Junctions between i-motif tetramers in supramolecular structures

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ABSTRACT

The symmetry of i-motif tetramers gives to cytidine-rich oligonucleotides the capacity to associate into supramolecular structures (sms). In order to determine how the tetramers are linked together in such structures, we have measured by gel filtration chromatography and NMR the formation and dissociation kinetics of sms built by oligonucleotides containing two short C stretches separated by a non-cytidine-base. We show that a stretch of only two cytidines either at the 3’- or 5’-end is long enough to link the tetramers into sms. The analysis of the properties of sms formed by oligonucleotides differing by the length of the oligo-C stretches, the sequence orientation and the nature of the non-C base provides a model of the junction connecting the tetramers in sms.

INTRODUCTION

The formation of hemiprotonated C•C+ pairs has been reported 50 years ago (1). At slightly acidic pH, the pairing of the neutral and protonated cytidines of C-rich oligonucleotides results in the formation of a four-stranded structure, the i-motif, including two parallel duplexes mutually intercalated in a head to tail orientation (2). I-motif structures reported by NMR (3) or X-ray (4) show that C•C+ pair intercalation and stacking produces a rigid core of long-lived base pairs. The symmetry of i-motif tetramers gives to C-rich oligonucleotides results in the formation of a four-stranded structure, the i-motif, including two parallel duplexes mutually intercalated in a head to tail orientation (2). I-motif structures reported by NMR (3) or X-ray (4) show that C•C+ pair intercalation and stacking produces a rigid core of long-lived base pairs. The symmetry of i-motif tetramers gives to C-rich oligonucleotides the self-associative properties allowing the formation of supramolecular structures (sms) with potential technological applications (5–7). This property has been first mentioned in a study reporting on the association of C7 oligonucleotides into i-motif wires (8). Using AFM microscopy, a recent publication has described the self-assembly of poly C into nanospheres (9).

Oligonucleotides containing two cytidine stretches separated by a single non-cytidine base associate into sms (10). The model derived from this study shows that C7GC4 for example forms a building block including the cytidines of the longest C stretch associated into an i-motif tetramer and two overhanging shorter C stretches at each end available for self-association. The end symmetry gives to this structure the capacity to build up sms by successive block addition and by association of short assemblies. In order to characterize the number of C•C+ pairs required to link i-motif tetramers into sms and to determine how much the sms stability depends on the number of C•C+ pairs involved in the junction between tetramers, we have investigated the formation and dissociation of sms formed by oligonucleotides with cytidine tracts as sort as possible. We observe that oligonucleotides with a C2 tract at the 3’- or 5’-end can associate into sms. However in all the conditions tested, the sms are too large to be investigated by NMR. Nevertheless, the comparison of the properties of sms built by oligonucleotides differing by the length of the oligo-C stretches, by the sequence orientation and by the nature of the non-C residue gives indirect information about the conditions required for tetramer association. A model of the junction linking i-motif tetramers into sms is presented.

MATERIALS AND METHODS

Oligonucleotide synthesis and sample preparation

The oligonucleotides were synthesized and purified as previously described (11). The oligonucleotide concentrations were determined using the A260 values computed according to a nearest neighbor model (12).

Formation and dissociation rate measurements of sms

The formation of sms was always measured with samples initially melted at 100°C and rapidly cooled at the incubation temperature. To measure the sms dissociation
kinetics, we used concentrated samples with a maximal sms proportion. The kinetics was initiated by diluting the samples in such a way as to allow complete sms dissociation at equilibrium. In order to avoid evaporation during incubation, the samples (50–200 ml) were always prepared in vials closed by airtight twisted caps with o-ring. They were incubated in a mastercycler Eppendorf® PCR incubator.

