Supporting Information

Macrocyclic host-dye reporter for sensitive sandwich-type fluorescent aptamer sensor

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Table of content.

Section 1: Labeled nucleic acid synthesis 3

1.1. Abbreviations 3

1.2. Materials and General Methods. 3

1.3. Syntheses of conjugates. 4

1.4. Synthesis and characterisations for mono conjugates. 5

1.4.1. Synthesis of H2-dansyl. 5

1.4.2. Characterisations for H2-dansyl. 5

1.4.3. Synthesis of CD-H1. 6

1.4.4. Characterisations for CD-H1. 6

1.5. Synthesis and characterisations for homo bisconjugates. 7

1.5.1. Synthesis of dansyl-H2-dansyl. 7

1.5.2. Characterisations for dansyl-H2-dansyl. 7

1.5.3. Synthesis of CD-H1-CD. 8

1.5.4. Characterisations for CD-H1-CD. 8

1.6. Synthesis and characterizations hetero bisconjugates. 9

1.6.1. Synthesis of alkyne-H1-dansyl. 9

1.6.2. Synthesis of CD-H1-dansyl. 10

1.6.3. Synthesis of alkyne-H2-dansyl. 11

1.6.4. Synthesis of CD-H2-dansyl. 12

1.6.5. Synthesis of alkyne-apt-dansyl. 13

1.6.6. Characterisations for alkyne-apt-dansyl. 13

1.6.7. Synthesis of CD-apt-dansyl. 14

1.6.8. Characterisations for CD-apt-dansyl. 14

Section 2: Sandwich-type aptamer biosensing of adenosine. 15
2.1. Chemicals and Materials. 15
2.2. Instrumentation. 15
2.3. Methods. 15
2.4. No effect of the adenosine target on the dansyl/β-CD interaction. 16
2.5. Monovalent system. 17
2.5.1. Fluorescence-monitored melting curves of CD-H1/H2-dansyl. 17
2.6. Bivalent system. 17
2.6.1. 'Head-to-tail' design for the bivalent approach. 17
2.6.2. Optimization of the CD-H1-CD:dansyl-H2-dansyl ratio. 18
2.6.3. Optimization of the NaCl concentration. 18
2.6.4. Optimization of the $MgCl_2$ concentration. 19
2.6.5. Influence of temperature on the S/B ratio. 19
2.6.6. Dose-response curves over the 5-20°C temperature range. 20
2.6.7. Comparison of detection limits. 21
2.6.8. Effect of the human serum dilution factor on the assay response. 21
2.6.9. Recoveries in 50-fold diluted human serum samples. 22

Section 1: Labeled nucleic acid synthesis

1.1. Abbreviations

CPG: Controlled pore glass; CuAAC: Copper-Catalysed Azide-Alkyne Cycloaddition; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MALDI-TOF: Matrix-assisted laser desorption/ionization-Time of Flight mass spectrometry; RP-HPLC: Reversed phase high pressure liquid chromatography; THPTA: tris(3-hydroxypropyltriazolylmethyl)amine; ε: molar extinction coefficient

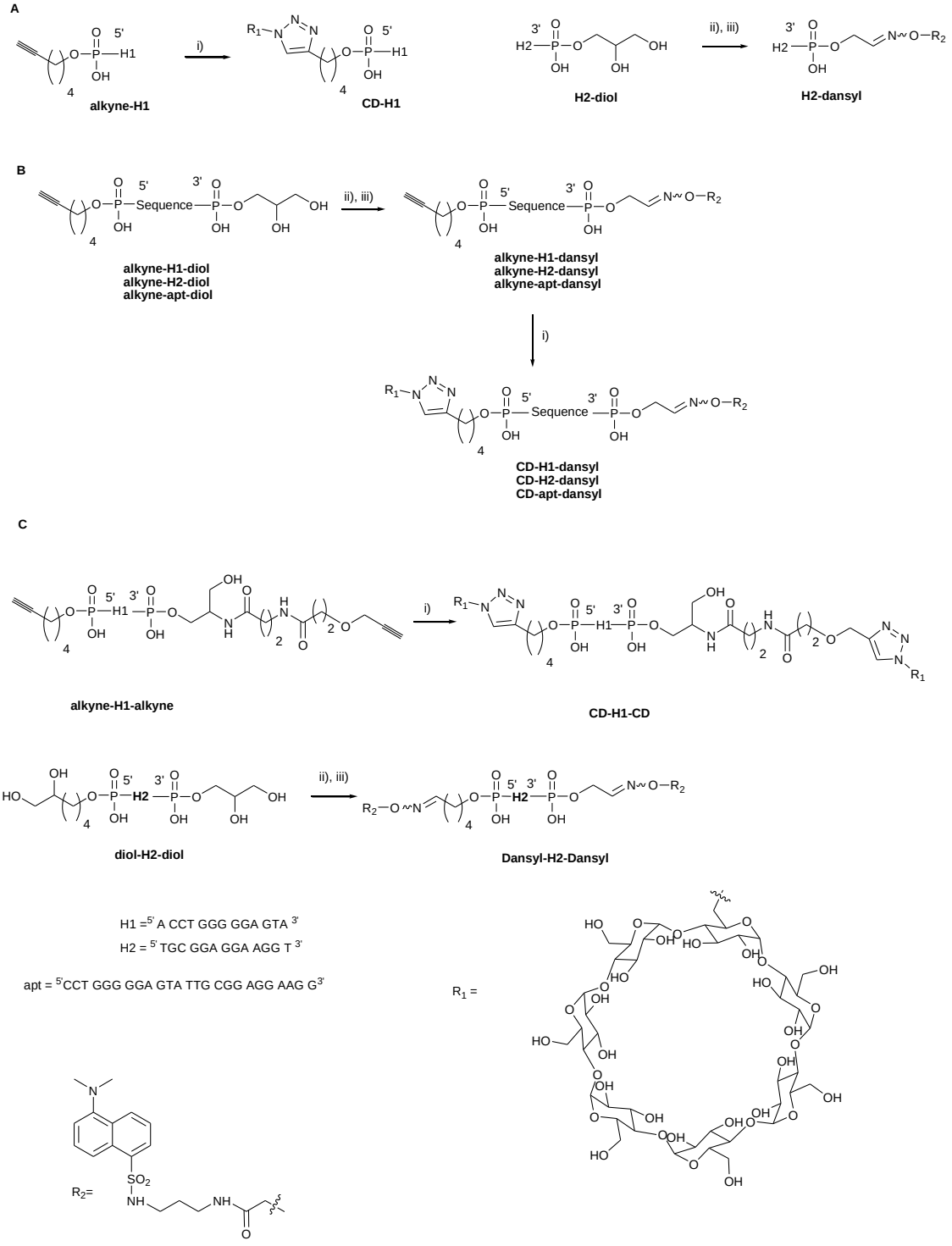
1.2. Materials and General Methods.

All solvents and reagents used were of the highest purity available. Syntheses of functionalized oligonucleotides (**alkyne-H1; H2-diol; alkyne-H1-diol; alkyne-H2-diol; alkyne-apt-diol; alkyne-H1-alkyne** and **diol-H2-diol**) were carried out on an ABI 3400 DNA synthesizer (Applied Biosystems) using standard β-cyanoethyl nucleoside phosphoramidite chemistry at 1 µM scale. 3' diol functionality was obtained using (3-[(4,4'-dimethoxytrityl)-glyceryl-1-succinyl] long chain alkylamino controlled pore glass (glyceryl-CPG); 3' alkyne functionality was obtained using 3'-alkyne-modifier serinol CPG ; 5' alkyne was obtained using hexynyl phosphoramidite and 5' diol was obtained using published procedure.[1] Glyceryl-CPG ; 3'-alkyne-modifier serinol CPG and hexynyl phosphoramidite were obtained from Eurogentec (Seraing, Belgium). After elongation ODNs were cleaved from solid support and released into solution by treatment with 28% ammonia (1.5 mL) for 2 h and finally deprotected by keeping in ammonia solution for 16 h at 55 °C. Quantification of oligonucleotides and conjugates was performed at 260nm using CARY 400 Scan UV-Visible Spectrometer (**apt**: ε= 251000 $M^{-1}.cm^{-1}$; **H1**: ε= 133400 $M^{-1}.cm^{-1}$; **H2**: ε= 133100 $M^{-1}.cm^{-1}$); ε were estimated according to the nearest neighbor model. Mono-6-azide-deoxy-6-β-cyclodextrin (R_2-N_3)[2] and 2-aminooxy-*N*-[3-(5-dimethylamino-naphtalene-1-sulfonylamino)-propyl]-acetamide (R_1-ONH_2) [3] were synthesized using published protocols. RP-HPLC analyses were performed on systems with dual wavelength detector out on a Nucleosil C18 column (Macherey Nagel, 250 x 4.6 mm, 5 µm) using the following solvent system: solvent A, 50 mM triethylammonium acetate (TEAAc) buffer containing 5% acetonitrile; solvent B, acetonitrile containing 5% water; flow rate of 1 mL min^{-1}; a linear gradient of 0-30 % B was applied in 20 minutes. Chromatograms were performed on a Gilson system for **CD-H1-CD** and **dansyl-H2-dansyl** and on a waters system for other conjugates. The oligonucleotides and conjugates were purified on a µ-Bondapak C-18 column (Macherey-Nagel Nucleosil, 10 x 250 mm, 7 µm) with similar gradients at a flow rate of 4 $mL.min^{-1}$ on a Gilson HPLC system with dual wavelength detector and a fraction collector (prep Fc). NAP-10 columns were purchased from GE-healthcare. MALDI-ToF mass spectra were performed in negative mode on an Autoflex Bruker using hydropiccolinic acid (HPA, 45 mg; ammonium citrate 4 mg in 500 µL H_2O/CH_3CN) as matrix.

[1] O. P. Edupuganti, Y. Singh, E. Defrancq, P. Dumy, *Chem. Eur. J.* **2004**, *10*, 5988.
[2] W. Tang, S.-C. Ng, *Nature protocols* **2008**, *3*, 691.
[3] D. Boturyn, A. Boudali, J.F. Constant, E. Defrancq, J. Lhomme, *Tetrahedron* **1997**, *53*, 5485.

