at room temperature protected from light for 30 min. Remaining fixative was quenched by adding 1 mL of 0.1 M glycine in DPBS and incubating at room temperature protected from light for 15 min. Cells were then washed three times with DPBS and coverslips were embedded in Mowiol mounting media consisting of 6 g glycerol, 6 mL deionized water, 12 mL 0.2 M Tris buffer pH 8.5 and 2.4 g Mowiol® 4–88 (Merck Millipore, Darmstadt, Germany) for imaging.

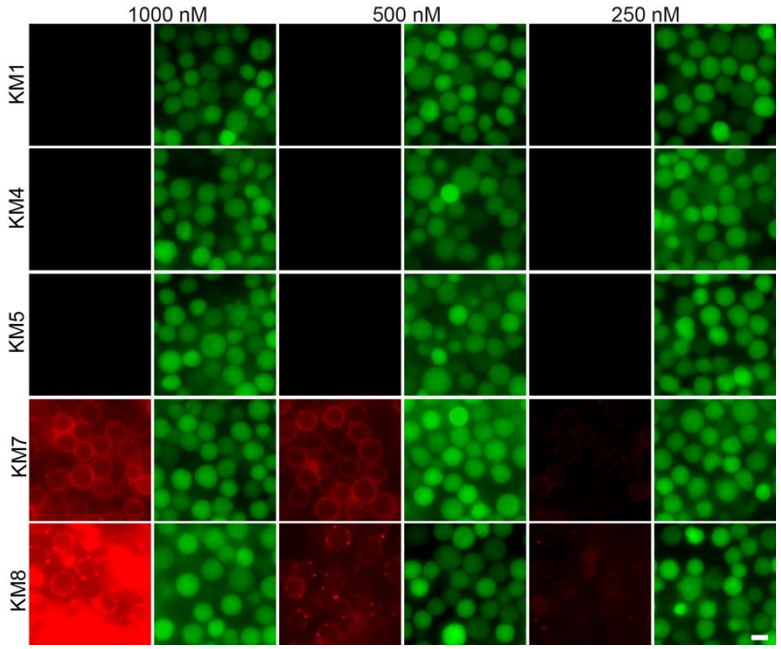
5. Supporting Figures



Supporting Fig. S1: Cell surface expression of IgD on Ramos IgD^{NIP} cells. Ramos and Ramos IgD^{NIP} cells were stained stepwise with 0.5 ng/ml biotinylated rat anti-mouse IgD, clone 11-26 (Southern Biotech, Birmingham, AL, USA) and with 0.2 ng/ml APC-conjugated streptavidin (BD Biosciences). After staining, all cells were washed extensively with cold PBS and analysed by flow cytometry. Unstained cells served as an additional control.

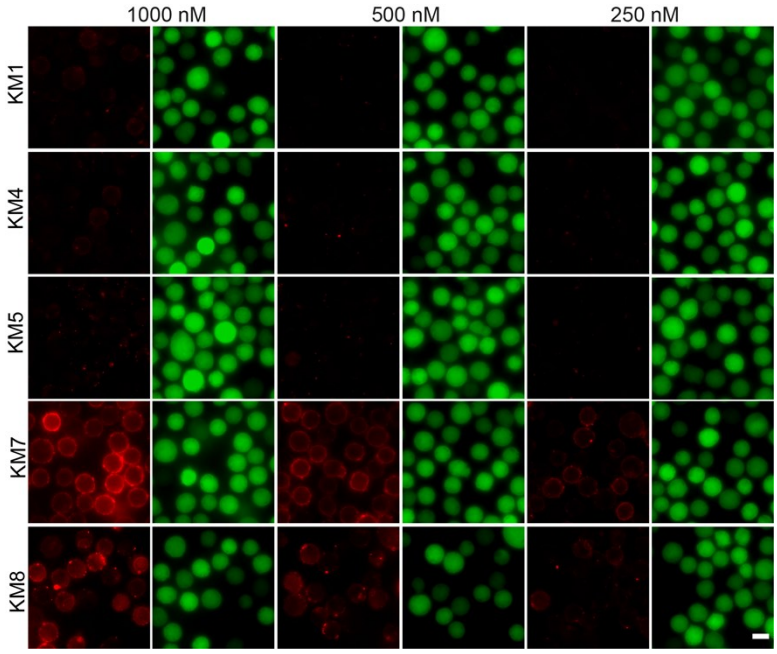


Supporting Fig. S2: Normalized absorption and emission spectra of the probes functionalized with **A** Atto647N and **B** Star635P fluorophores in PBS. For comparable reasons, it was assumed that the extinction coefficient of the dyes does not change after conjugation, as it is a standard procedure for commercially purchased dyes. The fluorescence intensities were normalized to their reference dyes, Atto647N Maleimide (Mal) and Star635P Mal, respectively. Emission spectra were recorded for **A** at λ_{ex} = 596 nm and **B** at λ_{ex} = 585 nm.

