Supporting Information for

Peptide-directed folding of the elusive RNA i-motif

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1. Materials & Methods

1.1 General Material / Instrument use

All solvents and reagents were purchased from chemical supply companies and used without further purification, unless otherwise stated. i-Motif oligonucleotides were synthesised by the UNSW RNA Institute on an OligoPilot 10 DNA/RNA Synthesizer, purified by Strong Anion Exchange High Performance Liquid Chromatography (SAX-HPLC), and desalted using Amicon® Ultra Centrifugal Filters. Peptides were synthesised by Solid Phase Peptide Synthesis (SPPS) and purified by Reverse-Phase High Performance Liquid Chromatography (RP-HPLC). Samples were characterised and purity confirmed by Liquid Chromatography Mass Spectrometry (LC-MS), Nuclear Magnetic Resonance (NMR), Ultraviolet-Visible Spectroscopy (UV-Vis), and Infrared Spectroscopy (IR).

1.2 Buffer and other conditions

NMR binding studies were performed in sodium phosphate/acetate buffer (50 mM), with added sodium chloride (50 mM) unless otherwise stated and pH adjusted with concentrated aqueous HCl / NaOH solutions.

1.3 Secondary Structure Assembly

RNA secondary structures were annealed in the desired buffer using an Applied Biosystems ProFlex PCR system. Oligonucleotides (20 μM) along with peptide (0 – 320 μM) were heated at 85 °C for 15 min, followed by cooling at 0.1 °C/min to 20 °C, unless otherwise stated. Experiments were performed in 50 mM sodium phosphate/acetate buffers, with added NaCl (50 mM), and pH adjusted using HCl/NaOH unless otherwise stated. Unless otherwise stated, experiments were performed at 25 °C.

1.4 pH measurements

The pH of the buffers and samples were measured with a Hanna Instruments edge^{pH} pH meter, fitted with a Hanna Instruments HI11310 pH-probe.

1.5 Circular Dichroism spectroscopy

Circular Dichroism (CD) spectra were obtained on an Applied Photophysics Chirascan Plus CD Spectrometer, fitted with a variable temperature unit. Samples were prepared in a 1 mm path length quartz cuvette. Characterisation spectra were taken at 25 °C unless otherwise stated. Spectra were obtained with a scan speed of 100 nm/min, with a bandwidth of 1 nm and step of 1 nm. Oligonucleotides (20 μM) along

with peptide (0 – 320 μM) were dissolved in appropriate buffer (200 μL) and annealed via the annealing protocol (Supplementary Information 1.3) prior to data collection. Data was analysed in GraphPad Prism.

1.6 Thiazole Orange fluorescence spectroscopy

Fluorescence spectra was obtained on a Varioskan LUX plate reader in a Corning 384 well low-volume flat-bottom fluorescence plate. Oligonucleotides (20 μM) in the presence or absence of peptide (320 μM) were heated at 85 °C for 15 min, followed by cooling at 0.1 °C/min to 20 °C. 10 wells were prepared in triplicate. Different concentrations of Thiazole Orange (0-320 μM, 0-16 eq.) were added to appropriate wells. The system was allowed to equilibrate for 30 minutes at room temperature, before being excited at 512 nm with a bandwidth of 4 nm, and corresponding fluorescence emission spectra measured from 535 – 750 nm. Data was analysed in GraphPad Prism.

1.7 NMR

Peptide and oligonucleotide ^{1}H NMR spectra were obtained on a Bruker Avance III 600 MHz Cryo NMR Spectrometer. Peptide (125 μM) and oligonucleotide (0 – 500 μM) were dissolved in 10% deuterium oxide in appropriate aqueous buffer and left to equilibrate at room temperature overnight. Solvent peaks at a chemical shift of ~4.70 ppm were suppressed using excitation sculpting. Unless otherwise stated, spectra were collected at 25 °C.

1.8 HPLC

1.8.1 Purification

Semi-Preparative (Peptide): The purification of peptide **RGGFGGRGG** was performed on a Shimadzu Prominence UFLC HPLC fitted with a Shimadzu 20AB pump and an SPD-20A photodiode array detector. An Intersil ODS-4 5 μm 20 × 150 mm column with a C18 stationary phase was used. The mobile phase was A: MilliQ™ water with formic acid (0.1%, v/v), and B: acetonitrile with formic acid (0.1%, v/v). The concentration of mobile phase B was increased from 10-95% over 35 min, with a flow rate of 5 μL/min and monitored at 254 nm. Samples were lyophilised to afford the purified peptide as a fluffy white powder.

Preparative (Oligonucleotide): Purification of oligonucleotides was performed via anion exchange HPLC using a ThermoScientific™ DNAPac™ PA 100 BioLC™ 22 × 250 mm column on a Shimadzu LC-20AP system fitted with a Shimadzu 20AB pump and an SPD-M40 photo diode array detector. The mobile phase was A: Milli-Q™ water with sodium hydroxide (5 mM), B: Milli-Q™ water with sodium hydroxide (5

mM) and sodium chloride (2 M). The concentration of mobile phase B was increased from 5%-80%-95% over 60 minutes with a flow rate of 5 mL/min and monitored at 260 nm.

1.8.2 Analytical HPLC

Analytical HPLC was performed on a Shimadzu Prominence UFLC HPLC system, using the same mobile phase as preparative, with a flow rate of 1 mL/min. A ThermoScientific™ DNAPac™ PA 100 analytical column was used.

1.8.3 LC-MS

Peptide: A Shimazdu LC/MS 2020 system was used, fitted with a reverse-phase C-18 analytical column. The mobile phase was A: Milli-Q™ water with formic acid (0.1% v/v), and B: acetonitrile with formic acid (0.1% v/v). The concentration of mobile phase B was increased from 10-95% over 35 minutes. Peptide samples were prepared to a concentration of 1 mg/mL in a solution of Milli-Q™ water and acetonitrile (1:1 v/v) and filtered.

Oligonucleotide: RNA samples were analysed by the UNSW RNA Institute on a Thermo Vanquish Flex UHPLC fitted with an Orbitrap Exploris 120. A DNAPac RP 50 × 2.1 mm column was used. The mobile phase consisted of two solvents, A: Milli-Q™ water with hexafluoro-2-propanol (2%, v/v) and *N*,*N*-diisopropylethylamine (0.1%, v/v), and B: Methanol with hexafluoro-2-propanol (0.075%, v/v) and *N*,*N*-diisopropylethylamine (0.0375%, v/v). A flow rate of 0.4 mL/min was used. Oligonucleotide samples were prepared in Milli-Q™ water (0.1 mg/mL).

1.9 Ultraviolet-visible spectroscopy (UV-Vis):

NanoDrop: The purity of oligonucleotides was assessed using a ThermoFisher NanoDrop One/OneC Microvolume UV Spectrophotometer. 1.0 µL of sample dissolved in Milli-Q™ water was added to the plate using an Eppendorf Research Plus® P10 pipette. The absorbance was measured at 220 and 260 nm, to give the 260:220 nm ratio. Any ratio above 1.80 was considered pure. Oligonucleotide concentration and yield was derived from the Beer-Lambert law. Integrated DNA Technologies Oligoanalyzer software was used to calculate the molar extinction coefficient.

1.10 Solid Phase Peptide Synthesis (SPPS)

Peptides were synthesised via literature procedure as previously published.[6] Peptide characterisation matched literature data.

1.11 Oligonucleotide Synthesis

RNA oligonucleotides were synthesized by the UNSW RNA Institute on an ÄKTA Oligopilot Plus 10 synthesizer at a 50 μmol scale. Phosphoramidites (Thermo Fisher Scientific) were dissolved to 0.1 M in anhydrous acetonitrile (Merck). All reagents were used as supplied without further purification.

Oligonucleotides were cleaved and deprotected from the solid support in a mixture of aqueous ammonia (40%, 2 mL) and methylamine (33%, 2 mL) (55 °C, 50 min). Secondary deprotection of TBDMS groups was achieved with TEA•3HF (1 mL) in dimethyl sulfoxide (1 mL) (65 °C, 3 h), followed by precipitation and washing in ice-cold butanol.

Purification was performed by strong anion exchange HPLC (SAX-HPLC) using a ThermoScientific™ DNAPac™ PA 100 BioLC™ column (22 × 250 mm) on a Shimadzu LC-20AP system with a Shimadzu 20AB pump and an SPD-M40 photodiode array detector with mobile phases: A: Milli-Q™ water with sodium hydroxide (5 mM, pH 12), and B: Milli-Q™ water with sodium chloride (1 M) with sodium hydroxide (5 mM, pH 12). The concentration of mobile phase B was increased from 20% to 80% to 95% over 60 min with a flow rate of 5 mL min^{-1} and monitored at 260 nm. Desired fractions were collected, neutralized with dilute HCl, lyophilized, and desalted using Amicon Ultra 3k centrifugal filters.

1.12 NMR Titrations

NMR titration procedure: A series of 11 samples were prepared, with a constant concentration of peptide, and increasing equivalents of oligonucleotide. Unless otherwise stated, the peptide concentration was 125 μM, with oligonucleotide concentrations of 0, 12.5, 25, 50, 75, 100, 125, 150, 200, 250 and 500 μM, for a final saturation point of 4× equivalents. Samples were prepared in 160 μL 3 mm NMR tubes in 90% phosphate buffer, 10% D_2O. A minimal concentration of 3-(trimethylsilyl)propinoic-2,2,3,3-d^4 acid sodium salt was included as an internal standard. Samples were mixed and allowed to sit at room temperature for at least 3 hours before spectra were collected, to allow for folding equilibrium.

Data analysis:

The online tool supramolecular.org[1] developed by us[2] was used for data analysis. Below is a description of how the data analysis was carried out and the underlying theory and equations used in the supramolecular.org online tool under the “Bindfit” algorithm. Full source code for Bindfit is also available at:

https://github.com/echus/supramolecular-apps

The chemical shift data from the NMR titration was imported into supramolecular.org. A global fit (which greatly improves the fit) model of 3 shifts within the phenylalanine aromatic region was used to produce

binding isotherms, as this region was well-defined and exhibited a significant shift. In titrations above pH 5.5, additional resonances resolved at ~7.4 ppm. These peaks are assigned as cytosine base H_6 protons for the single-stranded RNA. At lower pH, cytosine becomes hemi-protonated, inducing formation of the i-motif structure, increasing the deshielding of the cytosine. These protons appear to vanish, however they are re-emerging at ~7.95 ppm, where they become poorly defined and heavily overlapped. These resonances were thus not used for the global fit model. All titrations were run in duplicate, with the weighted average reported. The data was fitted to 1:1 and 2:1 equilibria, with five different binding models considered in each case as detailed below. Preliminary attempts to fit the data to 1:2 binding model did not yield any meaningfully good fit or physically impossible results (e.g. chemical shifts > 100s of ppm) and hence the 1:2 binding model was not considered further in this work. The focus was therefore on comparing the different 2:1 binding model and see if any or all of them where superior to the simple 1:1 model. Overall, the best fitting model in nearly cases was a 2:1 peptide:oligonucleotide statistical fit which is the focus of discussion in the main text. All fitting parameters and models are reported in Supplementary Tables 2-4. Full details on the equations and terminology used here for the binding models used have been published previously.[3] Below the most important equations referred to in this work here are summarized:

a) Key terminology: In the equations below, H = host (peptide), G = guest (RNA or DNA), $[X]_0$ total concentration of species X, and K = association constants) as discussed further in below. N.b. the peptide is defined as the "host" because we are adding RNA or DNA to the peptide solution.

b) Free energy (ΔG) changes: The association constants, also be known as the equilibrium constants (K_a), for the simple 1:1 H:G complexation as according to equation (S1) can be expressed in free energy (ΔG_a) according to equation (S2).[1]

$$K_a = \frac{[HG]}{[H][G]} \qquad \text{Eq. (S1)} \qquad\qquad \Delta G_a = -RT \ln K_a \qquad \text{Eq. (S2)}$$

For 2:1 H_2G complexation is described according to equation (S3) and (S4). We can now express the stepwise association constants (K_1 and K_2) can be expressed in terms of the free energy changes (ΔG_1 and ΔG_1) according to equations (S5) and (S6) using the microscopic stepwise association constants (K_{1m} and K_{2m}) which are derived from the stepwise association constants after correcting for statistical factors as $K_{1m} = K_1/2$ and $K_{2m} = 2K_2$.[4]

$$K_1 = \frac{[HG]}{[H][G]} \quad \text{Eq. (S3)} \qquad\qquad K_2 = \frac{[H_2G]}{[HG][H]} \quad \text{Eq. (S4)}$$

$$\Delta G_1 = -RT\ln K_{1m} = -RT\ln\left(\frac{K_1}{2}\right) \quad \text{Eq. (S5)}$$

$$\Delta G_2 = -RT\ln K_{2m} = -RT\ln(2K_2) \quad \text{Eq. (S6)}$$

c) Binding models: In the data below five different binding models are usually compared.

1. The first on is classical **1:1** equilibria. Here, we define the NMR resonance for the host as δ_H, the guest as δ_G and the host-guest complex as δ_{HG}. From this, we can also define the change in resonance for the host-guest complexation as $\delta_{\Delta HG} = \delta_{HG} - \delta_H$. If we then define δ_0 = NMR resonance of the host before the guest is added (before the start of titration) we can define the change in in resonance as $\Delta\delta = \delta - \delta_0$. We can now write the NMR version of our simple 1:1 equilibria according to equation (S9) which is derived from the generic quadratic equation used to calculate the concentration of host-guest complex [HG] as previously described. [3]

$$\Delta\delta = \frac{\delta_{\Delta HG}}{[H]_0}\left(\frac{1}{2}\left\{\left([G]_0 + [H]_0 + \frac{1}{K_a}\right) - \sqrt{\left([G]_0 + [H]_0 + \frac{1}{K_a}\right)^2 - 4[H]_0[G]_0}\right\}\right) \quad \text{Eq. (S7)}$$

2. The second one is the stepwise (non-degenerate) "**full 2:1**" binding model. This model assumes two non-identical two binding sites per molecule of "guest" (RNA) that allows for cooperativity (negative or positive). As with the 1:1 equilibria we first define $\delta_{\Delta HG_2}$ as the difference between in NMR resonance on the peptide "host" between the 2:1 host-guest complex (δ_{H_2G}) and the host NMR resonance (δ_H), that is $\delta_{\Delta H_2G} = \delta_{H_2G} - \delta_H$. Using $\delta_{\Delta HG} = \delta_{HG} - \delta_H$ for the change in NMR resonance for the 1:1 complex formation and the observed change in resonance as $\Delta\delta = \delta - \delta_0$ as before with the 1:1 equilibria, we obtain equation (S8).

$$\Delta\delta = \frac{\delta_{\Delta HG}K_1[G]_0[H] + 2\delta_{\Delta H_2G}K_1K_2[G]_0[H]^2}{[H]_0(1 + K_1[H] + K_1K_2[H]^2)} \quad \text{Eq. (S8)}$$

Here the guest [H] concentration is obtained from the cubic equation (S9). [3]

$$[H]^3(K_1K_2) + [H]^2\{K_1(2K_2[G]_0 - K_2[H]_0 + 1)\} + [G]\{K_1([G]_0 - [H]_0) + 1\} - [H]_0 = 0 \quad \text{Eq. (S9)}$$

Notably, for the "**full 2:1**" model we make no assumptions about the correlation between either K_1 and K_2 ($K_1 \neq 4K_2$) or $\delta_{\Delta H_2G}$ and $\delta_{\Delta HG}$ ($\delta_{\Delta H_2G} \neq 2\delta_{\Delta HG}$).