1.3. Syntheses of conjugates.

Synthesis of conjugates is depicted in scheme S1.



Scheme S1. *Synthesis of conjugated oligonucleotides used in this work. (A) mono conjugates* ***alkyne H1*** *and* ***CD-H2 .*** (B) *hetero bisconjugates* ***CD-H1-dansyl****;* ***CD-H2-dansyl*** *and* ***CD-apt-dansyl*** *(C) homo bisconjugates* ***CD-H1-CD*** *and* ***dansyl-H2-dansyl****. Reagents and conditions. (i)* R_1*-*N_3*, sodium ascorbate,* $CuSO_4$*, THPTA, HEPES; (ii)* $NaIO_4$ *water; (iii)* R_2*-*ONH_2*,* $AcONH_4$ *0.4M pH 4.5.*

1.4. Synthesis and characterisations for mono conjugates.

1.4.1. Synthesis of H2-dansyl.

H2-diol (0.55 µmol) was subjected to periodate oxidation with a 0.02M aqueous sodium *m*-periodate solution (550 µL). After desalting on NAP10 cartridge and evaporation, it was solubilized in 550 µL of ammonium acetate buffer (0.4M, pH 4.6) and 50 µL of a 22mM solution of 2-aminooxy-*N*-[3-(5-dimethylamino-naphtalene-1-sulfonylamino)-propyl]-acetamide in water was added. Reaction was stirred a R.T during 2 hour and was monitored by RP-HPLC. **H2-dansyl** (0.24 µmol, 43%) was obtained after RP-HPLC purification and desalting twice on NAP10. MALDI-ToF MS (-) m/z calcd. for $C_{148}H_{183}N_{61}O_{82}P_{13}S$: 4563.1; found: 4564.3.

1.4.2. Characterisations for H2-dansyl.

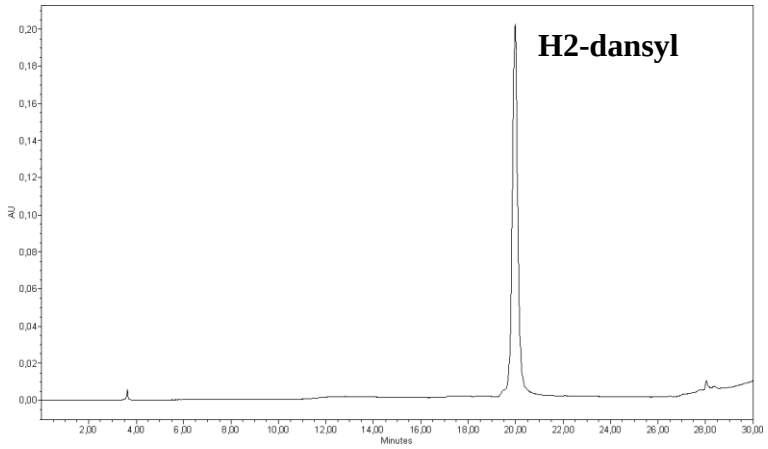


Figure S1. *RP-HPLC (260nm) chromatogram of purified* ***H2-dansyl****.*

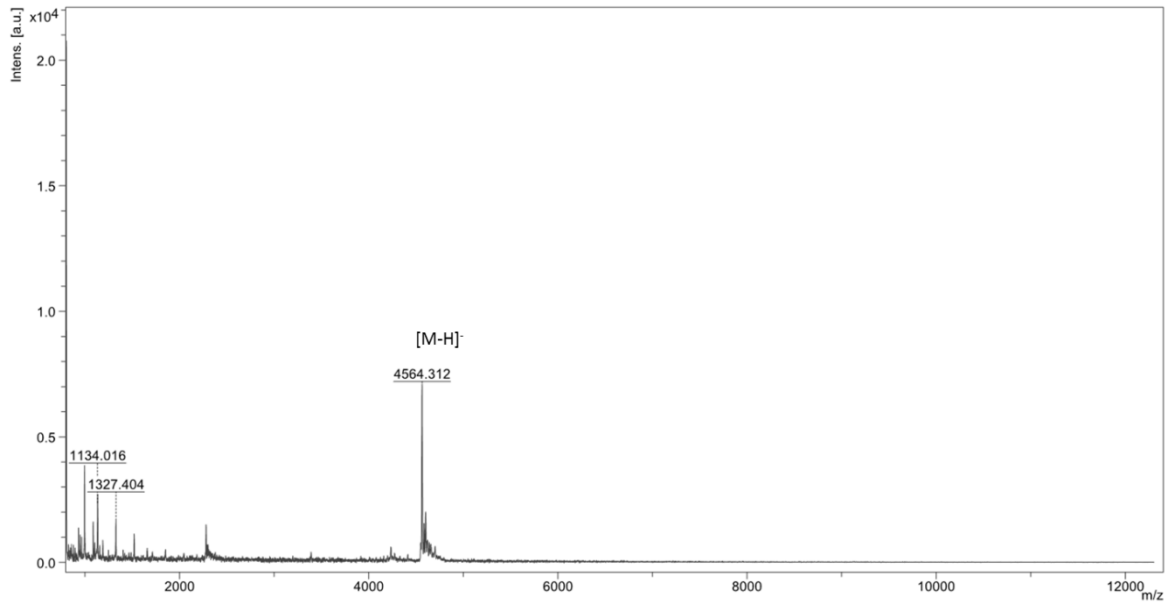


Figure S2. *MALDI-ToF (-) mass spectrum of purified conjugate* ***H2-dansyl****. m/z calcd. for* $C_{148}H_{183}N_{61}O_{82}P_{13}S$*: 4563.1; found: 4564.3.*

1.4.3. Synthesis of CD-H1.

All the solutions were freshly degased. To a solution of oligonucleotide **alkyne-H1** (0.35 µmol) in a mixture of 0.1 M HEPES buffer (330 µL, pH7.4) and DMF 20 µL was added mono-6-azide-deoxy-6-β-cyclodextrin (1.75 µmol, 2 mg), $CuSO_4$ (0.7 µmol,0.17 mg), THPTA (3.5 µmol, 1.5 mg) and sodium ascorbate (3.5 µmol, 0.7 mg). Reaction was stirred 2h at R.T and was monitored by RP-HPLC. EDTA solution in water was then added (10 µL, 0.5 M) and reaction was stirred 0.5h. **CD-H1** (0.25 µmol, 71 %) was obtained after RP-HPLC purification and desalting on NAP 10. MALDI-ToF MS (-) m/z calcd. for $C_{230}H_{325}N_{63}O_{153}P_{14}$: 6853.6 found: 6854.9.

1.4.4. Characterisations for CD-H1.

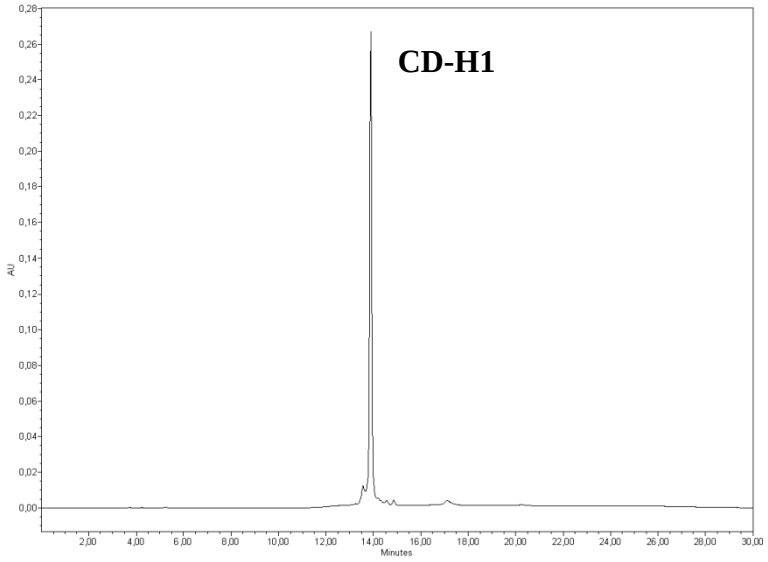


Figure S3. *RP-HPLC (260nm) chromatogram of purified* ***CD-H1****.*

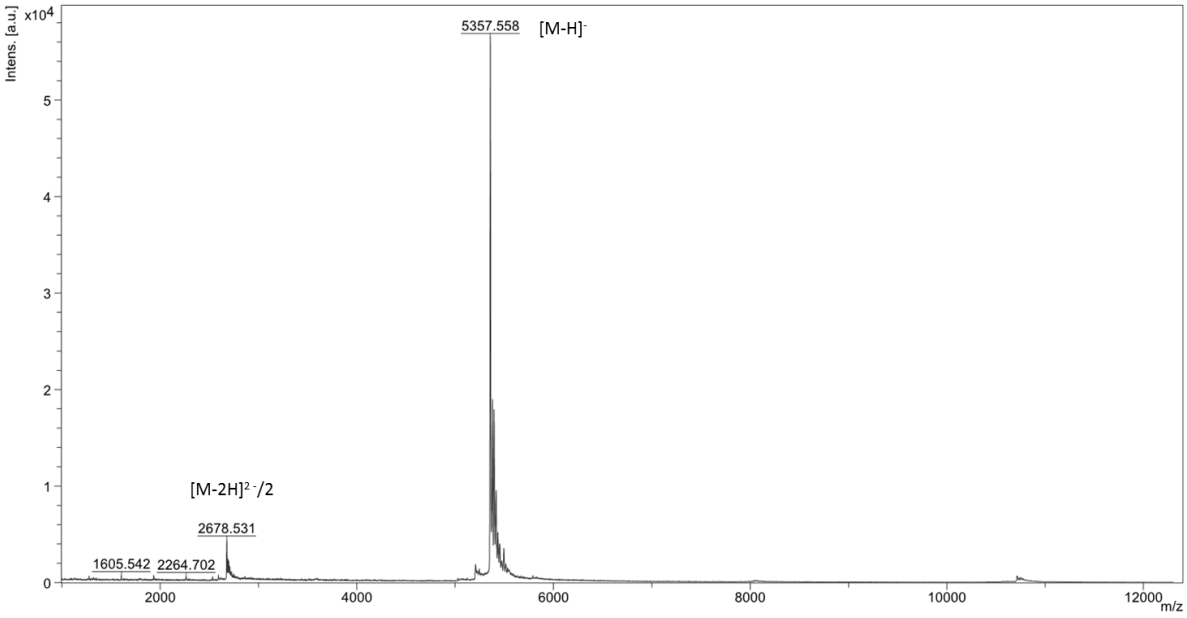


Figure S4*. MALDI-ToF (-) mass spectrum of purified* ***CD-H1****. m/z calcd. for* $C_{176}H_{236}N_{58}O_{112}P_{13}$*: 5358.7 found: 5357.6.*

1.5. Synthesis and characterisations for homo bisconjugates.

1.5.1. Synthesis of dansyl-H2-dansyl.

Dansyl-H2-dansyl (0.25µmol; 62%) was obtained using same procedure than for **H2-dansyl** starting form 0.4µmol of **diol-H2-diol.** After treatment with 250µL of 0.02 M sodium *m*-periodate, it was solubilized in 400µl ammonium acetate (0.4M, pH 4.6) and 73µL of a 22mM solution of 2-aminooxy-*N*-[3-(5-dimethylamino-naphtalene-1-sulfonylamino)-propyl]-acetamide were added. MALDI-ToF MS (-) m/z calcd. for $C_{170}H_{215}N_{65}O_{89}P_{14}S_2$: 5090.1 found: 5090.7.

