Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

A *de novo* Self-Assembling Peptide Hydrogel Biosensor with Covalently Immobilised DNA-Recognising Motifs

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Materials and Methods

Materials. Peptide Val-Lys-Val-Lys-Val-Glu-Val-Lys was purchased from Biomatik and excess trifluoroacetic acid (TFA) was removed via lyophilisation from 10 % aqueous acetic acid (w/v). Oligonucleotides F-CGATTCGCCAAACACAGAATCG-3'-D (MB), and thiol-modified **(**disulphide protected**)** *thiol*-CGATTCTGTGTT (partner) were purchased from ATDBio Ltd. Where required, these oligonucleotides were purified using reverse-phase HPLC using the same protocol as described below for peptide-oligonucleotide conjugate products.

Instrumentation. HPLC purifications were carried out on an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a diode array detector and Rheodyne (3725i) manual injector. Purifications were performed using a semi-preparative Phenomenex Luna C-18 column (5 µm, 4.6 × 10 x 250 mm, 100 Å, Phenomenex; CA, USA), with a flow rate of 2 mL min^{-1}. Analytical HPLC was used to determine product purity, using an Agilent Eclipse C-18 column (XDB-C18, 5 µm, 4.6 × 5 x 150 mm, 100 Å, Agilent Technologies Inc., CA, USA) with a flow rate of 0.5 mL min^{-1}. ESI mass spectral data was collected on a Thermo Scientific LTQ Orbitrap XL (MA, USA) at the EPSRC National Mass Spectrometry Facility, (Swansea, UK). MALDI (Matrix-assisted laser desorption ionization) mass spectral data was collected on a Bruker Daltonics Ultraflex TOF/TOF mass spectrometer (MA, USA), at the Manchester Interdisciplinary Biocentre, University of Manchester. NMR spectra (^{1}H and ^{31}P) were recorded using Bruker Avance II+ spectrometers operating at proton frequencies of 400 MHz and at phosphorous frequencies of 162 MHz. In all cases, spectra were acquired using BBI ^{1}H/D-BB Z-GRD Z8202/0347 probe and processed using Bruker software Topspin v2.0 or v2.1. UV and visible measurements were carried out using a Cary-400 UV-Visible spectrophotometer from Varian (Australia) connected to a Cary Peltier temperature controller, operating under Varian Cary WinUV software. All the spectra were recorded at 5 °C using 1 cm path-length quartz cuvettes. The wavelength range was from 200 to 800 nm. Fluorescence excitation and emission spectra were recorded in 1 cm path-length quartz cuvettes using a Cary-Eclipse fluorescence spectrophotometer operated with Cary Eclipse software, and equipped with a Cary Peltier-thermostatted cuvette holder. In some cases fluorescence spectra were recorded using a Tecan Safire plate reader (Salzburg, Austria) equipped with Magellan Data Analysis Software (V.7). For negative staining transmission electron microscopy (TEM), hydrogel samples were formed from 20 mg ml^{-1} Val-Lys-Val-Lys-Val-Glu-Val-Lys at pH 7, and incubated at 20 °C for 24 hours. Samples were then diluted 100-fold in doubly distilled water and placed onto glow-discharged 400 mesh carbon coated grids (Agar Scientific, Stansted, UK) for one minute, then washed three times with doubly distilled water, and negatively stained with freshly prepared and filtered 2% (w/v) uranyl acetate (Agar Scientific, Stansted, UK) for one minute, blotting at each stage using Whatman filter paper. Samples were viewed and recorded on a Tecnai Biotwin (FEI, Oregon, USA) under an accelerating voltage of 100 KV, and imaged with a GATAN Orius CCD (Gatan, Oxford, UK). Fibre width and morphology analysis was performed using ImageJ.

Peptide Synthesis. MAL-Gly-Gly-Val-Lys-Val-Lys-Val-Glu-Val-Lys was synthesized on a pre-loaded low-loading Wang resin (0.25 mmol g^{-1}), using standard Fmoc-based chemistry, on a CEM liberty microwave automated peptide synthesizer (CEM, USA). The maleimide group was introduced at the N-terminus, on-resin using N-Maleoyl-β-alanine. Coupling agents used were diisopropylcarbodiimide (DIC) and hydroxybenzotriazole (HOBt). All reagents used were purchased from Sigma-Aldrich Company Ltd. (Gillingham, UK), Novabiochem (Merck, Germany), or AGTC Ltd. (FL, USA), and were of the highest grade available. Peptide was cleaved from the solid phase resin using 2.5 % water and 2.5 % triisopropylsilane in TFA, precipitated in diethyl ether (2 hrs at −20°C), and purified using reverse-phase HPLC using 0.1 % TFA/water as eluent A and 0.1 % TFA/AcCN as eluent B. The flow rate was maintained at 1.5 mL min^{-1}, with a gradient of 10 to 35 % buffer B in A (peptide elution at 28 %).

