Cation-Responsive and Photocleavable Hydrogels from Noncanonical Amphiphilic DNA Nanostructures: Supporting Information

Giacomo Fabrini,† Aisling Minard,† Ryan A. Brady,‡ Marco Di Antonio,*† and Lorenzo Di Michele*†¶

†Department of Chemistry, Imperial College London, London W12 0BZ, UK
‡Department of Chemistry, King’s College London, London SE1 1DB, UK
¶Department of Physics - Cavendish Laboratory, University of Cambridge, Cambridge CB3 0HE, UK

E-mail: m.di-antonio@imperial.ac.uk; l.di-michele@imperial.ac.uk

Methods

Quad-Star design and oligonucleotide preparation.

The design of Quad-Stars was adapted from C-Stars[1] using NUPACK.[2] All sequences are provided in Table S1.

Oligonucleotides were purchased from and purified by Integrated DNA Technologies (IDT). Functionalised strands (cholesterol-tagged TerminalStrand_Chol and Alexa488-labelled Arm_duplex) were purified by HPLC, other oligonucleotides with standard desalting.

Samples were received de-hydrated and reconstituted in syringe-filtered (see below) TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0, obtained by diluting Tris-EDTA 100x, Sigma-Aldrich).

The concentration of reconstituted DNA strands was determined by measuring absorbance at 260 nm using a Thermo Scientific Nanodrop One Microvolume UV-Vis spectrophotometer and employing extinction coefficients provided by the supplier. Five repeated measurements were performed and averaged.

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Quad-Star sample preparation.

Soluble (non-cholesterolised) and cholesterolised Quad-Star samples for DLS, CD, AGE and bulk fluorimetry measurements were prepared by stoichiometrically mixing the required components in Eppendorf vials to give a final \([\text{Quad-Star}] = 5 \mu\text{M}\) (or equivalently \([\text{Oligonucleotide}] = 20 \mu\text{M}\)). Soluble Quad-Stars were obtained by replacing the cholesterol-bearing strand (TerminalStrand\_Chol) with one featuring identical sequence but lacking the cholesterol moiety (TerminalStrand\_NoChol). For cholesterolised Quad-Stars, cholesterol-bearing TerminalStrand\_Chol strand was heated at 90°C for around 3-5 min prior to mixing with other strands and buffer to break apart eventual aggregates.

For saline buffers, KCl, LiCl, NaCl and MgCl\(_2\) anhydrous were purchased from Sigma-Aldrich and dissolved in TE buffer to reach the desired concentration. Tris-Borate-EDTA (TBE) 1× (via 10× dilution from 10x TBE Buffer, Thermo Scientific) was used as gel loading and gel running buffer. Fresh buffers were prepared on a weekly basis to avoid degrading. All buffers were syringe-filtered (Fisherbrand sterile PES syringe filters, 0.2 \(\mu\text{m}\) pore size, 33 mm diameter, Fisher Scientific).

The strand mixtures were annealed in a Bio-Rad C1000 Touch Thermal Cycler according to the following protocol: incubation at 95°C for 3 min, cooling from 90°C to 4°C at \(-0.25°C\ \text{min}^{-1}\), incubation at 4°C for 8-10 hours prior to usage. Annealed samples were stored at 4°C and used within four days.

Agarose Gel Electrophoresis (AGE) and gel image analysis.

Agarose gels were prepared at 1.5% weight/volume agarose (Sigma-Aldrich) in TBE 1× buffer, using GelRed nucleic acid gel stain (3×, Biotium). The 10 \(\mu\text{L}\) wells were loaded with \(\approx 750-800\ \text{ng}\) DNA samples using loading tips and dye (TrackIt, 6x, Thermo Scientific). DNA ladders were purchased from Thermo Scientific (invitrogen’s Ultra Low Range DNA Ladder or molecular biology-grade GeneRuler Ultra Low Range DNA Ladder).

Gels were run for 90 min at 70 V (7 V cm\(^{-1}\)) and then imaged using either a Syngene G:BOX Chemi XRQ gel documentation system or a Syngene Dyversity 4 gel imager. The latter had been modified to replace the original camera and optics with a BFS-U3-63S4M-C USB 3.1 Blackfly® S, Monochrome Camera with a 12 mm UC Series fixed focal length lens (Edmund Optics).

The obtained images were analysed through a Python script to obtain the lane intensity profiles upon manual cropping and indication of the number of lanes (Figures 1d-e, S1, S3 and S5).

Dynamic Light Scattering (DLS).

DLS measurements shown in Figures 1f, S2, S4, S6 and S9 were performed on a Malvern Zetasizer Ultra using
Low Volume Disposable Sizing Cell Kit (ZSU1002) and 1 mm² glass capillaries (ZSU0003). The capillaries were filled with about 4 𝛅L of sample, and sealed at the bottom to prevent leakage using the clay sealing provided by the supplier. Samples for such measurements were syringe-filtered prior to annealing via 4 mm, 0.2 μm pore size, PES filters (Captiva Premium, Agilent Technologies).

The setup only allows side-scattering size measurements. Three to five independent measurements per sample were performed and averaged. Distribution by intensity was converted into distribution by volume in Malvern’s ZS Explorer. Average hydrodynamic diameter was obtained by fit (in Python) to lognormal distributions (either mono- or bi-modal).

**Circular Dichroism (CD).**

CD measurements shown in Figures 1g and S5 were performed via a J-715 spectropolarimeter from JASCO Corporation. 200 μL of each sample at 5 μM Quad-Star concentration were loaded in a 1 mm path-length, stoppered quartz cuvette. Spectra were acquired between 200 and 320 nm, with a 0.5 nm bandwidth and a 1 nm data pitch. Three to five spectra were accumulated for each sample and automatically averaged by the spectropolarimeter software. The data were then exported and processed in Python. Processing included blank correction by subtracting the spectrum of saline buffers (TE, 300 mM KCl or LiCl). Spectra were cut at 220 nm due to saturation of the applied voltage. All plotted spectra were processed via a Savitzky-Golay filter (scipy.signal.savgol_filter Python implementation) with window length equal to 17 datapoints and using a 3rd order polynomial.