1.5.2. Characterisations for dansyl-H2-dansyl.

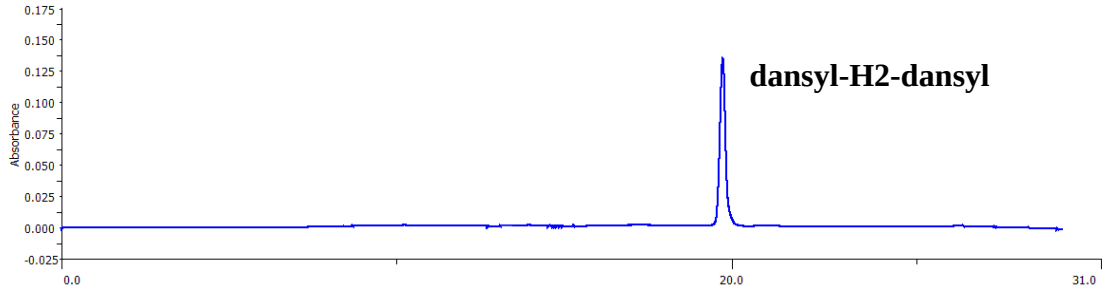


Figure S5. *RP-HPLC (260nm) chromatogram of purified* ***dansyl-H2-dansyl****.*

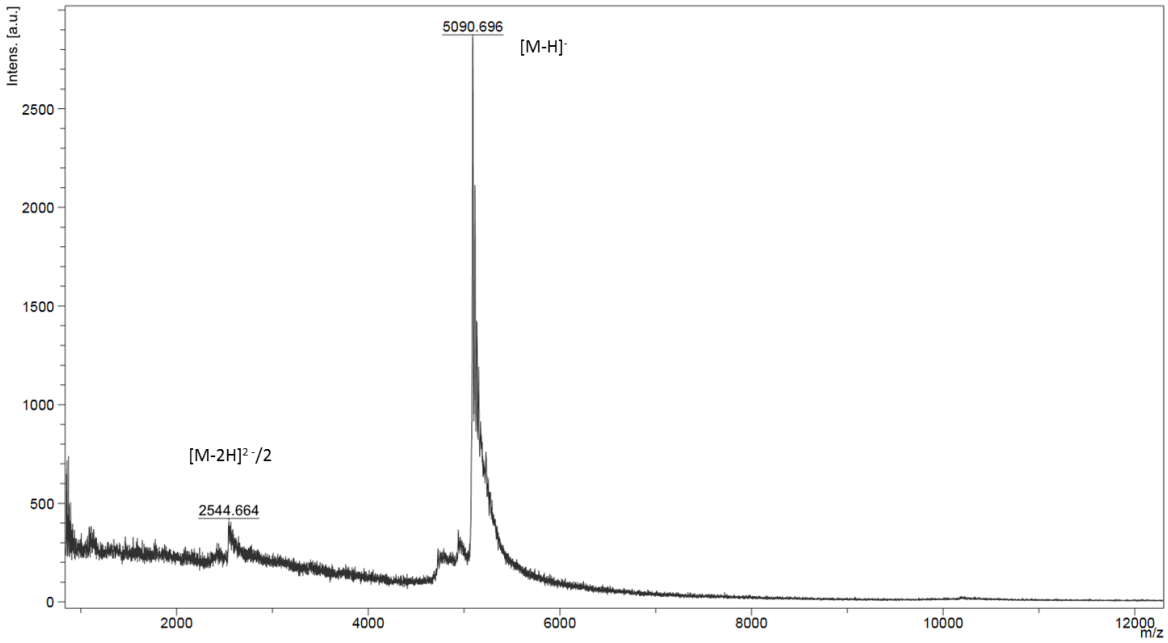


Figure S6. *MALDI-ToF (-) mass spectrum of purified* ***dansyl-H2-dansyl****. m/z calcd. for* $C_{170}H_{215}N_{65}O_{89}P_{14}S_2$ *: 5090.1 found: 5090.7.*

1.5.3. Synthesis of CD-H1-CD.

CD-H1-CD (0.06µmol; 24%) was obtained using same procedure than for **CD-H1** starting form 0.25µmol of **alkyne-H1-alkyne** in 240 µL HEPES buffer (0.1M; pH 7.4) and 30 µL DMF using 2.9 mg (2.5 µmol) mono-6-azide-deoxy-6-β-cyclodextrin ; 2.2 mg (5 µmol) THPTA; 0.25 mg (1 µmol) $CuSO_4$ and 1mg (5µmol) sodium ascorbate. MALDI-ToF MS (-) m/z calcd. for $C_{230}H_{325}N_{63}O_{153}P_{14}$: 6853.6 found: 6854.9

1.5.4. Characterisations for CD-H1-CD.

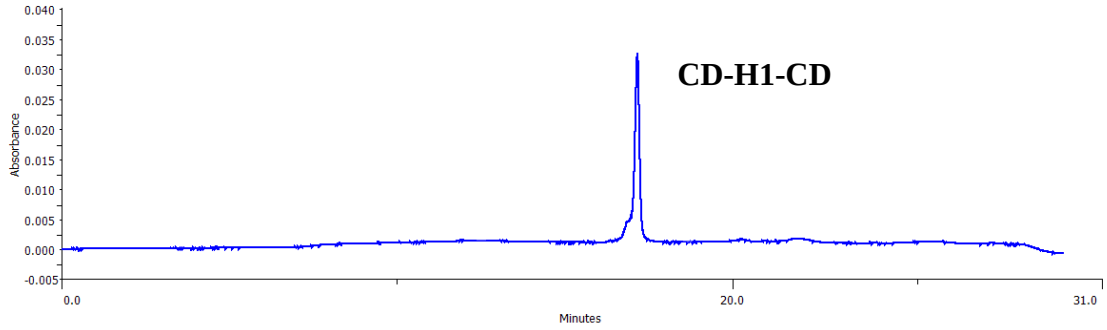


Figure S7. *RP-HPLC (260nm) chromatogram of purified* ***CD-H1-CD****.*

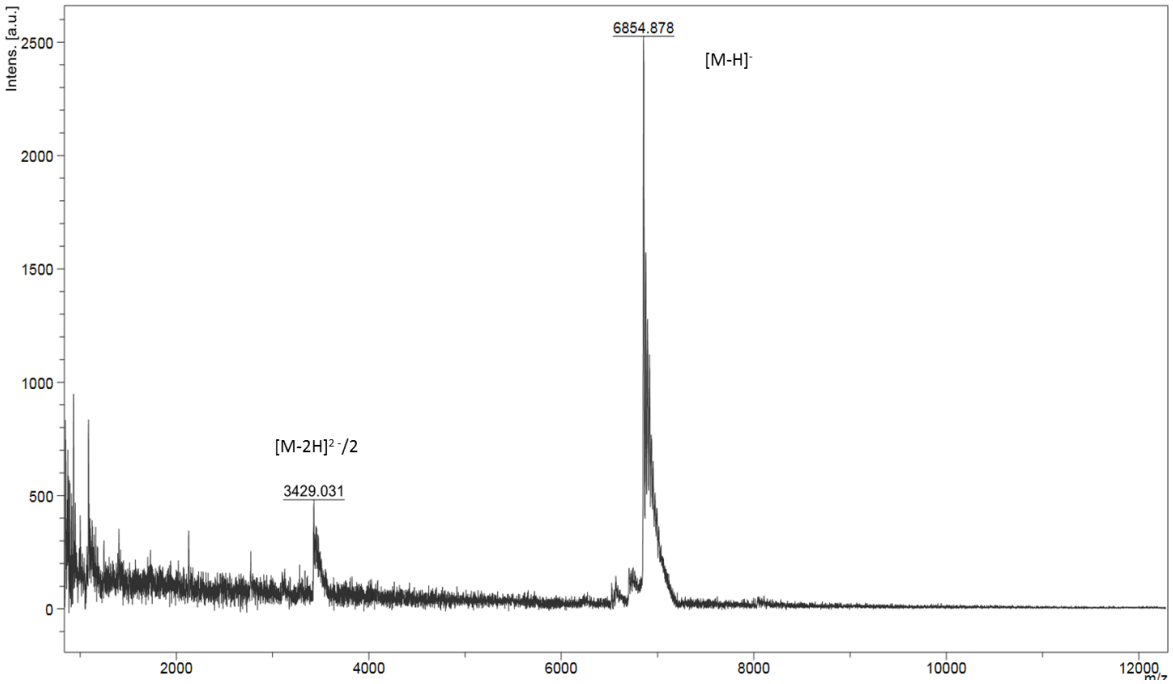


Figure S8*. MALDI-ToF (-) mass spectrum of purified* ***CD-H1-CD****.* m/z calcd. for $C_{230}H_{325}N_{63}O_{153}P_{14}$: 6853.6 found: 6854.9.

