DNA-driven assembly of micron-sized colloids
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A DNA-string quartet

Non-spherical colloidal building blocks can lead to the development of complex colloidal crystals of lower symmetry than is possible with the use of isotropic spherical colloids. In this chapter we describe an exploratory study of a viable strategy to assemble four DNA-coated colloids via a Holliday junction. A Holliday junction consists of four DNA double helices, with a branch point discontinuity at the intersection of the component strands. By the use of this structure we aim to construct tetrahedral colloidal assemblies that are not limited by specific colloid sizes or materials.

6.1 Introduction

Alternate nucleic-acid structures play an important role in biological processes. Multi-branched structures, where three or more duplexes intersect at a single point, serve as possible intermediates in DNA recombination and important permanent structural elements of naturally occurring RNA molecules [162]. Helical junctions may be defined as branchpoints where double-helical segments intersect with axial discontinuities, such that strands are exchanged between the different helical sections. Thus the integrity of junctions is maintained by the covalent continuity of the component strands and the perfect base pairing between the DNA strands.

The four-way DNA junction (see Figure 6.1a for an example) is generally accepted to be the central intermediate in homologous genetic recombination [163–166]. As the structure was first mentioned by R. Holliday [163] it is also commonly referred to as a Holliday junction. Homologous genetic recombination is important in the repair of double-strand breaks in DNA, and in providing the genetic diversity that is necessary for Darwinian evolution. A significant distinction among different four-way junctions lies in their potential ability to undergo branch migration. When a junction is formed by two DNA strands that are homologous (largely identical) it can undergo a sequential exchange of basepairing in which a branchpoint becomes effectively displaced along the DNA sequence. An extreme case is shown in Figure 6.1b. Here a schematic representation of junction formation from an inverted repeat sequence in dsDNA is shown. Further migration is still possible as all bases surrounding the cross-section can also pair. The same could occur in a four-way junction made from four DNA strands. The length of the region of homology will limit the number of steps of branch migration that are possible, from zero (sometimes called an immobile junction; Figure 6.1a) to thousands.
The structure of the four-way DNA junction is dependent on the presence of divalent metal ions. In the absence of ions or at low salt conditions, the four helices remain unstacked, and the junction is extended [167] (Figure 6.2a). In the presence of divalent metal ions and, to a lesser extent under high-salt conditions, the helices undergo pairwise coaxial stacking into a right-handed anti-parallel structure, termed the stacked X-structure (Figure 6.2b). This structure was first proposed in 1988 [167], on the basis of comparative gel electrophoresis experiments, and confirmed later by fluorescence resonance energy transfer (FRET) analysis [168].

While branched DNA motifs have been used to design objects [20, 21] and periodic arrays [18, 169], they have not been utilized to drive the assembly of colloids. The only literature relevant for this topic that we know of, is a study on the structural integrity of DNA motifs by Seeman and coworkers [117]. In this study, gold nanoparticles are used to test whether multiple-junction-DNA motifs can withstand stresses without changing their geometrical structure.

Assembling a limited number of colloids in a specific geometrical shape has been achieved before. One of the first experimental articles on DNA-driven assembly of micron sized colloids already reported on this very topic [119]. It describes a method to prepare mesoscale polyhedral structures from binary mixtures of microspheres of specific size ratios using DNA as a molecular bridge. The interest in such non-spherical building blocks comes from the fact that their assembly can lead to the development of complex colloidal crystals of lower symmetry than is possible with uniform spherical colloids.

Using a complete different approach, Pine and coworkers [170] were able to assemble four to fifteen colloids together. To this end they mixed particles dissolved in toluene with water, once mixed small numbers of colloidal microspheres are attached to the surfaces of liquid emulsion droplets. After removal of the fluid from the droplets, these...
Figure 6.2: Illustration of the ion-dependent folding equilibrium of the four-way DNA junction. At low salt concentrations the junction exists in an extended, unstacked conformation. Upon addition of divalent metal ions, such as magnesium ions, the junction folds into the stacked X-structure [162]

spheres pack in a manner that minimizes the area of the toluene-water interface during drying. The finite-sized clusters that we discussed in chapter 3 were made using λ-DNA coated colloids. Using such building blocks, it is possible to create clusters that contain a limited number of linked colloids. Unfortunately, the resulting clusters display a wide range of sizes and shapes.