3. The third one is the stepwise (non-degenerate) "**additive 2:1**" binding model. To reduce the number of fitted parameters we note that in many circumstances it can be assumed that the induced chemical shifts of the protons being monitored in the NMR experiment are simply additive, *i.e.*, for proton resonance Y, the shift caused by the second binding event is exactly the same as from the first binding. It then follows that $\delta_{\Delta H_2G} = 2\delta_{\Delta HG}$ and we can simplify equation (S8) to yield equation (S10).

$$\Delta\delta = \frac{\delta_{\Delta HG} K_1 [G]_0 [H]\{1+2K_2[H]\}}{[H]_0(1+ K_1[H]+K_1K_2[H]^2)} \quad \text{Eq. (S10)}$$

We have for the "**additive 2:1**" model therefore made the assumption that $\delta_{\Delta H_2G} = 2\delta_{\Delta HG}$, whilst not making any assumptions about the correlation between either K_1 and K_2 ($K_1 \neq 4K_2$).

4. The fourth model is the stepwise "**non-cooperative 2:1**" model. Here we revert back to noting that the chemical shift differences between the first and second binding may not be correlated ($\delta_{\Delta H_2G} \neq 2\delta_{\Delta HG}$) but instead we make the assumption that the 2:1 binding is non-cooperative and therefore that $K_1 = 4K_2$. We can then use this to rewrite equation (S8) and replace K_2 with $K_1/4$ to obtain equation (S11). If desired, K_2 can be calculated back from K_1 as $K_2 = K_1/4$.

$$\Delta\delta = \frac{\delta_{\Delta HG} K_1 [G]_0 [H]\left\{1+\frac{\delta_{\Delta H_2G} K_1[H]}{2}\right\}}{[H]_0\left(1+ K_1[H]+\frac{(K_1[H])^2}{2}\right)} \quad \text{Eq. (S11)}$$

We have in "**non-cooperative 2:1**" model made the assumption that $K_1 = 4K_2$ whilst making no assumption about the correlation between ($\delta_{\Delta H_2G}$ and $\delta_{\Delta HG}$ (($\delta_{\Delta H_2G} \neq 2\delta_{\Delta HG}$).

5. The fifth model is the "**statistical 2:1**" model. Here we not only make the assumption that the binding is non-cooperative ($K_1 = 4K_2$) but also that the chemical shift changes are simply additive ($\delta_{\Delta H_2G} = 2\delta_{\Delta HG}$. This means in other words we make the assumption that the two binding site behave like two independent hosts. This leads to a further simplification of equation (S8) to equation (S12).

$$\Delta\delta = \frac{\delta_{\Delta HG} K_1 [G]_0 [H]\left\{1+\frac{K_1[H]}{2}\right\}}{[H]_0\left(1+ K_1[H]+\frac{(K_1[H])^2}{2}\right)} \quad \text{Eq. (S12)}$$

In this "**statistical 2:1**" model we have therefore made the assumptions that $K_1 = 4K_2$ and that ($\delta_{\Delta H_2G} = 2\delta_{\Delta HG}$. It should also be noted that in this situation, the data could also be fitted to the simple **1:1** model according to Equation (S8) by simply multiply the total host concentration $[H]_0$ by a factor of 2. The resulting association constant K_a is then equal to the non-cooperative microscopic binding constants, *i.e.*, $K_a = K_{1m} = K_{2m}$, which means $K_1 = K_a/2$ and $K_2 = 2K_a$. As discussed, the statistical 2:1 model always gave the best fit and hence for brevity in some of the Figures further below, only the K_1 values are shown as K_2 can easily calculated from with $K_2 = K_1/4$.

d) *Comparing the models:*

To analyse and compare the model used we used the Bayesian Information Criteria (BIC)[5] as a robust method for model comparison. The challenge is that the more complicated the model (= higher number of parameters), the better the fit is likely to be. However, generally we should only pick a more complicated model if the fit is significantly better when compared to a simpler model. The BIC based on the calculated log-likelihood, the number of parameters, and the number of data points, whereby an increase in the number of parameters leads to a penalty (increase) in the BIC value. A low BIC is generally better and when comparing two models, and a difference of more than 6-10 is usually considered as strong evidence that there is a significant difference between the two models being compared.

2. Synthesis

2.1 Synthesis of Oligonucleotides

Oligonucleotide Synthesis

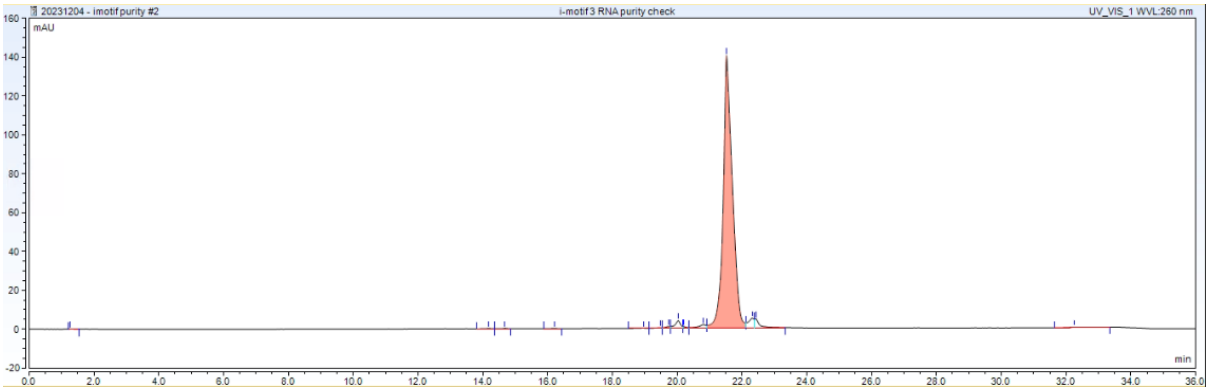
Synthesis of i-Motif DNA/RNA oligonucleotides was performed at the UNSW RNA Institute on a Cytiva ÄKTA Oligopilot™ 10 Plus oligonucleotide synthesiser. Synthesis was performed on preloaded Cytiva primer support Ribo 300 polymer resin in a 1.2 mL stainless steel column. Synthesis was carried out under an atmosphere of dry argon. Phosphoramidites were purchased from ThermoFisher and used without further purification. The solid-phase resin was purchased from Cytiva. The remaining reagents were purchased from Sigma Aldrich and Tedia High Purity Solvents and used without additional purification. SAX-HPLC purification was performed as per section 1.5.1. Millipore Amicon® Ultra-0.5 3 kDa centrifugal filters were purchased from Sigma and used for desalting.

Supplementary Table 1. i-Motif RNA & DNA sequences used

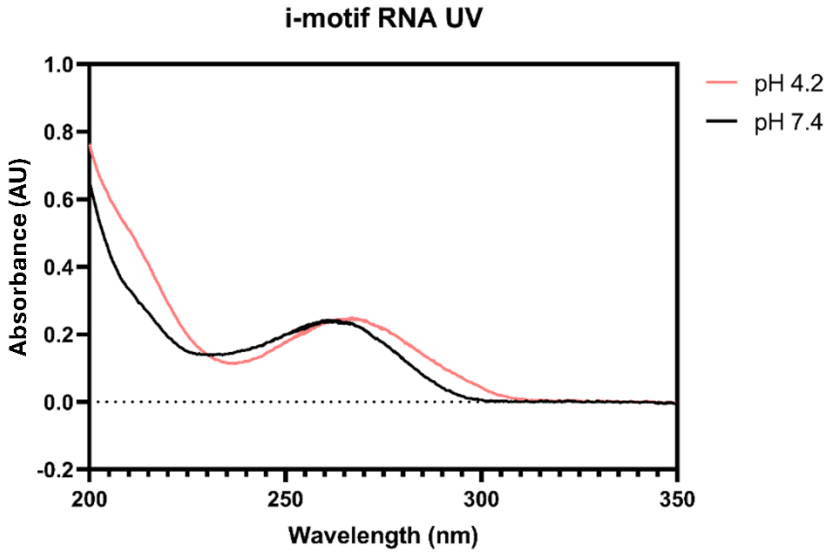
Code	**Sequence (5'-3')**
i-Motif hTeloC RNA	CCCUAACCCUAACCCUAACCC
i-Motif hTeloC DNA	CCCTAACCCTAACCCTAACCC

3. Oligonucleotide Characterization

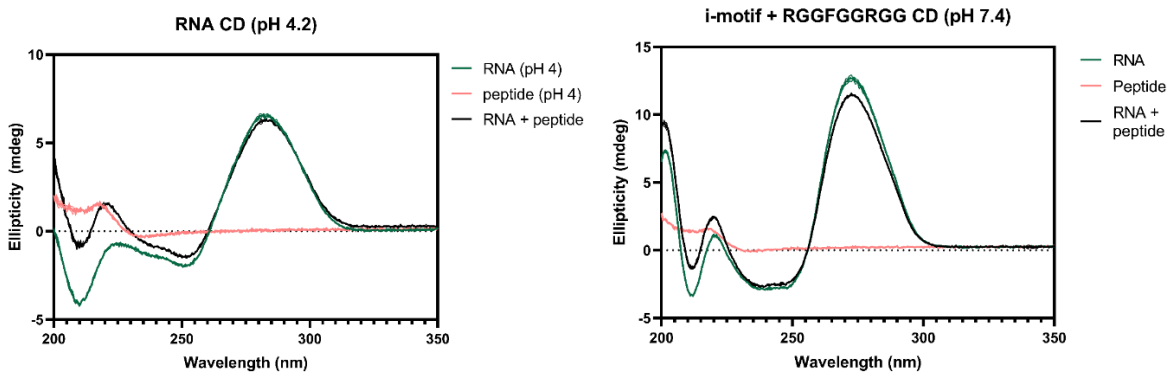
3.1 hTeloC RNA Characterization



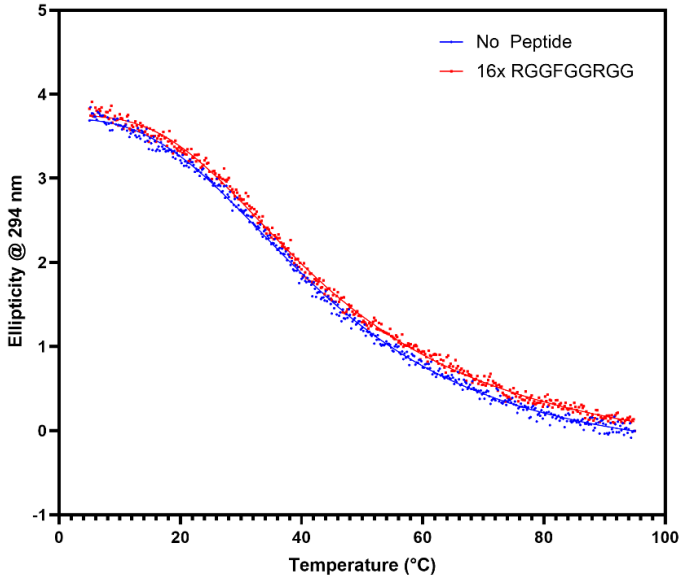
Supplementary Figure 1*: Analytical HPLC spectrum of 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) RNA.*



Supplementary Figure 2: *Ultraviolet-visible spectrum of 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) RNA, showing the difference between the hemi-protonated folded structure (pH 4.2) and single stranded RNA (pH 7.4).*

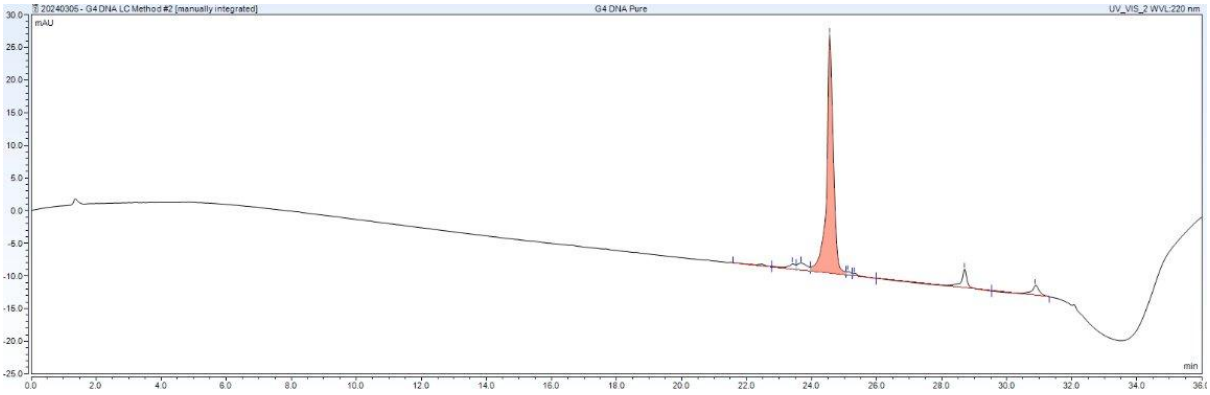


Supplementary Figure 3: *Circular Dichroism spectra of 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) RNA (20 μM) with and without 16 equivalents of the* ***RGGFGGRGG*** *peptide at pH 4.2 (left) and 7.4 (right).*