Gel filtration chromatography
The sample composition during sms association and dissociation was followed by gel filtration chromatography using GPC 100 and GPC 1000 columns (250 × 4.6 mm I.D.) provided by Eprogen®. Except otherwise stated, the chromatography were always performed at room temperature in 0.4 M NaCl, 10 mM Na–acetate and 10 mM Na–phosphate, pH 4.6. The volume of the injection loop was 25 μl. Systematic addition of thymidine at a final concentration of ~5 μM to the injected samples provided a reference marker. The GPC-1000 column, whose permeation and exclusion volumes correspond to oligonucleotides containing 50 and 10^4 residues respectively, was calibrated as previously reported (10). The GPC-100 was calibrated with i-motif tetramers and dimers, with monomeric C-rich oligonucleotides, un-fractioned tRNA, CC and TC dinucleotides, and thymidine. The comparison of the calibration curves obtained using i-motif structures and C-rich non-structured monomers shows that the elution volume of i-motif structures is somewhat larger than that of the monomers of identical molecular weights (Supplementary Figure S1). This indicates that the hydrodynamic radii of i-motif structures are smaller than those of monomers containing the same nucleotide number. The molecular weights of i-motif multimers were therefore estimated accordingly to the calibration curves obtained with i-motif structures. When this was required, the multimer stoichiometry was unambiguously determined by measuring the equilibrium multimer and monomer concentrations versus the oligonucleotide concentration.

Electrophoresis
The sms were also analyzed by a native polyacrylamide (PA) electrophoresis run on 6% and 10% gels containing 20 mM Robinson–Britton buffer, pH 4.6. During the electrophoresis the gel temperature was kept constant to 14°C with a waterbath. The gels were stained by Stains-All (Sigma), prepared as a 0.01% solution in 50% formamide.

NMR method
The NMR experiments were performed using a 500 MHz Varian Inova spectrometer with the jump and return sequence for water suppression (13). The magnetization transfer experiments performed to investigate the conformational exchange of the duplexes intercalated in the tetramers of C3TC2, C4TC2 and C3TC3 have been described (14).

Multimer dissociation constant
The dissociation constant of a multimer may be expressed as a function of αeq, the monomer equilibrium fraction of an oligonucleotide solution at concentration [M0] by:
\[ K_{\text{dis}} = s \alpha_{\text{eq}}^n [M_0]^{n-1} (1 - \alpha_{\text{eq}})^2 \]
where s are w in the multimer stoichiometry. We will characterize the multimers stability by Fi, a parameter whose dimension is independent of the stoichiometry which is equal to the free monomer concentration for which \( \alpha_{\text{eq}} = 0.5 \). Fi is related to the multimer dissociation constant by:
\[ Fi = \left( K_{\text{dis}}/s \right)^{1/(n-1)} \]
and it will be designated as the reduced dissociation constant.

RESULTS
We will consider as an sms all the species formed by association of at least two i-motif tetramers. The oligonucleotides investigated are sorted into four groups according to their capacity to associate into sms.

i-motif tetramer that cannot associate into sms
The oligonucleotides of this group associate into fully intercalated tetramers independently of the salt concentration. The group includes oligonucleotides with a thymidine at the 5‘- or 3‘-end (TCh and CnT, n = 2 and 5 (2,11); C2TC2 (14) and C2TC2T and short homopolymers: Cn (n = 2 to 5). The chromatograms of 2–5 mM solutions of these oligonucleotides show the equilibrium between the monomer and tetrameric structures. As previously shown (15), the salt concentration influences the intercalation topology of Cn tetramers but the association of these sequences into sms was negligible in the chromatograms of C3 or C5 recorded in 0.05, 0.4 or 1 M NaCl solutions.

This is also the case of oligonucleotides with a CT-sequence at the 5‘-end (CTC2 for example) or with a TC 3‘-end as in C5TC. It is noteworthy that at the extreme concentration of 25 mM, the chromatogram of C2TC2T shows that ~4% of the sample is associated in structures larger than tetramer.