By utilizing a Holliday junction we aim to make quartets of colloids. It is expected that, as all constructs use the same DNA-linkage, the aggregates will be very similar in shape as well. In principle, if the system behaves as predicted, we could selectively link colloids of different sizes or compositions. Such selective design is at present not possible with the two systems described above [119, 170].

In order to connect four DNA-coated colloids with a Holliday junction we must first choose an appropriate structure for the junction. As we are interested in assembly, it is important that branch migration is not possible as this would make the aggregates less stable. We therefore used a junction structure (Figure 6.1a) that is known for its high stability [160]. First, we tested the stability of the junction as well as appropriate buffer conditions for both the colloids and the junction. Next, we designed a DNA construct in order to assemble four colloids with one junction (due to spatial constraints a spacer is needed). Then the individual junction-DNA coated colloids were tested on their DNA quality. Finally, we present a first experimental attempt to obtain quartets of colloids connected by a Holliday junction.

6.2 Results and Discussion

6.2.1 Ionic stability of four-way junctions

The important role of metal ions on the stability of four-way DNA junctions has been established by experimental studies [162]. Under low salt conditions or in the absence of metal ions an extended planar cross-shaped geometry predominates whereas an X-shape
Figure 6.3: Thermal transition profiles of the quaternary complex or two- and three strand compositions. A: With additional sodium (100 mM) the junction is not stable. All strand combinations result in similar curves. Curves were measured from 90 °C to 5 °C and back to 90 °C. For clarity, only one reverse curve is shown (light gray). B: When a divalent salt is used (MgCl$_2$; 50 mM) the four-armed junction is more stable than the junctions missing one or two arms. This is reflected in the higher melting temperature. As before, only one reverse curve is shown.

with two inter-arm angles of 60° prevails in the presence of metal ions [162]. Higher salt conditions bring about at least a partial folding, but not as effective as with metal ions. Given that a higher stability is attributed to the X-structures, it seemed logical to use either high salt concentrations or to add a metal ion to our buffer. To test the formation of our junction at different salt conditions, UV melting studies were carried out.

First, the junction stability is tested in a buffer containing 100 mM salt (NaCl). The melting curves (Figure 6.3a) show that this buffer does not improve the junction stability. This can be deduced from Figure 6.3a as the pairwise combination, three-way structures and the complete four-way junction all show the same melting profile ($T_m \approx 34 ^\circ C$; this value can be read off from the midpoint of the curve or derived from the first derivative$^1$). In contrast to the results for the samples in high salt buffers, the greater stability of a junction formed in the presence of a metal ion (Mg$^{2+}$) is evident (Figure 6.3b). Whereas the pairwise combination and the three-way structure give the same melting profile, a clear shift is visible for the profile of the complete four-way junction. Here, the melting temperature of the complete junction is significantly higher than that of the incomplete structures ($T_m \approx 45 ^\circ C$ (4-way junction) vs $T_m \approx 36 ^\circ C$ (2 or 3 strand structure)). Pairwise structures already appear stable. This could later prove useful, as then two sets of dimers can be formed, to later combine into the complete junction.

Initially we chose the MgCl$_2$ concentration of 50 mM, to compare similar ionic strengths. However, the available literature suggests that a lower concentration of MgCl$_2$

$^1$The melting curves are measured by Linda Payet (Figure 6.3) and Sabrina Jahn (Figure 6.4) (Cambridge University), Linda Payet derived the first derivative as well (using Spectro software).
Figure 6.4: Thermal transition profiles of the quaternary complex extended with cos1-overhangs. Two concentrations of divalent salt (MgCl\(_2\)) are used (5 mM (gray) and 50 mM (black)). The four-armed junction dissociates at a higher melting temperature for higher MgCl\(_2\) concentrations.

should be possible as well. Therefore, we also measured a melting profile of the complete junction at a far lower MgCl\(_2\) concentration (5 mM). Comparing the results of the melting profiles of the extended oligonucleotides (the junction sequence attached to a cos1-end) at 5 and 50 mM MgCl\(_2\) (Figure 6.4) indicates that at lower MgCl\(_2\) the melting temperature of the junction decreases (T\(_m\) 55 °C (50 mM) vs T\(_m\) 48 °C (5 mM)).