Conjugate synthesis. The peptide-oligonucleotide conjugate was synthesised as described by the reaction route in **Figure 2** using a thiol-maleimide coupling reaction. Thiol-modified oligonucleotide was purchased from ATDbio Ltd. with a protecting group to prevent unwanted interference during synthesis, and also to prevent the spontaneous formation of disulfide bonds. Prior to conjugation of the MAL-Gly-Gly-Val-Lys-Val-Lys-Val-Glu-Val-Lys to the thiol-modified oligonucleotide *thiol*-CGATTCTGTGTT, the protecting group was removed using a 10-fold excess of tris(2-chloroethyl) phosphate (TCEP) in aqueous conditions at pH 3 for 30 mins at room temperature. Maleimide-modified peptide, MAL-Gly-Gly-Val-Lys-Val-Lys-Val-Glu-Val-Lys (1.68 µmol) was dissolved in a minimal volume of 1:1 DMSO and water (30 µL) and added dropwise to the oligonucleotide (0.07 µmol) in 70 µl in phosphate-buffered saline with gentle agitation. Conjugation of the thiol to maleimide proceeded spontaneously in aqueous media, adjusted to pH 7.0 with 0.5M NaOH.[23] 15 % DMSO was added to disrupt fibre formation and minimize self-assembly of Val-Lys-Val-Lys-Val-Glu-Val-Lys under these conditions. Reaction with a 25-fold excess of maleimide- Gly-Gly-Val-Lys-Val-Lys-Val-Glu-Val-Lys for 6 hrs at room temperature gave the desired product in 55 % yield. Crude material was purified by reverse-phase HPLC using a semi-preparative Phenomenex Luna C-18 column. Buffers used included (A) 5 % acetonitrile in water, and (B) 5 % water in acetonitrile, each containing 0.05 % $LiClO_4$ with a gradient of between 5 and 50 % buffer B in A over 60 mins. Desired oligonucleotide fractions were identified by UV-VIS absorbance at 260 nm. The collected fractions containing peptide-oligonucleotide conjugates

were lyophilised and finally precipitated in 4 % $LiClO_4$ in acetone overnight at −80°C. The precipitate was washed carefully with 3 × 1 mL acetone, dried, dissolved in D_2O, and re-lyophilized prior to ^1H NMR characterization. The successful conjugation of the MAL-Gly-Gly-Val-Lys-Val-Lys-Val-Glu-Val-Lys to the oligonucleotide was confirmed (i) by the change of the HPLC retention time from 22.8 min (unmodified thiol-oligonucleotide), to 19.5 min (conjugate), (ii) by mass spectrometry data for the purified product (see below), (iii) by disappearance of the ^1H NMR signal at 6.78 ppm, which was previously observed for the maleimide-CH=CH protons, and (iv) by comparison of the ^1H NMR spectrum recorded for the reaction product with those of the starting materials (**Figures S2 and S3**). In all cases oligonucleotide concentrations were measured using UV absorbance at 260 nm (Varian Cary 4000 UV-Vis spectrophotometer; Australia) using extinction coefficient ε_{260} of 249.043 mM^{-1} cm^{-1} for F-CGATTCGCCAAACACAGAATCG-3'-D and 105.210 mM^{-1} cm^{-1} for *thiol*-CGATTCTGTGTT.

Peptide characterization (Figure S5 A): NH_2-Val-Lys-Val-Lys-Val-Glu-Val-Lys-OH (MALDI-MS: m/z = 929.2 for $[M+H]^+$ (MW = 928.2 g mol^{-1} calcd. for $[C_{43}H_{81}N_{11}O_{11}]$). ^1H NMR (D_2O with TSP (0.1 µM), 400 MHz): δ 0.80-0.95 (d, 24H, 4×Val-$(CH_3^{\gamma})_2$), 1.25-1.49 (m; 6H; 3×Lys-CH_2^{γ}), 1.55-1.65 (apparent m, 6H, 3× Lys-CH_2^{δ}), 1.65-1.78 (apparent m, 6H; 3×Lys-CH_2^{β}), 1.81-2.05 (m, 3H, 3×Val-CH^{β}), 1.98 (apparent m, 2H, Glu-CH_2^{β}), 2.15 (m, 1H, Val-CH^{β}), 2.38 (apparent m, 2H, Glu-(CH_2^{γ}), 2.94 (t, 6H, 3×Lys-CH_2^{ε}), 3.77 (t, 1H, Glu-CH^{α}), 4.01-4.09 (d, 4H; 4×Val-CH^{α}), 4.21-4.43 (t; apparent 1.7H due to suppression of HOD signal (expected 3H), t, 3×Lys-CH^{α}).

N-maleoyl-β-alanine-Gly-Gly-Val-Lys-Val-Lys-Val-Glu-Val-Lys (Figure S5 B): (MALDI-MS: m/z = 1192.8 for $[M+H]^+$ (MW = 1191.7 g mol^{-1} calcd. for $[C_{53}H_{90}N_{14}O_{16}]$). ^1H NMR (D2O with TSP (0.1 µM), 400 MHz): δ 0.78-0.92 (d, 24H, 4× Val-$(CH_3^{\gamma})_2$, 1.25-1.49 (m; 6H; 3×Lys-CH_2^{γ}), 1.55-1.65 (apparent m, 6H, 3× Lys-CH_2^{δ}), 1.65-1.78 (apparent m, 6H; 3×Lys-CH_2^{β}), 1.78-2.08 (m, 6H, 4×Val-CH^{β} and Glu-CH_2^{β}), 2.38 (apparent m, 2H, Glu-CH_2^{γ}), 2.54 (t, 2H, maleimide-CH_2), 2.92 (t, 6H, 3×Lys-CH_2^{ε}), 3.75 (t, 2H, maleimide-N-CH_2), 3.82 (s, 2H, Gly-H), 3.91 (s, 2H, Gly-H), 4.01-4.09 (d, 5H from 4×Val-CH^{α} and t, 1H, Glu-CH^{α}), 4.21-4.43 (t, apparent 1.7H due to suppression of neighboring HOD signal (expected 3H), 3×Lys-CH^{α}), 6.78 (s; 2H, maleimide-CH=CH).