1.6. Synthesis and characterizations hetero bisconjugates.

1.6.1.Synthesis of alkyne-H1-dansyl.

Alkyne-H1-dansyl (0.13 µmol, 44 %) was obtained using same procedure than for **H2-dansyl** starting from 0.296 µmol of **alkyne-H1-diol.** After treatment with 300 µL of 0.02 M sodium *m*-periodate, it was solubilized in 300 µL of ammonium acetate buffer (0.4M, pH 4.6) and **30** µL of a 22mM solution of 2-aminooxy-*N*-[3-(5-dimethylamino-naphtalene-1-sulfonylamino)-propyl]-acetamide were added. MALDI-ToF MS (-) m/z calcd. for $C_{138}H_{174}N_{57}O_{83}P_{14}$: 4392.8; found: 4392.7.

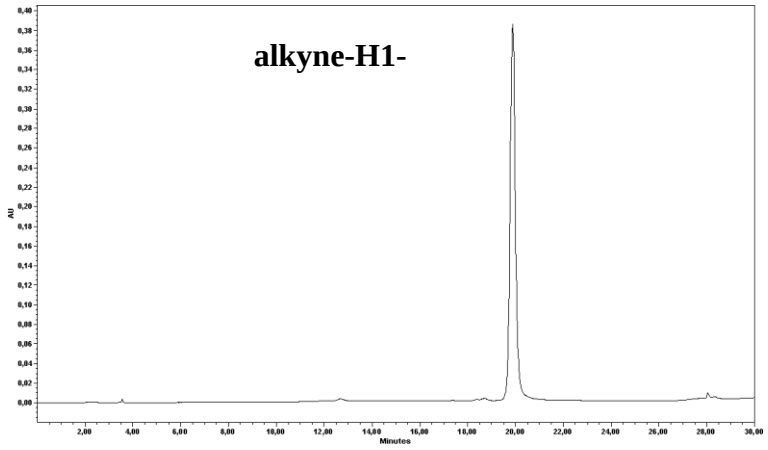


Figure S9. *RP-HPLC (260nm) chromatogram of purified conjugate* ***alkyne-H1-dansyl****.*

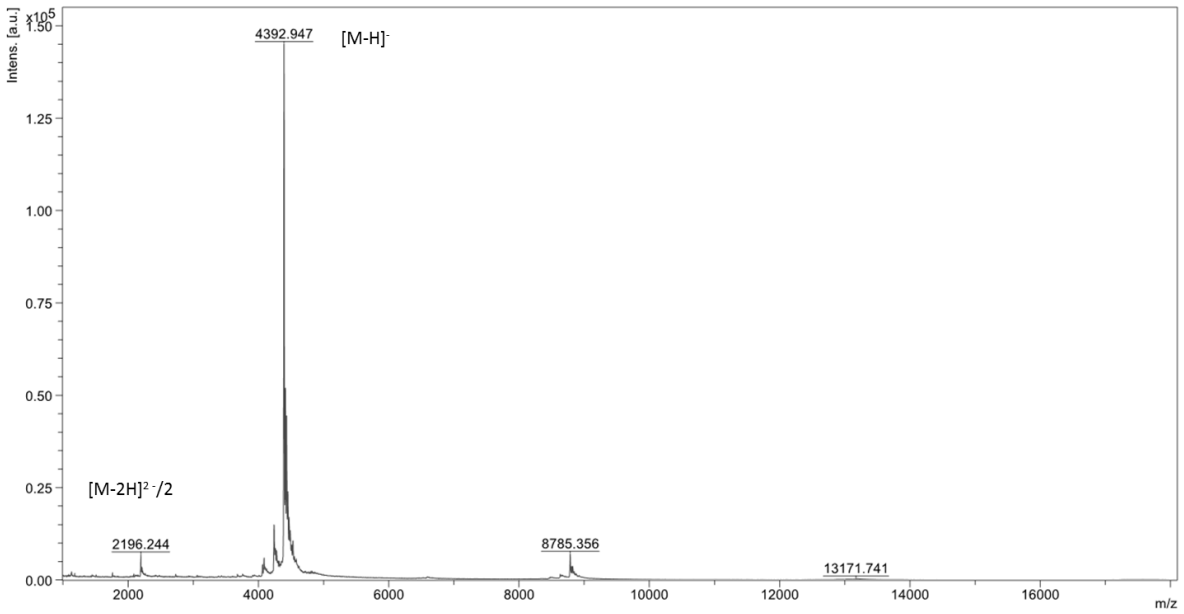


Figure S10. *MALDI-ToF (-) mass spectrum of* ***alkyne-H1-dansyl****.* m/z calcd. for $C_{138}H_{174}N_{57}O_{83}P_{14}$: 4392.8; found: 4392.7.

1.6.2. Synthesis of CD-H1-dansyl.

CD-H1-dansyl (0.044 µmol, 34%) was obtained using same procedure than for **CD-H1** starting from 0.13 µmol of **alkyne-H1-dansyl** in 150 µL HEPES buffer and 10 µL of DMF with 0.9 mg (0.75 µmol) mono-6-azide-deoxy-6-β-cyclodextrin, 0.07 mg (0.26 µmol) $CuSO_4$, 0.56 mg (1.3 µmol) THPTA and 0.3 mg (1.3 µmol) sodium ascorbate. MALDI-ToF MS (-) m/z calcd. for $C_{154}H_{192}N_{61}O_{85}P_{14}S$: 4723.2; found: 4721.8.

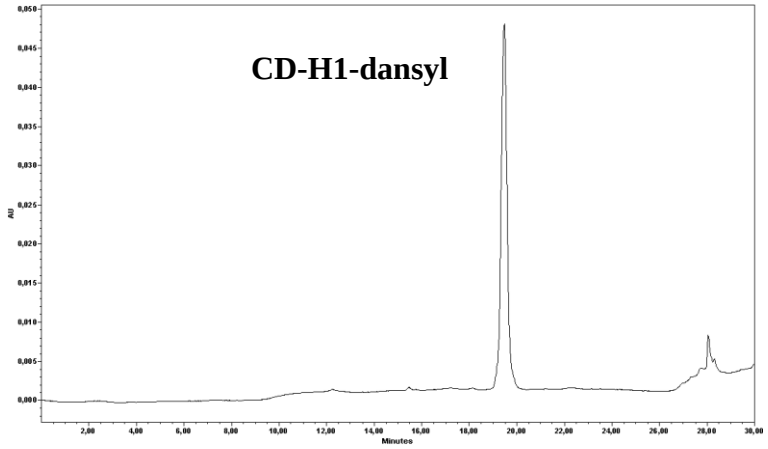


Figure S11. *RP-HPLC (260nm) chromatogram of purified conjugate* ***alkyne-H1-dansyl****.*

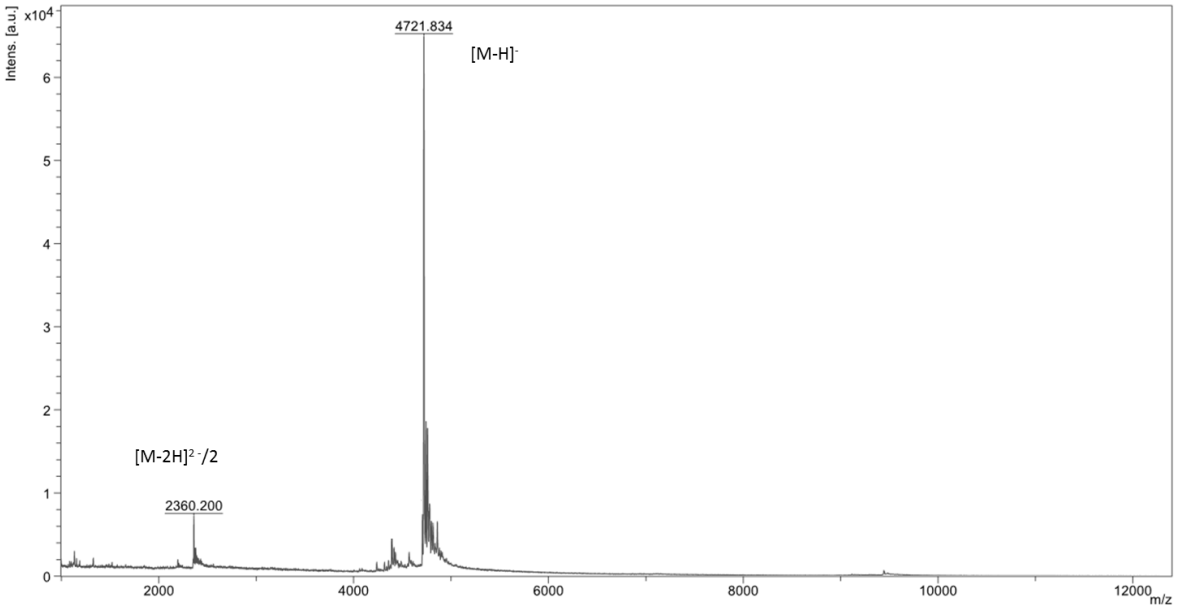


Figure S12. *MALDI-ToF (-) mass spectrum of* ***CD-H1-dansyl****.* m/z calcd. for $C_{154}H_{192}N_{61}O_{85}P_{14}S$: 4723.2; found: 4721.8.

1.6.3. Synthesis of alkyne-H2-dansyl.

Alkyne-H2-dansyl (0.18 µmol, 42.2 %) was obtained using same procedure than for **H2-dansyl** starting from 0.426 µmol of **alkyne-H2-diol.** After treatment with 500 µL of 0.02 M sodium *m*-periodate, it was solubilized in 500 µL of ammonium acetate buffer (0.4M, pH 4.6) and **50** µL of a 22mM solution of 2-aminooxy-*N*-[3-(5-dimethylamino-naphtalene-1-sulfonylamino)-propyl]-acetamide were added. MALDI-ToF MS (-) m/z calcd. for $C_{137}H_{174}N_{55}O_{83}P_{14}$: 4353.8; found: 4354.1.