We note that ref. [171] reports on the melting profiles of five-way junctions. Ref. [171] claims that higher melting temperatures are found for strands that did not form a junction, than for those that did: this finding is explained by comparing the experimental results for the “five-way junction” to the profiles of consistent parts of the junction. It turned out that it is possible that a superposition of the melting curves of all possible adjacent two strand combinations leads to the observed melting curve. We believe our results on the four-way junction have a different explanation as the cos1-sequences are not complementary and internal looping of this 12 base sequence is not possible. However, the study of ref. [171] makes it clear that UV measurements can sometimes be misleading and need to be interpreted with caution. Therefore, to have a clearer picture of the stability of the four-way junctions and all possible intermediates we also performed gel-electrophoresis.

6.2.2 Stability of the four-way junction and its intermediates tested by gel electrophoresis

Gel-electrophoresis experiments were performed\(^2\) on the four-way junction and its intermediates. On a gel, assembled structures migrate slower than single oligonucleotides, junction formation is therefore visible on a gel.

\(^2\)The gel-electrophoresis experiments are performed by Sabrina Jahn (Cambridge University).
Figure 6.5: Gel electrophoreses of two-, three- and the complete four-stranded junction (samples are loaded in duplicates). A: When MgCl$_2$ is present, the four-armed junction is more stable than the junctions with one or two arms less (strands loaded on gel are depicted in white in the cartoons). This is reflected in the difference in migration speed on the gel. B: Without MgCl$_2$ present in the gel or in the running buffer the junctions are all unstable. A 20 base dsDNA is used as a reference (faint band above the arrow-head)
Two experiments are performed. For both experiments, the junctions or intermediate structures were assembled at equimolar concentrations beforehand (4 micromol; Tris 100 mM; pH 8 containing 50 mM MgCl\textsubscript{2}). The samples were then loaded and run on a gel with either a buffer containing MgCl\textsubscript{2} or without MgCl\textsubscript{2}.

The results shown in Figure 6.5a refer to samples where MgCl\textsubscript{2} is always present (samples loaded in duplo). Whereas all intermediate structures display a smear of products (lane 1-7), the complete four-way junction results in a sharp band (lane 8). The predominant junction band migrates slower than the dimerized (lane 1-4) or three strands structures (lane 5-7). As observed in the UV-measurement adjacent pairwise combinations or three-strand structures yield comparable results. All bands end at similar heights on the gel. Note the difference in stability between adjacent pairwise combinations (8 base complementary; lane 2 and 4) and opposite pairs (close to zero complementary sequences; lane 1 and 3). The opposite pairs penetrate further into the gel, indicating (mostly) single stranded structures rather than assemblies. Interestingly, the minor difference in complementary base pairs between the two opposite pairs 2+4 and 1+3 is also reflected in the results. Pair 2+4 migrates slightly slower (lane 1) as this pair has more complementary bases (7 vs 3) than pair 1+3 (lane 3).

In Figure 6.5b the junctions were loaded without the presence of Mg\textsuperscript{2+}-ions. Instead of showing the pattern of Figure 6.5a, now all samples show a smear rather than sharp bands (lane 1-8). All constructs penetrate as deep as the individual oligonucleotide indicating little construct formation. These results strongly suggest that the ions that keep the junction in its stable form can diffuse away in time, resulting in the subsequent disassembly of the junction.

### 6.2.3 Optimizing buffer conditions for our colloids

To ensure our colloids are stable within the buffer (100 mM Tris, 5 or 50 mM MgCl\textsubscript{2}, pH 8) control experiments were performed. To this end, neutravidin colloids were added to Tris buffers with a range of MgCl\textsubscript{2} concentrations (ranging from 0 to 150 mM). Images were taken after a day and after five days. Figure 6.6a shows that after just one day at 50 mM Mg\textsuperscript{2+}-ions, small clusters appear, indicating that the colloids are not stable in this solution. The severity of this effect becomes more visible after five days, with all colloids assembled in a gel-like structure (Figure 6.6b). Within the concentration range, colloids in solutions with concentrations below 50 mM (0, 10 and 20 mM) remained stable, whereas at higher concentrations (70, 100 and 150) the colloids gelated within a day (data not shown).