Thiol-modified oligonucleotide CGATTCTGTGTT (Figure S6 A): MALDI-MS: m/z = 3969.6 $[M-H]^-$ MW = 3966.7 g mol^{-1} calcd. for $[C_{130}H_{176}N_{38}O_{79}P_{12}S_2]$). ^1H NMR (D2O with TSP (0.1 µM), 400 MHz): δ 1.15 (m, 4H, 2×CH_2 (linker)), 1.22 (m, 4H, 2×CH_2 (linker)), 1.36-1.52 (m, 8H, 4×CH_2 (linker)), 1.69 (s, 3H, T-CH_3), 1.72 (s, 3H, T-CH_3), 1.78 (s, 3H, T-CH_3), 1.82 (s, 3H, T-CH_3), 1.84 (s, 3H, T-CH_3), 1.88 (s, 3H, T-CH_3), 1.90-2.85 (overlapped region, 24H, 12×H2" and 12×H2'), 2.50 (apparent m, 4H, 2×S-CH_2 (linker)), 3.54 (t, 2H, 2×O-CH_2 (linker)), 3.73 (m, 2H, 2×-PO_4^- CH_2 (linker)), 3.81-4.62 (overlapped region; 36H from 12×H4', 12×H5' and 12×H5", 5.71 (dd, 1H, H1'), 5.96 (d, 1H, C-H_5(Ar)), 5.97 (d, 1H, C-H_5(Ar)), 6.02-6.37 (dd, 11H, H1'), 7.39 (bs; 1H, T-H6(Ar)), 7.43 (bs; 1H, T-H6(Ar)), 7.45 (bs; 1H, T-H6(Ar)), 7.56 (bs; 1H, T-H6(Ar)), 7.58 (bs; 1H, T-H6(Ar)), 7.62 (d; 1H, C-H6(Ar)), 7.67 (bs; 1H, T-H6(Ar)), 7.74 (d; 1H, C-H6(Ar)), 7.86 (s; 1H, G-H8(Ar)), 7.97 (s; 1H, G-H8(Ar)), 7.99 (s; 1H, G-H8(Ar)), 8.07 (s; 1H, A-H2(Ar)), 8.36 (s; 1H, A-H8(Ar)). Chemical shifts for 3H' sugar ring protons are not reported due to spectral distortion caused by pre-saturation of HOD NMR signal.

Peptide-oligonucleotide conjugate (Figure S6 B): MALDI-MS: m/z = 5018.0 $[M-H]^-$ MW = 5016.4 g mol^{-1} calcd. for $[C_{178}H_{245}N_{53}O_{93}P_{12}S]$). ^1H NMR (D2O with TSP (0.1 µM), 400 MHz): δ 0.9-3.1 (overlapped region; 94H including 8H, 4×CH_2 (linker), 12×H2", 12×H2', 2H from -S-CH_2 (linker), 24H from 4×Val-$(CH_3^{\gamma})_2$, 6H from 3×Lys-CH_2^{γ}, 6H from 3× Lys-CH_2^{δ}, 6H from 3×Lys-CH_2^{β}, 4H from 4×Val-CH^{β}, 2H from Glu-CH_2^{β}, 2H from Glu-CH_2^{γ}, 2H from maleimide-CH_2, 2H from maleimide-CH_2-CON-, 6H from 3×Lys-CH_2^{ε}), 1.69 (s, 3H, T-CH_3), 1.71 (s, 3H, T-CH_3), 1.77 (s, 3H, T-CH_3), 1.83 (s, 3H, T-CH_3), 1.87 (s, 3H, T-CH_3), 1.89 (s, 3H, T-CH_3), 3.73 (m, 2H, 2×-PO_4-CH_2 (linker)), 3.81-4.62 (overlapped region; 51H including 12×H4', 12×H5' and 12×H5", 2H from maleimide-N-CH_2, 1H from maleimide-S-CH, 4H from 2×Gly-H, 4H from 4×Val-CH^{α}, 1H from Glu-CH^{α} and 3H from 3×Lys-CH^{α}), 5.68 (t, 1H, H1'), 5.99 (d, 1H, C-H5(Ar)), 6.92 (d, 1H, C-H5(Ar)), 6.05-6.39 (apparent m, 11H, H1'), 7.39 (bs; 1H, T-H6(Ar)), 7.43 (bs; 1H, T-H6(Ar)), 7.45 (bs; 1H, T-H6(Ar)), 7.56 (bs; 1H, T-H6(Ar)), 7.58 (bs; 1H, T-H6(Ar)), 7.62 (d; 1H, C-H6(Ar)), 7.67 (bs; 1H, T-H6(Ar)), 7.74 (d; 1H, C-H6(Ar)), 7.86 (s; 1H, G-H8(Ar)), 7.97 (s; 1H, G-H8(Ar)), 7.99 (s; 1H, G-H8(Ar)), 8.07 (s; 1H, A-H2(Ar)), 8.36 (s; 1H, A-H8(Ar)). Chemical shifts for 3H' sugar ring protons are not reported due to spectral distortion caused by pre-saturation of HOD NMR signal.

While full identification of the ^1H-NMR protons of the conjugate was difficult due to strong overlap of the signals in the aliphatic region (0.5 - 4.7 ppm), it was possible to confirm conjugation by comparing the ^1H-NMR spectra of the conjugate with those of the starting materials, *i.e.* comparing the starting materials N-Maleoyl-β-Alanine N-terminally labelled peptide (**Figure S5 B**) and the thiol modified oligonucleotide (**Figure S6 A)** with that of the conjugate **Figure S6 B**. The appearance of new distinctive peptide signals in the area of 0.4-3.2 ppm and 3.5-4.7 ppm, in addition to the oligonucleotide signals, signify the presence of both components in the reaction product (**Figure S6 B)**. The disappearance of the distinctive signal at 6.79 pm from the -CH=CH- group in the N-Maleoyl-β-Alanine fragment, which was previously detected in the NMR spectrum of the peptide precursor (**Figure S5 B**), confirmed a completion of the conjugation reaction. Despite the majority of the peptide protons strongly overlapping with the methyl and sugar ring protons of the oligonucleotide component, the appearance of these new signals provided strong evidence of successful incorporation of the peptide component into the conjugate

structure. Careful integration of the ^{1}H-NMR signals in the oligonucleotide aromatic (7.3-8.5 ppm) and H1'/Ar-H5 (5.5-6.5 ppm) regions, as well as those generated in the aliphatic area of the ^{1}H NMR spectrum (0.4-3.2 and 3.5-4.7 ppm), confirmed a 1:1 molar ratio between oligonucleotide and peptide components.