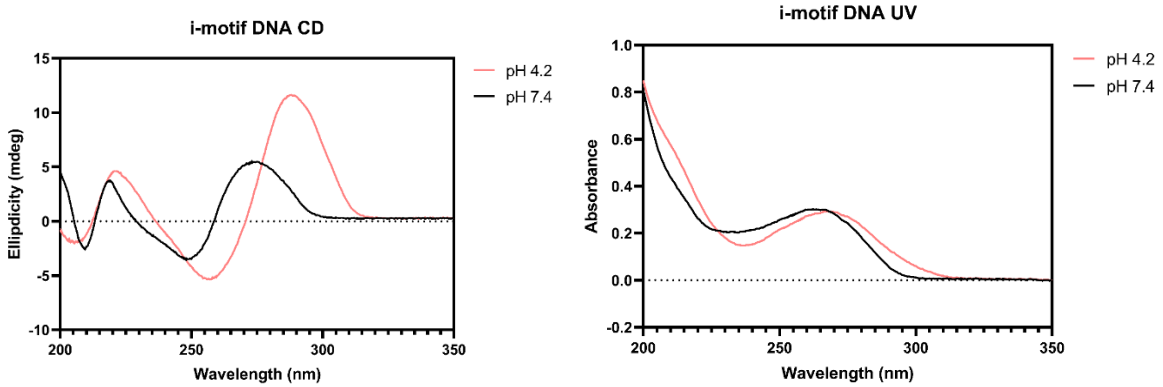


Supplementary Figure 4: *CD melting curve of the i-motif RNA (20 μM) 5'-CCC UAA CCC UAA CCC UAA CCC-3' in the absence and presence of the* ***RGGFGGRGG*** *peptide (320 μM) at pH 4.0 (sodium acetate, 50 mM, sodium chloride, 50 mM). Peptide does not statistically alter the melting point of the i-motif structure at pH 4.0.*

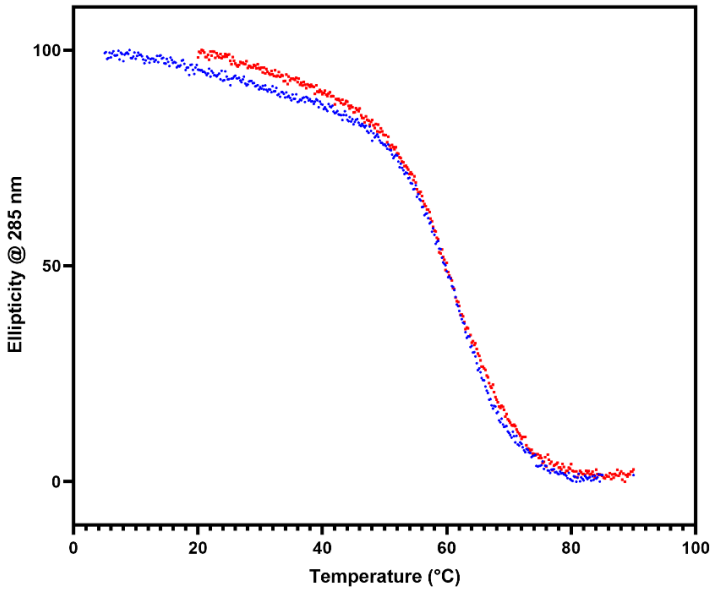
3.2 hTeloC DNA Characterization



Supplementary Figure 5*: Analytical HPLC spectrum for 5'-TAA CCC TAA CCC TAA CCC-3' (i-Motif) DNA.*



Supplementary Figure 6: *Circular Dichroism (left) and Ultraviolet-visible (right) spectra of 5'-CCC TAA CCC TAA CCC TAA CCC-3' (i-Motif) DNA, showing the difference between the hemi-protonated folded structure (pH 4.2) and single stranded DNA (pH 7.4).*



Supplementary Figure 7: *CD melting curve of the i-motif DNA (20 μM) 5'-CCC TAA CCC TAA CCC TAA CCC-3' in the absence and presence of the* ***RGGFGGRGG*** *peptide (320 μM) at pH 4.0 (sodium acetate, 50 mM, sodium chloride, 50 mM). Peptide does not statistically alter the melting point of the i-motif structure at pH 4.0.*

4. Binding model statistics

Raw NMR titration data is available on FigShare: 10.6084/m9.figshare.30881207

Supplementary Table 2: *Association constants of folded and unfolded hTeloC i-motif RNA and DNA, derived from NMR titrations, fit to a range of 1:1 and 2:1 (peptide:oligonucleotide) fitting models as explained in Section 1.9 above. K_1 = association constant for first binding event (M^{-1}). K_2 = association constant for second binding event. BIC = Bayesian Information Criterion, a statistic used for fitting model selection. Lower (more negative) numbers are indicative of a more successful fitting model. Shown in bold is the statistical 2:1 model, which is the best fitting model for the majority of titrations and for consistency, all the data in our work is then compared using this model.*

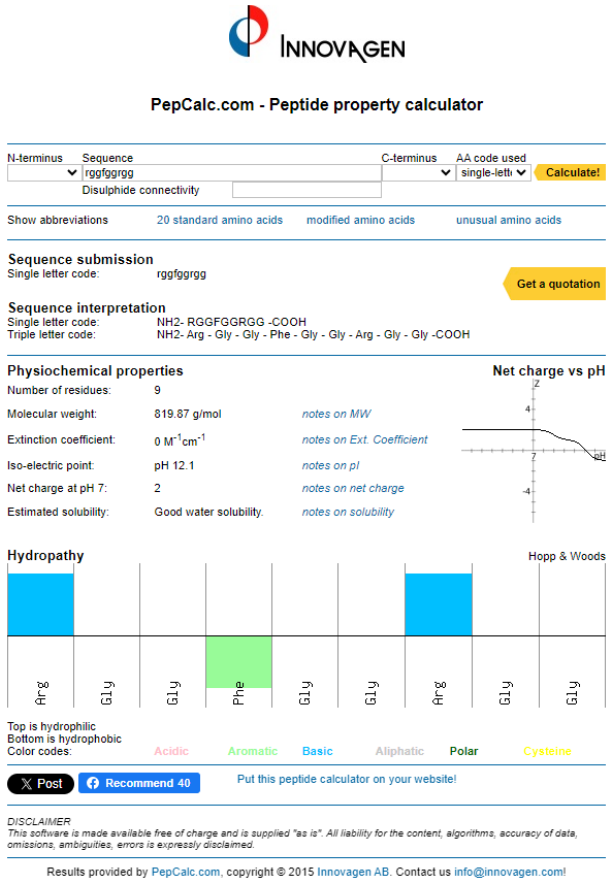
	RNA			***DNA***		
Binding Model	K_1	K_2	BIC	K_1	K_2	BIC
i-Motif (Folded)						
1:1	2.22×10^6	n/a	-366	1.04×10^5	n/a	-358
Full 2:1	1.36×10^1	5.61×10^7	-377	9.87×10^0	1.13×10^7	-337
Additive 2:1	5.11×10^4	4.41×10^4	-438	1.05×10^2	1.37×10^6	-406
Non-Coop 2:1	6.69×10^4	1.67×10^4	-433	1.76×10^4	4.39×10^3	-386
Statistical 2:1	**9.25×10^4**	**2.31×10^4**	**-441**	**1.63×10^4**	**4.09×10^3**	**-396**
i-Motif (Unfolded)						
1:1	1.35×10^4	n/a	-442	4.81×10^3	n/a	-261
Full 2:1	5.28×10^0	1.00×10^7	-387	7.28×10^0	5.68×10^5	-212
Additive 2:1	2.96×10^1	8.39×10^5	-452	1.87×10^3	2.01×10^3	-259
Non-Coop 2:1	5.88×10^3	1.47×10^3	-443	1.61×10^3	4.02×10^2	-256
Statistical 2:1	**6.05×10^3**	**1.51×10^3**	**-450**	**3.00×10^3**	**7.49×10^2**	**-262**

Supplementary Table 3: *Association constants derived from NMR titrations, fit to a 2:1 (peptide:oligonucleotide) statistical fitting model, including free energy of the interaction.*

	RNA			***DNA***		
Oligonucleotide Species	K_1	K_2	Free Energy (kJ/mol)	K_1	K_2	Free Energy (kJ/mol)
i-Motif (Folded)	9.3×10^4	2.3×10^4	-26.6	1.6×10^4	4.1×10^3	-22.3
i-Motif (Unfolded)	6.1×10^3	1.5×10^3	-19.9	3.0×10^3	7.5×10^2	-18.1

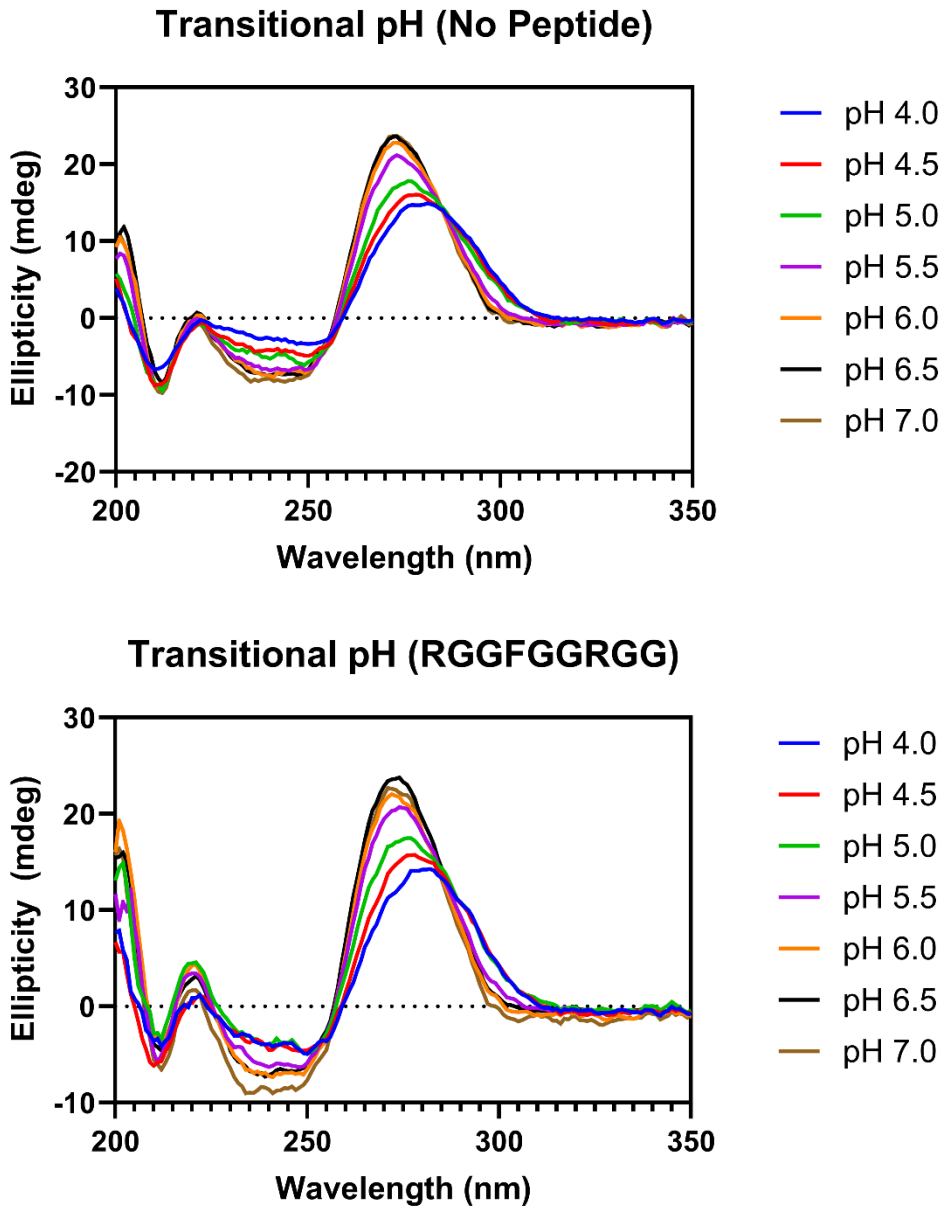
Supplementary Table 4: *Association constants of the hTeloC i-Motif RNA across a pH gradient, derived from NMR titrations, fit to a range of 1:1 and 2:1 (peptide:oligo) fitting models. Reported: K_1 = association constant for first binding event (M^{-1}). Shown in bold is the statistical 2:1 model, which is the best fitting model for the majority of titrations and for consistency, all the data in our work is then compared using this model.*

	pH 4.0	*pH 4.5*	*pH 5.0*	*pH 5.5*	*pH 6.0*	*pH 6.5*	*pH 7.0*	*pH 9.0*
i-Motif RNA								
1:1	2.2×10^{6}	2.5×10^{6}	2.2×10^{6}	4.6×10^{5}	3.0×10^{5}	4.6×10^{4}	1.4×10^{4}	1.9×10^{3}
Full 2:1	1.4×10^{1}	1.6×10^{1}	4.5×10^{4}	9.2×10^{2}	5.1×10^{0}	5.2×10^{4}	5.3×10^{0}	5.6×10^{3}
Additive 2:1	5.1×10^{4}	3.0×10^{4}	4.2×10^{4}	1.1×10^{4}	5.5×10^{1}	4.2×10^{5}	3.0×10^{1}	4.8×10^{3}
Non-Coop 2:1	6.7×10^{4}	6.4×10^{4}	4.9×10^{4}	2.7×10^{4}	3.1×10^{4}	7.7×10^{1}	5.9×10^{3}	5.7×10^{2}
Statistical 2:1	$\mathbf{9.3 \times 10^{4}}$	$\mathbf{7.9 \times 10^{4}}$	$\mathbf{6.3 \times 10^{4}}$	$\mathbf{4.0 \times 10^{4}}$	$\mathbf{2.6 \times 10^{4}}$	$\mathbf{1.2 \times 10^{4}}$	$\mathbf{6.1 \times 10^{3}}$	$\mathbf{5.7 \times 10^{2}}$