**G4 formation kinetics in Quad-Stars by NMM fluorimetry.**

Fluorescence kinetics assays shown in Figures 2, 3b-g, S8 and S11 were performed using a BMG CLARIOstar Plus plate reader equipped with a single injector, using UV-Star 384 well-Plates (Greiner Bio-One). N-methyl-mesoporphyrin IX (NMM) was purchased from Cambridge Bioscience, Ltd. A 10 mM stock solution was prepared by dissolving the as-received powder in DMSO, and stored at -25°C. Lower concentration solutions were prepared as needed via dilution in DMSO, and temporarily stored at 4°C. Excitation was set to 388 ± 15 nm. NMM displays two emission peaks at around 608 and 670 nm. As the two chromatics provide comparable insights, only the peak at 670 ± 15 nm was monitored.

Wells were initially filled with mixtures of 30 μL DNA sample, pre-annealed as discussed above in TE buffer supplied with either lithium (300 mM, for soluble Quad-Stars) or magnesium (2 mM, for cholesterolised Quad-Stars), and 2 μL NMM solution. Across all assays, NMM concentration was adjusted to stoichiometrically match G4 concentration ([NMM] = [G4] = [Quad-Star]). Prior to running an assay, the thermal stage of the instrument was set to 25°C and the plate was allowed to equilibrate for 45 min. After thermalisation, the
signal was recorded for some time to obtain an initial baseline (15 min for soluble and 30 s for cholesterolised Quad-Stars, see below) before injecting the wells with 10 µL KCl (1.26 M) solution to induce Quad-Star assembly. Unless otherwise specified, final Quad-Star and KCl concentrations were 5 µM and 300 mM, respectively. When investigating [KCl] dependency, different KCl concentrations (Figure 2b-(ii)) were achieved by reducing the injected volume while replacing injection reductions with an equivalent amount of plain TE buffer.

Data were acquired in triplicates for all samples in varying [KCl] (apart from 6G5T and 6G7T at 30 mM KCl, which were measured in duplicates only) and in triplicates/quintuplicates for 5G/6G, respectively, in varying [Oligo]. Negative controls (Figure 2, S8, S11) were performed (duplicates), in which KCl injections were replaced with plain TE buffer (manually pipetted prior to starting the run), to ensure that no fluorescence increase or unexpected trends were observed in the absence of the cation trigger. All data were blank-subtracted using samples of TE + NMM as reference, as NMM’s emission is non-zero in plain buffers. Negative controls are reported after normalisation by mean subtraction and division, unless otherwise stated.

Owing to the slower assembly kinetics, soluble Quad-Stars were characterised in “plate kinetics” mode, where the whole plate is scanned at each timepoint. Shaking was enabled after every reading, at 600 rpm for 1-2 s. Post-shaking settling time was set to 0.1 s. Runs were briefly paused soon after the injection to cover the plate with an adhesive film (MicroAmp optical adhesive films, Applied Biosystems, ThermoFisher Scientific) and reduce evaporation over the 22 h of the measurements. To better sample assembly kinetics, measurements were acquired as frequently as possible (30-40 s intervals) at the beginning of the transient, and more sparsely later. Measurements were carried out using bottom optics readout due to the optical adhesive covering the top of the plate.

Conversely, due to their rapid assembly, cholesterolised Quad-Stars (9T spacer only, [Quad-Stars] = 5 µM, [KCl] = 300 mM) were tested in “well kinetics” mode, where each well is injected and then measured for the prescribed number of cycles before moving on to the next one. Samples were measured in four/ten repeats for 5G/6G, respectively. Shaking was only performed right after injection (1-2 s at 600 rpm and 0.1 s settling time). Measurements were acquired at uniform 0.48 s intervals (unless otherwise specified). Given the much shorter duration, no optical adhesive was required. To prevent interactions between the hydrophobic plastic of the well-plates and the cholesterol moieties, wells were passivated with Bovine Serum Albumin (BSA, lyophylized powder, BioReagent, Sigma). To this end, wells were filled with 70-100 µL of a 1% w/v BSA solution, and then allowed to evaporate overnight at 65 °C. Prior to using the plate, each well was thoroughly washed with MilliQ water and nitrogen-dried. Due to the bottom BSA coating and the absence of the optical adhesive, top optics readout was used.

The Extended Dynamic Range mode of the instrument was enabled in all measurements, avoiding signal
saturation.

**Disassembly of cholesterolised Quad-Stars via NMM fluorimetry.**

A 2 M solution of chelating agent [2.2.2] cryptand (4,7,13,16,21,24-Hexaoxa-1,10-diazabicyclo[8.8.8] hexacosane)(98 % purity, Sigma-Aldrich) was prepared in Milli-Q water, syringe filtered (0.2 µm pore PES filters) and stored at 4 °C.

Subsequent assembly/disassembly experiments were performed on 6G cholesterolised Quad-Stars only, under the same conditions of the folding experiments described above (“well kinetics” mode, time step = 0.48 s, duration = 995 cycles). Injection took place after ≈45 s from the start of the disassembly run, to ensure prior stability of fluorescence signal. The injected volume was adjusted to obtain a 320 mM final cryptand concentration, completely chelating the available 300 mM K⁺ ions. Sample was analysed in quadruplicates. Resulting curves were background corrected and normalised as described below.