Sample preparation. All samples for hybridisation assays were prepared in aqueous 100 mM TRIS buffer (pH 7.2) containing 200 mM KCl. Final peptide concentrations of 20 mg mL^{-1} (21.5 mM) were used for hydrogel samples unless otherwise stated. These were prepared by dissolving the peptide in half the total volume of water (to avoid any undesirable precipitations) followed by adding hybridization buffer with NaOH to increase the pH to 5. The peptide-oligonucleotide conjugate was subsequently added and the pH adjusted to 7.2 with NaOH, prior to incubating samples for 24 hours at room temperature. The buffer concentration in hydrogel samples (50 mM TRIS buffer (pH 7.2); 100 mM KCl) was therefore reduced by 2×fold as compared with that used for the experiments in solutions. However, this did not affect hybridisation and the resulting fluorescent signal (Figure 3).

Hybridization experiments. Oligonucleotide hybridization was assessed through an increase in fluorescence at 517 nm (λ_{ex} 494 nm) at 20°C (Varian Cary Eclipse, with a temperature controller), or a plate reader (Tecan Safire). All spectra were collected and processed using Magellan Data Analysis Software (V.7). Hybridization was detected at 1–10 µM and 0.02–200 nM concentration ranges for the peptide-oligonucleotide conjugates on these devices, respectively. All samples were incubated overnight at 20°C.

Estimation of the Limit of Detection. The limit of detection (LoD) in this system was estimated using a Tecan Safire plate reader in accordance with the established protocol.[1] Equations (1) and (2) were used to estimate the mean and SD values for 'blank' samples (n = 132), and low concentration (50 pM) samples (n = 88) of the hydrogel biosensor with an equal amount of MB analyte. The fluorescence measurements were recorded at λ_{em} of 517 nm following excitation at 494 nm.

Biosensor signal development. The kinetics of hybridisation between MB and target was monitored in parallel and in triplicate for samples in TRIS buffer and in 21.5 mM hydrogel, by recording fluorescence intensity at 494 nm over 120 minutes, using a Cary-Eclipse fluorescence spectrophotometer (1 cm curvettes, 20°C, pH 7.2, **Figure S7**).

Peptide charge calculation. Peptide charge was calculated from pH 1 to 14 using the following expression, derived from the Henderson-Hasselbalch equation:[2]

$$Q_{VKVKVEVK} = \left(\frac{+1}{1+10^{-(pH-9.72)}}\right) + \left(\frac{+3}{1+10^{-(pH-10.53)}}\right) + \left(\frac{-1}{1+10^{-(pH-4.07)}}\right) + \left(\frac{-1}{1+10^{-(pH-2.18)}}\right)$$

(N-Terminus) (Lysine) (Glutamic acid) (C-Terminus)

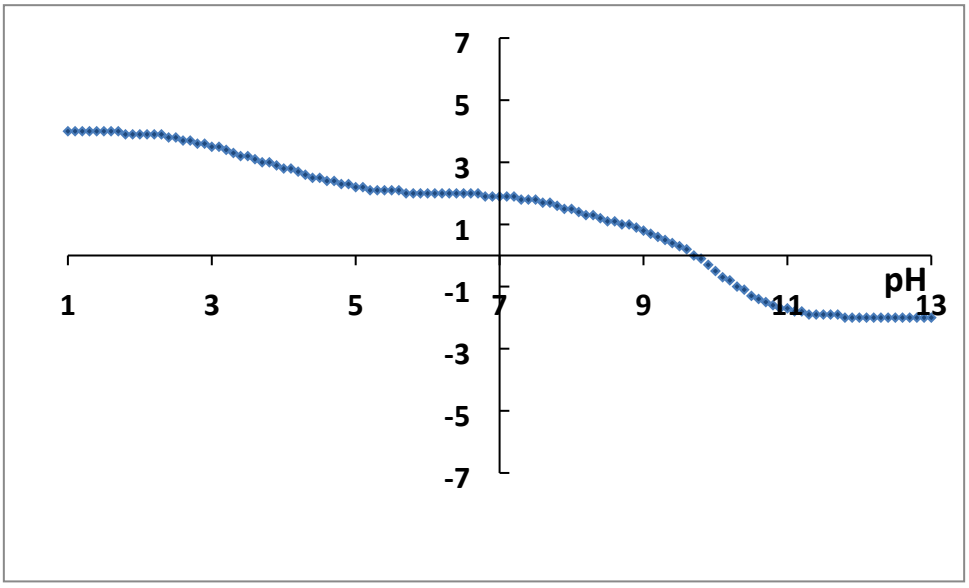

Figure S1: Peptide Val-Lys-Val-Lys-Val-Glu-Val-Lys overall charge with respect to pH. Peptide carries a net charge of +2 under biosensor working conditions (pH 7.0). Peptide charge calculated as described in materials and methods.