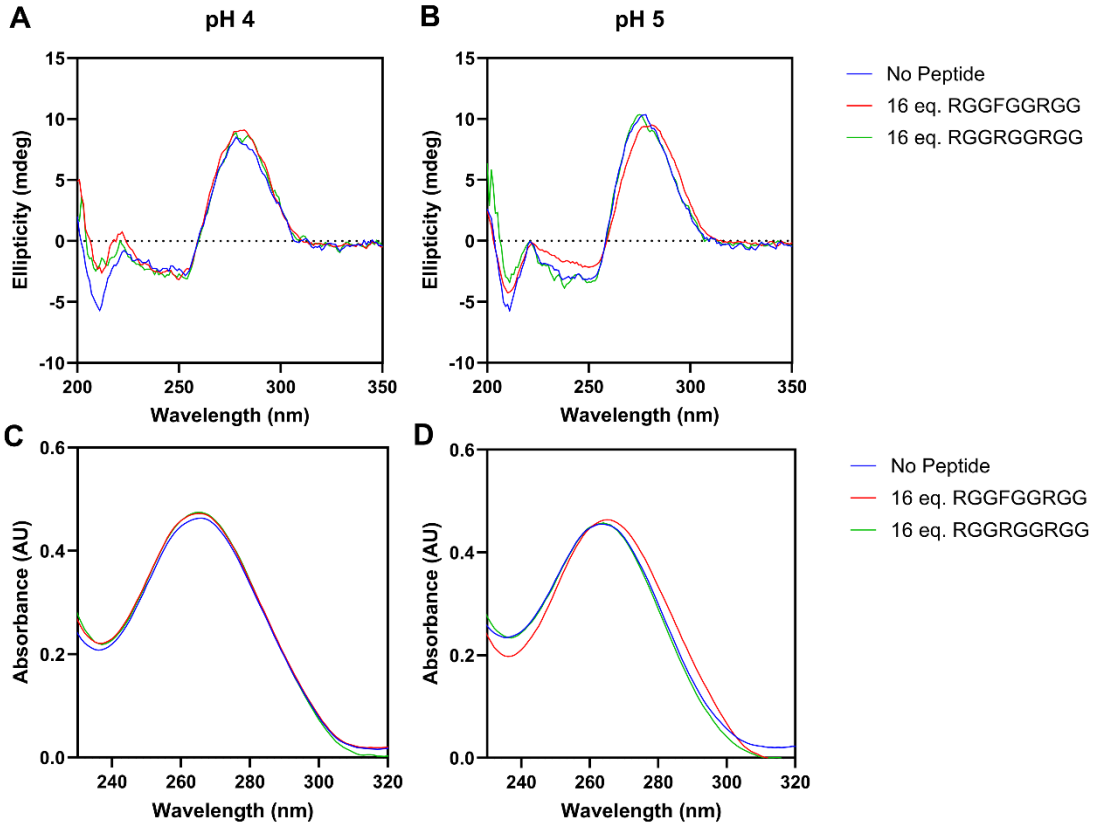
Supplementary Figure 8: *Calculated isoelectric point of the* ***RGGFGGRGG*** *peptide using Innovagen pepcalc.com. Based on this calculation the* ***RGGFGGRGG*** *peptide should remain at 2+ charge across the tested pH range.*

5. i-Motif Transitional pH

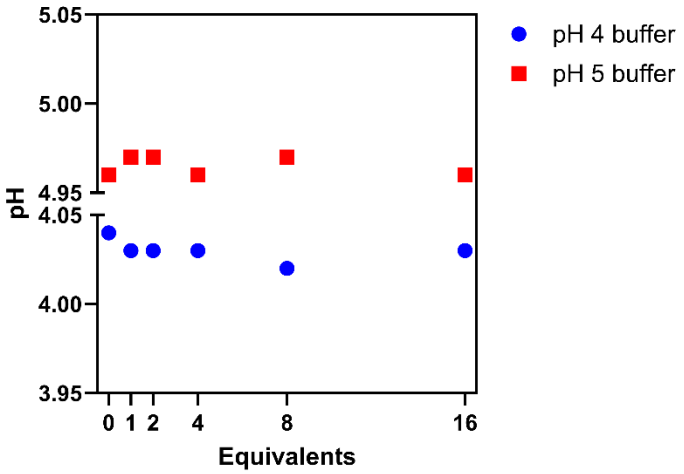


Supplementary Figure 9: *Circular Dichroism spectra of 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) RNA (20 μM) across a pH gradient, in the absence (top) and presence (bottom) of the* ***RGGFGGRGG*** *peptide (320 μM). Our peptide alters the transitional pH of the RNA i-motif structure.*

6. Negative Controls



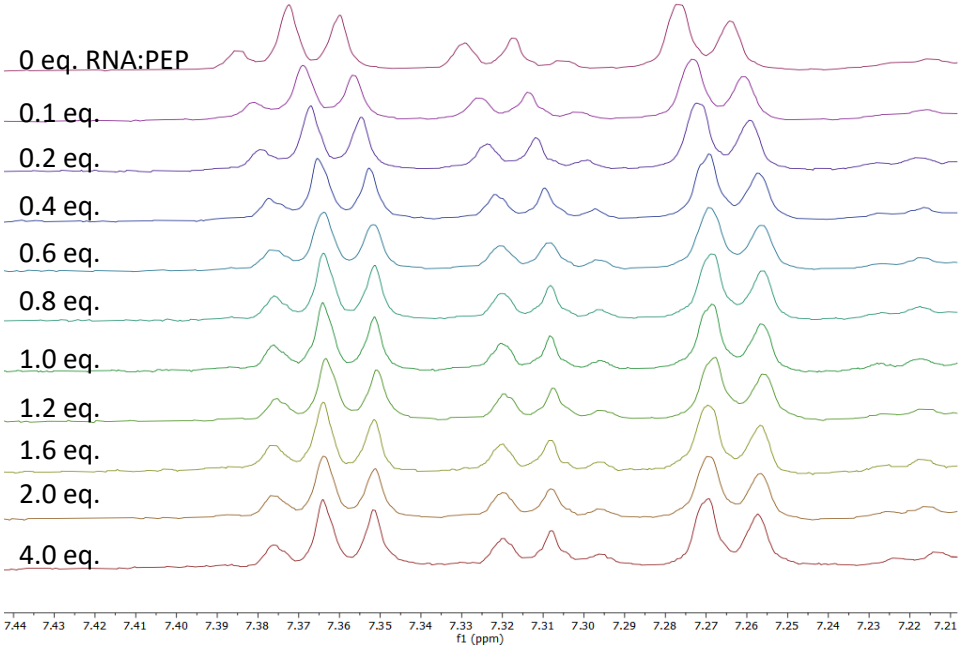
Supplementary Figure 10: *Circular dichroism spectra of 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) RNA at pH (left): 4 and (right): 5 in the absence and presence of 16 equivalents of the* ***RGGFGGRGG*** *peptide and a mutated sequence* ***RGGRGGRGG****. (A,C): At pH 4.0, the i-motif remains folded, with neither peptide inducing any structural change. (B, D): At pH 5, the i-motif structure begins to denature in the absence of peptide, or with 16 equivalents of the negative control* ***RGGRGGRGG****. (C): Conversely, additions of* ***RGGFGGRGG*** *induce a shift in ellipticity towards 294 nm, indicative of an increase in folded i-motif structure. (D): Additionally, a bathochromic shift is observed in a UV-Vis spectrum, indicating increased base-stacking, suggesting formation of the RNA i-motif.*



Supplementary Figure 11: *pH measurements of buffer systems at pH 4 and pH 5 containing 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) RNA (20 μM) upon titration of the* ***RGGFGGRGG*** *peptide. No observable change in pH was detected.*

7. Supplemental NMR titration data

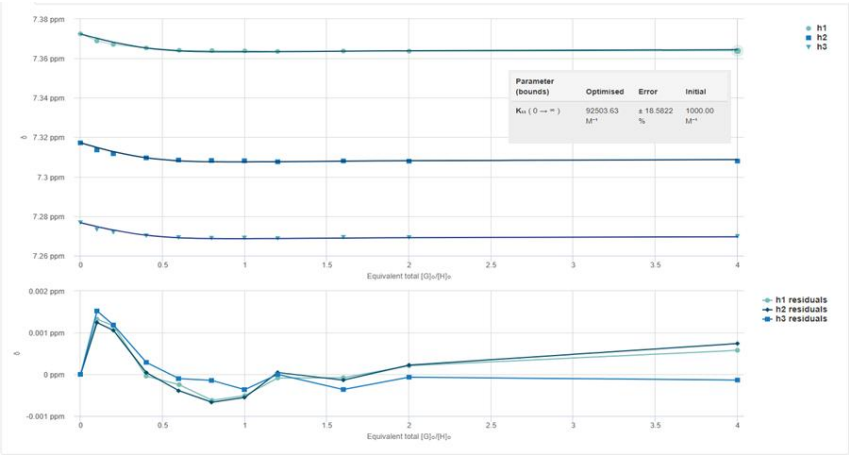
7.1 i-Motif RNA+ RGGFGGRGG (pH 4.0)



Supplementary Figure 12: *^{1}H NMR titration (600 MHz, 25 °C, 90% H_2O, 10% D_2O) of 5'-(CCCUAA)$_3$CCC-5' (i-Motif) RNA into a solution of **RGGFGGRGG** peptide (125 μM) at pH 4.0. Shown are phenylalanine aromatic shifts. Equivalents of RNA are labelled on spectra.*

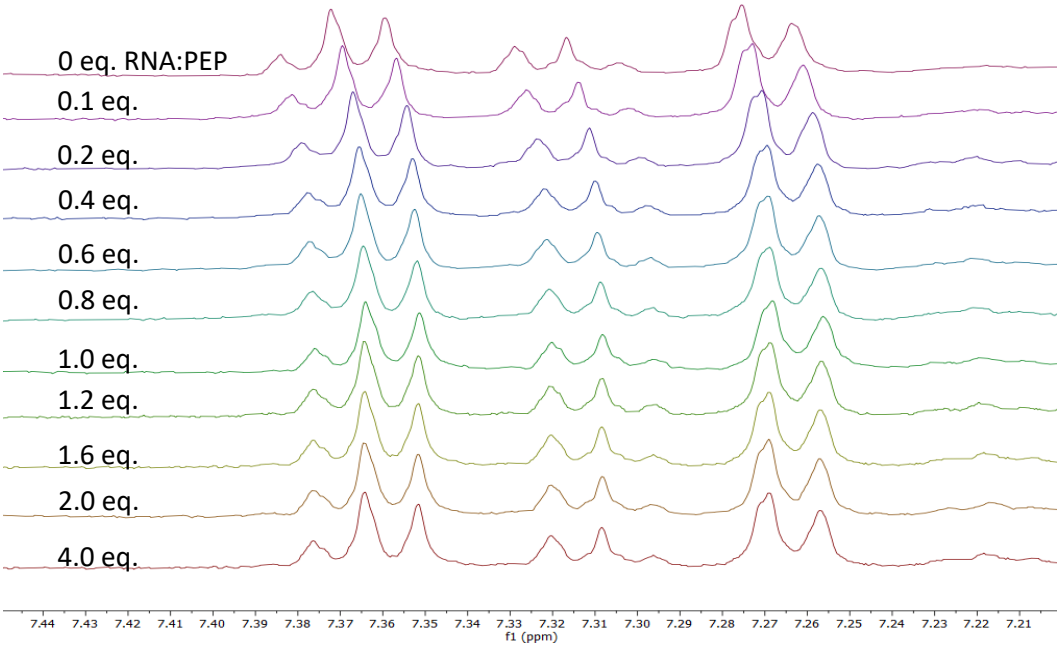
Supramolecular.org (Bindfit) permanent url hyperlinks to raw data and results

2:1 Model – full	http://app.supramolecular.org/bindfit/view/69831f8a-c9ca-4087-a0b8-58e9a6f1ba65
2:1 Model – additive	http://app.supramolecular.org/bindfit/view/4f12c61e-6ab7-4d19-9d67-2759f904d03f
2:1 Model – non cooperative	http://app.supramolecular.org/bindfit/view/b13c2c9b-55e5-493e-9963-fa4fcc97194e
2:1 Model – statistical	http://app.supramolecular.org/bindfit/view/867cc05e-6b45-421b-aed4-21fe1853200a
1:1 Model	http://app.supramolecular.org/bindfit/view/76e0e121-5848-4522-ad42-007028ba5f9c



Supplementary Figure 13: *Binding isotherm (2:1 stoichiometry) of 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) RNA (pH 4.0) fitted to the chemical shift of the aromatic phenylalanine of the **RGGFGGRGG** peptide. (Top): K_1 = 92504 M^{-1}. (Bottom): Residual plot from the fit.*

7.2 i-Motif RNA + RGGFGGRGG (pH 4.5)



Supplementary Figure 14: *^{1}H NMR titration (600 MHz, 25 °C, 90% H_2O, 10% D_2O) of 5'-$(CCCUAA)_3$CCC-5' (i-Motif) RNA into a solution of* ***RGGFGGRGG*** *peptide (125 μM) at pH 4.5. Shown are phenylalanine aromatic shifts. Equivalents of RNA are labelled on spectra.*

Supramolecular.org (Bindfit) permanent url hyperlinks to raw data and results

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2:1 Model – additive	http://app.supramolecular.org/bindfit/view/235d4daf-c1d5-4514-b501-85cc1a6a6ee3
2:1 Model – non cooperative	http://app.supramolecular.org/bindfit/view/1ec98134-d81f-4305-812b-da7b035c0c7c
2:1 Model – statistical	http://app.supramolecular.org/bindfit/view/3989f965-9fa8-4870-92fd-dee052685681
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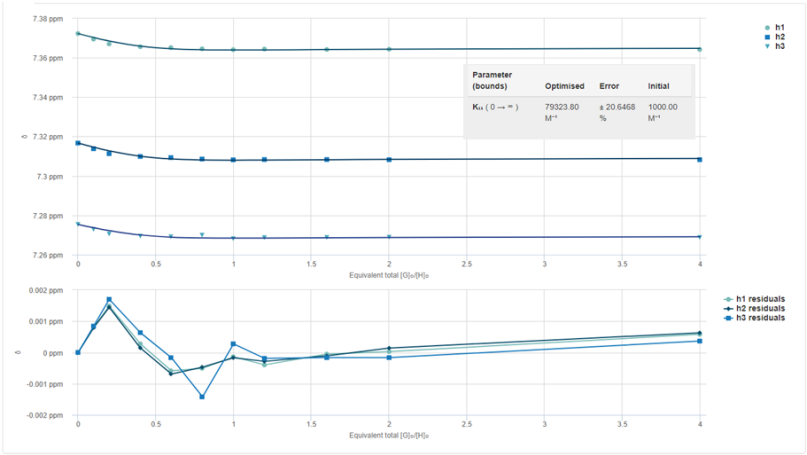
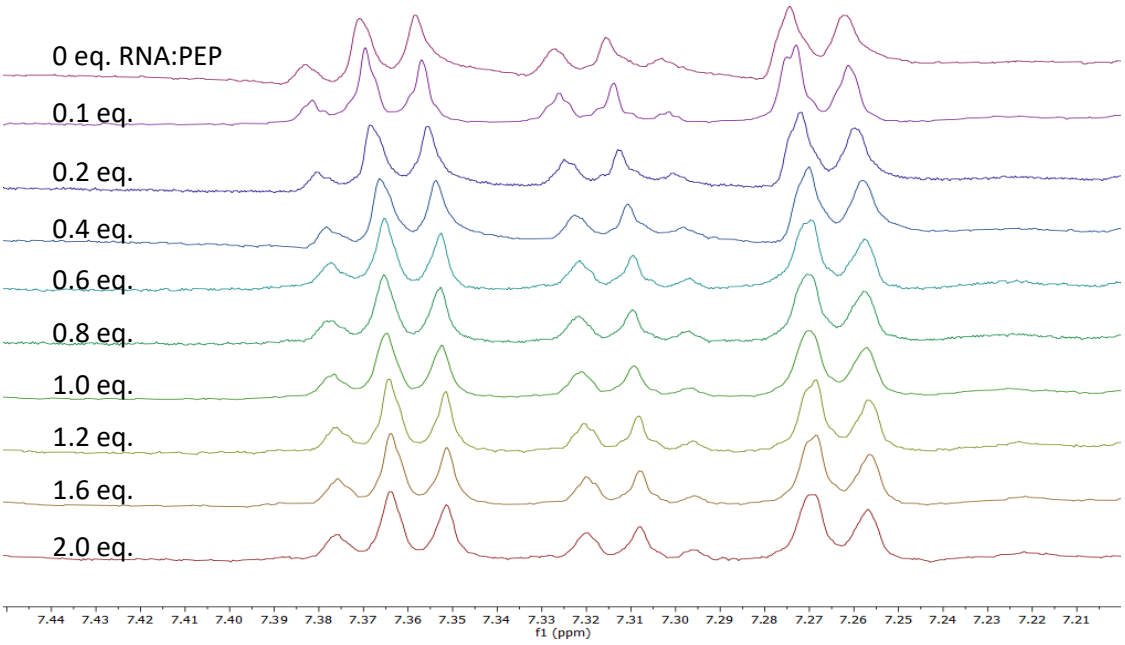