**Fitting fluorimetry data.**

Data processing and fitting was performed in Python. Folding kinetics of tetramolecular G4s were previously modelled with the following expression \(^3\) for the fraction of folded strands \(\alpha_f\)

\[
\alpha_f = 1 - \left[1 + C_0^{(n-1)} (n-1) k_{on} t\right]^{1/n}
\]

where \(C_0, n, k_{on}\) and \(t\) are the starting oligonucleotide concentration, the reaction order, the folding/association rate constant and time, respectively. The recorded fluorescence intensity (F.I.), pre-processed by subtracting blank data and translating the time origin \((t_{injection} = 0 s)\), can thus be fitted as \(F.I. = a \cdot \alpha_f\), where \(a\) is a scaling (fitting) parameter. The aggregation half-times shown in Figures 2, 3, S6 and S9 can be extracted from the fitted \(k_{on}\) and \(n\) as

\[
t_{1/2} = \frac{(\frac{1}{2})^{1-n} - 1}{(n-1)k_{on}C_0^{(n-1)}}.
\]

Best fit was obtained by minimisation of batch Mean Squared Error (scipy.optimize.minimize implementation in Python, using Limited-memory BFGS (L-BFGS) with parameter bounds (min = 2, max = 5) for \(n\)). Batch fitting was employed to improve fits by taking into account data for all examined spacer lengths, as these have no theoretical impact on the reaction order \(n\). For the folding kinetics of soluble 5G and 6G Quad-Stars in varying [KCl] at fixed 20 µM [Oligo], curves corresponding to the same G-run length (5G/6G) at all spacer lengths were fitted in [KCl] batches. Specifically, all curves having a given G-run length and [KCl] were fitted with the same value of \(n\) regardless of spacer length, while keeping other fitting parameters...
free. For kinetics of soluble Quad-Stars with different [Oligo] at fixed 300 mM [KCl], all motifs with the same
G-run length were similarly fitted in [Oligo] batches. The reaction order, $n$, was calculated as $1 - m$, with
$m$ being the slope of the linear fit of $\ln t_{1/2}$ vs $\ln [\text{Oligo}]$, weighted by the standard deviations (Figure S8c).
Reported uncertainties are calculated from the covariance matrix of the fit. For cholesterolised Quad-Stars,
curves were fitted in batches corresponding to G-run length.

**Isothermal reversible assembly via optical microscopy.**

For isothermal assembly experiments (Figure 3c), samples were annealed overnight in 2 mM MgCl$_2$ buffer, at
a lower [Oligo] = 5.6 µM to enable the formation of discrete aggregates and simplify quantification by image
segmentation. To enable fluorescence imaging, 25% of Arm$_{duplex}$ strands were replaced with an Alexa488
fluorophore-labelled version of the same strands (see Table S1).

Imaging was performed via a Nikon Eclipse Ti2-E microscope with Perfect Focus System (PFS), equipped
with a CFI Plan Apochromat λ 40× (NA 0.95, WD 0.21) objective lens, a Hamamatsu Orca-Flash4.0v3
camera and a Lumencor SPECTRA X LED engine. A 510/25 nm SPECTRA X LED was used to excite the
Alexa488 fluorophores. Samples were imaged in FlexWell incubation chambers (6.5 mm × 6.5 mm × 3.2
mm, 8 wells, Grace Bio-Labs) attached on top of glass coverslips (24 mm × 60 mm, Menzel-Glæser) via the
built-in adhesive strip.

Imaging was automated via JOBS in Nikon NIS Elements, with PFS enabled to ensure constant z-height
during the timelapse. Both bright-field and epifluorescence z-stacks (42 µm height, 2 µm step) were captured
at each timepoint. Examples of unprocessed timelapses are provided as supporting data (5G: videos S1-S2,
6G: videos S3-S4, MP4 format, 7 fps playback speed).

Samples were imaged in triplicates/quadruplicates (5G/6G) for 2.5 h, with no delay among timelapse acquisi-
tions and three non-overlapping fields of view (FOVs) per well. Initial sample volume was 90 µL. After 3
timepoints from the start (≈ 15-20 min), the run was paused and 6.5 µL of 4.5 M KCl solution (in TE with
2 mM MgCl$_2$) were injected. Chambers were sealed right after KCl injection by placing a second coverslip
on top to reduce evaporation.

For isothermal disassembly, a 2 M [2.2.2] cryptand solution (in TE with 2 mM MgCl$_2$) was used. Multiple
injections (2 × 5 µL + 1 × 10 µL + 1 × 20 µL) were administered in each well, while monitoring several FOVs
via bright-field or epifluorescence timelapses for around 1-2 min each at the highest possible framerate. Split-
ting the injected volume allowed better visualisation of the disassembly.

For multiple cycles of reversible assembly-disassembly (Figure 3h), the first was performed as described above.
Some of the supernatant was carefully removed to prevent chambers from overflowing after the following
KCl injection (10/15 µL for the second/third assemblies). Injected volume of cryptand solution was kept at
40 μL. The multiple injections lead to considerable sample dilution, likely causing the decrease in aggregate formation (both size and number) observed in Figure 3h. Three FOVs per well in quadruplicate/duplicate 6G Quad-Star wells were imaged for the second/third cycles. Epifluorescence micrographs in the insets of Figure 3h were background-corrected in FIJI via Rolling Ball algorithm (radius = 100 px, px = pixels).

Assembly control samples (negative control in 2 mM MgCl₂, 2 mM MgCl₂ + 300 mM LiCl, 300 mM LiCl + 300 mM LiCl or KCl, respectively) in Figure S12 have been imaged via the same protocol and for the same time interval (2.5 h, with injection after 2 cycles = 10 min from run start), with a single well per control sample, 3 FOVs per well. Only exception was 5G, LiCl + KCl control, which was imaged in only 2 FOVs. As each control was imaged separately, standard timelapse acquisition was used rather than JOBS setup (same dual channels, time interval = 5 min). Injected buffer solutions were 2 M LiCl and 4.5 M KCl (same as above); volumes were adjusted to produce the described final concentrations. No buffer solution was injected in the 2 mM MgCl₂ negative control. Imaging was performed in incubation chambers obtained by carving circular holes (8 mm diameter) in a PDMS spacer later attached to a glass coverslip.

Segmentation pipeline for area fraction determination.

A Python script was used to extract the in focus z-planes from Nikon’s ND2 files (via ND2Reader) and save them as TIFFs, then automatically opened and analysed via the following macro pipeline in FIJI (ImageJ):⁴ background subtraction via Rolling Ball (radius = 50-100 px, depending on the size of the aggregates) to compensate for uneven illumination; noise reduction via median filter (radius = 5 px) and minimum filter (radius = 1 px); unsupervised Li thresholding and binary conversion; watershed to break clusters of aggregates; Particle Analysis with size (> 100 px²) and circularity (> 60 %) filters. The pipeline automatically produced the area fraction (%) data, which we used as a proxy to estimate the kinetics of isothermal aggregation.