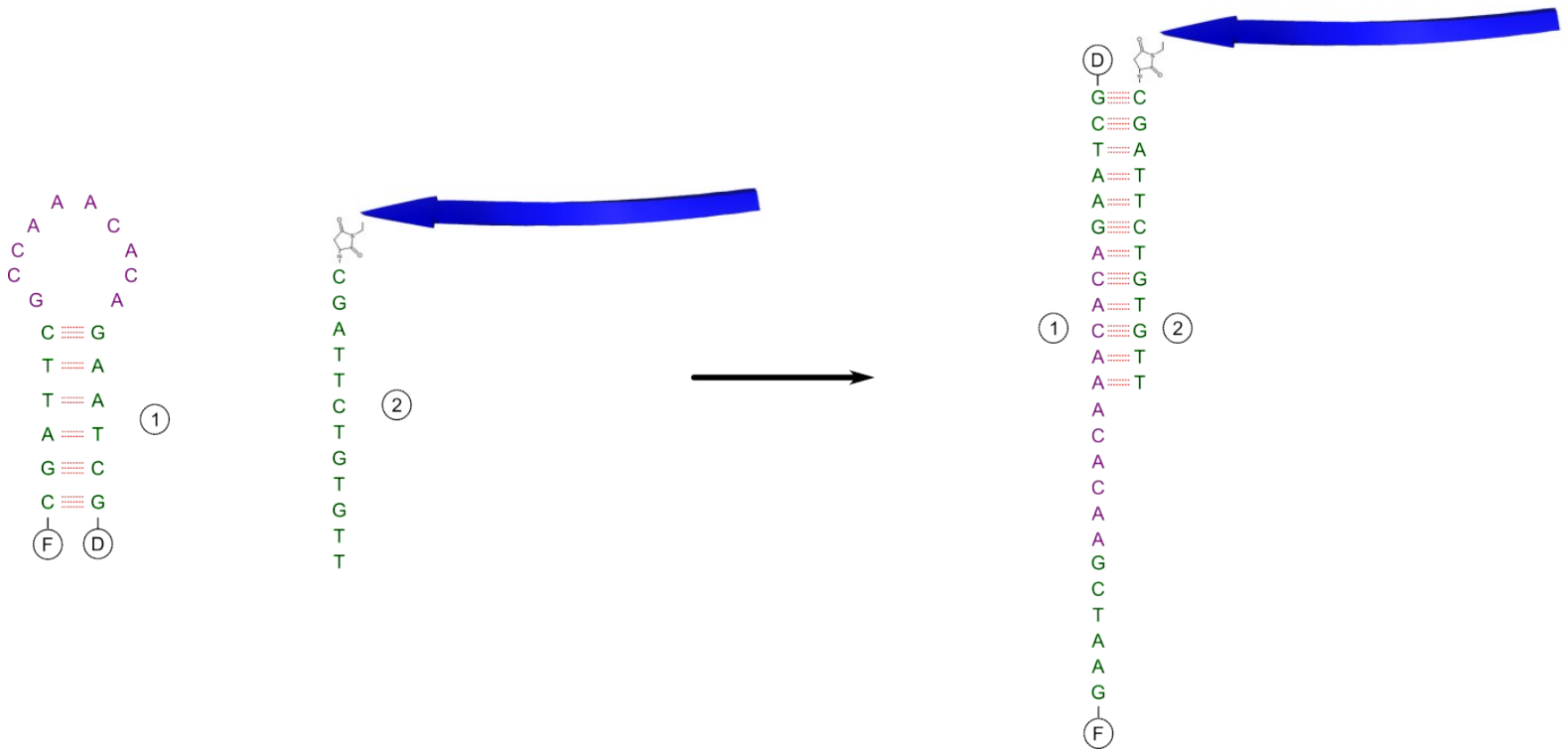

Figure S2. Diagram showing the MB probe (**1**), the DNA recognition motif CGATTCTGTGTT (**2**) and their hybridisation when (1) and (2) are mixed together. One-letter oligonucleotide sequences are shown. Blue and green signify peptide and oligonucleotide, and spheres with the letter 'F' and 'D' signify fluorophore (fluorescein) and quencher (dabcyl), respectively. The loop region of the MB is shown in purple.