C7 and C12 assembly into supramolecular structures
These two oligonucleotides have the same properties. A few minutes after melting at 100°C and fast cooling at 20°C, we observe that 1.5 mM C7 or C12 solutions become opalescent and precipitate. The chromatogram of a C7 solution displayed in Figure 1 shows two very weak components. According to the column calibration (Supplementary Figure S1), one is eluted at a tetramer; the other may be either the unstructured monomer, or/and an i-motif dimer formed by the association of two C7 hairpins. The same solution injected immediately after melting at 100°C shows an intense (monomer + dimer) peak. The comparison of both chromatograms establishes that a fraction of ~95% of the sample is missing on the chromatogram of the non-melted sample and therefore that C7 associates into extremely large sms that are trapped on the column. It is remarkable that the sms of C7 are also trapped in a GPC-1000 column.
whose exclusion size corresponds to structures including about 10⁴ nucleotides. The behavior of C7 depends on the NaCl concentration. We do not observe precipitation in a 1.5 mM C7 solution incubated in a buffer containing only 10 mM Na–acetate and 10 mM Na–phosphate, pH 4.6. In this buffer, the sms and tetramer fractions are 10% and 90% respectively at equilibrium, the sms half formation time is \(\frac{1}{2} \text{C24} \) 30 min and the elution time of sms is centered on a position corresponding to the assembly of 20 tetramers.

\[ \text{C2TCn (n = 3, 4, 5) and C5PurC2 (Pur = A or G)} \]

These oligonucleotides associate into sms. The chromatograms indicate common properties: (i) the formation and dissociation times of sms are comparable to the elution times, 4–6 min, in the GPC-100 column; (ii) sms dissociation induced by sample dilution results in the formation of tetramers. The sms proportion was determined by the comparison of chromatograms recorded before and after melting as in the left panel. The exclusion and permeation times are indicated by dashed lines.

dissociation constant is \(1.3 \times 10^{-7} \text{ M} \). It is noteworthy that incubation during days is required to reach the equilibrium between M and Te. The chromatograms recorded with concentrated C2TC5 solutions show a peak corresponding to sms and a broad shoulder indicated by arrows in Figure 2. The elution times of both peaks decrease continuously when the sample concentration increases.

Supplementary Figure S2 shows that all the oligonucleotides of this family exhibit the same characteristic elution profile. For these oligonucleotides, we observe that the chromatogram of a solution incubated at concentration C and diluted by a factor of 10 immediately before injection is similar (except for the Te/M ratio) to that of a sample incubated at concentration C/10. This observation indicates that the sms formation and dissociation times are comparable to or shorter than the elution time. According to this interpretation, the elution time of Te, the sms formed by association of n tetramers, is averaged by exchange between Te and [Te(n–m) + Tem] during the migration. The broad component designated by arrows in Figure 2 and Supplementary Figure S2 is attributed to Te, the tetramers involved in sms formation. The elution time of Te depends on the fraction of the time during which it is associated to sms and on the sms elution time. For this reason, the elution peak of Te is shifted in a direction indicating an apparent larger molecular weight when the oligonucleotide concentration increases. Chromatography performed as a function of temperature support this interpretation (Supplementary Figure S3). The chromatograms of a C5AC2 solution incubated at 20°C and injected in the column at controlled
temperatures show that at 0°C the sms are eluted in the exclusion volume. At 20°C the sms elution time is that expected for the assembly of three tetramers. Sms are dissociated into tetramer during the chromatography at 42°C. At 56°C the solution is eluted as a monomer. In relation with exchange between different species and with the structural heterogeneity of these samples, the NMR spectra of 0.5–1 mM C2TCn and C5PurC2 solutions have extremely broad lines unfavorable to structural investigations.

**CnTC2 (n = 3, 4, 5), C3TC3, C4TC4 and C5AC3**

These oligonucleotides associate into sms with the following common properties: (i) the sms formation and dissociation times are much longer than the elution time in the chromatography column, (ii) sms dissociation does not releases tetramers but monomers.