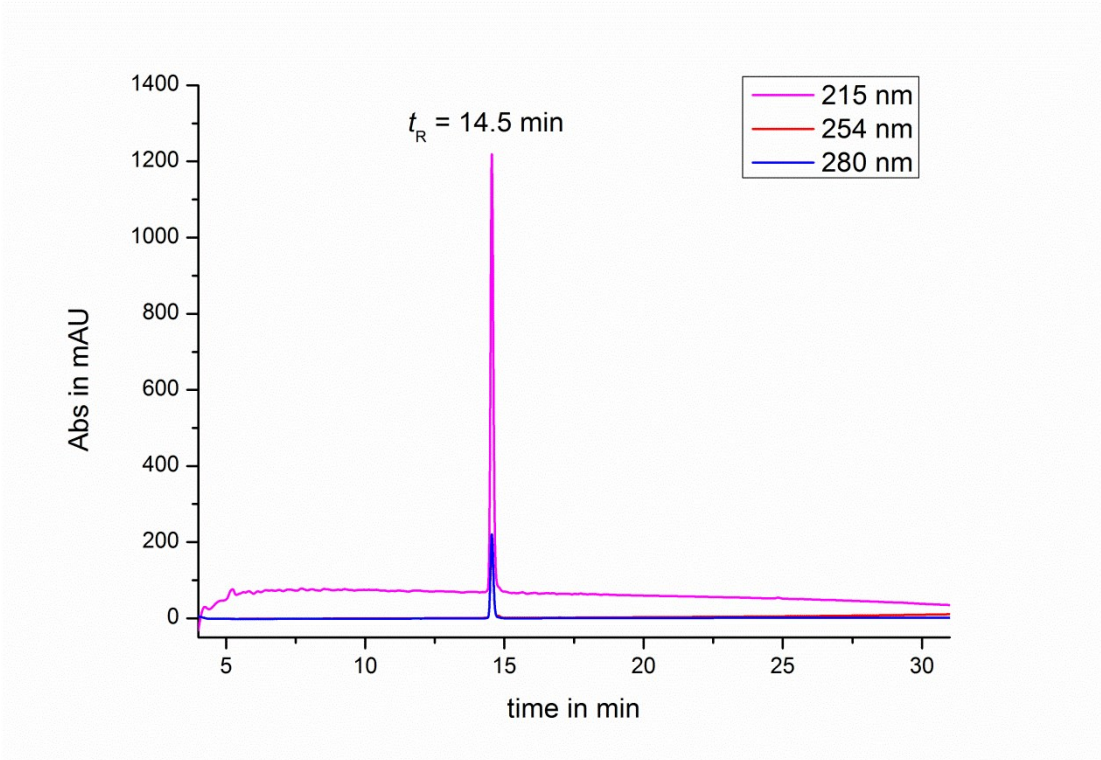


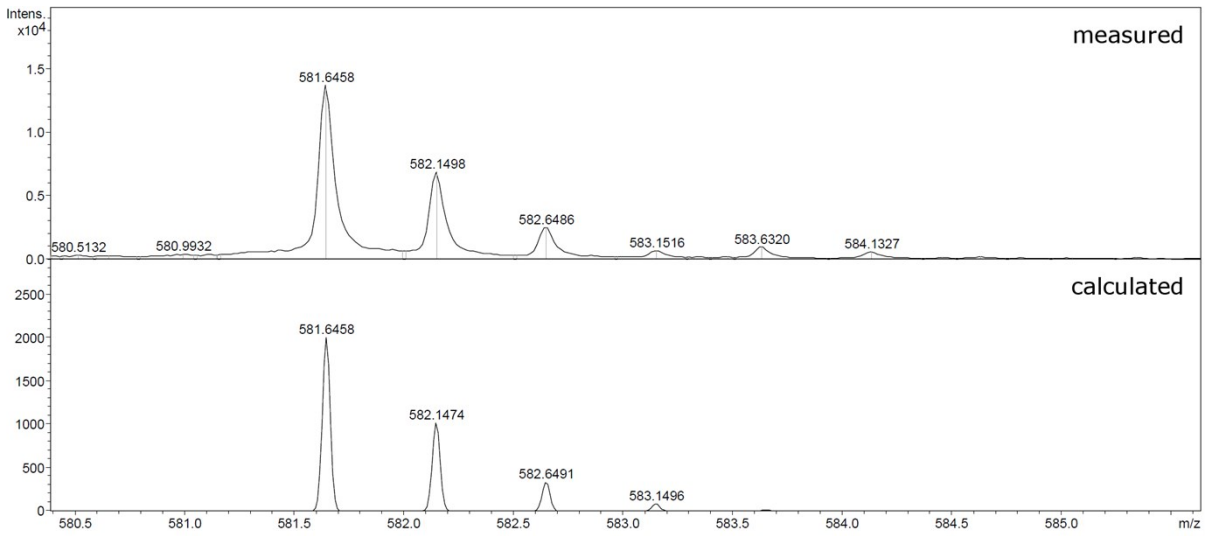
Supporting Fig. S3: Direct comparison between the different NIP probes in non-fixed cells. Epifluorescence images of Ramos IgD^{NIP} cells stained 1000 nM, 500 nM and 250 nM NIP probes (pseudo-coloured in red) and the corresponding nuclear GFP expression images (pseudo-coloured in green) as marker for cell localization. All images are equally scaled (considering KM7 at 1000 nM as reference) to allow direct signal intensity comparison. Scale bar represents 10 µm.

Supporting Fig. S4: Direct comparison between the different NIP probes in fixed cells. Epifluorescence images of and Ramos IgD^{NIP} cells stained 1000 nM 500 nM and 250 nM NIP probes (pseudo-coloured in red) and the corresponding nuclear GFP expression images (pseudo-coloured in green) as marker for cell localization. All images are equally scaled (considering KM7 at 1000 nM as reference) to allow direct signal intensity comparison. Scale bar represents 10 µm.