Supplementary Figure 15: *Binding isotherm (2:1 stoichiometry) of 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) (pH 4.5) fitted to the chemical shift of the aromatic phenylalanine of the* ***RGGFGGRGG*** *peptide. (Top): K_1 = 79324 M^{-1}. (Bottom): Residual plot from the fit.*

7.3 i-Motif RNA + RGGFGGRGG (pH 5.0)



Supplementary Figure 16: *^{1}H NMR titration (600 MHz, 25 °C, 90% H_2O, 10% D_2O) of 5'-(CCCUAA)$_3$CCC-5' (i-Motif) RNA into a solution of* ***RGGFGGRGG*** *peptide (125 μM) at pH 5.0. Shown are phenylalanine aromatic shifts. Equivalents of RNA are labelled on spectra.*

Supramolecular.org (Bindfit) permanent url hyperlinks to raw data and results

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2:1 Model – additive	https://app.supramolecular.org/bindfit/view/33520c43-8b48-4977-998c-e7bb00e8f0f9
2:1 Model – non cooperative	https://app.supramolecular.org/bindfit/view/ec175578-43bc-4f42-88c3-878607fcf624
2:1 Model – statistical	https://app.supramolecular.org/bindfit/view/7a131e95-2687-4fc2-a7e8-43a7084e51ee
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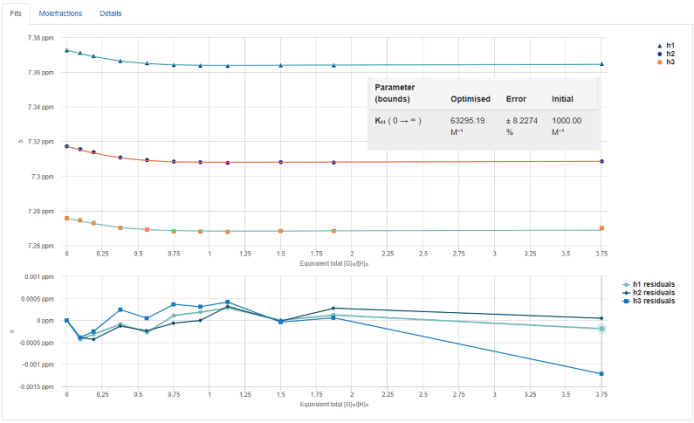
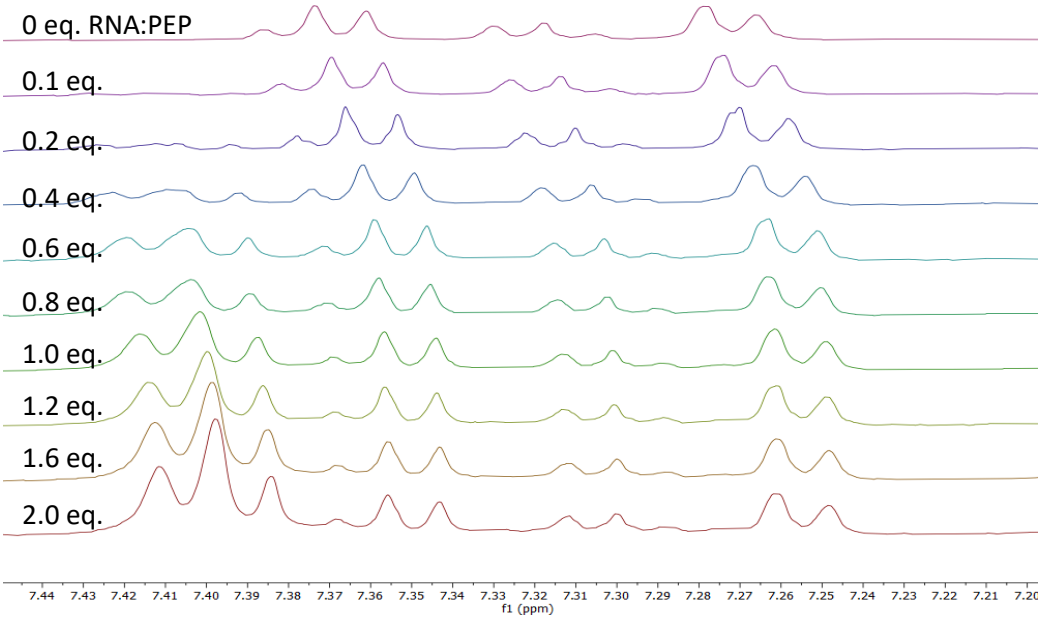


Supplementary Figure 17: *Binding isotherm (2:1 stoichiometry) of 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) (pH 5.0) fitted to the chemical shift of the aromatic phenylalanine of the* ***RGGFGGRGG*** *peptide. (Top): K_1 = 63295.19 M^{-1}. (Bottom): Residual plot from the fit.*

7.4 i-Motif RNA + RGGFGGRGG (pH 5.5)



Supplementary Figure 18: *^{1}H NMR titration (600 MHz, 25 °C, 90% H_2O, 10% D_2O) of 5'-(CCCUAA)$_3$CCC-5' (i-Motif) RNA into a solution of* ***RGGFGGRGG*** *peptide (125 μM) at pH 5.5. Shown are phenylalanine aromatic shifts. Equivalents of RNA are labelled on spectra.*

Supramolecular.org (Bindfit) permanent url hyperlinks to raw data and results

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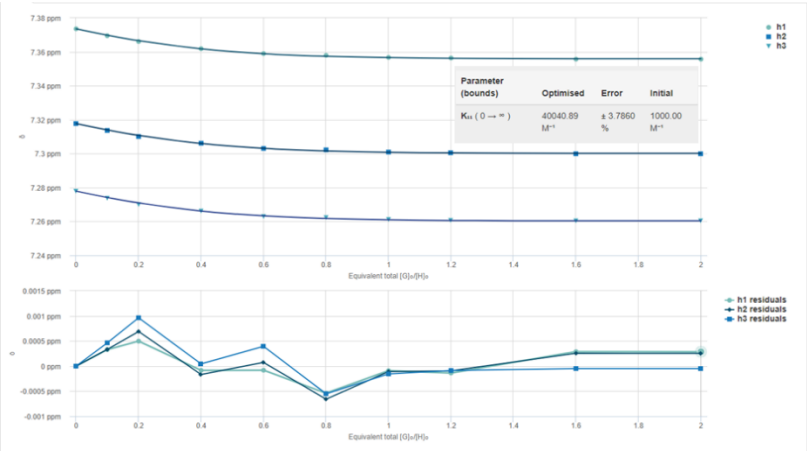
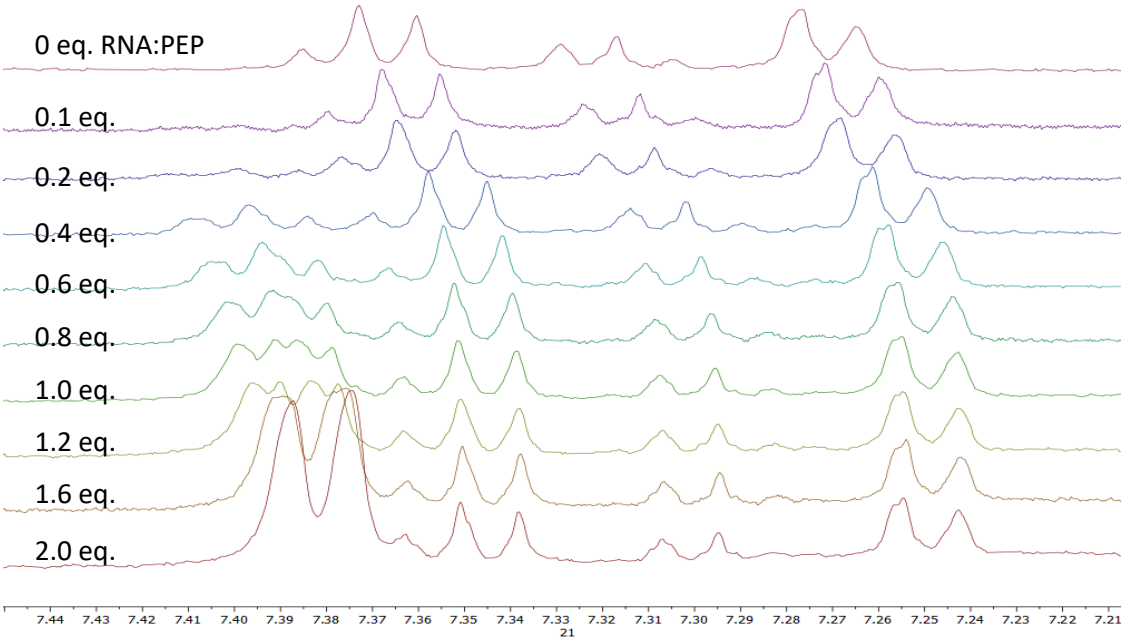
2:1 Model – additive	http://app.supramolecular.org/bindfit/view/10d46130-8699-44ce-8772-b610ecc91cff
2:1 Model – non cooperative	http://app.supramolecular.org/bindfit/view/1c500861-d167-4328-8814-c92322ebf4c9
2:1 Model – statistical	http://app.supramolecular.org/bindfit/view/697dd038-033d-43ab-affd-33426c13e5ad
1:1 Model	http://app.supramolecular.org/bindfit/view/b5fc1da2-f795-476b-bcf6-1d6862f63976



Supplementary Figure 19: *Binding isotherm (2:1 stoichiometry) of the 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) (pH 5.5) fitted to the chemical shift of the aromatic phenylalanine of the* ***RGGFGGRGG*** *peptide. (Top): K_1 = 40041 M^{-1}. (Bottom): Residual plot from the fit.*

7.5 i-Motif RNA + RGGFGGRGG (pH 6.0)



Supplementary Figure 20: *^{1}H NMR titration (600 MHz, 25 °C, 90% H_2O, 10% D_2O) of 5'-(CCCUAA)$_3$CCC-5' (i-Motif) RNA into a solution of* ***RGGFGGRGG*** *peptide (125 μM) at pH 6.0. Shown are phenylalanine aromatic shifts. Equivalents of RNA are labelled on spectra.*

Supramolecular.org (Bindfit) permanent url hyperlinks to raw data and results

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2:1 Model – statistical	https://app.supramolecular.org/bindfit/view/dccbb615-f14d-4166-a896-7052d234a484
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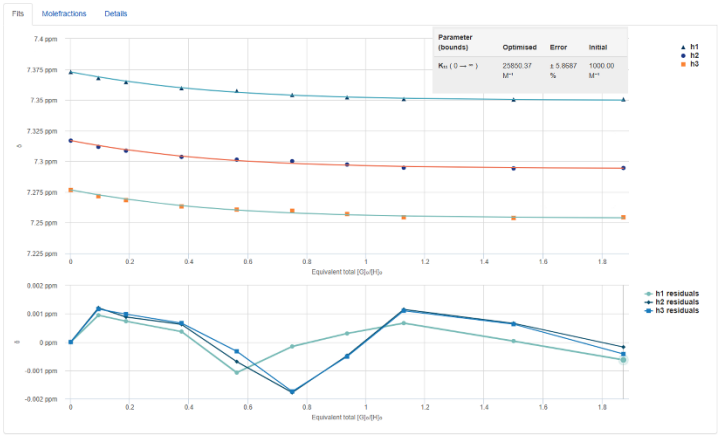
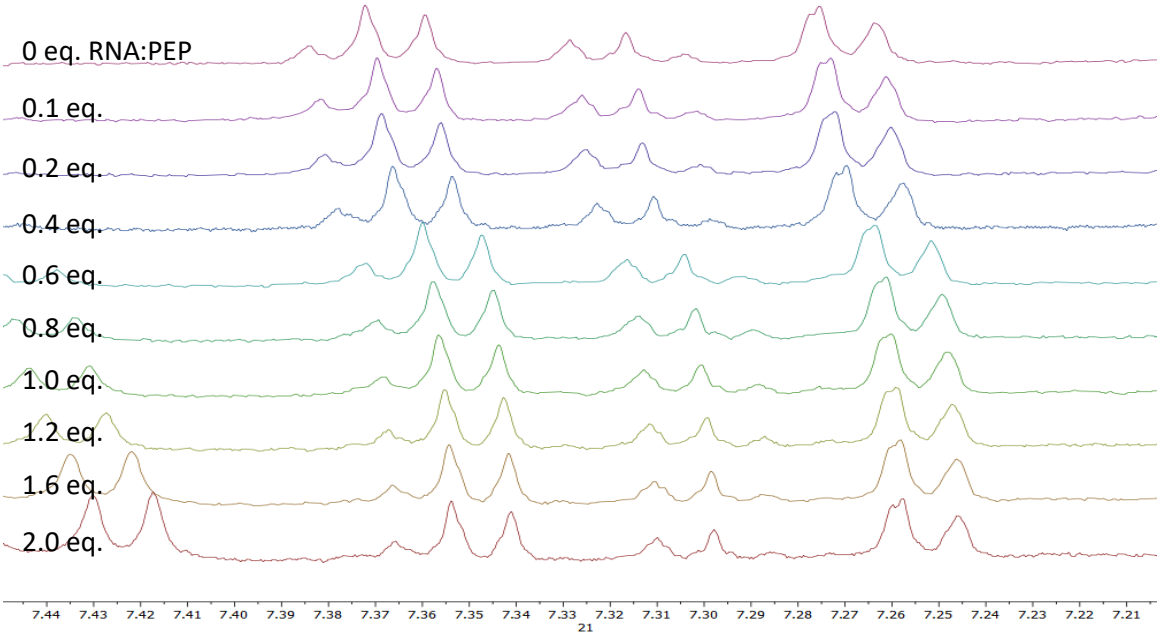