**C5TC2.** The evolution during incubation at 20°C of the composition of a C5TC2 solution is displayed in Figure 3. The chromatogram recorded immediately after melting shows two poorly resolved components labeled M and D. The measure of the equilibrium concentration of M and D as a function of the oligonucleotide concentration establishes that peak D increases as the square of peak M and thus identifies M to a monomer and D to a dimer (Supplementary Figure S4). The calibration curves of Supplementary Figure S1 show that the anomalously long elution time of the dimer, by comparison to that of the unstructured monomer, seems to be a common feature in relation with the compact character of i-motif. The dimer reduced dissociation constant estimated from Supplementary Figure S4 is \(8 \times 10^{-8}\) M at 20°C and \(9 \times 10^{-6}\) M at 42°C. The chromatograms recorded after several hours show the slow apparition of sms peaks whose elution times correspond to the assemblies of 2, 3 and 4 tetramers. Lastly the chromatograms show larger sms and the decrease of the species including a small number of tetramers. C5TC2 association into sms was also followed by NMR (Supplementary Figure S5). It must be reminded that the imino proton peak of non-paired cytidines is broadened out by exchange with water at pH 4.6 (16). Hence, the narrow imino proton peaks (15.3 ppm) of the first spectrum recorded after melting demonstrate the early formation of a structured hemiprotonated species corresponding to that detected as a dimer by chromatography. The spectrum recorded at equilibrium shows extremely broad NMR lines indicative of large sms. The proportion of short sms is always too small to be detected by NMR. Supplementary Figure S6 show that the sms half formation time decreases approximately as the power of \(-1\) of the oligonucleotide concentration in contrast to the tetramer half formation time that decreases as the power of \(-2\). The half formation times of C5TC2 and C3TC3 sms decrease when the temperature is raised with comparable activation energies of \(-190\) kJ/M (Supplementary Figure S7).

**C4TC2, C3TC2 and C3TC3.** The sms, tetramer and (dimer + monomer) fractions measured as a function of the oligonucleotide concentrations are comparable to those reported above for C5TC2 (Supplementary Figure S8). Nevertheless, these oligonucleotides show specific points that are described below.

The evolution of the species observed in a 1 mM C3TC3 solution during incubation at 42°C gives a valuable indication about the sms formation pathway (Figure 4). The sms fraction first increases with an initial formation rate about one-half that of the tetramer, reaches a maximum value of 20% and slowly decreases to zero while the tetramer fraction increases towards a limit value of ~75%.

The evolution in opposite directions of the tetramer and sms fractions demonstrates that the sms are not built by association of this tetramer species. The NMR spectra of C4TC2, C3TC2 and C3TC3 recorded during sms built-up present a common characteristic features providing key information about the intercalation topology of the corresponding tetramers. The spectra of C3TC3 are exemplary (Figure 5). They show two thymidine imino proton peaks of comparable intensities (11.2–10.95 ppm) that increase as a function of the time as the tetramer fraction measured by chromatography. These thymidine imino protons were therefore assigned to the tetramer. Magnetization transfer experiments show that selective saturation of either the 11.2 or the 10.95 ppm peak results in saturation of both protons (Figure 5). This demonstrates that the thymidine exchanges within the tetramer between two non-equivalent environments. The inter-conversion rate between the two environments of