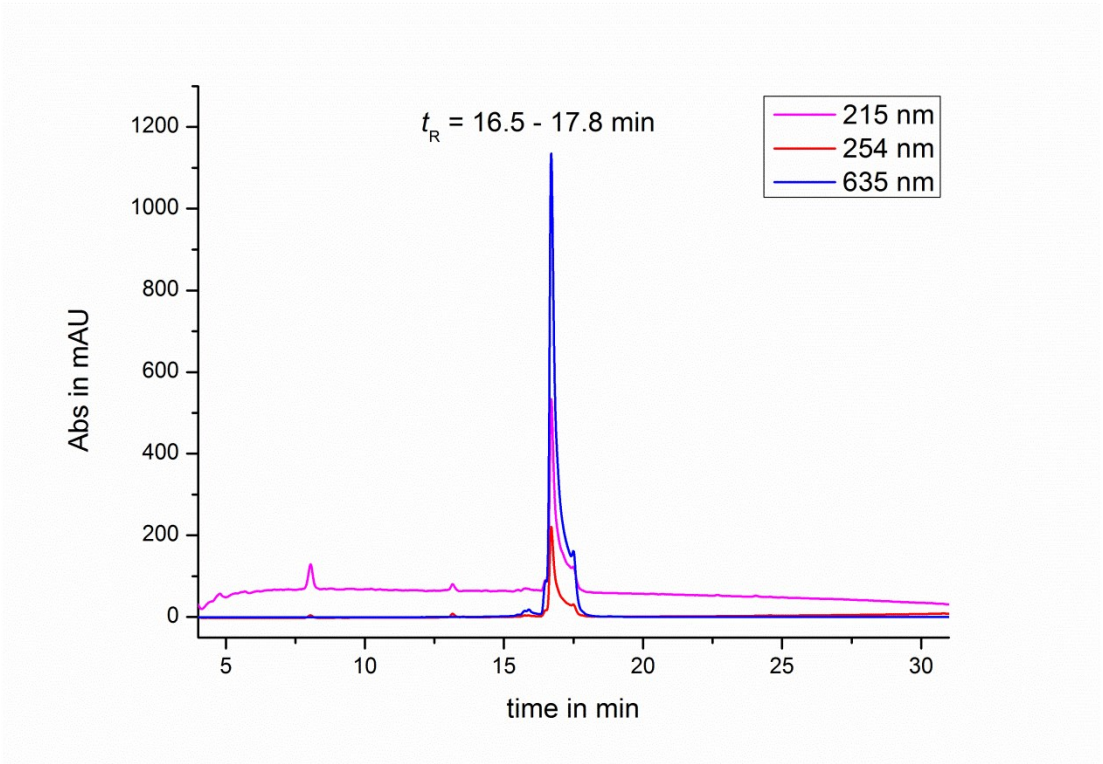


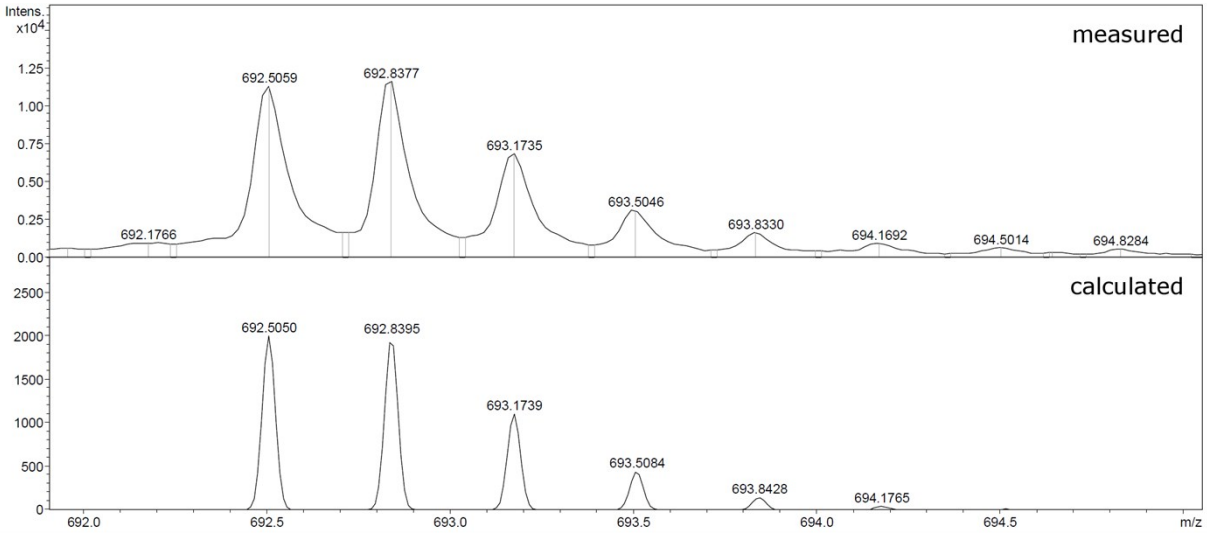
6. Supporting Spectra



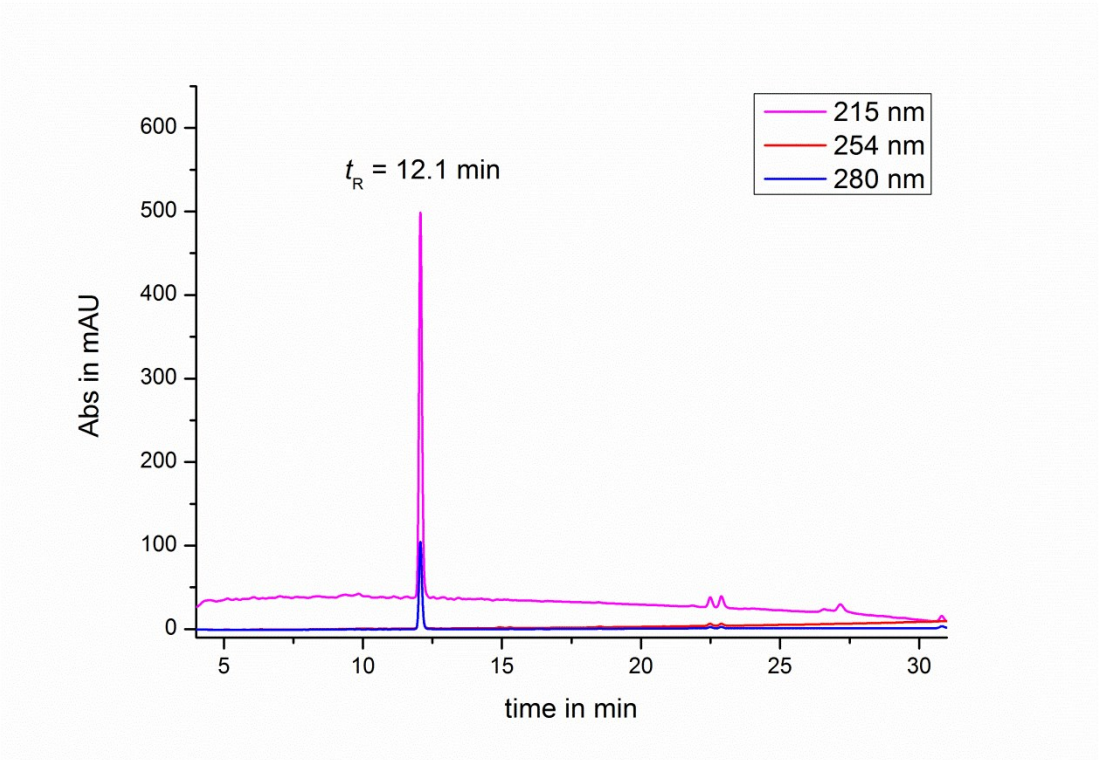


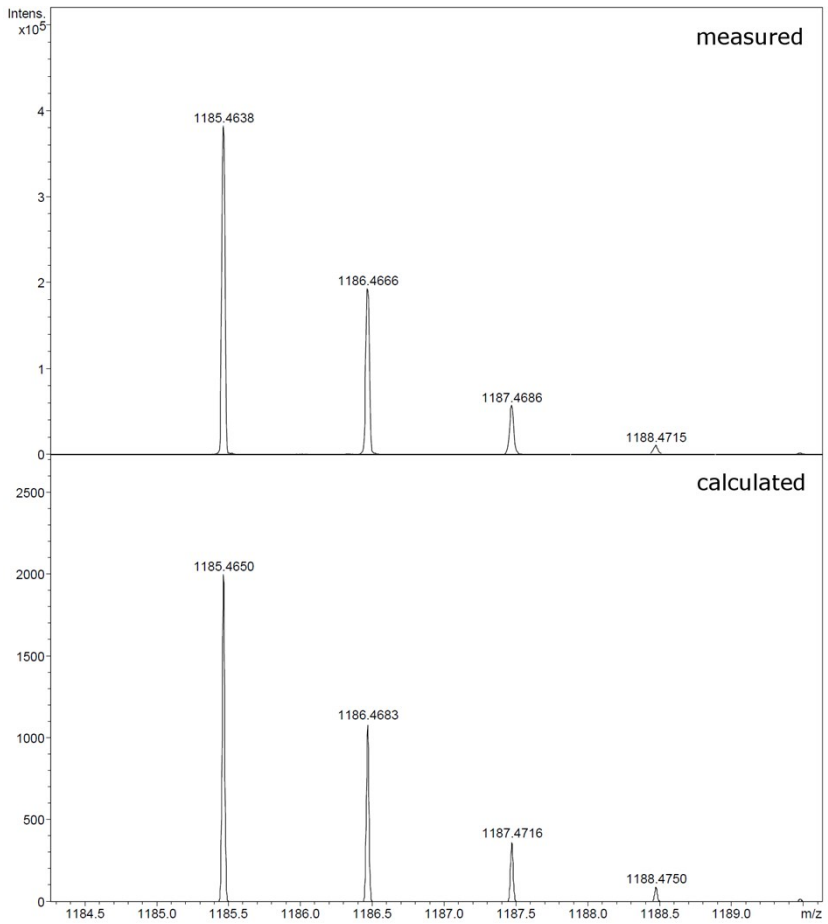
Supporting Spectra S1: HPLC chromatogram of compound **1** (top) and HR-ESI-MS spectra of compound **1**, $[M - 3H]^{2-}$ selected as example (bottom).