Although it is possible to perform the experiments at 5 mM MgCl\textsubscript{2}, we prefer to work under conditions where the construct is as stable as possible. Therefore, we add a blocking agent (κ-casein; 0.1 mg/ml) to the tris-MgCl\textsubscript{2} buffer. As κ-casein prevents the neutravidin colloids from aggregating after one day (Figure 6.6c) as well as after five days (Figure 6.6d), a concentration of 50 mM of MgCl\textsubscript{2} can be used.
Figure 6.6: Colloid stability tested in buffers containing MgCl$_2$. A: Neutravidin-colloids in TRIS-HCl buffer with 50 mM MgCl$_2$. After one day small clusters appear. B: The same sample as in A, four days later. Neutravidin-colloids are not stable over time in a TRIS-HCl buffer with 50 mM MgCl$_2$. C: Neutravidin-colloids in TRIS-HCl buffer with 50 mM MgCl$_2$ and 0.1 mg/ml $\kappa$-casein. After one day colloids are now still stable. D: The same sample as in C, four days later. With the addition of $\kappa$-casein colloids are now stable over time in a buffer containing MgCl$_2$. All scale bars measure 10 $\mu$m.
6.2 Results and Discussion

Figure 6.7: DNA construct used to string four colloids together with a DNA-junction. A fragment of the pBelo-DNA is restricted at two sides. One carries a cos2-end, which can be used to attach the junction overhang (the AAA nucleotides are added for more flexibility). The opposite end contains a 4-base overhang. This is filled in with 4 nucleotides (TGCT); one labeled with a biotin-tag (dCTP). For clarity the eventual junction is depicted with dotted lines.

6.2.4 Designing a construct to string four colloids together with DNA

A certain minimum length of DNA is required to bind four micron-sized colloids. We estimated this length to be at least $\sim 450$ bp by drawing a close packed tetrahedral structure and assuming near stretched DNA conformations. As plasmids of this size are not available and ordering a construct of this size is not possible we used the pBeloBac11 plasmid (New England Biolabs). Sufficient amounts of DNA were obtained by amplifying a short fragment of the pBelo DNA (700 bp)$^3$. To ensure the calculation is not underestimating the minimum length we decided to use a 540 bp DNA construct as shown in Figure 6.7. One side is used to bind to the colloid by a biotin-neutravidin bond, the other side contains the junction overhang. Note that we added three nucleotides (adenine) to render the overhang more flexible. This idea was inspired by other studies [34, 62], which showed the use of a “flexor” molecule (nucleotide or a short polymer fragment (PEG)) given that greater flexibility can enhance the assembly process. As our construct might be under stress once formed we considered adding a “flexor” could prove useful.

6.2.5 Testing the individual DNA-coated colloids

All junction-DNA coated colloids were tested individually, to ensure that the colloids are coated with the desired DNA construct. The hybridization and ligation of the junction overhangs to the dsDNA is not necessarily a reaction with a 100% yield. To test to what extent the overhang is present, colloids are coated with the complementary 16 base ssDNA strands. Next, the four junction-DNA coated colloids are mixed one-on-one

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$^3$The amplification of DNA (PCR) was performed by Sabrina Jahn (University of Cambridge).
with these colloids (Figure 6.8a). The cluster in Figure 6.8b shows that the colloids indeed display the junction overhang.

While we now know that the junction-overhang is present this does not imply that all DNA on the colloid contains the overhang. It is possible that a small fraction of DNA did not hybridize to a junction overhang. If this would be the case, our colloids would also display cos2-overhangs (Figure 6.7).

To test whether cos2-overhangs are indeed present on our colloids, we also let our colloids aggregate with cos1-coated colloids (Figure 6.8c). Figure 6.8d shows that the hybridization and ligation of the junction overhangs to the DNA spacer worked well, as practically no small clusters are present.