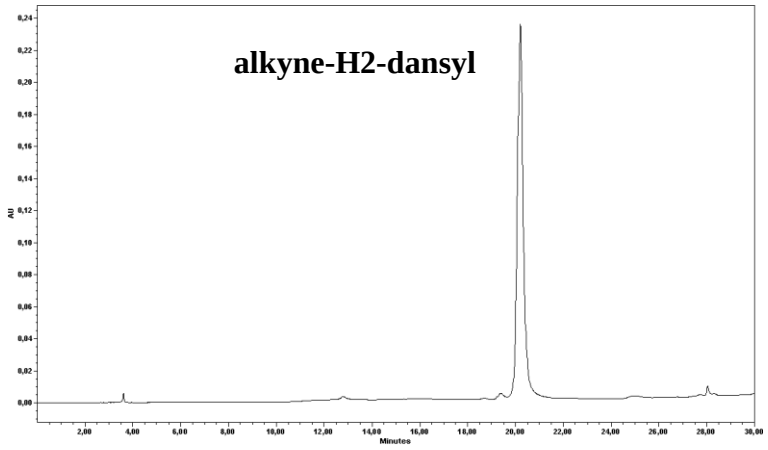


Figure S13. *RP-HPLC (260nm) chromatogram of purified conjugate* ***alkyne-H2-dansyl****.*

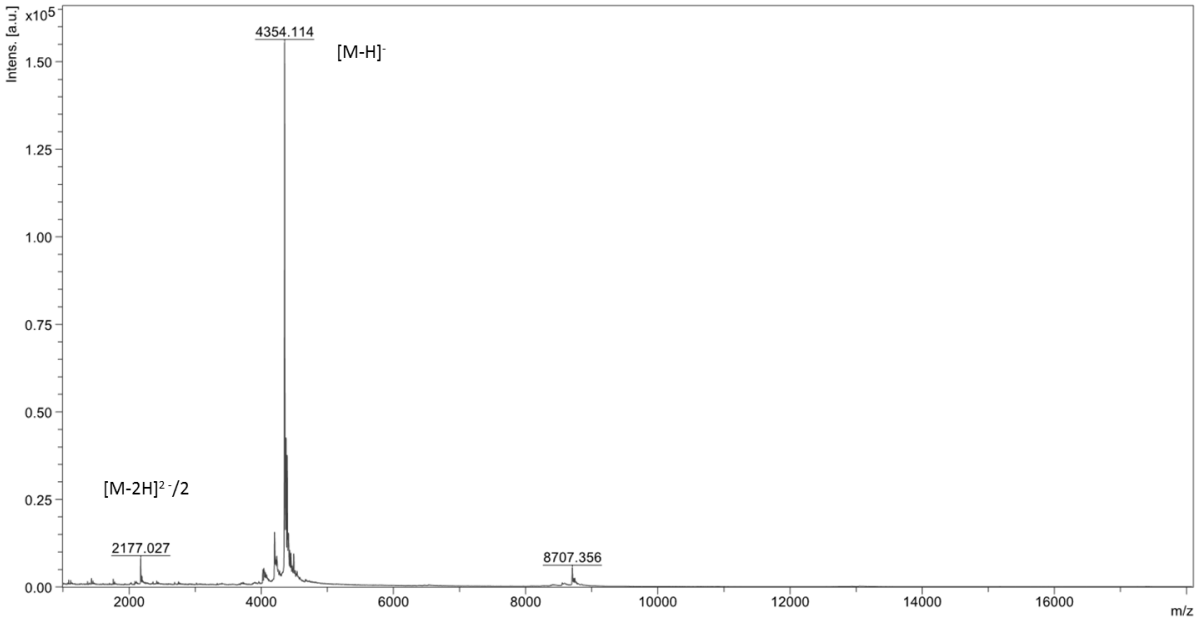


Figure S14. *MALDI-ToF (-) mass spectrum of* ***alkyne-H2-dansyl****.* m/z calcd. for $C_{137}H_{174}N_{55}O_{83}P_{14}$: 4353.8; found: 4354.1.

1.6.4. Synthesis of CD-H2-dansyl.

CD-H2-dansyl (0.067 µmol,37 %) was obtained using same procedure than for **CD-H1** starting from 0.180 µmol of **alkyne-H2-dansyl** in 200 µL HEPES buffer and 15 µL of DMF with 1mg (0.9µmol) mono-6-azide-deoxy-6-β-cyclodextrin, 0.09 mg (0.36 µmol) $CuSO_4$, 0.8mg (1.8µmol) THPTA and 0.4mg (1.8µmol) sodium ascorbate. MALDI-ToF MS (-)m/z calcd. for $C_{153}H_{192}N_{59}O_{85}P_{14}S$: 4683.2; found: 4682.2.

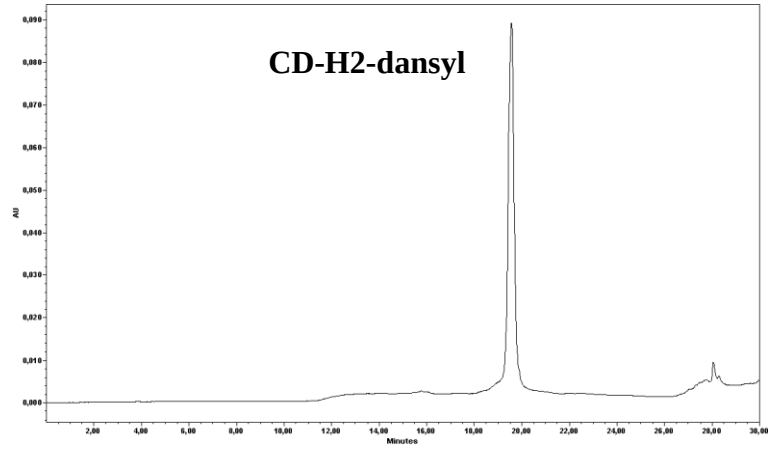


Figure S15. *RP-HPLC (260nm) chromatogram of purified conjugate* ***CD-H2-dansyl****.*

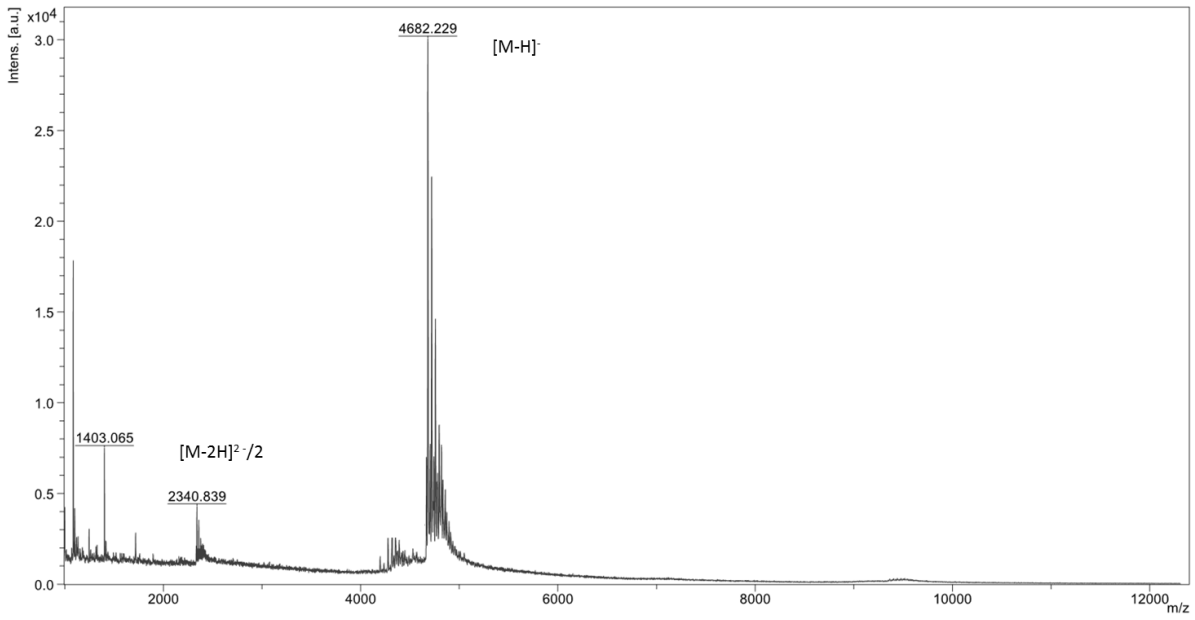


Figure S16. *MALDI-ToF (-) mass spectrum of* ***CD-H2-dansyl****.* m/z calcd. for $C_{153}H_{192}N_{59}O_{85}P_{14}S$: 4683.2; found: 4682.2.

1.6.5. Synthesis of alkyne-apt-dansyl.

Alkyne-apt-dansyl (0.22 µmol, 51 %) was obtained using same procedure than for **H2-dansyl** starting from 0.43 µmol of **alkyne-apt-diol.** After treatment with 220µL of 0.02 M sodium *m*-periodate, it was solubilized in 430 µL of ammonium acetate buffer (0.4M, pH 4.6) and 39 µL of a 22mM solution of 2-aminooxy-*N*-[3-(5-dimethylamino-naphtalene-1-sulfonylamino)-propyl]-acetamide were added. MALDI-ToF MS (-) m/z calcd. for $C_{272}H_{338}N_{111}O_{157}P_{26}S$: 8511.6; found: 8510.4.