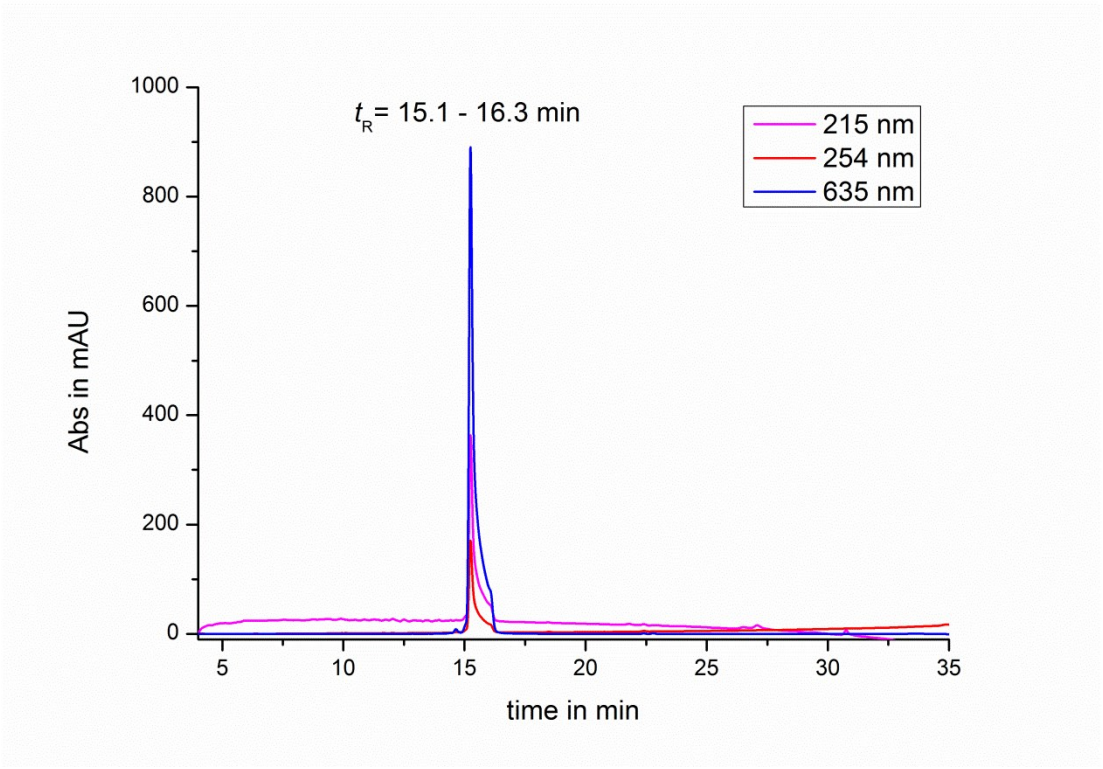


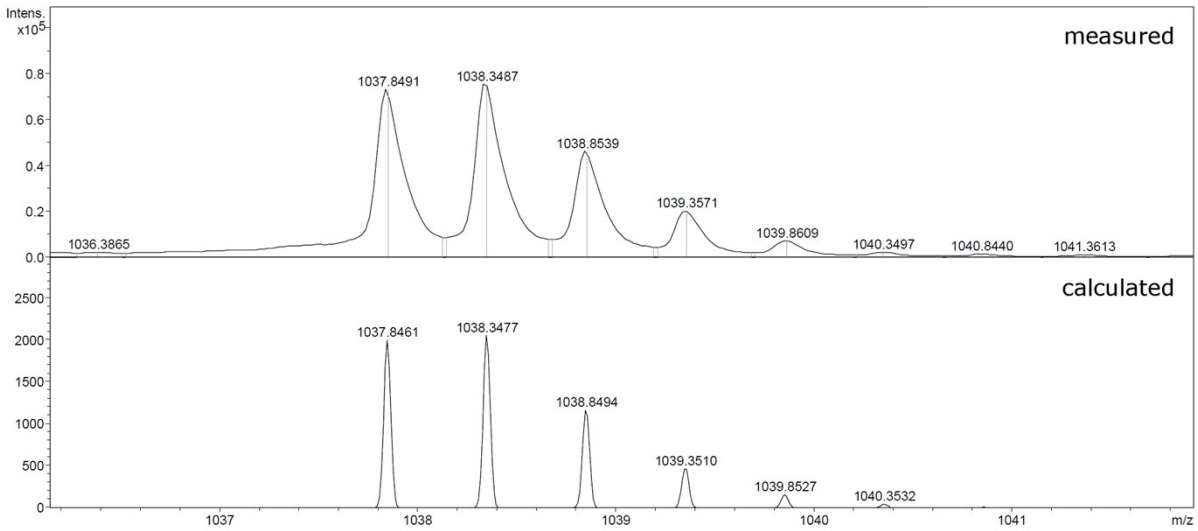
Supporting Spectra S2: HPLC chromatogram of KM1 (**2**, top) and HR-ESI-MS spectra of probe KM1 (**2**, top), $[M - 3H]^{3-}$ selected as example (bottom). The broad product peak in the HPLC chromatogram is specific for the fluorophore Star635P under applied conditions.