Supplementary Figure 21: *Binding isotherm (2:1 stoichiometry) of 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) RNA (pH 6.0) fitted to the chemical shift of the aromatic phenylalanine of the* ***RGGFGGRGG*** *peptide. (Top): K_1 = 25850.37 M^{-1}. (Bottom): Residual plot from the fit.*

7.6 i-Motif RNA + RGGFGGRGG (pH 6.5)



Supplementary Figure 22: *^{1}H NMR titration (600 MHz, 25 °C, 90% H_2O, 10% D_2O) of 5'-(CCCUAA)$_3$CCC-5' (i-Motif) RNA into a solution of* ***RGGFGGRGG*** *peptide (125 μM) at pH 6.5. Shown are phenylalanine aromatic shifts. Equivalents of RNA are labelled on spectra.*

Supramolecular.org (Bindfit) permanent url hyperlinks to raw data and results

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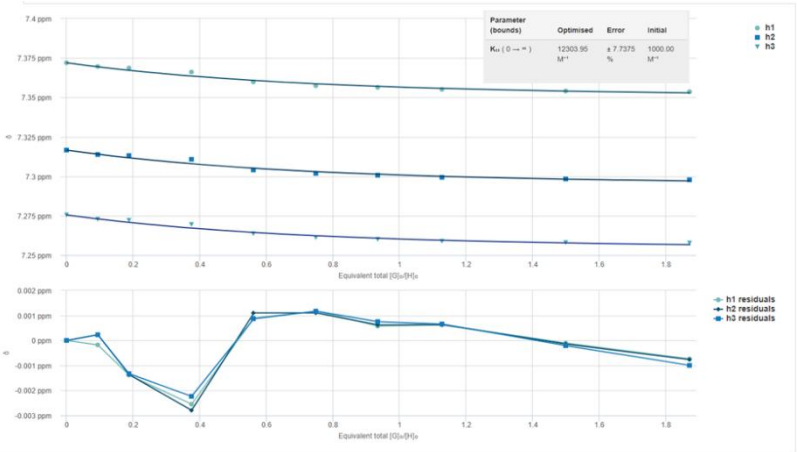
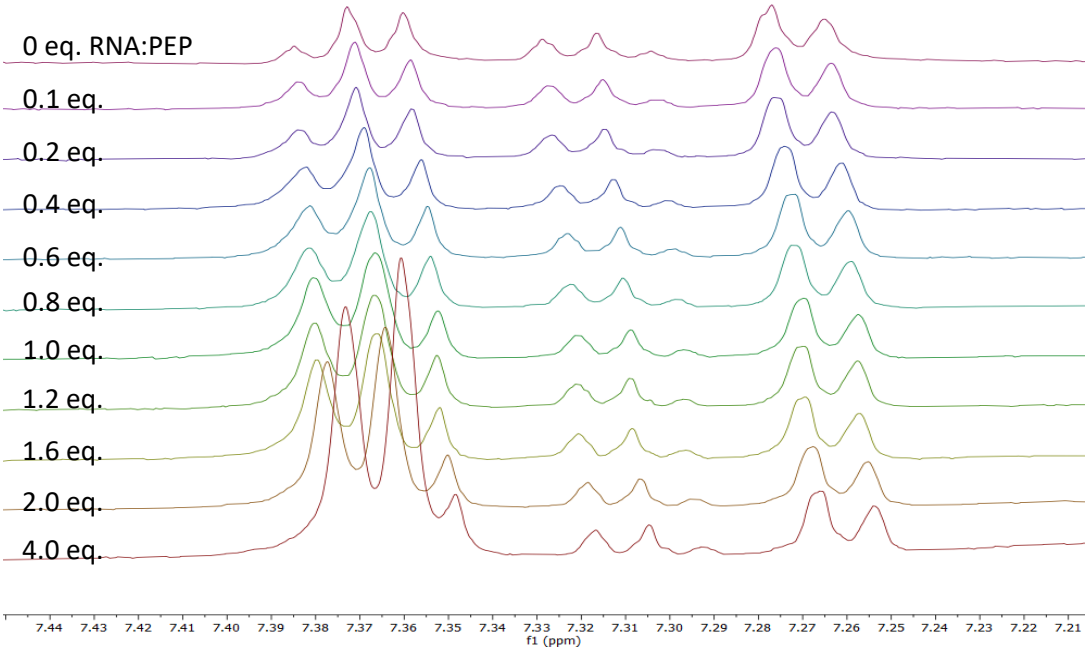


Supplementary Figure 23: *Binding isotherm (2:1 stoichiometry) of 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) RNA (pH 6.5) fitted to the chemical shift of the aromatic phenylalanine of the* ***RGGFGGRGG*** *peptide. (Top): K_1 = 12304 M^{-1}. (Bottom): Residual plot from the fit.*

7.7 i-Motif RNA + RGGFGGRGG (pH 7.0)



Supplementary Figure 24: *^{1}H NMR titration (600 MHz, 25 °C, 90% H_2O, 10% D_2O) of 5'-(CCCUAA)$_3$CCC-5' (i-Motif) RNA into a solution of* ***RGGFGGRGG*** *peptide (125 μM) at pH 7.0. Shown are phenylalanine aromatic shifts. Equivalents of RNA are labelled on spectra.*

Supramolecular.org (Bindfit) permanent url hyperlinks to raw data and results

2:1 Model – full	http://app.supramolecular.org/bindfit/view/80b6f807-8f15-4eee-a396-b8d08c64fd5e
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2:1 Model – non cooperative	http://app.supramolecular.org/bindfit/view/e96cb717-37a6-4fd0-8363-334a0a5f8d58
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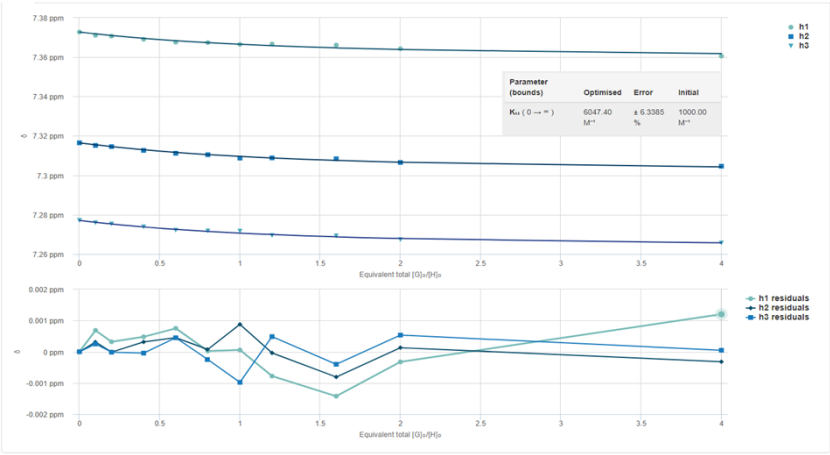
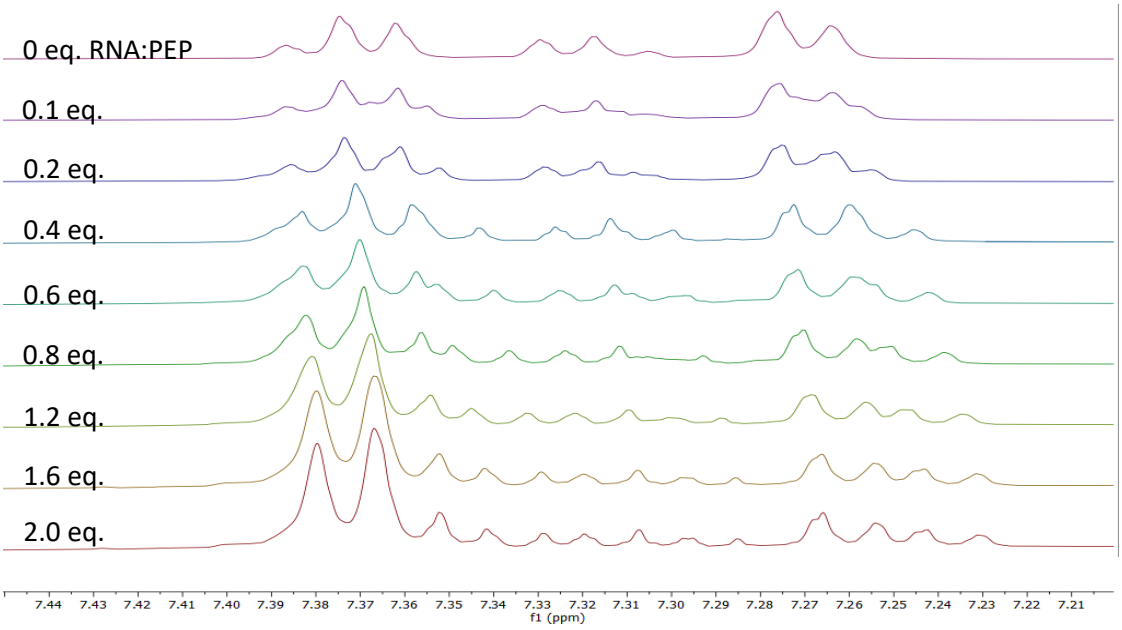


Supplementary Figure 25: *Binding isotherm (2:1 stoichiometry) of 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) RNA (pH 7.0) fitted to the chemical shift of the aromatic phenylalanine of the* ***RGGFGGRGG*** *peptide. (Top): K_1 = 6047 M^{-1}. (Bottom): Residual plot from the fit.*

7.8 i-Motif RNA + RGGFGGRGG (pH 9.0)



Supplementary Figure 26: *^{1}H NMR titration (600 MHz, 25 °C, 90% H_2O, 10% D_2O) of 5'-$(CCCUAA)_3CCC$-5' (i-Motif) RNA into a solution of* ***RGGFGGRGG*** *peptide (125 μM) at pH 9.0. Shown are phenylalanine aromatic shifts. Equivalents of RNA are labelled on spectra.*

Supramolecular.org (Bindfit) permanent url hyperlinks to raw data and results

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2:1 Model – non cooperative	http://app.supramolecular.org/bindfit/view/1ac74a3f-36bc-41c4-849f-4e4eada1ad81
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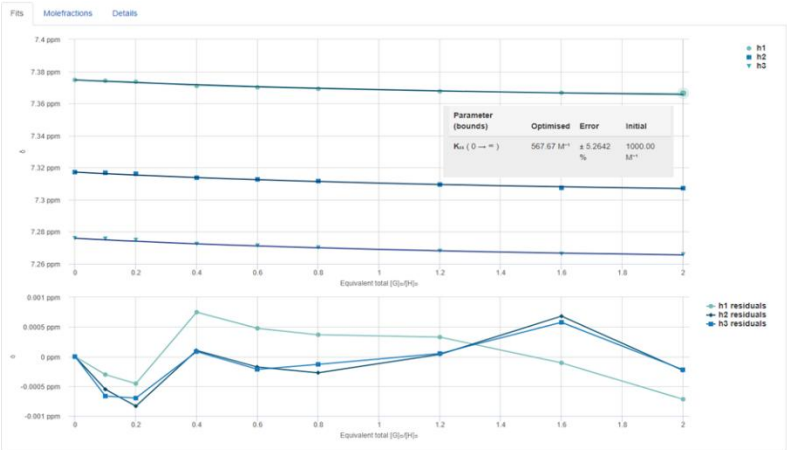
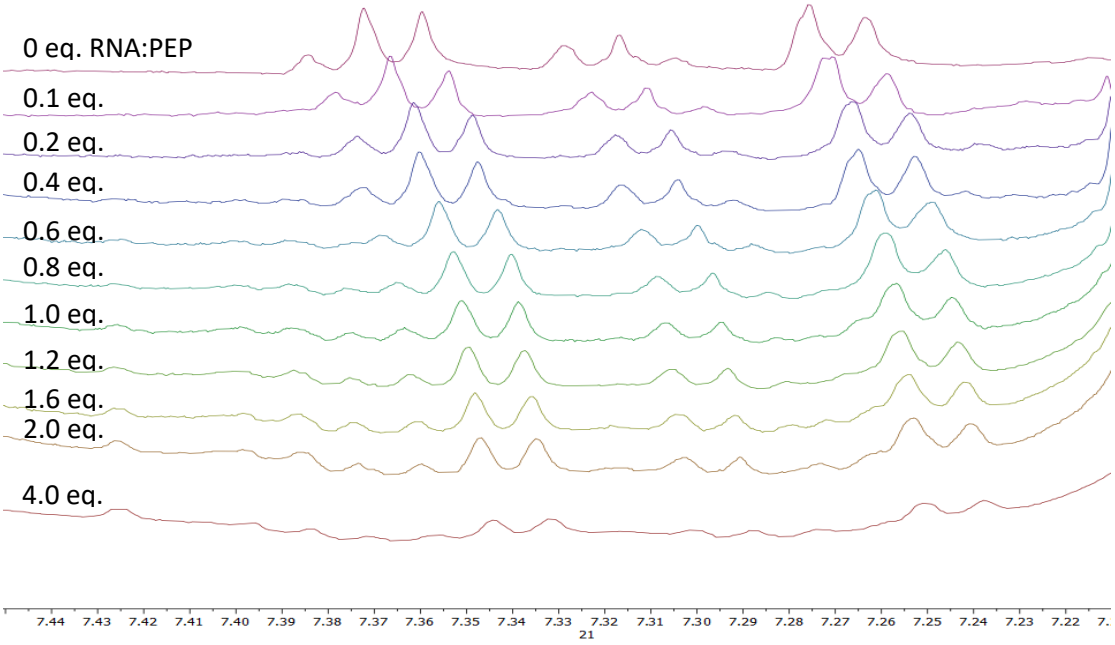