1.6.6. Characterisations for alkyne-apt-dansyl.

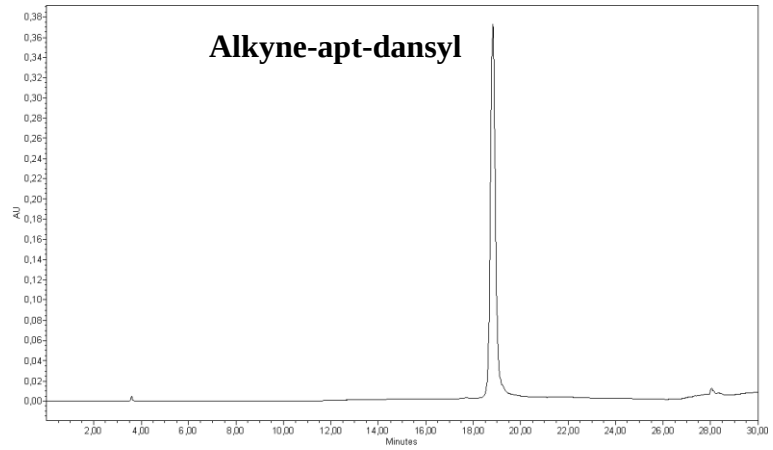


Figure S17. *RP-HPLC (260nm) chromatogram of purified conjugate* ***alkyne-apt-dansyl****.*

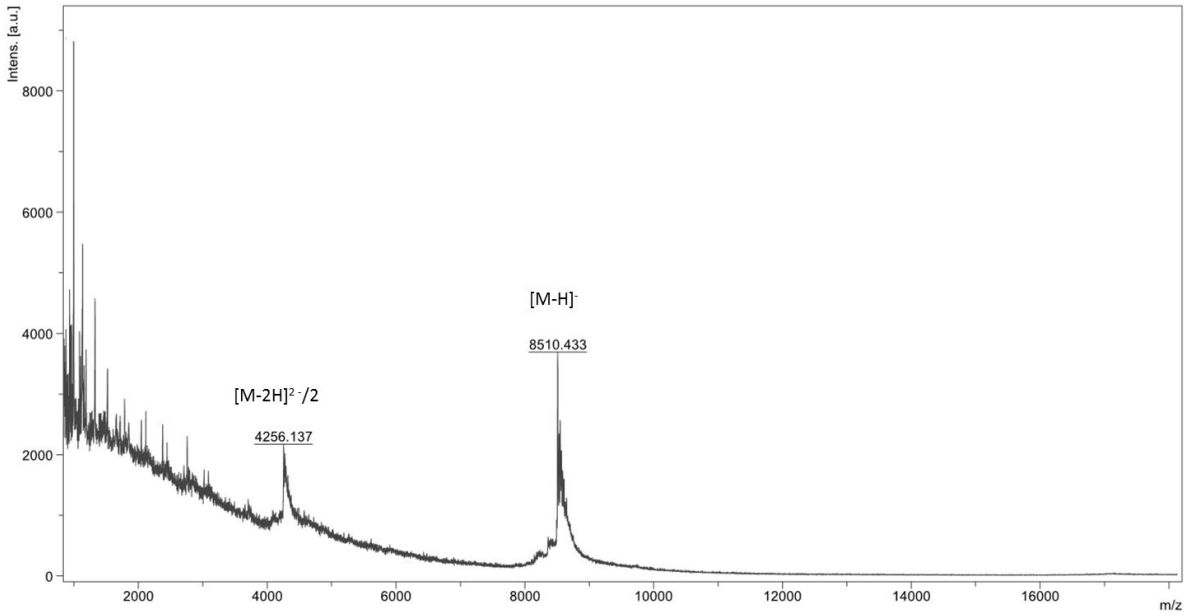


Figure S18. *MALDI-ToF (-) mass spectrum of* ***alkyne-apt-dansyl****. m/z calcd. for* $C_{272}H_{338}N_{111}O_{157}P_{26}S$*: 8511.6; found: 8510.4.*

1.6.7. Synthesis of CD-apt-dansyl.

CD-apt-dansyl (0.13µmol, 59%) was obtained using same procedure than for **CD-H1** starting from 0.22 µmol of **alkyne-apt-dansyl** in 130 µL HEPES buffer and 11 µL of DMF with 1.28 mg (1.10 µmol) mono-6-azide-deoxy-6-β-cyclodextrin, 0.11 mg (0.44 µmol) $CuSO_4$, 0.96 mg (2.21 µmol) THPTA and 0.44 mg (2.21 µmol) sodium ascorbate. MALDI-ToF MS (-) m/z calcd. for $C_{314}H_{407}N_{114}O_{191}P_{26}S$: 9671.6; found: 9678.2.

1.6.8. Characterisations for CD-apt-dansyl.

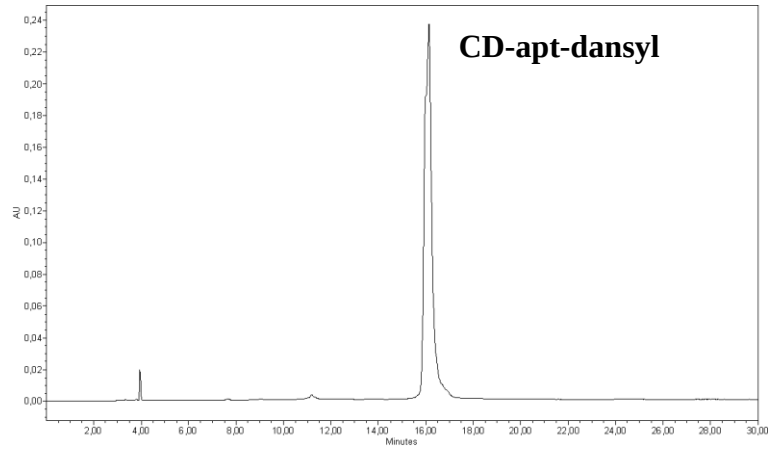


Figure S19. *RP-HPLC (260nm) chromatogram of purified conjugate* ***CD-apt-dansyl****.*

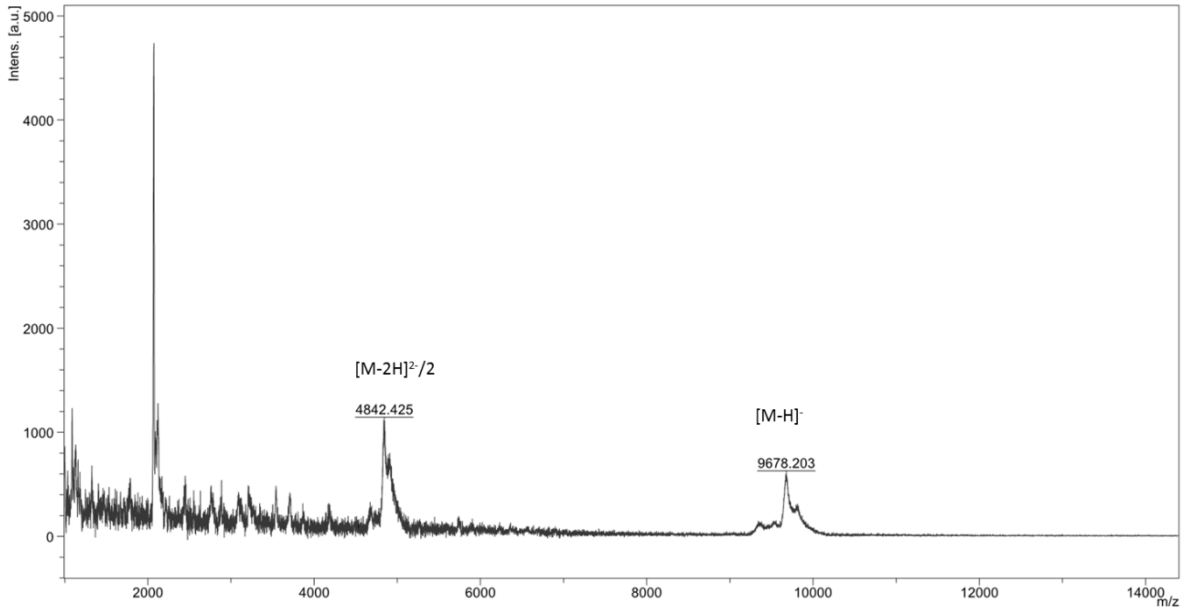


Figure S20 . *MALDI-ToF (-) mass spectrum of* ***CD-apt-dansyl****. m/z calcd. for* $C_{314}H_{407}N_{114}O_{191}P_{26}S$*: 9671.6; found: 9678.2.*

Section 2: Sandwich-type aptamer biosensing of adenosine.

2.1. Chemicals and Materials.

Name	Sequence (5' to 3')
H1	ACCTGGGGGAGTA
alkyne-H1	alkyne-ACCTGGGGGAGTA
CD-H1	CD-ACCTGGGGGAGTA
H2	TGCGGAGGAAGGT
H2-diol	TGCGGAGGAAGGT-diol
H2-dansyl	TGCGGAGGAAGGT-dansyl
CD-H1-dansyl	CD-ACCTGGGGGAGTA-dansyl
CD-H2-dansyl	CD-TGCGGAGGAAGGT-dansyl
CD-H1-CD	CD-ACCTGGGGGAGTA-CD
dansyl-H2-dansyl	dansyl-TGCGGAGGAAGGT-dansyl
apt	CCTGGGGGAGTATTGCGGAGGAAGG
CD-apt-dansyl	CD-CCTGGGGGAGTATTGCGGAGGAAGG-dansyl
C9	GAGGAAGGT

The following were obtained from Sigma-Aldrich (Saint-Quentin, France): β-D-adenosine (A), tris(hydroxymethyl)aminomethane (Tris), human serum(from human male AB plasma). Sodium chloride (NaCl) and Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) were purchased from Chimie-Plus laboratories (Bruyères de Pouilly, France) and Panreac Quimica (Barcelona, Spain), respectively. HCl and NaOH were provided by Carlo Erba (Val de Reuil, France). All chemicals were at least of analytical grade. Water was purified using a Purite Still Plus system (Thame, U.K.) fitted with a reverse osmosis cartridge. The unmodified ssDNA oligonucleotides were synthesized by Eurogentec (Belgium).

2.2. Instrumentation.

Fluorescence measurements were performed using a FluoroMax®-4 (HORIBA Scientific, France) equipped with a Peltier temperature controller (Hellma® fluorescence cuvettes, ultra Micro path length 3x3 mm, chamber volume 70 µL, Hellma GmbH, Germany). Circular dichroism spectra were recorded on a Jasco J-810 circular dichroism spectropolarimeter (Tokyo, Japan) using 10 mm path length quartz cuvette.