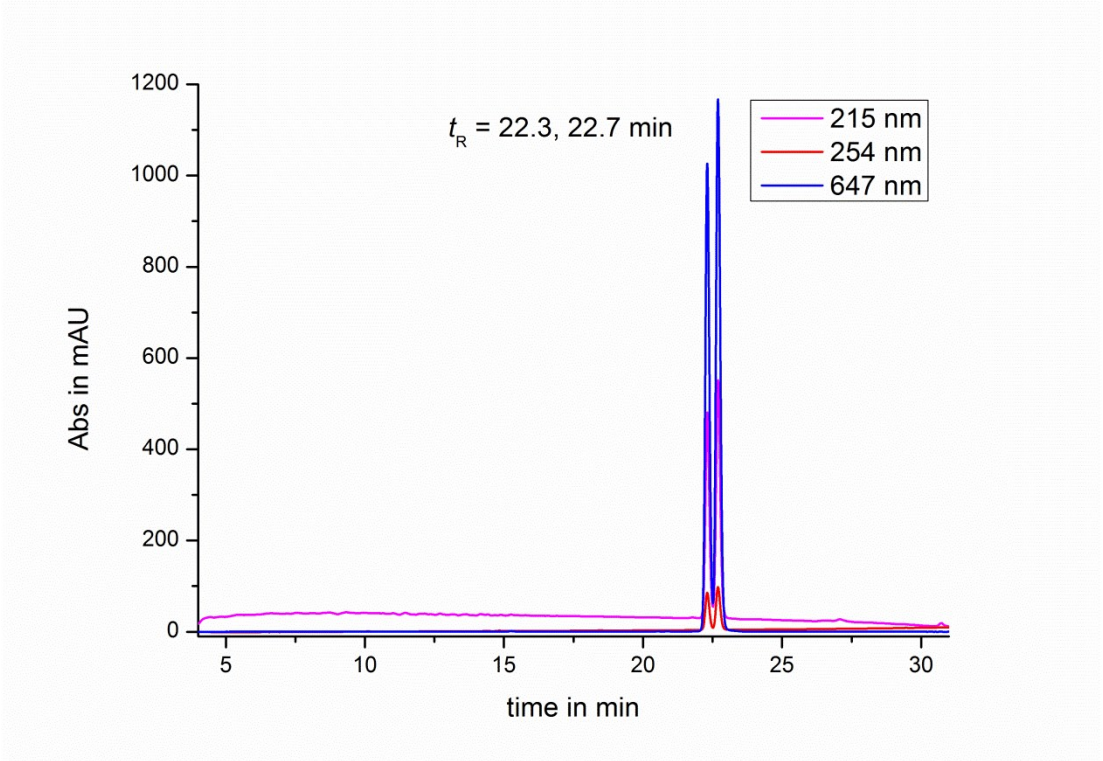


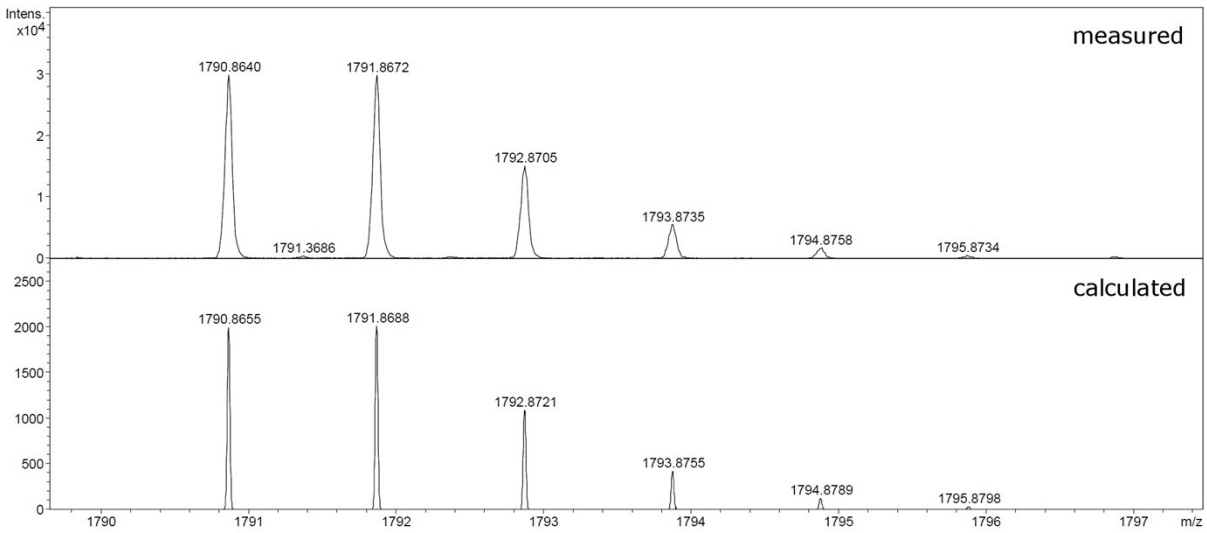
Supporting Spectra S3: HPLC chromatogram of compound **3** (top) and HR-ESI-MS spectra of compound **3**, $[M + Na]^+$ selected as example (bottom).



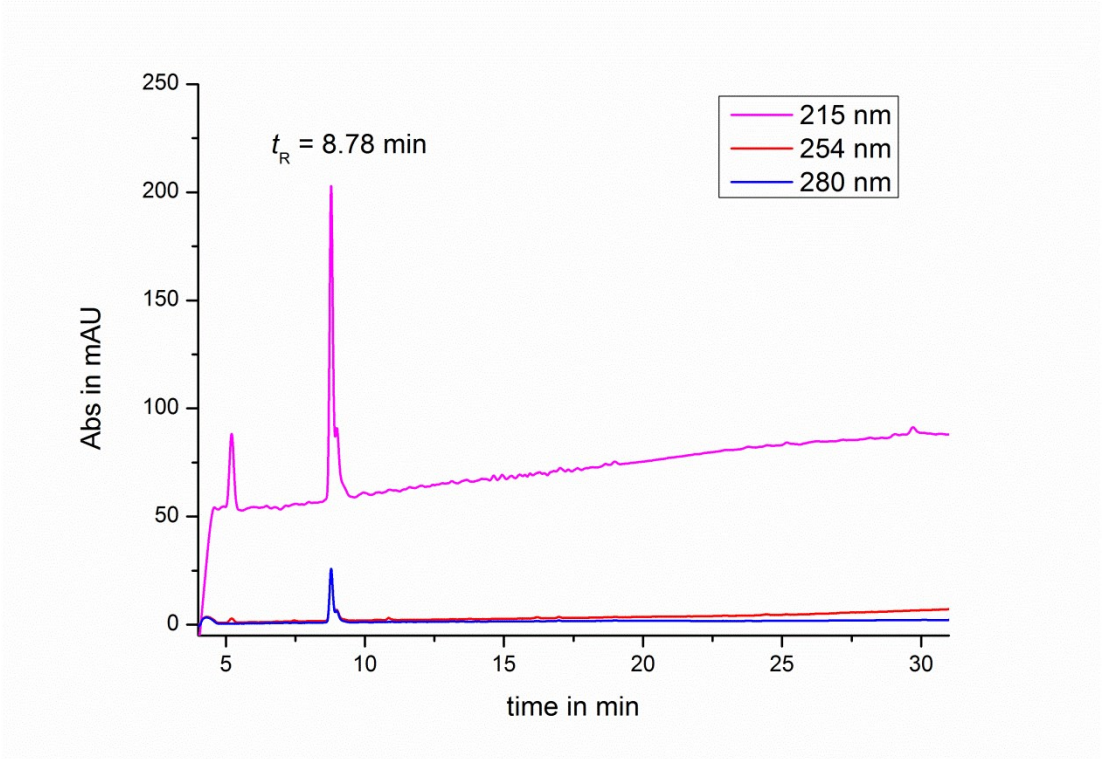


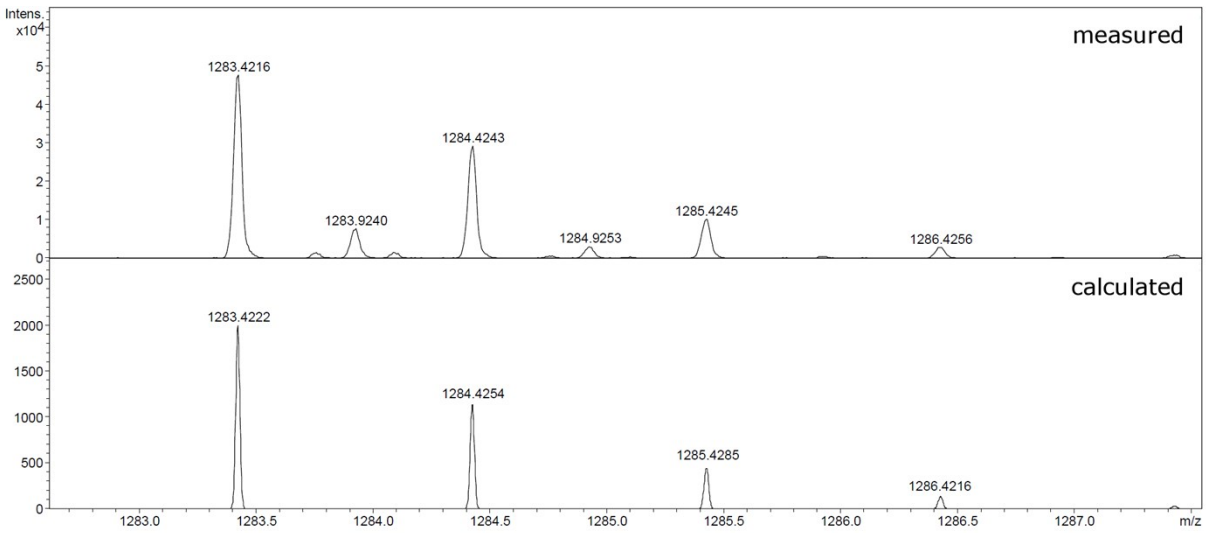
Supporting Spectra S4: HPLC chromatogram of KM4 (**4**, top) and HR-ESI-MS spectra of KM4 (**4**), $[M - 2H]^{2-}$ selected as example (bottom). The broad product peak in the HPLC chromatogram is specific for the fluorophore Star635P under applied conditions.