### 6.2.6 First experiments on binding four colloids simultaneously by a Holliday junction

As the dimer structures with adjacent overhangs are stable, a high concentration of colloids could lead to percolation. Therefore, binding four colloids simultaneously by a Holliday junction requires a lower density than used in the λ, pBelo and cos experiments (volume fraction of $\phi \approx 0.004$). Presently, we work with a six times lower density. The results from the first experiments are promising (Figure 6.9a), but the desired conformation was not yet found. One day after mixing equal amounts of the four types of colloids, dimers and trimers are present (white arrows). The fact that most dimers consist of colloids with both colors, indicates that the binding is most likely specific. However, the existence of one color dimers (black arrows), could indicate unspecific binding. As the non-adjacent junction arms contain some bases that are complementary (7 for 2+4 and 3 for 1+3; see insert), it could be specific as well.

After five days, imaging shows a similar picture (Figure 6.9b). Still, the sample contains mostly monomers, with some dimers and trimers. Although quartets are not visible in the figure, cluster analysis indicates they are present, be it at a very low concentration (0.1%). Compared to the results after one day of aggregation the percentages of monomers (90%) and dimers (3.5%) are similar. The number of trimers shows a small decrease, indicating that some of them found a fourth partner to bind with.

While results look promising, we cannot predict if the system will develop into a system with a higher percentage of quartets present. As bigger clusters are not observed, it may be that a prolonged aggregation time is all the system needs.

### 6.3 Conclusions

In this chapter we described an exploratory study of the possible assembly of four colloids by a Holliday junction. A junction was chosen from literature [160] and tested on its stability in different buffers. Both UV measurements as well as gel-electrophoreses experiments indicate that the junction is stable under experimental conditions.

After testing each type of junction-colloid individually, we concluded that we are indeed capable of constructing the DNA needed. The first experiments look promising, although longer aggregation times are needed. It may also be useful to use longer DNA
Figure 6.8: Testing the individual DNA constructs on colloids. A: Cartoon of colloids used to test the individual junction overhangs. Instead of combining four colloids by a DNA junction formation, the overhangs are tested individually against their complementary 16 bp sequence directly coated on a colloid. The sequence shown represents junction overhang 1. B: Confocal image of the clustering with colloids as shown in A. C: Cartoon of colloids used to test the individual junction overhangs. If for any reason the hybridization or ligation of the junction overhang to the dsDNA spacer failed, the colloid is left with cos2 overhangs instead. The presence of these (unwanted) overhangs can easily be detected by addition of cos1-coated colloids. D: Confocal image of clustering against cos1-oligonucleotide colloids to test if all DNA on our junction-colloids contain the junction overhang as shown in C. Both scale bars measure 10 µm.
Figure 6.9: First experiment on binding four colloids simultaneously by a Holliday junction. A: Aggregation after one day: both trimers and dimers are present. While some structure appear to be specific binding (white arrow-heads), others are less certain (black arrow-heads). Insert: a cartoon of the structure formed. It is depicted planar to visualize strand binding. B: Aggregation after five days: little change is observed. Cluster-size analysis indicates that 0.1% of all clusters present are tetramers.

“arms” in the Holliday junction as the present constructs are presumably fairly stretched in the tetramer. This is likely to increase the concentration of tetramers. This would be highly desirable as for this type of self-assembly to be useful percentages above ~ 25 are needed. Needless to say that additional experiments are definitely needed.

6.4 Materials and Methods

6.4.1 Thermal transition profiles of the quaternary complex

The junction sequence was taken from Seeman and coworkers [160] for its high stability. Also, the lack of 2-fold symmetry around the center will prevent migration of the junction.

16 bases junction strands

With UV absorbance measurements (240-260 nm) the melting profile of the junction can be measured. Three- or two-strand combinations were tested to examine the stability of the assemblies formed with these sequences as well. The first set of measurements were performed with the junction sequences themselves (16 base sequences; 1. 5′-CGCA ATCC TGAG CACG-3′ 2. 5′-CGTG CTCA CCGA ATGC-3′ 3. 5′-GCAT TCGG ACTA TGGC-3′ 4. 5′-GCCA TAGT GGAT TGCG-3′).