2.3. Methods.

Unless otherwise stated, the binding buffer for the adenosine sensing consisted of 10mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$ (reaction temperature = 10℃). The stock oligonucleotide solutions were prepared in water and stored at −20℃. The working oligonucleotide solutions were obtained by adequate dilution of the stock solution in 1.25× concentrated binding buffer. Prior to the first utilisation, the working solutions were heated at 80 ℃ for 5min and left to stand at room temperature for 30 min. The analyte solutions were prepared in water. All solutions were filtered prior to use through 0.22 µm membranes. The final working solutions (with or without analyte, final volume = 100 µL) were kept at the defined temperature for 5

min to attain mixture equilibrium prior to the fluorescence intensity (FI) measurement (λ_{ex} = 333 nm, slit = 5 nm; λ_{em} = 530nm, slit = 10 nm). We performed triplicate experiments. The signal-to-background (S/B) parameter is determined from the ratio of the FI value of samples containing the adenosine target to the FI value of samples without adenosine. The emission spectra were collected over the 400-640 nm wavelength range (scan speed of 0.5 nm/0.3s).
For the dose-response curves, 5400 µL of test solutions (1.11×) were prepared using 4800 µL of 1.25× binding buffer with 24 µL of 100 µM **CD-H1-CD** or **CD-H1** and 12 µL of 100 µM **dansyl-H2-dansyl.** Thereafter, we prepared 100µL of the sample solutions by mixing 90 µL of test solutions (1.11×) with 10 µL of solutions containing different amounts of target. Human serum samples were used to test the performance of the assay under biological conditions. Serum was first diluted to a ratio of 1/2 and the resulting diluted serum was then spiked with the stock solution of adenosine to obtain a final concentration of 75 µM and 125 µM of target. Adenosine-containing serum was heat-treated (90 °C for 10 min, centrifugation and supernatant recovery). Thereafter, for a final serum dilution of 1/50, we prepared 100µL of the sample solutions by mixing 90 µL of test solutions (1.11×) with 6 µL of water and 4 µL of treated serum (the same procedure was applied for samples with different serum dilution factors by adequately varying the proportions of water and treated serum).
Circular dichroism spectra were collected over the 220-340 nm range with a scanning speed of 100 nm per minute. Blank spectra of sample containing buffer were subtracted from collected data. The CD spectra represent an average of three scans. For the melting curves, a stepwise temperature increase from 5 to 60℃ at 2℃/step was carried out. The melting curves were constructed using the residue ellipticity at 263nm. The final melting curves were normalized. The melting temperatures were determined at 50% of the maximal signal.

2.4. No effect of the adenosine target on the dansyl/β-CD interaction.

FI of 1µM Dansyl dye was around 7,000 (condition: 50 mM Tris-HCl, 20 mM Mg^{2+}, pH=7.5, 250 mM Na^{+}, 20℃). Upon addition of 1mM *β*-CD, the FI increased to 13,000. But even with high concentration of adenosine (2 mM), there was no obvious difference in the fluorescence intensity signal (Fig. S21).

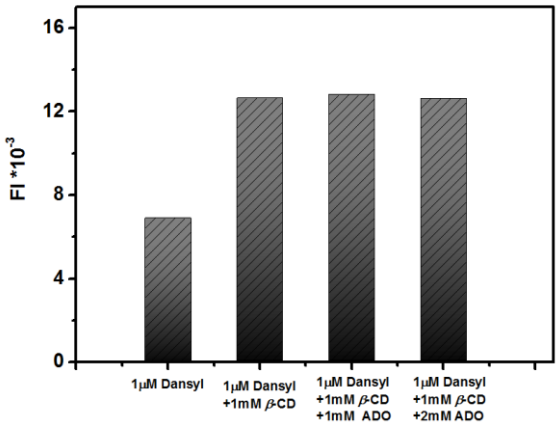


Fig. S21. *FI of dansyl, dansyl with β-CD and with different concentrations of* adenosine (ADO). *Experimental conditions: 1 µM Dansyl and 1 mM β-CD in 50 mM Tris-HCl, 20 mM Mg^{2+}, pH=7.5, 250 mM Na^{+}, 20 ℃. λ_{ex} = 333 nm, λ_{em} = 546nm.*

2.5. Monovalent system.

2.5.1. Fluorescence-monitored melting curves of CD-H1/H2-dansyl.

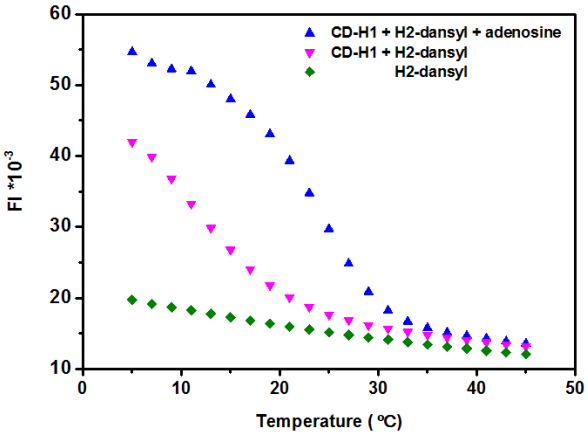


Fig. S22. *Fluorescence-monitored melting curves for* ***H2-dansyl*** *(400 nM)/****CD-H1*** *(800 nM) in absence and presence of 200 µM adenosine. Experimental conditions: 10 mM Tris-HCl, pH=7.5, 10mM* Mg^{2+}*,.* λ_{ex} *= 333 nm,* λ_{em} *= 530nm. Stepwise temperature increase from 5 to 45 ℃ at 2 ℃/step.*

2.6. Bivalent system.

2.6.1. 'Head-to-tail' design for the bivalent approach.

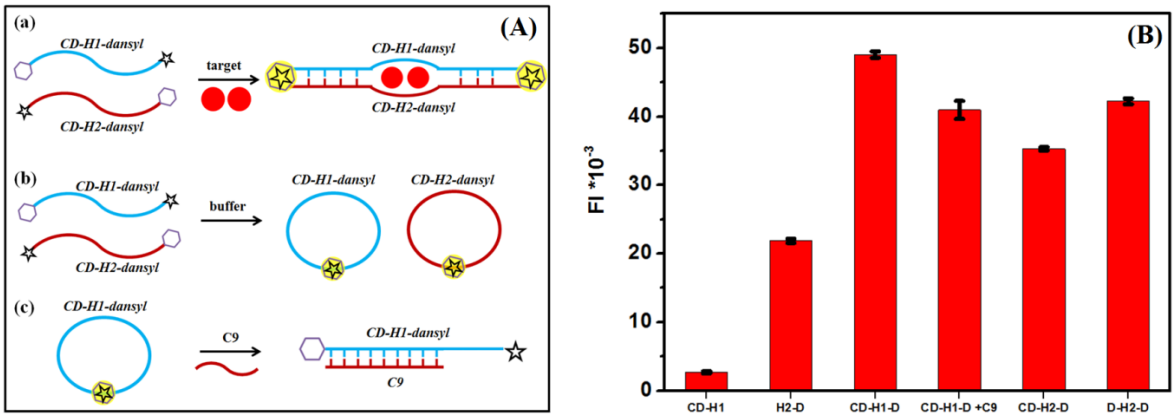


Fig. S23. (A) *Schematic illustration of (a) the 'head-to-tail' design of the bivalent signaling system, (b) the inclusion of the dye into the macrocycle for free hetero bis-conjugates and (c) the formation of hybrid between* ***CD-H1-dansyl*** *and its 9mer DNA complementary strand (C9).* ***(B)*** *FI of* ***H2-dansyl***, ***CD-H1-dansyl***, ***CD-H2-dansyl***, ***dansyl-H2-dansyl*** *and* ***CD-H1-dansyl****/C9 hybrid (400 nM for each oligonucleotide).*

2.6.2. Optimization of the CD-H1-CD:dansyl-H2-dansyl ratio.

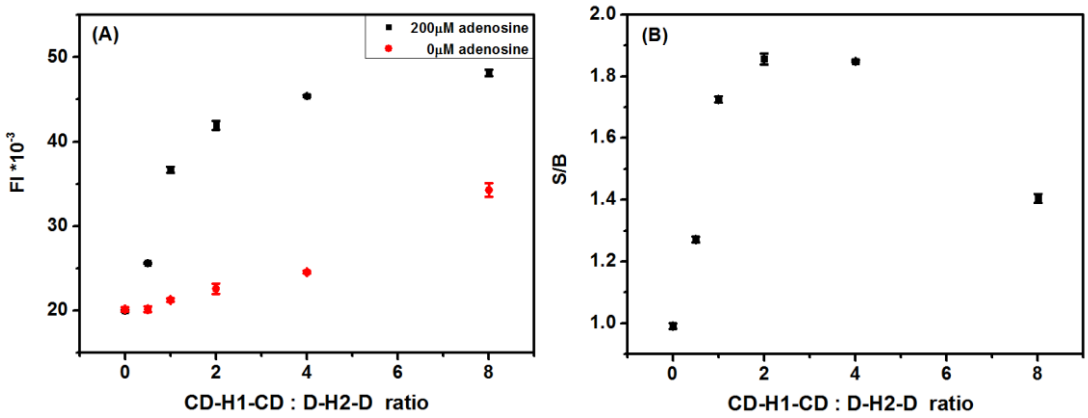


Fig. S24. *FI and S/B ratio (calculated from samples in absence and presence of 200 μM adenosine) for different ratio of* ***CD-H1-CD*** *to* ***dansyl-H2-dansyl****. Experimental conditions: 200nM of* ***dansyl-H2-dansyl*** *in 10 mM Tris-HCl, pH=7.5, 5 mM* Mg^{2+}*, 10 ℃.* λ_{ex} *= 333 nm,* λ_{em} *= 530nm.*