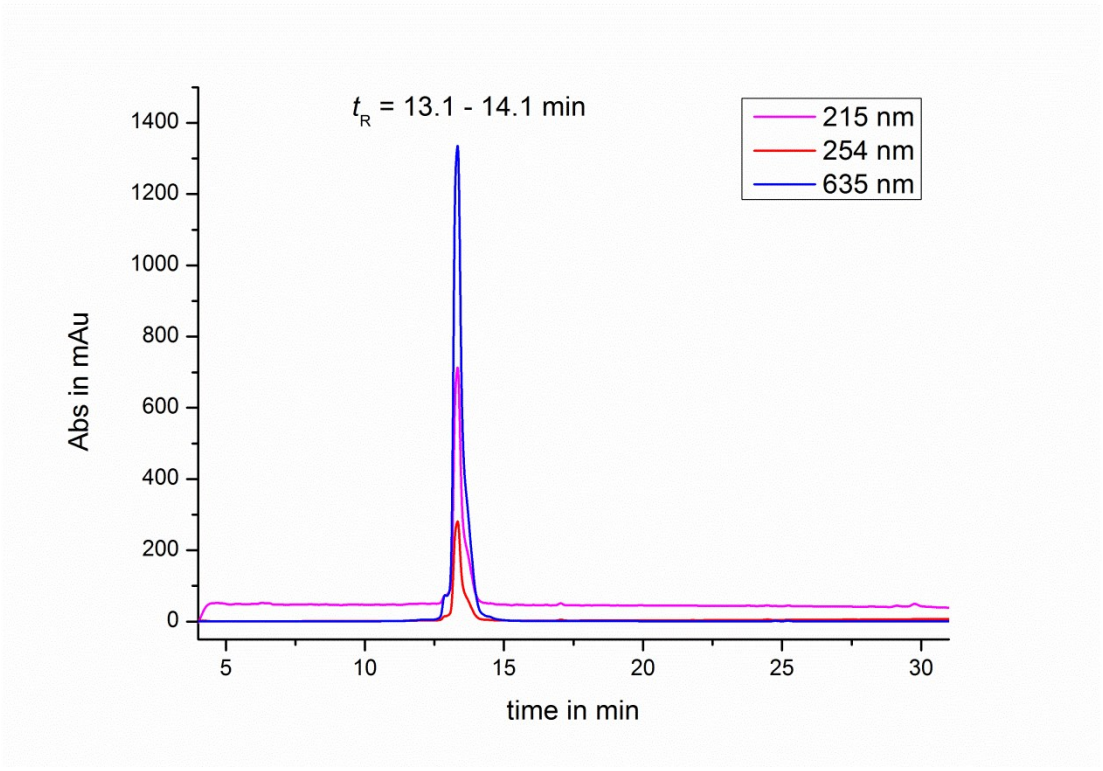


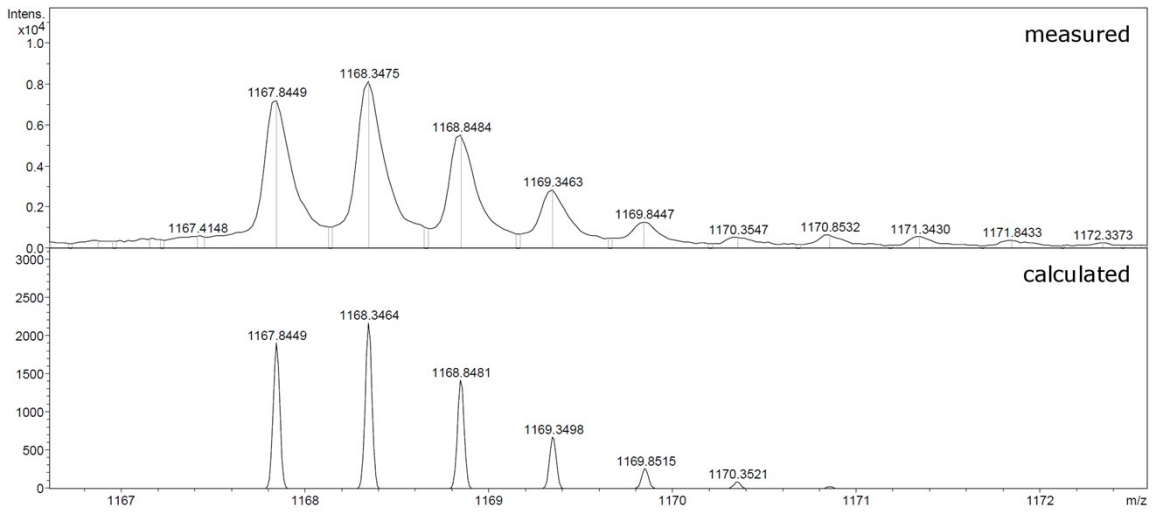
Supporting Spectra S5: HPLC chromatogram of KM5 (**5**, top) and HR-ESI-MS spectra of KM5 (**5**), [*M* + H]$^+$ selected as example (bottom). The two peaks in the HPLC chromatogram belong to the product (diastereomeric mixture) caused by the fluorophore Atto647N. Atto647N consists of a mixture of two isomers (described by the supplier), which are specified as atropisomers in the literature [2,3]. The peaks were confirmed by HR-ESI-MS.