As timelapses were run with no delay between adjacent timepoints, the time interval varied among different experiments depending on exposure, z-stack settings and the number of simultaneously imaged samples. In order to pool together data with different time intervals, cubic spline interpolation was performed in Python, with an overall 2.5 h duration (same for all runs) and desired number of equally spaced points equal to 30. Profiles were synchronised via the annotated injection timepoints. No interpolation was needed for curves in Figure 3h and Figure S12.

Rhodamine B uptake and release.

Samples were prepared as for assembly assays and, prior to initiating assembly, mixed with aliquots of 2.5 mM rhodamine B (Sigma-Aldrich) solution (in TE with 2 mM MgCl₂) for a final [rhodamine B] = 28 μM. Same sample and KCl injection volumes were used as for assembly assays. PDMS incubation chambers discussed
above for isothermal assembly control samples were employed. Samples were imaged 3.5 h after the injection with a CFI Planar APO λ 60× Oil (N.A. 1.40, W.D. 0.13 mm) immersion lens. Rhodamine B was excited via a 550/15 nm SPECTRA X LED.

Fluorescence intensity ratios between the inside of aggregates and the background were calculated as ratios of the average intensity inside and outside the selection obtained via the segmentation pipeline above. Both samples (Figure 4a-b) display a slightly higher coverage compared to previous assays, most likely due to the longer assembly duration.

Cargo release was triggered by 3 µL injection of the same cryptand solution described above. Volume was lowered compared to disassembly assays to slow down and better follow the release process, aided by administering the injection on the opposite side of the imaged FOV within each well. Disassembly of hydrogel particles and concomitant rhodamine B release were imaged via epifluorescence timelapses at 2 fps for 1-2 min. Unprocessed timelapses are provided as supporting data (videos S5-S6 for 5G and 6G, MP4 format, 7 fps playback speed).

Release profiles (Figure 4c) depict the integrated fluorescence intensity within the initial background regions, that is the inverted aggregate segmentation mask in the timepoints preceding cryptand injection (i.e. the regions with no aggregates to begin with). Area fraction profiles for disassembly were obtained via segmentation as above. Presented data corresponds to single, representative samples, due to the specific timescales strongly depending on the proximity between the injection location and the imaged FOV. The overall trend is repeatable, with closer proximity expectedly producing faster disassembly and release (Figure S15).

**UV-triggered disassembly in the presence of NMM.**

Samples for UV-triggered photodisassembly (Figure 4d) were prepared and imaged as for uptake and release. After 3.5 h from KCl addition, 6 µL of 105 µM NMM in DMSO were pipetted into the sample chambers (NMM:G4 ≈ 5:1). NMM was excited via a 395/25 nm Spectra X LED. Samples were imaged via epifluorescence and/or bright-field timelapses at 2 fps, for an overall duration of 2 min per FOV. Pristine bright-field and epifluorescence timelapses are provided as supporting data (videos S7-S8, MP4 format, 7 fps playback speed).

Disassembly profiles were obtained via the segmentation pipeline above. Bright-field insets (from representative samples) have been contrast-enhanced to aid visual detection of aggregates. Pristine images are provided in Figure S16. Engraving in Figure 4e has been obtained by programmed sample exposure at selected locations positioned along the desired path via Nikon’s JOBS. The image of the entire well (as the one in Figure S16c) was obtained via automated Large Image stitching in NIS Elements. Stitched FOVs were imaged through a CFI Planar APO λ 20× (N.A. 0.75, W.D. 1.00 mm).
Stability of hydrogel aggregates in model physiological conditions.

For stability experiments in Figures S17-S18 and videos S9-S16, samples were prepared as for isothermal assembly, albeit with only 10% of Arm_d duplex strands being replaced with the Alexa488 fluorophore-labelled version (see Table S1). The same KCl solution discussed above was injected and 3-4 h were allowed for the assembly of the aggregates. After assembly, samples were imaged and were either left undisturbed (negative controls - Figure S17a-b, videos S9-10 for 5G and 6G, respectively) or subjected to supernatant removal and replacement. For the latter, 75 µL out of the overall 96.5 µL sample volume were carefully removed with a pipette and replaced with either Phosphate Buffered Saline (PBS) 1× (from PBS tablets, Sigma-Aldrich) (Figure S17c-d, videos S11-12 for 5G and 6G, respectively) or Dulbecco’s Modified Eagle Medium (DMEM, high glucose, GlutaMAX, Gibco) 1× supplied with either 10% (Figure S18a-b, videos S13-14 for 5G and 6G, respectively) or 30% (Figure S18c-d, videos S15-16 for 5G and 6G, respectively) Fetal Bovine Serum (FBS, Gibco). Incubation chambers were sealed with an adhesive strip (FlexWell Seal Strips, Grace Bio-Labs) to reduce evaporation.