To samples (450 µl; 2 µM) 200 µl mineral oil was added (heated to 95 °C; air-bubble free). The final volume was adjusted to 1 ml (TRIS-HCl buffer 100 mM; pH 8). The
samples were measured in the thermal mode (Cary 300 Bio Visible UV spectrophotometer (Viaran); wavelength 240-260 nm). Two sets of experiments were performed. For the first set, sodium chloride (NaCl; 100 mM) was added to the samples. The second set of experiments used magnesium chloride (MgCl₂; 50 mM) instead. All samples were measured starting from 90 °C to 5 °C. To test if the junction formation is indeed a reversible process, “reverse” curves were measured (starting from 5 °C to 90 °C) as well.

6.4.2 Gel-electrophoresis of the quaternary complex

For gel-electrophoresis assays all samples were prepared at 4 µM (TRIS-HCl buffer 100 mM pH 8; 50 mM MgCl₂) in equimolar concentrations. Two 20% polyacrylamide gels were prepared. One containing 20 mM MgCl₂ and one without additional salt. The same was done with the running-buffer (sigma-powder T7525-4L). For all samples 20 µl (including loading dye) was loaded in the gel.

The magnesium containing gel samples were loaded in the following order: in pairwise combinations: 2+4, 2+3, 1+3 and 1+2; as three-way structures: 1+3+4, 2+3+4 and 1+2+3; and as the assembled 4-way junction: 1+2+3+4. As a reference a low range DNA ladder was added (Sigma Aldrich, PCR ladder 1000-20 bp). On the gel without additional salt samples were loaded in the following order: oligonucleotide 3 individually, in pairwise combinations, 2+4, 2+3, 1+3 and 1+2, as three-way structures, 1+3+4 and 1+2+3 and as the assembled 4-way junction, 1+2+3+4. As a reference a 20 base dsDNA piece (Sigma Aldrich) was added as a reference. Both gels were run for 7 hrs at 110 V. The gel was then scanned on a flatbed scanner (UVP, multi doc-it) to capture images.

6.4.3 Preparation of junction-DNA-constructs

A fragment around the cos-restriction site of the pBeloBac11 plasmid (New England Biolabs) is amplified by PCR. The pBeloBac11 plasmid was purchased as a strain (New England Biolabs, ER24208). To obtain DNA to amplify with a PCR-reaction, the strain was grown overnight at 37 °C in 50 ml LB medium (for 100 ml: Bacto-Tryptone 1 g; Bacto-Yeast extract 0.5 g; NaCl 1 g and ddH₂O to 100 ml; autoclave sterilized) in presence of chloramphenicol (20 µg/ml). With a spin miniprep-Kit (Qiagen) the plasmid DNA was then isolated. The purity of the DNA was checked by gel electrophoresis on a 1% agarose gel. Next, the pBelo-DNA is diluted 1000x and 1 µl of each primer is added (p1: 5'-TAGTCTGGAACCACGGTCCC-3' (placement on plasmid: base 6631); p2: 5'-GCTTTCAGCACCTGTCGTTCC-3' (placement on plasmid: base 7355); both 0.25 µM). For a sample, 10 µl of the DNA primer mixture is mixed with 10 µl of HotStarTaq Plus Master Mix (HotStarTaq Plus DNA Polymerase, PCR Buffer, and dNTPs; Qiagen). The solution provides a final concentration of 15 mM MgCl₂ and 200 µM of each dNTP. We used the following cycling protocol for our PCR reaction: 1) initial activation step; 5 min 95 °C. 2) denaturation; 45 sec 94 °C. 3) annealing; 45 sec 67.7 °C. 4) extension; 1 min 72 °C. Step 2 to 4 are repeated 27 times. Finally, the samples are kept at 72 °C for 7 more minutes.

The amplified piece of DNA (724 bp) was restricted at two sides. One side was restricted with BssSI (New England Biolabs), leaving a 4-base ssDNA end. The other
end of the amplified piece of DNA contains the cos-restriction site, that can be opened by restriction with λ-terminase (BIOzymTC), leaving a 12 base single strand (“sticky end”). The 4-base end can be filled up nucleotide by nucleotide with Klenow exo⁻. (To obtain biotinylated DNA regular dTTP and dGTP-nucleotides were used in combination with biotin-dCTP; all Invitrogen). The cos-end is used to hybridize our piece of junction-DNA to the dsDNA piece. For this end the DNA was divided in four batches and mixed with one of the four cos1-junction-overhang oligonucleotides solutions (5 µl; 20 µM; Eurogentec). To hybridize the oligonucleotides to the DNA, the solution was heated to 65 °C for ~30 minutes and then cooled overnight to room temperature. To improve the hybridization this reaction was performed in a special annealing buffer (50 mM TrisHCl pH 8, 1 M NaCl and 0.2 mM EDTA; 10x, diluted with TRIS-buffer pH 8). Subsequently, T4 DNA ligase (New England Biolabs) was added to ligate the DNA backbone. To remove the excess of oligonucleotides and enzyme the samples were centrifuged and washed three times on a Microcon YM100 membrane (Milipore) with Tris-HCl buffer (100 mM, pH = 8). The biotin-DNA-junction solutions were then recovered in a clean tube.