Supplementary Figure 27: *Binding isotherm (2:1 stoichiometry) of 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) RNA (pH 9.0) fitted to the chemical shift of the aromatic phenylalanine of the* ***RGGFGGRGG*** *peptide. (Top): K_1 = 568 M^{-1}. (Bottom): Residual plot from the fit.*

7.9 i-Motif DNA + RGGFGGRGG (pH 4.0)



Supplementary Figure 28: *^{1}H NMR titration (600 MHz, 25 °C, 90% H_2O, 10% D_2O) of 5'-(CCCTAA)$_3$CCC-5' (i-Motif) DNA into a solution of* ***RGGFGGRGG*** *peptide (125 μM) at pH 4.0. Shown are phenylalanine aromatic shifts. Equivalents of RNA are labelled on spectra.*

Supramolecular.org (Bindfit) permanent url hyperlinks to raw data and results

2:1 Model – full	http://app.supramolecular.org/bindfit/view/08468c4d-0d95-4615-82ea-75bdd6d0d53f
2:1 Model – additive	http://app.supramolecular.org/bindfit/view/689f8544-2464-4f75-a297-170e8e8f3f6f
2:1 Model – non cooperative	http://app.supramolecular.org/bindfit/view/e9c3a52e-949b-4595-adb2-60d6c569c1f7
2:1 Model – statistical	http://app.supramolecular.org/bindfit/view/dd8fe788-0553-46e9-bdd2-e160417a79a0
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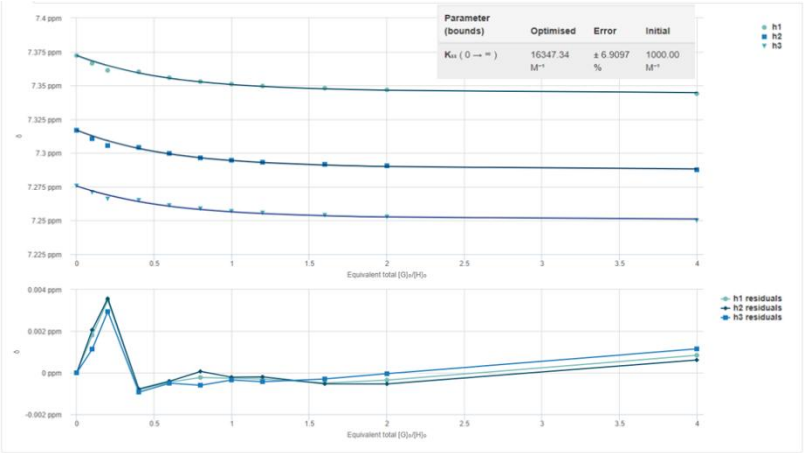
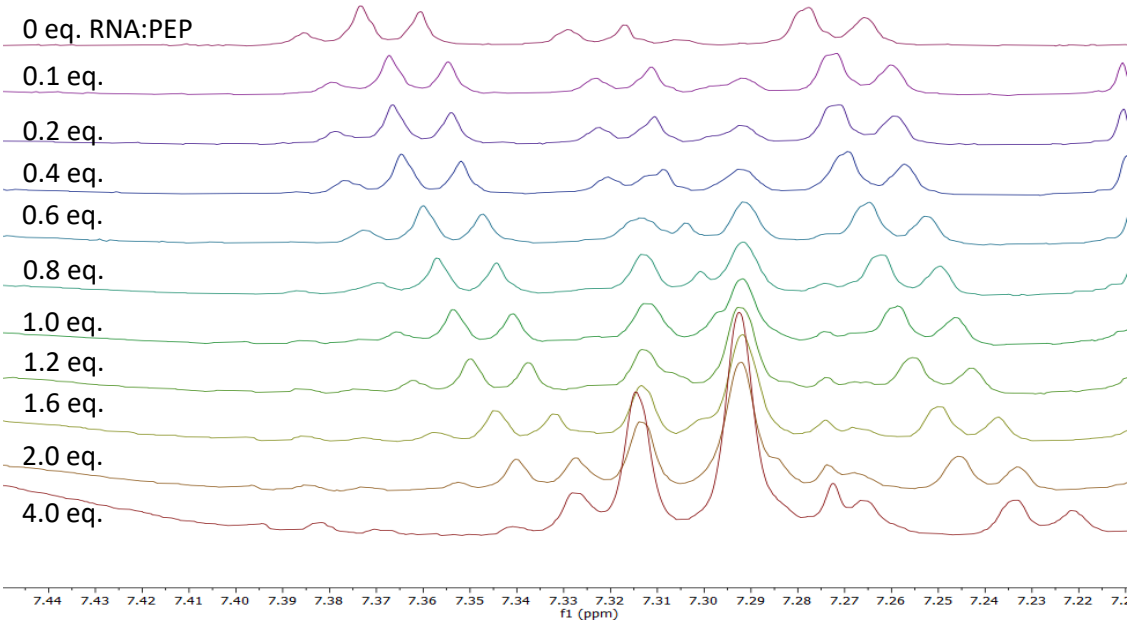


Supplementary Figure 29: *Binding isotherm (2:1 stoichiometry) of 5'-CCC TAA CCC TAA CCC TAA CCC-3' (i-Motif) DNA (pH 4.0) fitted to the chemical shift of the aromatic phenylalanine of the* ***RGGFGGRGG*** *peptide. (Top): K_1 = 16347 M^{-1}. (Bottom): Residual plot from the fit.*

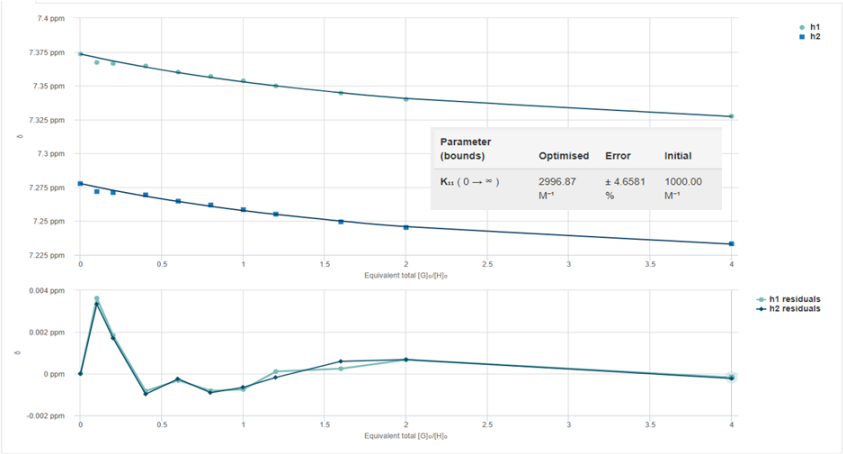
7.10 i-Motif DNA + RGGFGGRGG (pH 7.0)



Supplementary Figure 30: *^{1}H NMR titration (600 MHz, 25 °C, 90% H_2O, 10% D_2O) of 5'-(CCCTAA)$_3$CCC-5' (i-Motif) DNA into a solution of* ***RGGFGGRGG*** *peptide (125 μM) at pH 7.0. Shown are phenylalanine aromatic shifts. Equivalents of RNA are labelled on spectra.*

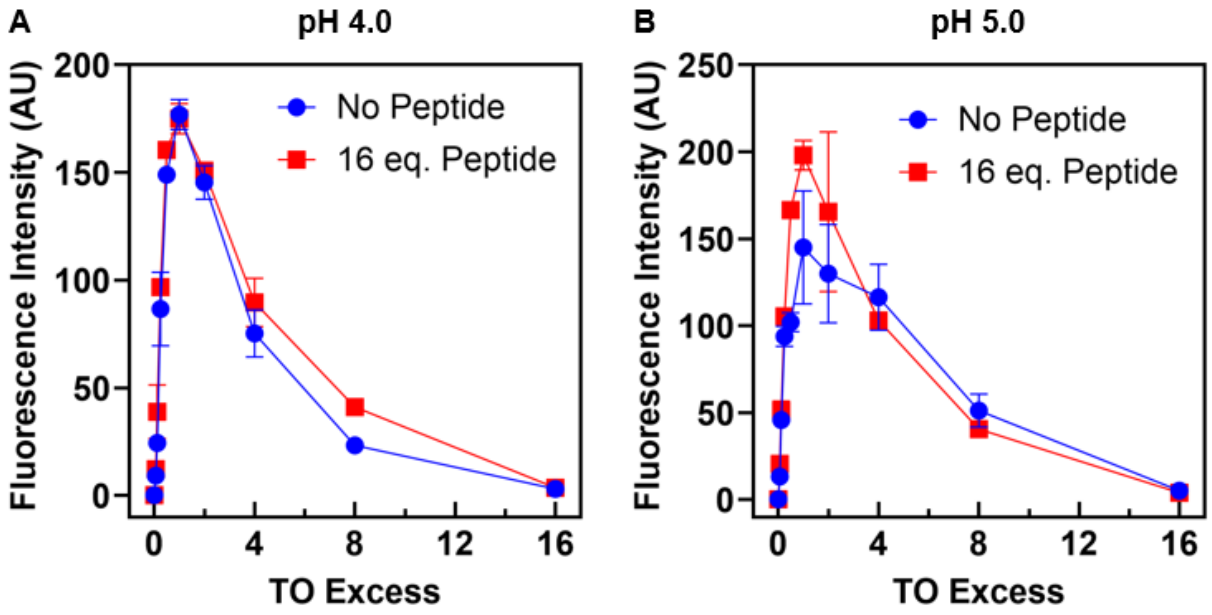
Supramolecular.org (Bindfit) permanent url hyperlinks to raw data and results

2:1 Model – full	http://app.supramolecular.org/bindfit/view/f8c4a047-f921-44ed-89aa-b80d1c96537f
2:1 Model – additive	http://app.supramolecular.org/bindfit/view/88b31725-1c34-46c1-9c03-1d16de56695a
2:1 Model – non cooperative	http://app.supramolecular.org/bindfit/view/41894f3b-5d4f-4a3b-880b-1bebcb7ced3e
2:1 Model – statistical	http://app.supramolecular.org/bindfit/view/46b851b7-168a-429c-83bb-7061d20afa9d
1:1 Model	http://app.supramolecular.org/bindfit/view/dcbc8d5c-8cf7-4033-8c44-16bca62f47ac

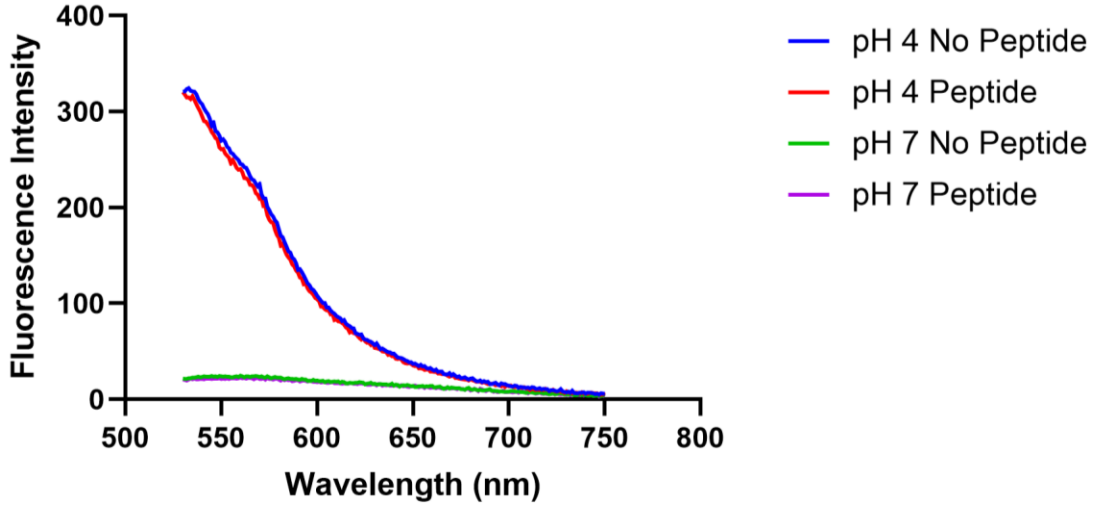


Supplementary Figure 31: *Binding isotherm (2:1 stoichiometry) of 5'-CCC TAA CCC TAA CCC TAA CCC-3' (i-Motif) DNA (pH 7.0) fitted to the chemical shift of the aromatic phenylalanine of the* ***RGGFGGRGG*** *peptide. (Top): K_1 = 2997 M^{-1}. (Bottom): Residual plot from the fit.*