Imaging was performed via an automated JOBS script in NIS Elements, capturing both bright-field and epifluorescence z-stacks every 20 min for 60 h. Every sample was analysed in triplicates, except for duplicates in PBS 1×, and three fields of view per well were monitored. Representative epifluorescence timelapses have been contrast enhanced in FIJI (see below), with selected timepoints (0, 20, 40 and 60 h) being provided in Figures S17 and S18 and the overall time evolution reported as supplementary videos S9-S16 at a playback speed of 21 fps in MP4 format. The aforementioned contrast enhancement (called Enhance Contrast in FIJI) rescales pixel values via histogram stretching to reach a defined percentage of saturated pixels (default 0.3% was chosen), by taking into account the stacked (global) histogram of the entire timelapse, rather than the histograms of individual timeframes. Bright-field micrographs have not been included due to condensation artifacts.
Figure S1: Self-assembly of non-cholesterolised 5G and 6G Quad-Stars in different ionic environments investigated via agarose gel electrophoresis. (a) Agarose gel (see Methods) comparing electrophoretic mobilities of reference 4-arm nanostars with arm length equal to 28 bp (annealed in 300 mM NaCl, orange), 5G (green) and 6G (blue) Quad-Stars in 2 mM MgCl₂, 300 mM LiCl or 300 mM KCl, respectively. Ultra-Low-Range (ULR) DNA reference ladder is used as electrophoresis control (red). (b) Intensity profiles extracted from agarose gel in (a). Visual inspection of the agarose gel, aided by the adjacent lane intensity profiles, confirms the lack/correct self-assembly of 5G and 6G samples in LiCl/KCl as previously demonstrated (Figure 1d-e). However, while 5G samples do not exhibit any retarded band when annealed in 2 mM MgCl₂, 6G Quad-Stars display an intermediate behaviour among the ones in LiCl/KCl, which indicates Mg²⁺ ions still allow for a certain degree of G4 formation, and thus Quad-Star motif assembly. The lower fraction of correctly assembled 6G Quad-Stars in Mg²⁺, signalled by the lower intensity of the retarded band, together with the lack of micelle formation in such lower ionic strength buffer (Figure S9), is likely the reason why we don’t experimentally observe any aggregation in our microscopy samples prior to KCl injection. All samples for the illustrated measurements were prepared at 20 µM oligonucleotide concentration.
Figure S2: Volume-based size distributions obtained via DLS for soluble 5G (left) and 6G (right) Quad-Stars in 2 mM MgCl$_2$, 300 mM LiCl and 300 mM KCl buffers support agarose gel electrophoresis results (Figure S1). 2 mM MgCl$_2$ buffers allow some degree of formation of G4s in 6G, but not in 5G samples, as indicated by the average hydrodynamic diameters ($d_H$, vertical lines, obtained via fitting to mono- or bi-modal lognormal distributions - fits not shown). 5G motifs display indeed similar size in Mg$^{2+}$ and Li$^+$ buffers (free, disjoint dsDNA arms), while 6G in Mg$^{2+}$ display a bimodal distribution, with the larger-sized peak shifted towards the size of fully assembled Quad-Stars. The smaller-sized peak for 6G is slightly shifted to lower size values compared to free dsDNA arms in Li$^+$ buffers, but the two $d_H$ lie within one standard deviation (see below). All profiles are averages of at least 5 batch runs. Mean hydrodynamic diameters and corresponding standard deviations (from covariance matrix of the fit) are hereby reported: 5G - 4.84 ± 1.33 nm in 300 mM LiCl, 5.31 ± 1.30 nm in 2 mM MgCl$_2$, 7.92 ± 1.49 nm in 300 mM KCl; 6G - 4.87 ± 1.36 nm in 300 mM LiCl, 2.64 ± 1.11 nm and 6.01 ± 1.40 nm in 2 mM MgCl$_2$, 8.51 ± 1.38 nm in 300 mM KCl.
Figure S3: Self-assembly of non-cholesterolised 5G and 6G Quad-Stars in different ionic environments investigated via agarose gel electrophoresis. (a) Agarose gel (see Methods) comparing electrophoretic mobilities of 5G (green) and 6G (blue) Quad-Stars in either 100 or 300 mM LiCl, NaCl or KCl, respectively. Ultra-Low-Range (ULR) DNA reference ladder is used as electrophoresis control (red). (b) Intensity profiles extracted from agarose gel in (a). Visual inspection of the agarose gel, aided by the lane intensity profiles below, confirms the lack/correct self-assembly of 5G and 6G samples in 300 mM LiCl/KCl as previously demonstrated (Figure 1d-e). A lower concentration of K\(^+\) ions, namely 100 mM, is only sufficient to induce the assembly of 6G, but not 5G Quad-Stars, due to the fast overnight quenching (see Methods). Moreover, Na\(^+\) ions appear to support the self-assembly of 6G Quad-Stars only at high ionic strength (300 mM), but fail at inducing a significant retarded band in 5G constructs at both examined cation concentrations. All samples for the illustrated measurements were prepared at 20 \(\mu\)M oligonucleotide concentration.
Figure S4: Volume-based size distributions obtained via DLS for soluble 5G (left) and 6G (right) Quad-Stars in 100 or 300 mM LiCl, NaCl or KCl buffers support agarose gel electrophoresis results (Figure S3). At lower cation concentration (100 mM, top panels), only 6G Quad-Stars assemble in the presence of K\(^+\) ions. At higher ionic strength (300 mM, bottom panels), both 5G and 6G self-assemble in KCl, whereas NaCl buffers allow G4 formation only in 6G, but not in 5G samples. Average hydrodynamic diameters (<d\(_H\)>), obtained via fitting to lognormal distributions (fits not shown) are indicated by vertical lines. All profiles are averages of at least 4 batch runs. Reported <d\(_H\)> values lie within one standard deviation from the ones presented in Figures 1, S2 and S6. Mean hydrodynamic diameters and corresponding standard deviations (from covariance matrix of the fit) are hereby reported: 5G - 5.17 ± 1.30 nm in 100 mM LiCl, 5.73 ± 1.24 nm in 100 mM NaCl, 5.17 ± 1.30 nm in 100 mM KCl, 5.02 ± 1.31 nm in 300 mM LiCl, 5.33 ± 1.30 nm in 300 mM NaCl, 6.97 ± 1.37 nm in 300 mM KCl; 6G - 5.16 ± 1.35 nm in 100 mM LiCl, 5.72 ± 1.32 nm in 100 mM NaCl, 7.48 ± 1.36 nm in 100 mM KCl, 5.55 ± 1.30 nm in 300 mM LiCl, 7.16 ± 1.36 nm in 300 mM NaCl, 9.20 ± 1.38 nm in 300 mM KCl.
Figure S5: Self-assembly of non-cholesterolised Quad-Stars investigated via agarose gel electrophoresis. (a, b) Agarose gels (see Methods) of all investigated Quad-Star motifs annealed in either 300 mM KCl (a) or LiCl (b). Ultra-Low-Range (ULR) DNA reference ladder is used as electrophoresis control (red). Nanostars (NS) with 28 base pairs (bp) arm length annealed in 300 mM NaCl are used as mobility reference for correctly assembled motifs (orange). Spacer lengths increase from left (3T) to right (9T) within each sample group (4G, 5G, 6G and no Gs), as schematised. (c, d) Intensity profiles extracted from agarose gels in (a) and (b), respectively. Visual inspection of the agarose gels, aided by the lane intensity profiles demonstrates that only 5G and 6G samples (at all poly-T spacer lengths) in K⁺-rich environments display a retarded band with slower mobility, comparable to that of nanostars with an arm length of 28 bp, signalling correct, K⁺-dependent self-assembly of the designed Quad-Stars. Samples with no Gs (7-13T) cannot form G4s, and are thus incapable of linking their four double-stranded DNA (dsDNA) arms. A similar behaviour holds for 4G samples, which, despite their potential for G4 formation, present a single band corresponding to free dsDNA arms, clearly indicating failed assembly into the designed nanostar geometry. All samples for the illustrated measurements were prepared at 20 µM oligonucleotide concentration.
Figure S6: (a) Volume-based size distributions obtained via DLS for all investigated Quad-Star motifs in 300 mM KCl buffer support agarose gel electrophoresis results (Figure S5). Only 5G and 6G Quad-Stars, regardless of poly-T spacer length display an average hydrodynamic diameter ($d_H$) close to that of correctly assembled 4-way nanostars with $l = 28$ bp ($<d_H> = 8.44$ nm, Figure 1f). Motifs with no Gs (replaced with Ts) or 4G display a consistently smaller hydrodynamic diameter, close to that of 5G and 6G motifs in absence of K$^+$ ions (300 mM LiCl buffer, Figure 1f). All profiles are averages of at least three independent repeats. Vertical dashed lines represent mean hydrodynamic diameters ($<d_H>$) obtained by fitting average profiles with lognormal distributions (fits not shown). The corresponding mean hydrodynamic diameters $<d_H>$, rounded to two significant figures, are reported in the legends on top of the corresponding panels. (b) Summarised mean hydrodynamic diameters for all structures in 300 mM KCl buffer against length of poly-T spacers highlight a negligible influence of the spacer length on the self-assembly of Quad-Stars, as anticipated by agarose gel electrophoresis results (Figure S5). Data are presented as mean hydrodynamic diameters (solid lines and markers) ± standard deviation from covariance matrix of the mentioned fit (error bars).