6.4.4 Preparation of junction-DNA coated colloids

In contrast to earlier experiments where a binary system in which DNA-mediated attractions are favorable only between heterogeneous colloids was used, we now have four different pieces of DNA that hybridize to form one construct (DNA-junction). Since we can only excite green and red fluorescent colloids, two batches of both colors were prepared. Green fluorescent colloids (diameter 1 µm, Molecular Probes; depicted in white in figures) were functionalized with junction-1-DNA or junction-3-DNA. Red fluorescent colloids were functionalized with junction-2-DNA or junction-4-DNA (depicted in black in figures).

Our conjugation protocol is based on the neutravidin-biotin coupling procedure described in Ref. [53]: the four batches of junction-DNA were separately mixed with either green or red fluorescently labeled neutravidin-colloids, that were dispersed in TRIS-HCl (100 mM, pH 8). In each case the DNA and colloids were left to react overnight, during which they were continuously tumbled. The next day, samples were pelleted and washed five times to remove excess of non-conjugated DNA. In between these washing steps, samples were heated once for 10 min. at 50 °C to remove poorly bound DNA.

The protocol above results in four batches of colloids coated with 517 bp dsDNA displaying a 16 base ssDNA “sticky end”. Between two colloids only partial hybridization is possible, so to hybridize all ssDNA four colloids have to bind together (two green and two red). To be able to test the constructs individually the four complementary strands of 16 bp were purchased from Eurogentec (complementary 16 base sequences; 1’. 5’-GGGT TAGG ACTC GTGC-3’ 2’. 5’-GACG GAGT GGCT TACG-3’ 3’. 5’-CGTA AGCC TGAT ACCG-3’ 4’. 5’-CGGT ATCA CCTA ACGC-3’). Colloids coated with this type of DNA were prepared in the same way as cos-oligonucleotide colloids (chapter 4).
6.4.5 Preparation and coating imaging chambers

To avoid any nonspecific interactions between DNA-coated colloids and glass surfaces of the imaging chambers, cover slips were coated with polyethylene glycol (PEG). The coating of glass slides with PEG is a two-step procedure. First the glass slides were silanized with 3-mercaptoptriethoxysilane (97%, Fluka), then a 5-kDa maleimide modified PEG (Laysan Bio Inc) was tethered to the terminal thiol group of the silane.

Prior to the silanization, the glass slides were cleaned in an UV/ozone box. The cleaned slides were placed in contact with silane vapor overnight. Subsequently, the slides were rinsed with Ethanol, dried with Nitrogen and left in an oven (100 °C) for 30 minutes. Next a drop of concentrated maleimide modified PEG (250 mg/ml in TRIS-HCl buffer) is trapped between two silanized slides and left overnight at room temperature. The excess of PEG is removed by rinsing with ddH₂O.

A sample chamber is made from two glass coverslips of different size separated by a parafilm spacer (≈ 100 µm). The parafilm sealed the chamber on three sides. After loading the chamber with the four populations of DNA-coated colloids the chamber is completely sealed with glue.

6.4.6 Confocal imaging

All suspensions were imaged with an inverted microscope (DMIRB, Leica) with a confocal spinning-disc scan head (CSU22, Yokogawa Electric Corp.) and a 60x water-immersion objective. Fluorescence of the two populations of colloids was excited at 488 nm and 512 nm. Emission was observed at 505 nm and above 600 nm respectively. The use of two types of fluorescent beads and confocal microscopy allowed us to extract particles coordinates and to reconstruct 3D images [2].

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