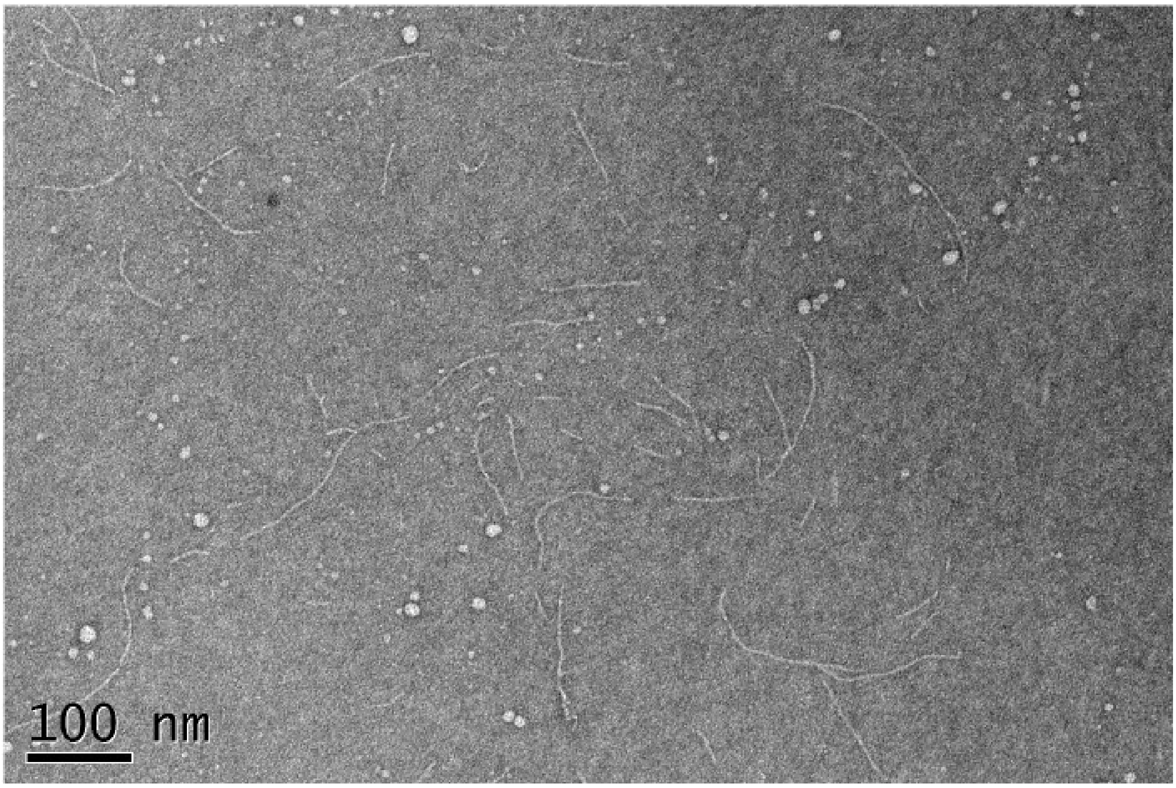

Figure S3. TEM image showing nanofibers that form Val-Lys-Val-Lys-Val-Glu-Val-Lys peptide hydrogels. Average fibre width 5.27 nm (SD = 0.54 nm, n = 50). Image analysis performed using ImageJ, applying intensity profiles taken perpendicular to the fibre long axis in order to provide accurate width measurements (Figure 4). Sample prepared and imaged as described in the materials and methods section. Peptide concentration was 20 mg ml^{-1}, pH 7. Samples diluted 10-fold before application to the grid.

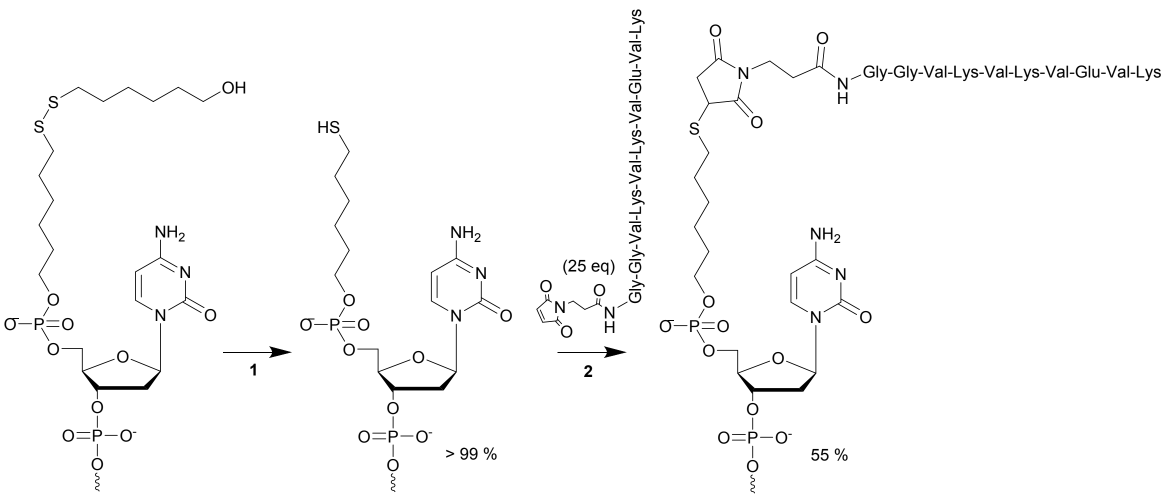

Figure S4: Synthesis of the peptide-oligonucleotide conjugate. Conjugate was synthesised by coupling a thiol-modified oligonucleotide and N-Maleoyl-β-Alanine N-terminally labelled peptide Gly-Gly-Val-Lys-Val-Lys-Val-Glu-Val-Lys. (**1**) 10 eq TCEP, 30 mins at RT, pH 3. (**2**) 25 eq maleimide-labelled peptide, 6 hrs at RT in 15 % aq. DMSO, pH 7.2.