Figure S7: Circular Dichroism (CD) spectra of all investigated Quad-Stars in 300 mM KCl (red) and LiCl (blue). Negative controls with guanines replaced by thymines (no Gs, first row) and 4G samples present the same signature in both ionic conditions, with only a slight hint of shift appearing for 4G9T, confirming no G4 formation as per AGE and DLS in Figures S5-S6. Only 5G and 6G samples display the clear, parallel G4 signature with characteristic shift in maximum and minimum towards $\approx 263$ and 240 nm, respectively, proving K$^+$-dependent assembly of Quad-Stars via tetramolecular parallel G4 formation, as per design. All samples were prepared at 20 $\mu$M oligonucleotide concentration.
Figure S8: Kinetics of G4-dependent formation of non-cholesterolised Quad-Stars for all spacer lengths investigated via NMM fluorimetry. (a) Kinetic profiles at target conditions ([Oligo] = 20 µM, [KCl] = 300 mM). (b) Profiles for Quad-Stars with 9T spacers for all examined [Oligo] (Oligo = oligonucleotide), corresponding to half-life values in Figure 2b-(i). (c) Half-life of formation vs [Oligo] at fixed [KCl] = 300 mM. Solid lines are best fit lines (least squares). Derived reaction orders, $n$, are hereby reported: 4.005 ± 0.747 for 5G3T ($R^2 = 0.880$), 4.613 ± 0.667 for 5G5T ($R^2 = 0.971$), 4.501 ± 0.787 for 5G7T ($R^2 = 0.958$), 3.605 ± 0.740 for 5G9T ($R^2 = 0.911$), 3.823 ± 0.086 for 6G3T ($R^2 = 0.990$), 3.467 ± 0.039 for 6G5T ($R^2 = 0.999$), 3.590 ± 0.030 for 6G7T ($R^2 = 0.999$), 3.711 ± 0.127 for 6G9T ($R^2 = 0.996$), with uncertainties representing standard deviations calculated from the covariance matrix of the fit. (d) Profiles for Quad-Stars with 9T spacers for all examined [KCl], corresponding to half-life values in Figure 2b-(ii). (e) Half-life of formation vs [KCl] at fixed [Oligo] = 20 µM. In (a), (b) and (d), curves with saturated colours (top) correspond to samples receiving K$^+$ cation trigger injection, whereas fainter curves (bottom) are negative controls (no KCl). Data are reported as mean (dots, one every 3 datapoints shown) ± standard deviation (shaded regions) and fits to theoretical tetramolecular kinetics$^3$(solid lines). For negative controls, only average datapoints are shown (see Methods for normalisation procedure). Vertical lines are mean half-life values, $t_{1/2}$, with the surrounding shaded region depicting the standard deviation. Data in (c), (e) are reported as mean (markers) ± standard deviation (error bars). Reported negative controls and 6G5T and 6G7T at 30 mM KCl have been averaged across independent duplicates. All other data have been averaged across at least three independent repeats (see Methods).
Figure S9: Volume-based size distributions obtained via DLS for cholesterolised 5G and 6G Quad-Stars in 2 mM MgCl$_2$ and 300 mM LiCl buffers, as well as negative controls (no G, where guanines are replaced by thymines, see Table S1) under the same conditions and in 300 mM KCl buffer. Data indicates that dsDNA arms fail to form micelles in lower ionic strength 2 mM MgCl$_2$ buffer, where average hydrodynamic diameters ($<d_H>$, vertical lines, extracted via fit to mono- or bi-modal lognormal distributions - fits not shown) closely resemble those of non-cholesterolised dsDNA arms, being considerably smaller than single assembled soluble Quad-Stars (see Figures 1, S2, S4). Higher ionic strength buffers, such as 300 mM LiCl or KCl, allow for micelle formation as indicated by the larger-sized peaks at $\approx$10-15 nm, while still displaying some amount of free dsDNA arms (smaller-sized peaks closely matching the ones in 2 mM MgCl$_2$). Data has been averaged across (at least) five independent measurements (solid profiles). Mean hydrodynamic diameters and standard deviations (from covariance matrix of fit) are hereby reported: 5G - 5.51 ± 1.29 nm in 2 mM MgCl$_2$, 5.96 ± 1.23 nm and 16.16 ± 1.36 nm in 300 mM LiCl; 6G - 5.78 ± 1.29 nm in 2 mM MgCl$_2$, 4.92 ± 1.20 nm and 9.56 ± 1.64 nm in 300 mM LiCl; 5G - no G - 5.34 ± 1.32 nm in 2 mM MgCl$_2$, 4.29 ± 1.40 nm and 14.68 ± 1.40 nm in 300 mM LiCl; 6G - no G - 5.20 ± 1.34 nm in 2 mM MgCl$_2$, 13.24 ± 1.44 nm in 300 mM LiCl, 4.70 ± 1.21 nm and 12.54 ± 1.51 nm in 300 mM KCl.
Figure S10: (a) Alexa488 epifluorescence and (b) corresponding bright-field micrographs depicting 5G (i) and 6G (ii) Quad-Star hydrogel aggregates formed after annealing in 300 mM LiCl buffers, therefore in absence of K⁺ ions. Micrographs are unprocessed and have been cropped for ease of visualisation. Scale bars = 50 µm.
Figure S11: (a) Raw fluorescence intensity (F.I.) of two independent repeats of both negative controls (NC) for 6G cholesterolised Quad-Stars, i.e. samples measured without the K⁺ cation trigger for assembly, and plain TE buffer + NMM (Blank) acquired via fast kinetics (see Methods) with time interval among datapoints = 1.3 s. Without the cation trigger, samples display a flat-line behaviour with no fluorescence increase, just like buffer + dye blanks. (b) Comparison among 6G cholesterolised Quad-Stars with cation trigger (bright curve, same as in Figure 3b) and negative controls and blanks lacking it. Average of negative control and blank duplicates have been normalised by mean subtraction and division, and their time evolution has been trimmed to match the one for 6G. Upon normalisation, negative controls clearly display the same flat-line behaviour as blanks completely lacking DNA nanostructures. In both (a) and (b), one marker every 3 datapoints has been displayed for clearer visualisation. (c) Cholesterolised Quad-Stars display much faster assembly kinetics compared to soluble, non-cholesterolised ones, as exemplified by their half-life values. As the folding of tetramolecular G4 motifs is highly concentration dependent (see Figure 2b-(i)), the reduction in half-life can be attributed to an increase in the local oligonucleotide concentration caused by the formation of cholesterol-induced micelle-like clusters upon increase in the ionic strength (see DLS in Figure S9). This half-life reduction is more dramatic, almost 100-fold, for the slower 5G sample. Reported samples are the ones discussed in the main text (9T spacer length). Data are reported as mean (filled bars) ± standard deviation (error bars) from the same number of repeats as data in Figure 3b (4/10 for 5G/6G).
Figure S12: Area fraction profiles corresponding to several control samples for both 5G (left) and 6G (right) cholesterolised Quad-Stars. While samples annealed in 2 mM MgCl$_2$ (blue) fail to form aggregates without addition of other cations (Mg$^{2+}$ Neg. Ctrl. = Negative Control), they undergo a certain degree of aggregation when provided with 300 mM Li$^+$ ions (orange), most likely due to the increase in ionic strength facilitating micelle formation (see Figure S9) and thus colocalisation of the dsDNA arms of Quad-Star motifs. The enhanced local concentration is likely enough to allow G4 formation despite the lack of stabilising cations. This also explains why samples annealed in 300 mM Li$^+$ present some aggregates, albeit way fewer than samples in 300 mM K$^+$ (Figure S10). When provided with a further 300 mM Li$^+$ (green) or K$^+$ (red), samples undergo much stronger and faster aggregation in the presence of K$^+$ ions. This confirms the presented hypothesis of aggregate formation relying on both micelle-induced colocalisation of dsDNA arms and K$^+$-promoted G4 formation. All data is presented as the average (solid line) ± standard deviation (shaded region) of three FOVs within single representative samples, apart from 5G - Li$^+$ + K$^+$ (red, left panel), which is reported as average of duplicate FOVs (solid line) and the interval among them (shaded region).