2.6.3. Optimization of the NaCl concentration.

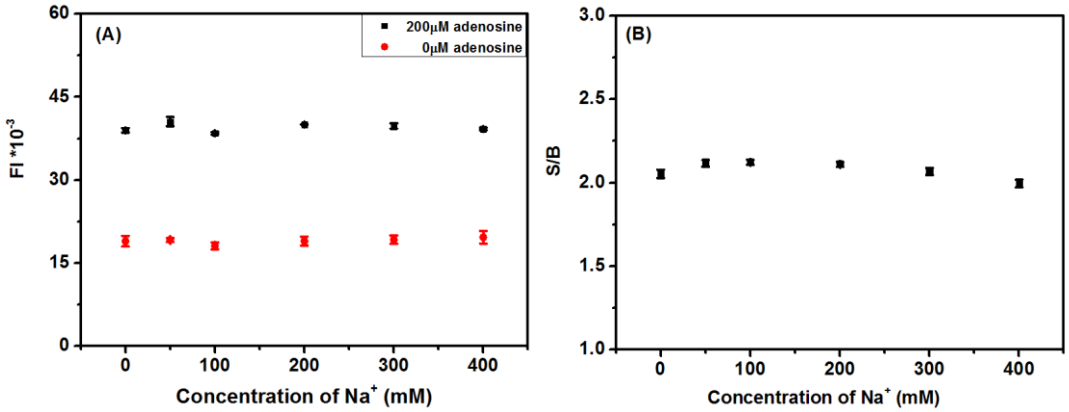


Fig. S25. *FI and S/B ratio (calculated from samples in absence and presence of 200 μM adenosine) at different concentrations of NaCl. Experimental conditions: 400 nM of* ***CD-H1-CD*** *and 200 nM of* ***dansyl-H2-dansyl*** *in 10 mM Tris-HCl, pH=7.5, 5mM* Mg^{2+}*, 15 ℃.* λ_{ex} *= 333 nm,* λ_{em} *= 530nm.*

2.6.4. Optimization of the $MgCl_2$ concentration.

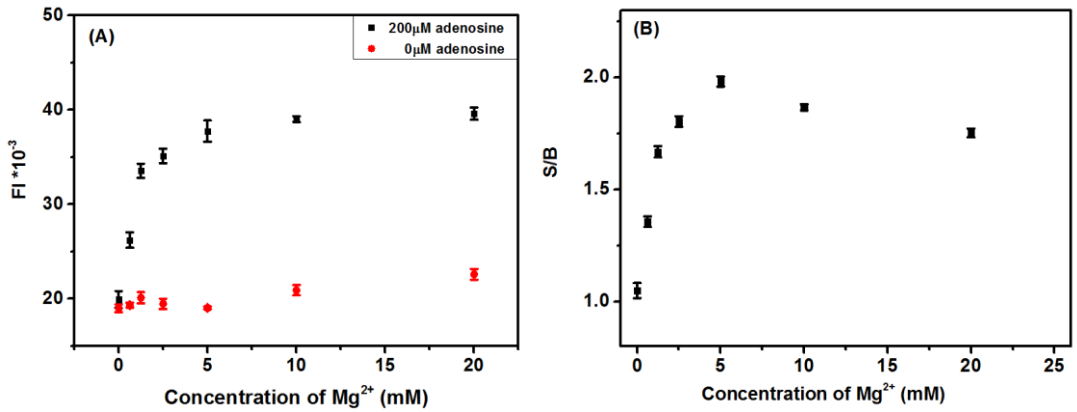


Fig. S26. *FI and S/B ratio (calculated from samples in absence and presence of 200 μM adenosine) at different concentrations of $MgCl_2$. Experimental conditions: 400 nM of* ***CD-H1-CD*** *and 200 nM of* ***dansyl-H2-dansyl*** *in 10 mM Tris-HCl, pH=7.5, 15 ℃. λ_{ex} = 333 nm, λ_{em} = 530nm.*

2.6.5. Influence of temperature on the S/B ratio.

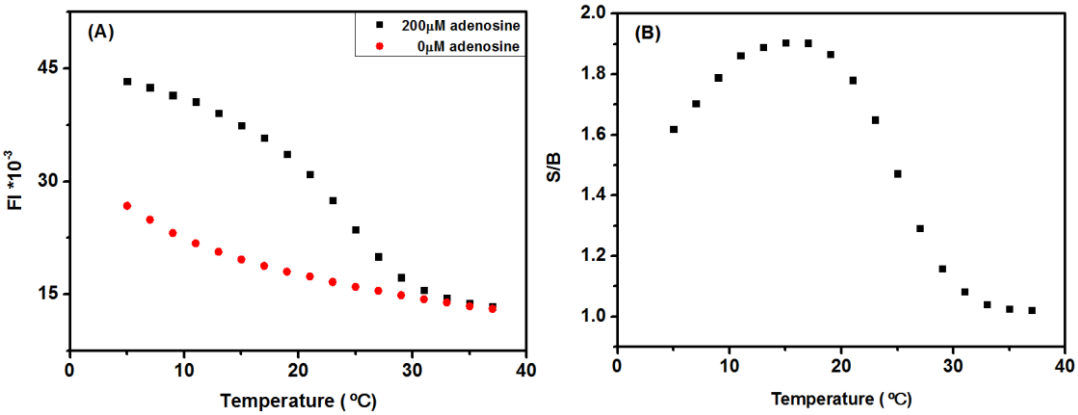


Fig. S27. *FI and S/B ratio (calculated from samples in absence and presence of 200 μM adenosine) at different temperatures. Experimental conditions: 400 nM of* ***CD-H1-CD*** *and 200 nM of* ***dansyl-H2-dansyl*** *in 10 mM Tris-HCl, pH=7.5, 5mM Mg^{2+}. λ_{ex} = 333 nm, λ_{em} = 530nm.*

2.6.6. Dose-response curves over the 5-20°C temperature range.

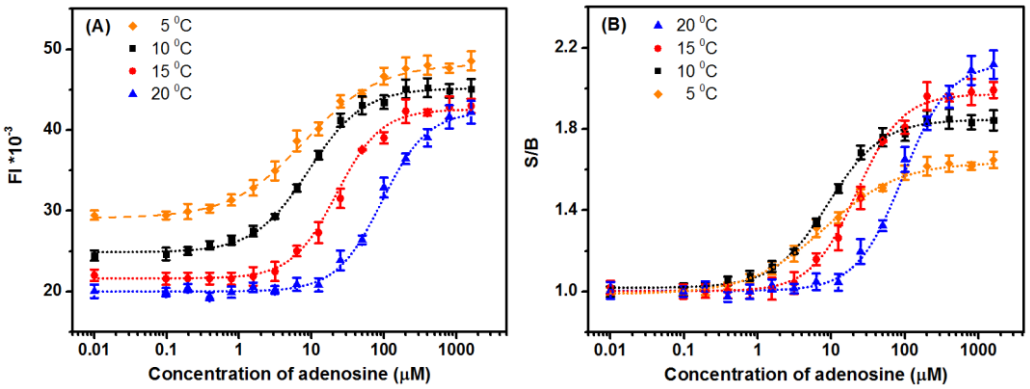


Fig. S28. *Dose-response curves for the adenosine ligand using the* ***dansyl-H2-dansyl/CD-H1-CD*** *system at 5°C, 10°C, 15°C, 20°C. Experimental conditions: 400 nM of* ***CD-H1-CD*** *and 200 nM of* ***dansyl-H2-dansyl****, in 10 mM Tris-HCl, pH=7.5, 5mM* Mg^{2+}*.* λ_{ex} *= 333 nm,* λ_{em} *= 530nm.*

The 15-20°C temperature range provided the highest S/B value (Fig. S27). However, from titration curves constructed over the 5-20°C range, we also observed a significant reduction of the half-saturation adenosine concentration with the temperature decreasing (Fig. S28). Thus, the 10°C temperature constituted the best compromise in terms of S/B change and sensitivity.

2.6.7. Comparison of detection limits.

Table S1. *Limit of detection (LOD) for small molecules using optical split aptasensors.*

Assay strategy/target	LOD	Ref
Host-dye reporter/adenosine	1 µM	This work
Fluorescence/cocaine	~50 µM	4
Fluorescence/ATP	~20 µM	4
Fluorescence anisotropy/adenosine	15 µM	9
Fluorescence anisotropy-mass amplifier/adenosine	10 µM	9
Excimer/cocaine	10 µM	16
Ligation/steroids	1-10 µM	13
Ligation/cocaine	1 µM	11
Fluorescence-competition/theophylline	1 µM	10
Enzyme-based amplification/cocaine	0.5 µM	15
Excimer-signal enhancement/ATP	0.08 µM	17
Quantum dot assembly/adenosine	0.01 µM	14

2.6.8. Effect of the human serum dilution factor on the assay response.

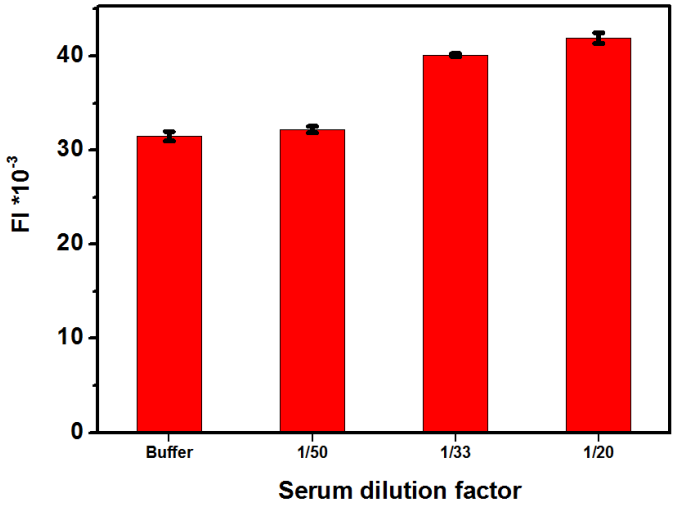


Fig. S29. *Assay response for 5 µM adenosine using the* ***dansyl-H2-dansyl/CD-H1-CD*** *system at 10°C in different diluted serum samples (and in buffer as control). Experimental conditions: 400 nM of* ***CD-H1-CD*** *and 200 nM of* ***dansyl-H2-dansyl****, in 10 mM Tris-HCl, pH=7.5, 5 mM* Mg^{2+}*.* λ_{ex} *= 333 nm,* λ_{em} *= 530nm.*

2.6.9. Recoveries in 50-fold diluted human serum samples.

Table S2. *Determination of adenosine recovery in heat-treated 50-fold diluted human serum samples. Results represent mean ± SD obtained from three independent experiments. RSD (relative standard deviation) = standard deviation/mean ×100.*

adenosine added μM	**adenosine found** μM	**RSD (%)**	**Recovery (%)**
3.0	3.4 ± 0.06	2.04	113.3
5.0	5.5 ± 0.05	1.02	110.9