**Figure 3.** Evolution during incubation at 20°C of the composition of a 0.3 mM C5TC2 solution initially melted. Left panel: The GPC-100 chromatograms are normalized to the same integrated area. The vertical scale of the chromatograms drawn in heavy lines are multiplied by a factor of five. On the chromatogram recorded at \(t = 0\), the oligonucleotide is eluted as a monomer (M) and as a dimer (D). The anomalous elution order of D and M reflects probably the hydrodynamic radius difference between the unstructured monomer and the compact i-motif (Supplementary Figure S1). The chromatograms recorded versus time show the early formation of a tetramer (Te), the transient apparition of species including 2, 3 and 4 tetramers and the accumulation of non-resolved sms. Right panel: Evolution of the sms (red), tetramer (blue) and (monomer + dimer) (green) fractions as a function of the incubation time. The dotted line shows the elution time.
was determined by measuring the magnetization transferred between the T imino protons as a function of the length of the saturation pulse (14). We find that it is equal to 1 s at 0° and to 50 ms at 20°C. It is noteworthy that the NMR properties of the thymidine imino proton of [C3TC3]₄, [C3TC2]₄ and [C4TC2]₄ are identical to those previously described for the thymidines of [C2TC2]₄ (14). This gives a strong argument to assume that these tetramers have an intercalation topology similar to that of [C2TC2]₄ (Figure 6) and that thymidine exchange reflects, as in [C2TC2]₄, duplexe inter-conversion within the tetramers.

**Sms dissociation and lifetimes**

We have shown above that the sms of C2TCn and C5PurC2 oligonucleotides dissociate in <6 min at 20°C into tetramers. The lifetime of the sms of C5AC3, 12 ± 3 h at 20°C, shows the dramatic effect of an additional cytidine.

The dissociation pathways of the sms of CnTC2 and C3TC3 have common characteristics. The chromatograms recorded as a function of the time after dilution of sms solutions show that the decrease of the sms fraction is approximately exponential and that the (dimer+monomer) peak increases consecutively to sms dissociation. The tetramer fraction decreases much more slowly (Supplementary Figure S9). The lifetimes of the tetramer of C3TC3 (Supplementary Figure S10) and CnTC2 prevents association into sms. This implies that the sms building block is a minor tetrameric species formed by a reaction parallel to that leading to the fully intercalated tetramer.

Figure 6. The intercalation topologies of the i-motif tetramers of C3TC3, C3TC2 and C4TC2. Their NMR spectra have characteristics indicating that as in [C2TC2]₄ (2), the tetramers include two intercalated duplexes, one (yellow) with a T•T pair (green) stacked on the 5’-adjacent C•C’ pair and the other (red) whose thymidines are looped out in the i-motif wide groove (blue circle). A black heavy line marks the face of the bases oriented in the 5’-direction. The magnetization transfer detected between the T imino protons of each duplex establishes that concerted opening/closing of the T•T pairs switches the duplex conformations. Full intercalation topology of the tetramers of C3TC3 and C3TC2 prevents association into sms. This implies that the sms building block is a minor tetrameric species formed by a reaction parallel to that leading to the fully intercalated tetramer.

[C3TC3]₄, [C3TC2]₄ and [C4TC2]₄ are identical to those previously described for the thymidines of [C2TC2]₄ (14). This gives a strong argument to assume that these tetramers have an intercalation topology similar to that of [C2TC2]₄ (Figure 6) and that thymidine exchange reflects, as in [C2TC2]₄, duplexe inter-conversion within the tetramers.
This may indicate either that the sms dissociate into monomers or that sms dissociation releases a short-lived tetramer that dissociates and does not accumulate. The sms lifetimes measured at 20°C and 42°C increase with the number of cytidines in the sequences (Supplementary Figure S11).

**Evaluation of the sms length**

The size of sms measured at equilibrium by gel filtration chromatography on GPC-1000 column (Figure 7) and by electrophoresis on PA gels (Figure 8) are in fairly good agreement. At the concentration of 1 mM, the equilibrium distributions of the sms of C5TC2 and C3TC3 are centered on molecular weights corresponding to the association of 20 and 15 tetramers respectively. Considering that the distance between sequentially adjacent i-motif C\(\cdot\)C\(^+\) pairs is 6.3 Å, the lengths of these structures should be \(\sim 70\) nm. The sms eluted in the rising edge of the chromatograms are about 10 times larger. The size of sms increases with the sample concentration, as shown with the example of a 9 mM C3TC3 solution whose sms distribution at equilibrium corresponds to the assembly of 170 tetramers (Figure 7). Due to exchange during chromatography, only averaged molecular weight are accessible at 20°C for the sms of C2TCn and C5PurC2 oligonucleotides. In 1–3 mM solutions, the elution times of the sms formed by these oligonucleotides correspond to structures including 5–8 tetramers (Supplementary Figure S2). In a 3 mM solution at equilibrium, the sms of C5AC3 (Figure 7) are about three times larger than those of C5AC2.