Figure S13: Area fraction profiles depicting disassembly of hydrogel aggregates (6G Quad-Stars) upon cryptand injection ($t = 0$ s). These are enlarged versions of the orange traces in Figure 3h. The reduction in the initial area fraction percentage reflects the decrease in aggregate formation with increasing assembly/disassembly cycle count, most likely due to sample dilution. Data is presented as mean (solid lines) ± standard deviation (shaded interval) of twelve (top) and six (bottom) kinetic curves (equivalent to 4 and 2 wells, respectively).
Figure S14: (a) Alexa488 epifluorescence and (b) corresponding bright-field micrographs depicting 5G (i) and 6G (ii) Quad-Star hydrogel aggregates after a representative 2.5 h run. Micrographs in (a) are the uncropped and unprocessed versions of Figure 3e-f. Scale bars = 50 µm.
Figure S15: Representative profiles illustrating aggregate disassembly (A.F. = Area Fraction) upon cryptand injection (red dashed vertical line), thereby causing the release of the rhodamine B cargo (N.B.I. = Normalised Background Intensity). Curves for the same sample are marker shape-matched (circles for 5G, squares for 6G). Profiles are extracted from single FOVs of single samples. These additional profiles directly supplement Figure 4c, demonstrating that the specific timescales strongly depend on the proximity of the injection to the FOV. In this case, cryptand was injected much closer to the imaged FOV, thus inducing a much faster (approximately 2-fold) and less temporally resolved disassembly of aggregates with consequent cargo release.
Figure S16: Raw (a) NMM epifluorescence and (b) bright-field micrographs corresponding to inset panels in Figure 4d, illustrating 6G Quad-Star aggregates disassembling upon UV exposure (395-400 nm) in the presence of NMM. Micrographs from different channels are number-matched, reflecting uppercase roman numerals in Figure 4d. Uneven focus in (b) is most likely due to uneven placement of the specimen on the microscope stage. Scale bars = 50 µm. (c) Unprocessed (left) and background corrected (right, via Rolling Ball algorithm in FIJI) micrograph depicting Alexa488 fluorophore-labelled 6G Quad-Star aggregates after localised exposure to UV light in the presence of NMM. The hole carved by NMM-mediated photocleavage can be observed via Alexa488 fluorophores, demonstrating that disappearance of aggregates observed via NMM emission is due to disassembly and not to photobleaching of the porphyrin dye, as substantiated by the same trend in bright-field micrographs in (b). The underlying square-lattice pattern in (c) is an artefact of slightly uneven illumination and FOV stitching (Large Image via NIS Elements), rather than representing a physical characteristic of the sample. Scale bars = 1000 µm.
Figure S17: Contrast-enhanced (see Methods) Alexa-488 epifluorescence micrographs monitoring the integrity of 5G (a, c) and 6G (b, d) Quad-Star aggregates after 3-4 h assembly, at room temperature over the course of 60 h: (a, b) in native assembly buffer (TE 1× supplied with 2 mM MgCl₂ and 300 mM KCl); (c, d) after supernatant removal and replacement (75 out of 96.5 µL) with Phosphate Buffered Saline (PBS) 1× at time 0 (i). We observe samples left in K⁺-rich assembly buffer (a, b) to keep growing, as expected. Conversely, samples for which the buffer was replaced with PBS 1× (c, d) coalesce into larger microgels, possibly due to a reduced viscosity. This might in turn be caused by the combination of a different, less G4-stabilising prevalent cation (Na⁺ rather than K⁺), and reduced overall ionic strength (from ≈300 mM down to ≈140 mM monovalent cation). Area coverage in (a, b) is higher due to longer assembly time allowed (4 h for (a, b), 3 h for (c, d)). Time is indicated by the indices (i-iv) above the micrographs. Scale bars = 50 µm. Entire contrast-enhanced timelapses are provided as supporting videos S9-S10 (corresponding to (a, b)) and S11-S12 (corresponding to (c, d)), respectively.
Figure S18: Contrast-enhanced (see Methods) Alexa-488 epifluorescence micrographs monitoring the biostability of 5G (a, c) and 6G (b, d) Quad-Star aggregates after 3-4 h assembly, at room temperature over the course of 60 h in Dulbecco’s Modified Eagle Medium (DMEM) 1× supplied with: (a, b) 10% Fetal Bovine Serum (FBS) or (c, d) 30% Fetal Bovine Serum (FBS). As for Figure S17, samples were allowed 3-4 h to assemble, then supernatant was removed (75 out of 96.5 µL) and replaced with DMEM 1× supplied with FBS at time 0 (i). Compared to samples in K⁺-rich assembly buffer or PBS 1× (Figure S17), which kept growing and coalescing, respectively, Quad-Star aggregates appear to maintain a fully stable morphology in DMEM + FBS buffers. This might be due to the difference in ionic conditions (cations - monovalent: 155.30 mM Na⁺, 5.33 mM K⁺; divalent: 1.80 mM Ca²⁺, 0.81 mM Mg²⁺; multi-valent: 0.248 µM Fe³⁺). It is also possible that serum proteins (e.g. albumin) play a role in granting additional stability to the aggregates. Area coverage in (c, d) is higher due to longer assembly time allowed (3 h for (a, b), 4 h for (c, d)). Time is indicated by the indices (i-iv) above the micrographs. Scale bars = 50 µm. Entire contrast-enhanced timelapses are provided as supporting videos S13-S14 (corresponding to (a, b)) and S15-S16 (corresponding to (c, d)), respectively.
Table S1: All strands in use and their sequences according to 5’-3’ convention