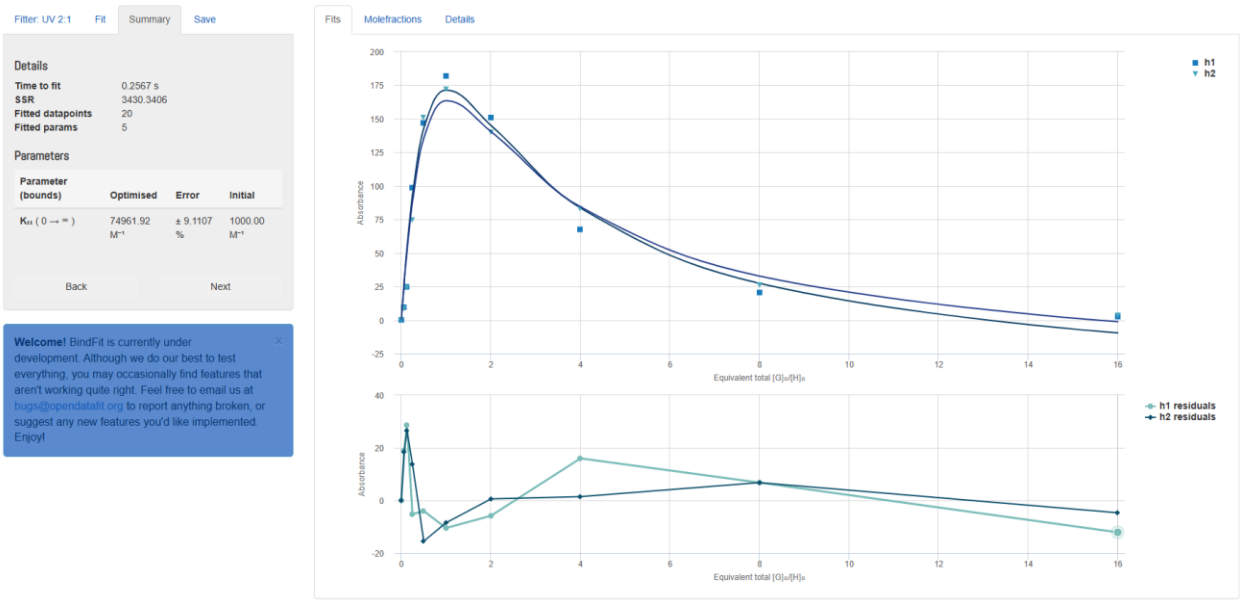
8. Thiazole Orange Titrations



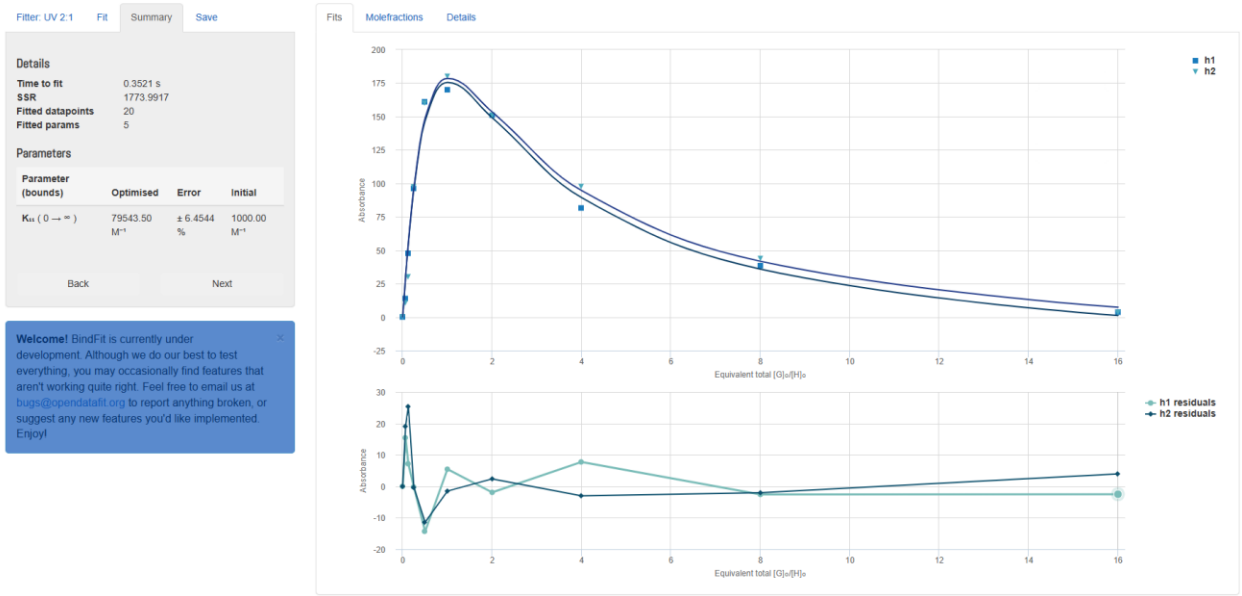
Supplementary Figure 32: *Thiazole Orange (TO) fluorescence titrations with 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) RNA (20 μM) in the absence (blue) and presence (red) of* ***RGGFGGRGG*** *(320 μM). Left: pH 4.0. Right: pH 5.0.*



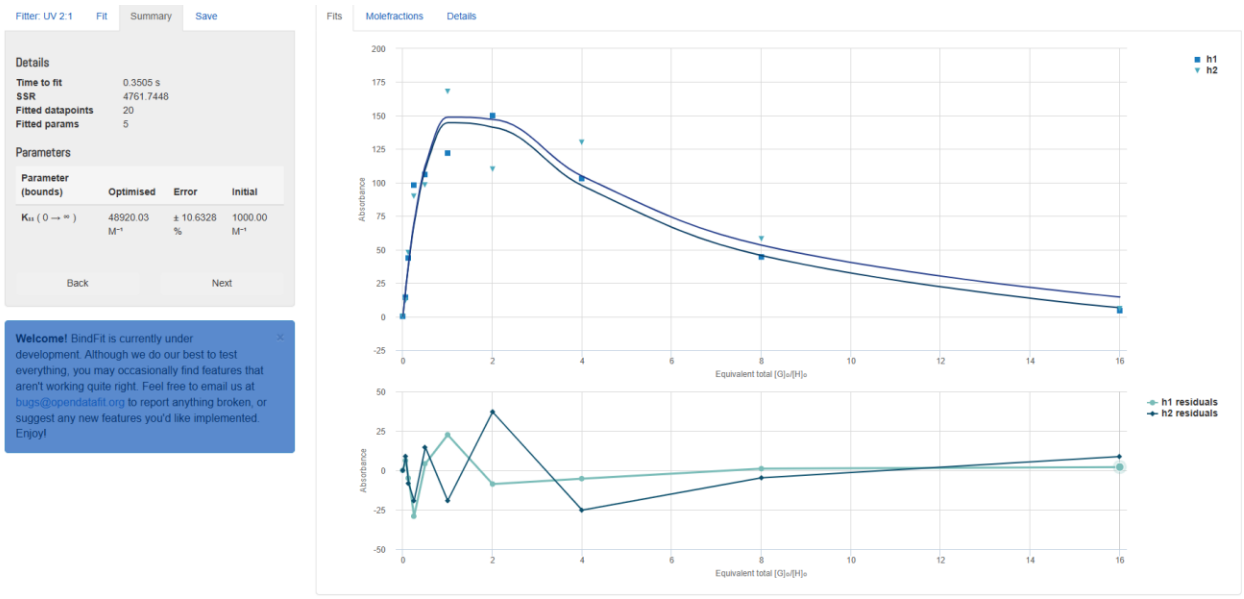
Supplementary Figure 33: *TO (40 μM) emission spectra with 5'-CCC TAA CCC TAA CCC TAA CCC-3' (i-Motif) DNA (20 μM), in the absence and presence of* ***RGGFGGRGG*** *(320 μM), at pH 4 and pH 7.* ***RGGFGGRGG*** *has no impact on fluorescence.*



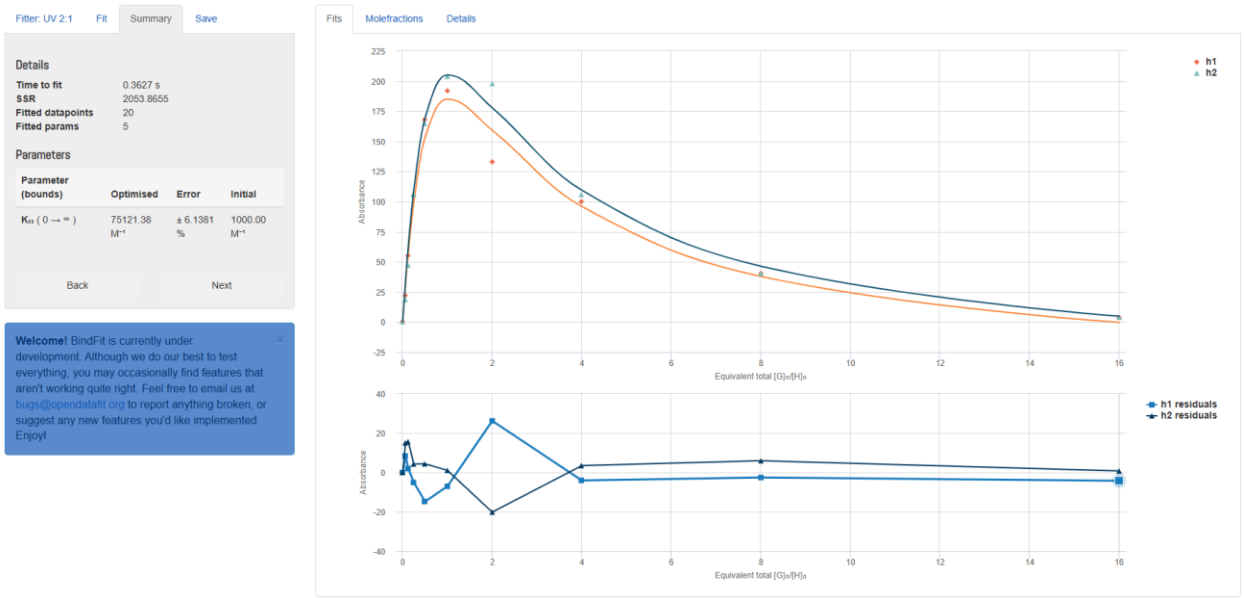
Supplementary Figure 34: *Binding isotherm (2:1 stoichiometry, non-cooperative model) of 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) RNA (pH 4.0) to Thiazole Orange in the absence of the* ***RGGFGGRGG*** *peptide. Titration performed in duplicate. (Top):* K_1 *= 74962* M^{-1}*. (Bottom): Residual plot from the fit. Fit available at http://app.supramolecular.org/bindfit/view/9ab1abb2-5ec1-4dd3-be5c-4e7b33e80e8e.*



Supplementary Figure 35: *Binding isotherm (2:1 stoichiometry, non-cooperative model) of 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) RNA (pH 4.0) to Thiazole Orange in the presence of the* ***RGGFGGRGG*** *peptide (16 eq.). Titration performed in duplicate. (Top):* K_1 *= 79544* M^{-1}*. (Bottom): Residual plot from the fit. Fit available at http://app.supramolecular.org/bindfit/view/fbc87cc5-16bb-4cd4-8683-2f7d712f4b0e.*

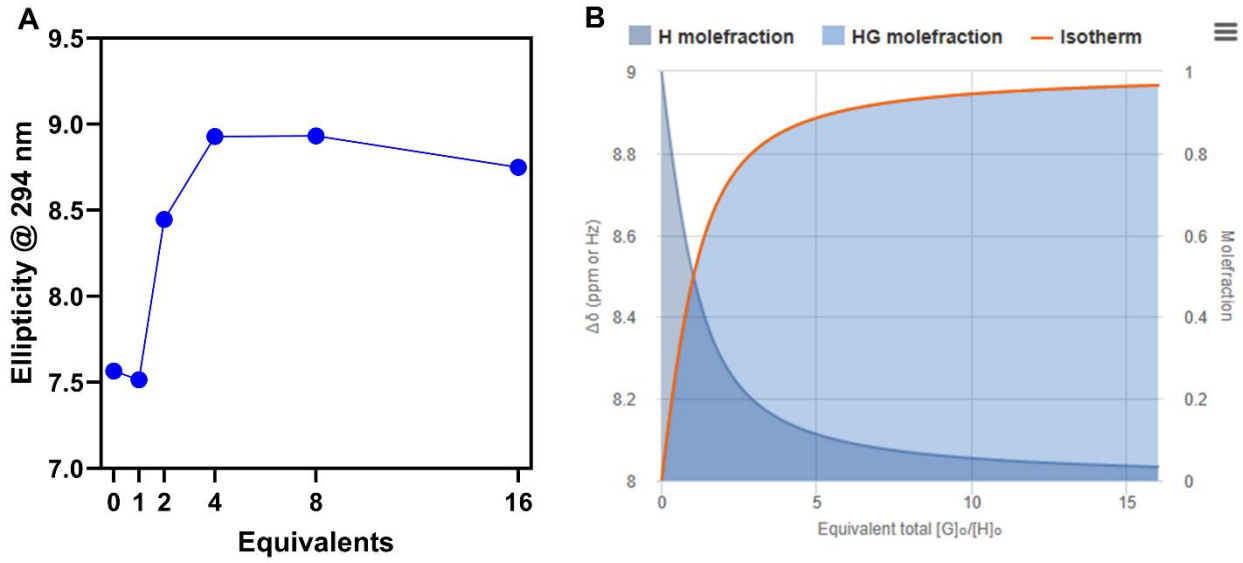


Supplementary Figure 36: *Binding isotherm (2:1 stoichiometry, non-cooperative model) of 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) RNA (pH 5.0) to Thiazole Orange in the absence of the* ***RGGFGGRGG*** *peptide. Titration performed in duplicate. (Top): K_1 = 48920 M^{-1}. (Bottom): Residual plot from the fit. Fit available at http://app.supramolecular.org/bindfit/view/cc5f290a-2d05-4182-910c-b5f5c82a6feb.*

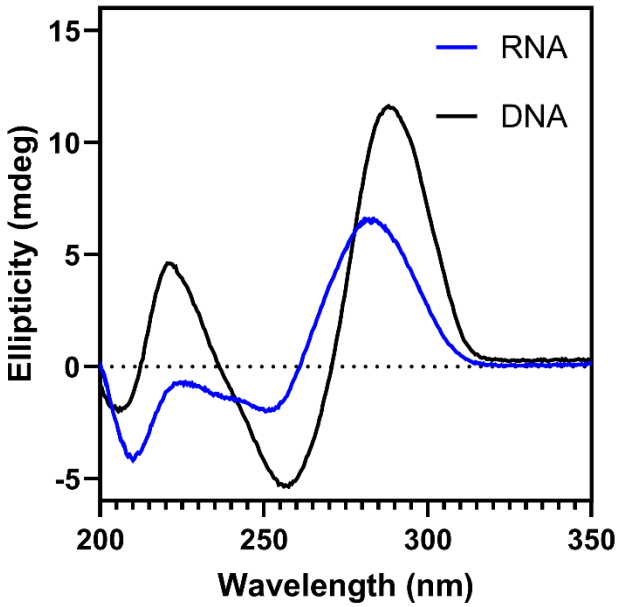


Supplementary Figure 37: *Binding isotherm (2:1 stoichiometry, non-cooperative model) of 5'-CCC UAA CCC UAA CCC UAA CCC-3' (i-Motif) RNA (pH 5.0) to Thiazole Orange in the presence of the* ***RGGFGGRGG*** *peptide (16 eq.). Titration performed in duplicate. (Top): K_1 = 75121 M^{-1}. (Bottom): Residual plot from the fit. Fit available at http://app.supramolecular.org/bindfit/view/13d23913-d09e-4971-b62e-62aac9433de3.*

9. Circular Dichroism Titrations



Supplementary Figure 38: *(A): Circular dichroism titration of the hTeloC RNA (20 μM) being titrated with* ***RGGFGGRGG*** *peptide (0 – 16 eq.) at pH 5.0. Spectra obtained in sodium acetate buffer (50 mM, pH 5) with sodium chloride (50 mM) at 25 °C. (B): Binding simulation performed for a 2:1 stoichiometry with* K_a *= 95,000* M^{-1} *at the supramolecular.org website using the Bindsim tool. Experimental data matches closely with simulated binding isotherm.*



Supplementary Figure 39: *Circular dichroism spectroscopy of the RNA vs DNA i-motif (10 μM) at pH 4.0. Spectra obtained in sodium acetate buffer (50 mM, pH 4) with sodium chloride (50 mM) at 25 °C.*

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