**DISCUSSION**

It emerges from this study that stretches of only two cytidines, either at the 3’- or 5’-oligonucleotide end, give to i-motif tetramers self-associative capacity and that the sms formation/dissociation kinetics is influenced by the sequence polarity. However, in the concentration range required for NMR studies, the sms are always too large to allow structural investigations. Our model of the junction linking the tetramers in sms is therefore based on the comparison the properties of sms built by different oligonucleotides.

**The sms formation pathway**

A characteristic feature of C-rich oligonucleotides is their ability to associate, via parallel reactions, into a variety of
i-motif structures including fully or partially intercalated tetramers and hairpin dimers. The formation rate of i-motif structures depends on the stoichiometry and on the oligonucleotide concentration. For solutions in the millimolar concentration range, the formation of dimer is faster than that of tetramer (17,18). Tetramers with full or partial intercalation topologies are built at comparable rates and their lifetimes increase by one or two magnitude orders for each additional stacked C+C+ pair (19). For these reasons, the thermodynamically stable tetramers are those with optimal intercalation topology.

The formation rate of C5T2C2 sms increases as the oligonucleotide concentration by contrast to that of [C2T2C]4, which increases as the square of the oligonucleotide concentration (Supplementary Figure S6). This suggests that sms formation does not proceed via consecutive incorporation of monomer, a process that should have stronger concentration dependence, but, by stepwise association of tetrameric building blocks and possibly by association of preformed short sms. The equilibrium of the sms length is reached when spontaneous sms dissociation counterbalances elongation. The sms formation rate may be extremely slow (Supplementary Figures S6 and S7). However, this does not seems to be an intrinsic property of sms as shown by the examples of C7, C2T2Cn and C5purC2 oligonucleotides (Figure 2, Supplementary Figures S2 and S3). It appears instead that the slow formation rate of CnT2C and C3T3 sms is due to competitive production of i-motif dimers or of fully intercalated tetramers that limit the availability of the sms building blocks and by that way controls the sms formation rates. The identification of the building block tetramer has therefore a crucial importance to understand how i-motif tetramers associate into sms.

The sms building blocks

The magnetization transfer experiments performed on the tetramers observed in C3T3, C4T2C and C3T2C solutions indicate structures similar to that of [C2T2C]4. However, as shown in Figure 6, the structures built on this model for C3T3C and C3T2C are fully intercalated and cannot associate into sms. This demonstrates that the sms are built by minor species, intrinsically less stable but which are stabilized into sms. We note that this conclusion is in line with the interpretation of the dissociation process of the sms of CnT2C and C3T3C suggesting that sms dissociation releases an unstable tetramer (see above). This also gives the following interpretation for the evolution after initial melting of the composition of C3T3C solutions at 42°C (Figure 4). Due to its long lifetime, the fully intercalated tetramer of C3T3C displayed in Figure 6 is the thermodynamically stable species at 42°C. Nevertheless, the building block and the fully intercalated tetramer are created at comparable rates. This induces the transient accumulation of kinetically trapped sms. At last, sms dissociation contributes to increase the fully intercalated tetramer fraction.

The comparison of the association kinetics of CnT2C and C2T2Cn oligonucleotides into sms is particularly instructive. What is the feature related to the sequence polarity that could account for the differences observed in sms formation and dissociation? Structural investigations by NMR have established that T+T pairs intercalated in i-motif adopt always the same structural arrangement (14,17,19,20). Let us consider the example of [C2T2C]4 (Figure 6). The tetramer is formed by two intercalated parallel duplexes. One whose thymines (T3) are unstacked and the other whose thymines (T3) are paired and stacked to the sequentially adjacent pair in the 5'-direction. The intercalation order, C5/C1/C4/C2/T3C2/C4/C1/C5, shows that pairs T3T3 and C2C2 are intercalated into the other duplex in the empty space between C2C2+ and C4C4+.