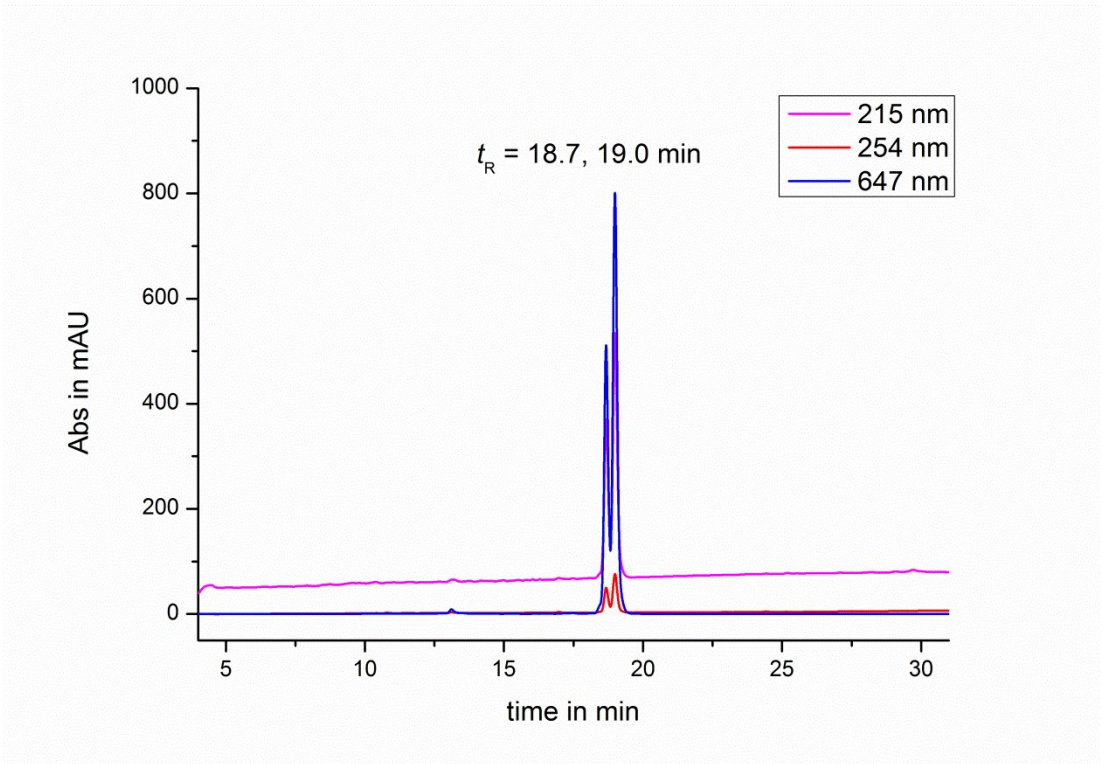


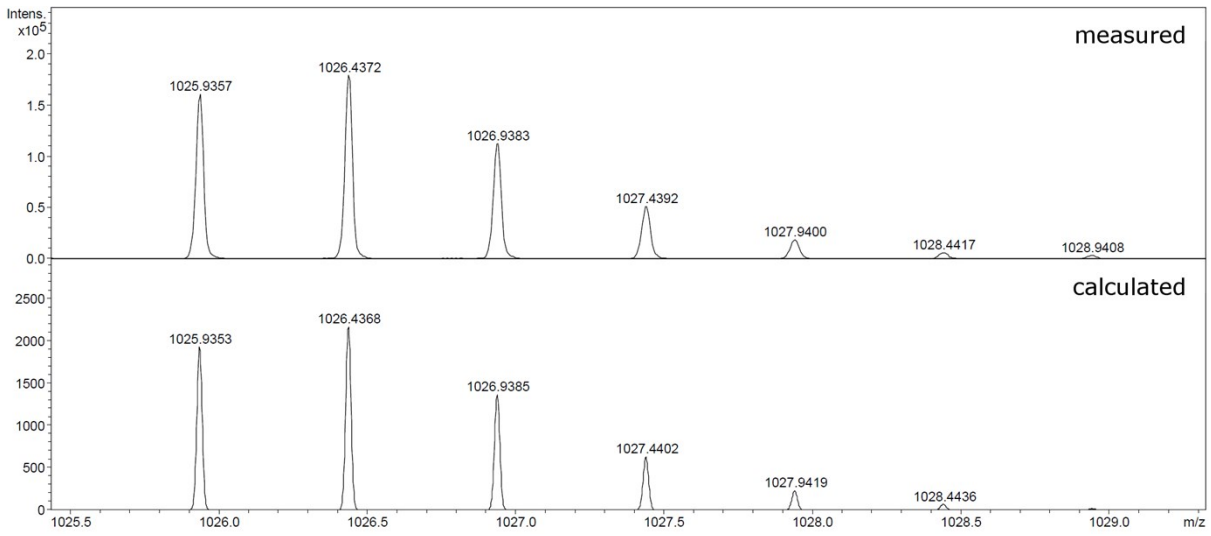
Supporting Spectra S6: HPLC chromatogram of compound **6** (top) and HR-ESI-MS spectra of compound **6**, $[M + H]^+$ selected as example (bottom).





Supporting Spectra S7: HPLC chromatogram of KM7 (**7**, top) and HR-ESI-MS spectra of KM7 (**7**), $[M - 2H]^{2-}$ selected as example (bottom). The broad product peak in the HPLC chromatogram is specific for the fluorophore Star635P under applied conditions.





Supporting Spectra S8: HPLC chromatogram of KM8 (**8,** top) and HR-ESI-MS spectra of KM8 (**8**), $[M + H]^{2+}$ selected as example (bottom). The two peaks in the HPLC chromatogram belong to the product (diastereomeric mixture) caused by the fluorophore Atto647N. Atto647N consists of a mixture of two isomers (described by the supplier), which are specified as atropisomers in the literature [2,3]. The peaks were confirmed by HR-ESI-MS.

7. References

[1] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, *Nat. Methods*, 2012, **9**, 676 – 682.

[2] C. Fischer, C. Sparr, *Synlett*, 2018, **29**, 2176 – 2180.

[3] J. L. Bachmann, C. I. Pavlich, A. J. Boley, E. M. Marcotte, E. V. Anslyn, *Org. Lett.*, 2020, **22**, 381–385.