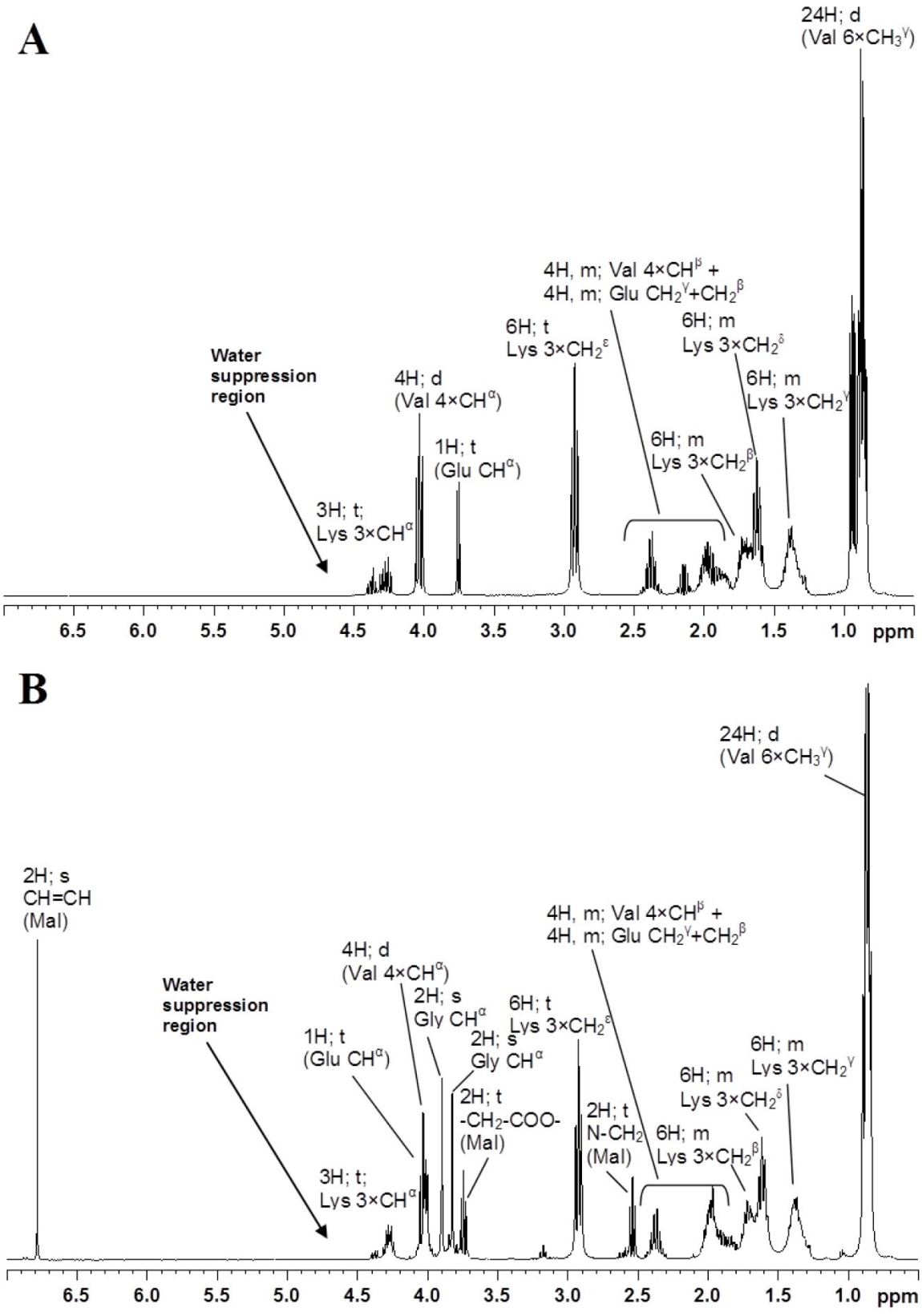

Figure S5. ^{1}H NMR spectrum of the peptide Val-Lys-Val-Lys-Val-Glu-Val-Lys (**A**) and MAL-Gly-Gly-Val-Lys-Val-Lys-Val-Glu-Val-Lys (**B**) recorded in D_2O at 25°C using Bruker Avance II+ spectrometers operating at proton frequencies of 400 MHz.