| Strand                  | Sequence                                      |
|-------------------------|-----------------------------------------------|
| Arm_duplex              | CGACGCCGTGACGCGTTGGACGCACTCG                  |
| Arm_duplex_A_Alexa488   | /5Alex488N/ACGACGCCGTGACGCGTTGGACGCACTCG      |
| TerminalStrand_NoChol   | GCGTCACGGCGTCGAA                             |
| TerminalStrand_Chol     | GCGTCACGGCGTCGAA/3CholTEG/                   |
| Arm_4G3T_NoG (7T)       | TTTTTTTTTTCGAGTGCGTCCAAC                     |
| Arm_4G5T_NoG (9T)       | TTTTTTTTTTTTCGAGTGCGTCCAAC                   |
| Arm_4G7T_NoG (11T)      | TTTTTTTTTTTTTTCGAGTGCGTCCAAC                 |
| Arm_4G9T_NoG (13T)      | TTTTTTTTTTTTTTTTCGAGTGCGTCCAAC               |
| Arm_5G9T_NoG (14T)      | TTTTTTTTTTTTTTTTTTCGAGTGCGTCCAAC             |
| Arm_6G9T_NoG (15T)      | TTTTTTTTTTTTTTTTTTTTTTCGAGTGCGTCCAAC         |
| Arm_4G3T                | TGGGGTTTCGAGTGCGTCCAAC                       |
| Arm_4G5T                | TGGGGTTTTTTTCGAGTGCGTCCAAC                   |
| Arm_4G7T                | TGGGGTTTTTTTTTCGAGTGCGTCCAAC                 |
| Arm_4G9T                | TGGGGTTTTTTTTTTTCGAGTGCGTCCAAC               |
| Arm_5G3T                | TGGGGGTTCGAGTGCGTCCAAC                       |
| Arm_5G5T                | TGGGGGTTTTTTTCGAGTGCGTCCAAC                  |
| Arm_5G7T                | TGGGGGTTTTTTTTCGAGTGCGTCCAAC                 |
| Arm_5G9T                | TGGGGGTTTTTTTTTTTCGAGTGCGTCCAAC              |
| Arm_6G3T                | TGGGGGGTTTCGAGTGCGTCCAAC                     |
| Arm_6G5T                | TGGGGGGTTTTTTTCGAGTGCGTCCAAC                 |
| Arm_6G7T                | TGGGGGGTTTTTTTTTCGAGTGCGTCCAAC               |
| Arm_6G9T                | TGGGGGGTTTTTTTTTTTTTCGAGTGCGTCCAAC           |

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