The building block models described below are based on the following assumptions: (i) the thymines in the building blocks are paired and intercalated with the same topology than the T+T pair intercalated in [C2T2C]4; (ii) the effects of the sequence polarity on the sms formation and dissociation kinetics originate from the stacking directionality of the T+T pair on the 5'-adjacent C+C+ pair. Using the example of C3T2C and C2T3C, Figure 9 shows that for CnT2C oligonucleotides, stacking of Tn+1 to Cn allows the formation of a i-motif core including the 2x n C+C+ pairs of the longest C stretches. At each end two empty intervals are available for intercalation of the two terminal C+C+ pairs of another identical building block. In the building block model of C2T2Cn, stacking of the thymidine to the 5'-adjacent C+C+ pair leaves a single empty interval available for intercalation of the terminal C+C+ pairs of another building block. According to this model, the number of intercalation sites in the junctions between the corresponding building blocks account for the lifetime differences of CnT2C and C2T2Cn sms. The slow formation rate of CnT2C and C3T3C sms is due to the weak proportion of the building block species (Figure 9) in competition with the more stable fully intercalated tetramers (Figure 6). It is also remarkable that the building blocks of C2T2Cn oligonucleotides have a larger number of stacked bases and therefore probably longer lifetimes than the building blocks of CnT2C. We suggest that this is exactly the reason why the sms of C2T2Cn oligonucleotides dissociate into long-lived tetramers in contrast to CnT2C sms that release unstable tetramers rapidly dissociated into monomers.

The C1'-C1' distance in purpur pairs (21) is certainly incompatible with purpur insertion in i-motif. We can therefore consider that the purines are unstacked in the building block of C5purC2 sms. Figure 9 shows that the tetramer built according to this assumption, by the cytidines of the longest C stretch, has also a single empty interval available for association into sms. The structural similarity of the terminal segments of the building blocks of C2T2Cn and C5purC2 sms is consistent with the similarity of their formation/dissociation kinetics. According to this model the building block of C5purC3 sms should provide two intercalation sites. This prediction is in agreement with the observation that the half dissociation time of C5AC3 sms is at least 120 times longer than that of the sms of C5AC2.
In contrast to short Cn oligonucleotides \((n \leq 5)\) that associate into fully intercalated tetramers, C7 and C12 aggregate into extremely large insoluble assemblies. We suggest that the presence of a non-C base in the oligonucleotide sequences prevents the formation of tetramers with staggered strands and that on the contrary, long Cn oligonucleotides associates into i-motif with staggered strands. Supplementary Figure S12 shows how staggered strands connecting together several i-motif cores can generate branched structures leading to the formation 2D networks or of 3D assemblies.

**CONCLUSION**

In summary, we have shown that C stretches as short as CC can link i-motif tetramers into sms. The sms grow in competition with i-motif tetramers or dimers and their formation rate is controlled by the availability of the building block involved in sms formation. The sms stability increases with the number of cytidines in the shorter C stretch. The comparison of the sms lifetimes with those of i-motif tetramers (19) shows that incorporation of tetramers into a larger structure has a stabilizing effect. The presence of a single non-C residue in the oligo-C sequences prevents the formation of structures with staggered strands, an effect certainly favorable to sms elongation into linear structures. Due to sugar backbone stretching induced by systematic intercalation, the i-motif is a stiff structure. Association of i-motif tetramers into sms can potentially form unbendable rods with a particularly large persistence length. This prediction is testable since the size of the sms examined in present work, typically \(600 \times 16 \times 10 \, \text{Å}\), is accessible to atomic force microscopy measurements.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online: Supplementary Figures 1–12.

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