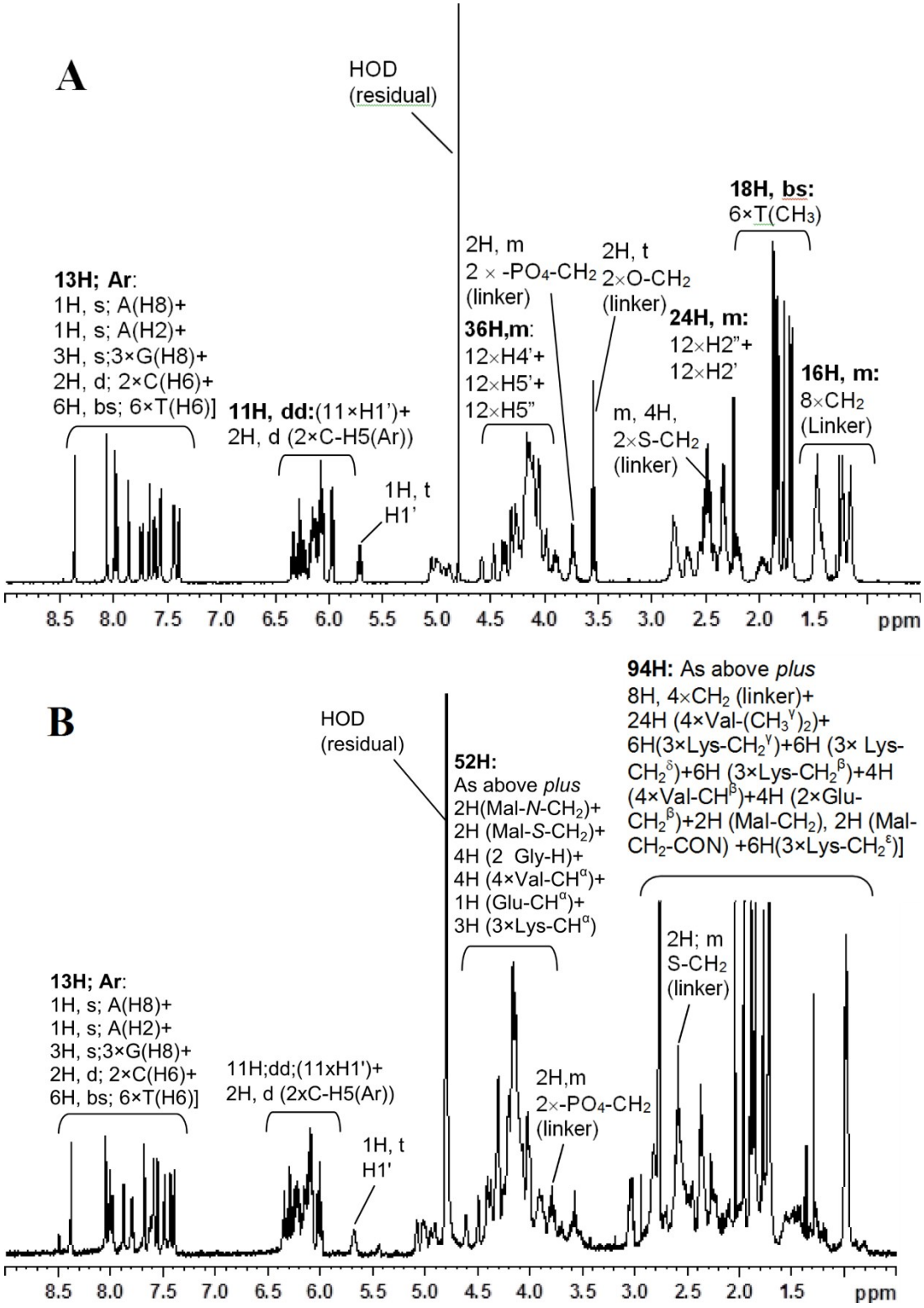

Figure S6. ^{1}H NMR spectra of the thiol-modified oligonucleotide CGATTCTGTGTT (**A**) and peptide-oligonucleotide conjugate (**B**) recorded in D_2O at 25°C using Bruker Avance II+ spectrometers operating at proton frequencies of 400 MHz.

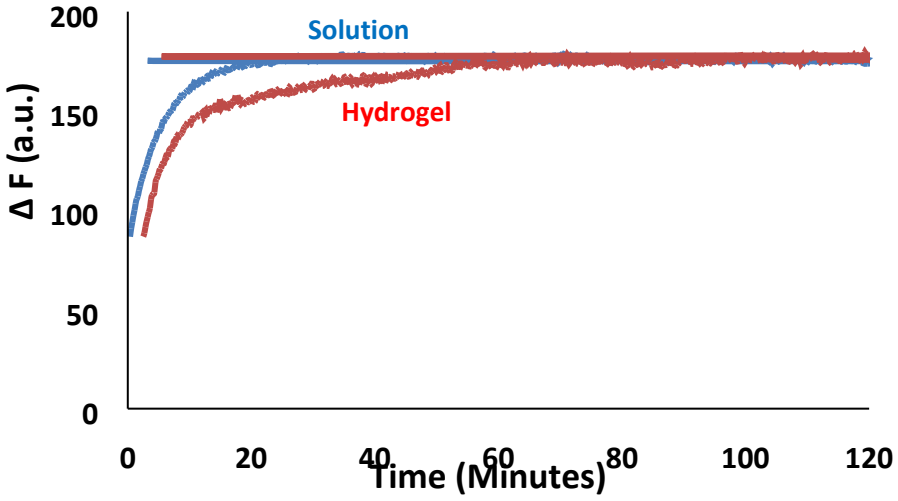

Figure S7: Change in fluorescence intensity (**ΔF**) over time in solution (red) and in hydrogel (black) as the result of hybridisation between the MB and peptide-oligonucleotide conjugate (averaged for 3 datasets). **ΔF** was measured at the emission λ_{max} against time following excitation at 494 nm (Solution: λ_{em} = 518 nm; Hydrogel: λ_{em} = 526 nm).

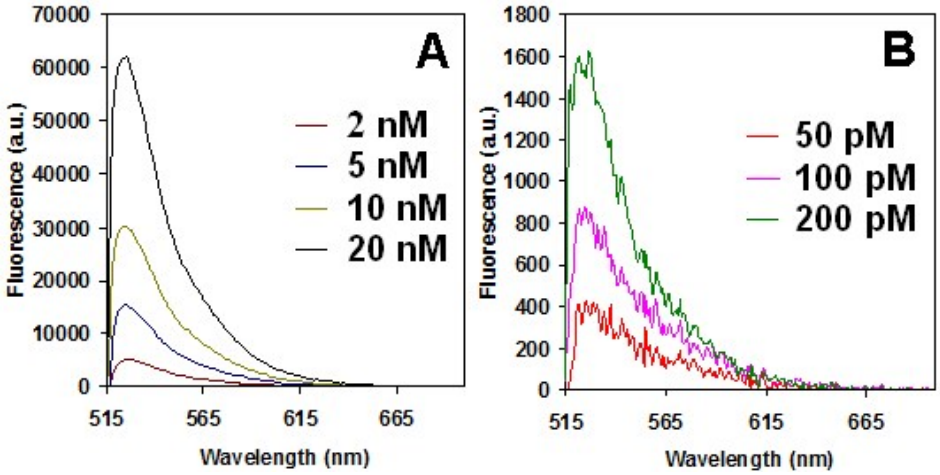

Figure S8: Detection limit of a 3D hydrogel-based DNA biosensor evaluated using a Tecan Safire plate reader. (A) Fluorescence spectra of the biosensor at 2 to 20 nM concentrations of immobilized conjugate and MB (conjugate doping level was 1.24×10^{-5} to 1.24×10^{-4} %). (B) Fluorescence spectra of the biosensor at 50 to 200 pM concentrations of both components (conjugate doping level was 3.11×10^{-7} to 1.24×10^{-6} %). MB and conjugate present at equimolar concentrations as indicated in figure legends. Hydrogels formed using 16.1 mM base peptide, pH 7.

Notes and references

1 D.A. Armbruster and T. Pry, *Clin Biochem Rev.*, 2008 (Suppl 1): S49–S52.
2 D.S. Moore, *Biochemical Education*, 1985, 13(1), 10-11.